Identification of a motor protein required for filamentous growth in *Ustilago maydis*

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The phytopathogenic fungus *Ustilago maydis* exists in two stages, the yeast-like haploid form and the filamentous dikaryon. Both pathogenicity and dimorphism are genetically controlled by two mating-type loci, with only the filamentous stage being pathogenic on corn. We have identified two genes (*kin1* and *kin2*) encoding motor proteins of the kinesin family. Kin1 is most similar to the human CENP-E gene product, while Kin2 is most closely related to the conventional kinesin Nkin of *Neurospora crassa*. Deletion mutants of *kin1* had no discernible phenotype; Δ *kin2* mutants, however, were severely affected in hyphal extension and pathogenicity. The wild-type dikaryon showed rapid tip growth, with all the cytoplasm being moved to the tip compartment. Left behind are septate cell wall tubes devoid of cytoplasm. In Δ *kin2* mutants, dikaryotic cells were formed after cell fusion, but these hyphal structures remained short and filled with cytoplasm. A functional green fluorescent protein (GFP)–Kin2 fusion was generated and used to determine the localization of the motor protein by fluorescence microscopy. Inspection of the hyphal tips by electron microscopy revealed a characteristic accumulation of darkly stained vesicles which was absent in mutant cells. We suggest that the motor protein Kin2 is involved in organizing this specialized growth zone at the hyphal tip, probably by affecting the vectorial transport of vesicles.

Keywords: dimorphism/kinesin/pathogenicity/*Ustilago maydis*/vesicle transport

Introduction

The phytopathogenic fungus *Ustilago maydis* can infect corn plants only in the dikaryotic stage of its life cycle (see Christensen, 1963). Formation of the dikaryon by fusion of yeast-like haploid cells is associated with a morphological switch to filamentous growth. Cell fusion, maintenance of hyphal growth and pathogenicity are controlled by the mating-type loci *a* and *b* (see Banuett, 1995). The biallelic *a* locus encodes a pheromone-based cell recognition system, the multiallelic *b* locus codes for homeodomain proteins that function as master regulators

of filamentous growth and pathogenicity (Bölker *et al.*, 1992; Gillissen *et al.*, 1992; Hartmann *et al.*, 1996). When two haploid cells of different *a* and *b* genotype (so-called compatible combinations) are mixed, the secreted pheromones bind to their cognate receptors and induce the formation of mating tubes that orient their growth towards the pheromone source and fuse at their tips (Snetselaar, 1993; Banuett and Herskowitz, 1994; Spellig *et al.*, 1994; Snetselaar *et al.*, 1996). In the common cytoplasm of the dikaryon, the products of the two different *b* alleles form heterodimers that control the switch to hyphal growth and pathogenicity (Kämper *et al.*, 1995). In the dikaryon, the pheromone genes continue to be expressed, leading to an autocrine stimulation of the pheromone signaling cascade that activates *b* gene expression (Hartmann *et al.*, 1996; Urban *et al.*, 1996). The dimorphic transition can be followed on solid media containing charcoal (Day and Anagnostakis, 1971; Holliday, 1974) or in microscopic assays (Snetselaar and Mims, 1992; Snetselaar *et al.*, 1997). After cell fusion, a straight hypha emerges and the nuclei migrate into the growing filament. The tip compartment containing the cytoplasm becomes delimited from the older vacuolated part of the hypha by a septum. During hyphal tip growth, new septae are built regularly to separate the growing tip zone from these vacuolated parts left behind (Snetselaar and Mims, 1992). This mode of growth is also characteristic for the early infection stages in planta (Snetselaar and Mims, 1992).

Since *U. maydis* is amenable to classical and molecular genetic analyses, it can be used as a model organism to study the intracellular processes underlying this morphogenetic program. In particular, we have become interested in the function of the cytoskeleton for hyphal tip growth. A characteristic feature of tip growth in fungi is the long distance transport and targeted fusion of vesicles containing cell wall precursors. It has been proposed that motor proteins acting on the cytoskeleton are involved in these processes, but their precise role remains to be elucidated (Heath, 1994). Since microtubules have been shown to play a role in fungal dimorphism (Harris *et al.*, 1989), a participation of kinesin- or dynein-like motor proteins appears likely. In fungi, several members of these protein families have been identified. They affect such diverse processes as karyogamy in *Saccharomyces cerevisiae* (Meluh and Rose, 1990), nuclear division in *Aspergillus nidulans* (Enos and Morris, 1990) and nuclear distribution and hyphal morphogenesis *Neurospora crassa* (Plamann *et al.*, 1994).

In this study, we have identified two genes encoding motor proteins of the kinesin family in *U. maydis*. We demonstrate that one of these proteins is required for hyphal extension and affects fungal virulence.

Results

Identification of genes encoding motor proteins of the kinesin family

We have used degenerate primers to amplify a highly conserved region within the motor domain of kinesins. Two products were identified that were used to isolate corresponding cosmid clones. Sequencing revealed open reading frames whose predicted amino acid sequences were highly similar to other members of the kinesin family. The corresponding genes were designated *kin1* and *kin2* and have been sequenced in their entirety.

For *kin1*, two in-frame translational start codons, both of which are embedded in a sequence context confined to the fungal consensus (Ballance, 1991), can be found upstream of the region encoding the conserved motor domain. The derived polypeptide sequence of Kin1 consists of 1459 amino acids if the first ATG codon is used for translation initiation (Figure 1A, DDBJ/EMBL/GenBank accession No. U92844). The motor domain of Kin1 (amino acids 237–649) is most similar to that of the human CENP-E protein (Yen *et al.*, 1991) with 40% identity (Figure 1A). The amino acid sequence of the C-terminal domain of Kin1 is predicted to adopt a coiled-coil structure but lacks significant sequence similarity to known proteins in the data base.

The predicted product of the *kin2* gene comprises 968 amino acids (Figure 1B, DDBJ/EMBL/GenBank accession No. U92845). The Kin2 protein has an N-terminal motor domain and displays 52% identity across the entire amino acid sequence to the recently identified Nkin protein from *N.crassa* (Steinberg and Schliwa, 1995) (Figure 1B).

Phenotype of $\Delta kin1$ and $\Delta kin2$ mutants

To analyze the function of the *kin1* and *kin2* genes, deletion constructs were generated (see Materials and methods). These mutant alleles were introduced by gene replacement into the haploid strains FB1 (*a1 b1*) and FB2 (*a2 b2*) which carry different *a* and *b* alleles. The absence of the wild-type allele was demonstrated by Southern analysis (Figure 2). Furthermore, this analysis did not reveal additional hybridization signals which could have indicated the existence of genes closely related to *kin1* or *kin2*. Western analysis using the polyclonal Nkc antibody directed against the C-terminus of the related *N.crassa* Nkin protein (Steinberg and Schliwa, 1995; Steinberg, 1997) identified a single band of the expected size in wild-type cells which is absent in FB1 $\Delta kin2$ and FB2 $\Delta kin2$ extracts (Figure 3). Both the $\Delta kin1$ and the $\Delta kin2$ mutants were viable, and neither cell morphology nor growth of haploid cells were significantly different from the parental strains (data not shown). When compatible mutant strains were mixed and co-spotted on charcoal plates, $\Delta kin1$ mutant combinations developed dikaryotic hyphae indistinguishable from those formed by compatible wild-type strains (Figure 4). This suggests that the *kin1* gene is not involved in cell fusion and hyphal growth. In crosses between a $\Delta kin2$ mutant strain and a compatible wild-type strain, long aerial hyphae were observed (Figure 4), but their appearance was somewhat delayed compared with filaments derived from a cross of compatible wild-type strains (not shown). In a mixture of compatible $\Delta kin2$ strains, however, formation of long aerial hyphae was

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Um-Kin1      MFLFRSSTSSPAPPPTPRKSSSLGSLPTISAGSEISSGSSSLASSPSSNSLSLQAHSS
Um-Kin1      VSSSSSVSPSPATCFATLSEPIILANALPTDSEPKAAATPARPTSPNRLTPTC
Um-Kin1      PRTSRATTTIKACPSFNTASRRSTISPSNTEHRPSKVTMAKAKVGSAAKSSPFTKLVA
Um-Kin1      ADTATKQSAVSATTDVETSLPTVAEDDAATPAATSNASTVNDVDTSTESKQNVVC
Hs-CENP-E     MAEEGAVAVC

Um-Kin1      VMRRSRASSSDSEASVWNCDEKRNIPTEHHPAIAKRTTSSERAGAGASIAAAPSSHD
Hs-CENP-E     VRVRPLNS-----RE-----ESLGETAQVYWKTDNNV

Um-Kin1      LDHEDPTSTYHFOCDKLITGAQTTDDMYHSHIAPVVRAAVEGYNGTIFAYGQTGSGKTH
Hs-CENP-E     IYQVDGSKS---FNEDRVFHGNETTKNVYEEIAAFIIDSALQGYNGTIFAYGQTGSGKTH

Um-Kin1      TMSGSDAEPGVIPRAVEQIFQMIKDEPDREPLLVSYLEYNETLKDLLAPLPPLTGSTG
Hs-CENP-E     TMSGSEDLGVIPRAIHDFQKIKKFPDREPLLVSYMEYNETITDLLC-----

Um-Kin1      SSLQTTDRPASPIKGGSSHAAGQSQCSTLRIEDQSSRVIITGLREEIVTDANTVILCLI
Hs-CENP-E     TTKMKPLIIEEDVNRN-VYVADTIEEVVYTESMALKWI

Um-Kin1      QRQDERHVGATDWNERSRSRSHVCPITTESRSP-APG-ASKEVRIISQNLNLDLAGSERA
Hs-CENP-E     TKGEKSRHYGETKMNORSSRSHITFERMILSESCEGSKVSHNLNLDLAGSERA

Um-Kin1      ASQAE---RRKEGAFINKSLTLGTVIGKLTPEVNGDAHIPIYRDSKLTRILQSTLSGNA
Hs-CENP-E     AQTGAAGVRLKEGCNINRSLFILGQVKKLSGDQVCG--PINYRDSKLTRILQSLGNGP

Um-Kin1      RIAVICTLSHTEHANETLSLTKFGKRCRLVVTAKKGTAMDKALLQYKRELDLAK
Hs-CENP-E     KTRICTITP---VSPDETALQFASATKYMKNIPYVNEVSTDEALLKRYRKE...

Um-Kin1      LEANGPSPNPNVMTVISAESAESQOKLDQLNQKEAAQREVEDMQKRSKSHLKAQIEHL
Um-Kin1      TRILITQSQVSDTADSNAAAGSPFPIRNRGAYASALARRGPRMSDLGGSCNPSILTPAP
Um-Kin1      LETTSLGAKPFLEAELALRQLRQIESRTALITAHATLARDARQAEQAEIRLINE
Um-Kin1      EELDEAAYKDLKAERDEARSIALKEQAKARENKLLLEQEBANRLKLVAKARAGEQD
Um-Kin1      QALQAEIKLKKLLESKEEELQVKKRALDEELREVRRAVEEKAARQAQGEINELKQAK
Um-Kin1      TALQELDQAIASVTELEKEKQDANKASRDDDEDDVKTFRNIVRGQVKKDDASQELK
Um-Kin1      QROLELQERAEQLERIGTELEQARRQLASQTCAPSPISLSELSTRLDELGASSEERQT
Um-Kin1      LOVELAKLRAVASGRERVTEKLASLETELSEANAKISLEKELELAEKASAEALKVSFP
Um-Kin1      LPVPLPTQGGSMQSPVSPFKALAGSKRLFGATPTCTTISGLNARAGSKVEYRYQSGA
Um-Kin1      DASSTPPSPSPASGSRVMLGHTPTTSPSSSLGSHPTSLAASPSTAQPAKAEARDEIDR
Um-Kin1      LNAVINSORAIMADLNSVSWKTRMKQAEILHKMLVNSGTSSAAGSPSSDAEADVEF
Um-Kin1      FGGGSPRRHKQAGQWQDQCHTLPTTHLALSTNNSVPTPYGAHTFNRPPSTISGPTKP
Um-Kin1      FSNSTADSWSYSSPHPLPLPGSGDSVSPSKRKRKRTIEMDLKLLKSSPRVESSEKRNFL
Um-Kin1      ENEVANSADTPTKRRTASAYYI

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B

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Um-Kin2      MSNNIKVVCFRPPNAIEQREGSDIVDFSDGSLVKLTRGVSTSGPEAGGFVDFKV
Nc-Kin       MSSANSIKVVARTRPQNRVIESGCOPIVTFQ--G---PDCTVDSK-EAQGSFTFDRV

Um-Kin2      FPMNIMQRDVFPEGIKETVEDLVNGYNGTIFAYGQTGSGKTFPMMSGDIDNDNLKGIIR
Nc-Kin       EDMSCQSDIRDFSIKPTVDLNGYNGTIFAYGQTGAGKSYTMMGTISIDDPDGRVIR

Um-Kin2      ITTQIFENIMASPHILEYLYKYSYMEIYMERIDRLDLPADNQLVHEKRNKRVYVYKGLSD
Nc-Kin       IYVEQFITSILSSAANIEYTVRVSMEIYMERIDRLDLPADNQLVHEKRNKRVYVYKGLLE

Um-Kin2      FVVGQADVVEIMRGGGLARAVSSTNNNAESSRSISIFLITICQRTNETGSAKTNGLYLV
Nc-Kin       IYVSVQVEVVEVMRGGNARAVATNNNDESSSHSIFVITITQKVNETSQAKSQGLFV

Um-Kin2      DLAGEKVGKTGAGSCQTELEAKKINKLSALGMVINALTDGKSSHPIYRDSKLTRILQES
Nc-Kin       DLAGEKVGKTGAGSCQTELEAKKINKLSALGMVINALTDGKSSHPIYRDSKLTRILQES

Um-Kin2      LQNSRTTLIVNASPCVYNADBTSLTRPGRVAKSINKKARVNAELSPBELKTLKKAKA
Nc-Kin       LQNSRTTLINCSFSSYNDAETSLTRPGRVAKSINKKARVNAELSPBELKTLKKAKA

Um-Kin2      DNERYOQYIVNLEAEKLVWRSGKVPESWADASKVVGOGAPIAATPTLAATQSOSRPIJ
Nc-Kin       QITSFENYIVNLESEVQVWRGGTVPKPKVWVPLELA---TFSKASATARPSTPSRLI

Um-Kin2      TPAVENRELASRPETPSAVSIDKDRDDPLRRENEISQIAEKEVALKSASALREABT
Nc-Kin       PESRAETPAISDRAGTF-SLPLDKDEREPLRRENEISQIAEKEVALKSASALREABTKE

Um-Kin2      ELAFYREQEATLSKDNKSMTSIBINIKQLERYVYEHKDATITDLIEKQMKDLAELEB
Nc-Kin       ELIALKDHDSKGGKENERLISESNBPKQLERLAPENAEQITIDQLKDANSELTAELDE

Um-Kin2      LRKSLADTAKTVKDPQOEG--KERKAEKMAOMADFNOC--IVSEKEQETRETSKLELT
Nc-Kin       VKQQLDMKMSAKETSAVLDEKKEKKAEMKMMAGDPLSGDVFSNDRAVADATQALDA

Um-Kin2      AGSTCSG--ALSADDLSLREQLLESQSLREQSERVPOAHEENELLHORROELESRPAT
Nc-Kin       LFEISSAGDAIPPEIKALREKLVETQGFVR-QAELSSFSAASSDAEARKRAELBARLEA

Um-Kin2      LEAEYBELLDKSIKAEANHVNDVTIQDLKAKLEQVASKRDAQTSEINDLKROTEFKA
Nc-Kin       KQAEHEBELLSRNLTADK-----EVKALLAKSLSDKSAVQVELVQDLKADIALKN

Um-Kin2      KEAILTNSNDNLKSTNEEKRAFAVTVTGVGEGGNLAESARELERVRKTMALQSEEDT
Nc-Kin       -----SETEHLKALVDDLQRRVKAAGASCHGQR-----EDR--PAAAEEDV

Um-Kin2      MKKSLMRDLQNRCEKVELEISLDESREQYNNVNRNSKAAQCKMAFLERNLEQLTNVQ
Nc-Kin       MKKSLMRDLQNRCEKVELEISLDESREQYNNVNRNSKAAQCKMAFLERNLEQLTNVQ

Um-Kin2      KQVLEQNSLSKKEVSTAEKLLARNERTQALEQHLMDGQDKMLQNRKPEEQQVAYKERI
Nc-Kin       KQVLEQNSALKKEVSTAEKLLARNERTQALEQHLMDGQDKMLQNRKPEEQQVAYKERI

Um-Kin2      HHANQMRASVAGSAAPGLGGFG--RIAKPLRGCGPTSGPQPGSVAAQVPGGSGVAGA
Nc-Kin       EAKAG--STRGLTDAGLGGFSGSRIAKPLRWRDAVAG-----ATATNP

Um-Kin2      GSSLGENSEPK-ARSSWFFSSK
Nc-Kin       IATLQONPPENKRGSWFFOKS

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Fig. 1. Deduced amino acid sequences of the kinesin motor proteins Kin1 and Kin2 from *U. maydis*. (A) Amino acid sequence of *U. maydis* Kin1. The methionine residue corresponding to the second potential translational start codon is underlined. The motor domain of Kin1 is aligned to that of human CENP-E protein (Swissprot accession No. Q02224). (B) Alignment of the *U. maydis* kinesin Kin2 with the conventional kinesin Nkin from *N. crassa* (DDBJ/EMBL/GenBank accession No. L47106). Identical amino acids are shaded, gaps are indicated by dashes.

abolished (Figure 4). Instead, colonies were covered with a loose fur of stunted aerial hyphae (Figure 4). This suggests that the $\Delta kin2$ mutation affects either the fusion step or the development of dikaryotic hyphae. To distin-

Fig. 2. Southern analysis of Δkin strains. In the lower parts, restriction maps of the wild-type and the Δkin mutant alleles are shown. The motor domains are shaded and the hygromycin (Hyg) and carboxin (Cbx) resistance cassettes are indicated as black bars. Restriction sites are shown for *HindIII* (H), *HincII* (Hi), *SalI* (S), *EcoRI* (E), *RsaI* (R) and *BamHI* (B). (A) DNA was isolated from the strains indicated above each slot, digested with *HindIII* and probed with the 3.6 kb *HindIII* fragment comprising the N-terminal portion of the *kin1* gene. (B) DNA of the strains indicated above each slot was cleaved with *EcoRI* and *BamHI* and probed with the 3.8 kb *EcoRI* fragment containing the *kin2* gene.

Fig. 3. Immunodetection of Kin2 and GFP-Kin2 fusion proteins. Protein extracts were prepared from the strains indicated above, and 75 μ g total protein per slot was separated on a 7.5% SDS-polyacrylamide gel. After blotting, Kin2 proteins were detected using the Nkc antibody. Size markers are indicated on the right. CL42 is FB2 $\Delta kin2$ /psGFP-Kin2#1 and CL53 is FB1 $\Delta kin2$ /pKin2-sGFP#3.

guish between these possibilities, we performed agar drop matings (Snetselaar *et al.*, 1996) to follow the mating reaction microscopically (Figure 5). When compatible wild-type strains FB1 and FB2 were placed in adjacent drops on water agar, formation of conjugation tubes could be observed already after 2 h. Their numbers had increased dramatically after 3 h, and after 5 h the first fusion events could be observed (Figure 5). After 12 h, straight dikaryotic hyphae could be detected (Figure 5). In mixtures of FB1 $\Delta kin2$ and FB2 $\Delta kin2$, the initial events appeared delayed for ~ 1 h. After 5 h, fusion events could be observed but the formation of extended straight hyphae did not occur even after 12 h incubation (Figure 5).

Next we have analyzed isolated mating structures for both compatible wild-type and compatible $\Delta kin2$ mutant

Fig. 4. Kin2 affects formation of aerial hyphae. Plate mating assays were performed on charcoal-containing media by co-spotting strains indicated on the left and on top of the upper panel. FB1 (*al b1*) and FB2 (*a2 b2*) were used as wild-type strains. The formation of dikaryotic aerial hyphae is visible as white mycelium. In the lower panels, the edges of colonies are shown at higher magnification for the cross of FB1 and FB2 wild-type strains and the cross between the $\Delta kin2$ mutant derivatives.

strain combinations. Cells were allowed to mate on water agar after mixing both mating partners, aliquots were removed at different times and analyzed microscopically (Figure 6). Nuclei were stained with 4',6'-diamidino-2-phenylindole (DAPI). In the combination of compatible wild-type strains FB1 (*al b1*) and FB2 (*a2 b2*), fused cells that have initiated hyphal development could be observed after 4.5 h (Figure 6). At this stage the length of the hyphae approximated 1–2 times the length of haploid cells, and the nuclei were beginning to invade the filament. After 6 h, the filaments had reached 6–10 times the length of a haploid cell, and most nuclei were found in the hyphal cells (Figure 6). Some of the haploid progenitor cells appeared empty, indicating that the cytoplasm had been translocated to the growing hypha. After prolonged incubation (20 h), the hyphae exceeded the length of the progenitor cells >20 -fold. The cytoplasm was restricted to the tip compartment, and empty cell structures divided by regularly spaced septa could be seen at the distal end of the hyphae (Figure 6).

When compatible $\Delta kin2$ mutant cells were mixed, cell fusion also occurred within 4.5 h (Figure 6). However, the emerging hyphae were much shorter than in the wild-type combination, and nearly all nuclei were still found in the progenitor cells. At 6 h after mixing, the filaments reached approximately twice the length of haploid cells and, in some cases, the nuclei had already migrated into the hyphae (Figure 6). Often, however, the nuclei could be found at the neck of the emerging filament. Even after

Fig. 5. Formation of conjugation tubes and mating reaction between compatible *U. maydis* wild-type and $\Delta kin2$ mutant sporidia. Small droplets of cells were placed on water agar in close proximity and overlaid with liquid paraffin. Mating reactions between the *U. maydis* cells were observed microscopically, and photographs were taken at the time points indicated on the left. In the schematic drawing on the top, colonies are indicated as shaded circles and the view shown in the micrographs is marked by a rectangle. Arrowheads point to the straight dikaryotic hyphae emerging from mating events between compatible wild-type cells after 20 h incubation. The bar corresponds to 50 μ m.

Fig. 6. DIC (differential interference contrast) and epifluorescence micrographs of DAPI-stained *U. maydis* wild-type and $\Delta kin2$ mating structures. Mixtures of cells were incubated on water agar under paraffin, and aliquots were removed at the time points indicated on the left. For each time point, one micrograph of wild-type cells (left) and two micrographs of $\Delta kin2$ mutant cells (right) are shown. The upper and lower panels show DIC and DAPI views, respectively, of the same field. Arrowheads point to regularly spaced septa in the empty cell wall structures of dikaryotic wild-type hyphae. The bar corresponds to 15 μ m.

20 h the length of the hyphae approximated at most 5 times the length of haploid cells (Figure 6). In general, the hyphae and progenitor cells appeared thicker and more irregular than in mating structures derived from wild-type strains. Nuclei were more rounded and more closely spaced than in wild-type hyphae, and progenitor cells often exhibited irregular projections which were empty in most cases (Figure 6). Most notably, however, long straight filaments with empty compartments at their distal ends could not be detected in the cross between $\Delta kin2$ mutant strains. This indicates that the rapid extension of dikaryotic hyphae and, in particular, the cytoplasmic movement to the tip cell compartment is impaired in $\Delta kin2$ mutants.

Deletion of *kin2* affects pathogenicity

To test the influence of the kinesin genes *kin1* and *kin2* on pathogenic development, pairwise combinations of wild-type and mutant strains were co-injected into maize plants. The combination of FB1 $\Delta kin1$ and FB2 $\Delta kin1$ was as effective in tumor induction as the respective wild-type combination (Table I). However, when two compatible $\Delta kin2$ mutant strains were crossed, tumor development

Table I. Pathogenicity of $\Delta kin1$ and $\Delta kin2$ mutants

Strain combination	No. of infected plants	Tumor formation
FB1 $\times$ FB2	19	18 (94%)
FB1 $\Delta kin1$ $\times$ FB2 $\Delta kin1$	12	9 (75%)
FB1 $\Delta kin2$ $\times$ FB2 $\Delta kin2$	51	4 (8%)
FBD11	33	14 (42%)
CLD1121	66	3 (5%)

was observed only in 8% of infected plants while for the wild-type combination of strains, 94% of infected plants developed tumors (Table I). To exclude the possibility that this effect is due to defects in cell fusion or nuclear migration, we have constructed a stable diploid strain CLD1121 (*a1 a2 b1 b2 $\Delta kin2$ -Cbx $\Delta kin2$ -Hyg*) where both alleles of *kin2* are disrupted (see Materials and methods). Since CLD1121 is heterozygous for *a* and *b*, this strain should be pathogenic without the requirement for cell fusion. Compared with the solopathogenic diploid strain FBD11 (*a1 a2 b1 b2*), CLD1121 was severely affected in its ability to induce tumors (Table I). This illustrates that Kin2 does not exert its effect during the cell fusion step but must play an important role during fungal development in planta.

Localization of GFP-Kin2 fusion proteins

To relate the observed phenotypes to the subcellular localization of Kin2, we have constructed translational fusions between Kin2 and the green fluorescent protein (GFP) from *Aequorea victoria* (Chalfie et al., 1994). Plasmids that express either an N-terminal (pSGFP-Kin2) or a C-terminal fusion protein (pKin2-SGFP) under the control of constitutive promoters were generated and transformed into haploid $\Delta kin2$ mutant strains (Materials and methods). Transformants that carry ectopically integrated plasmids were tested for mating behavior in crosses with compatible wild-type and $\Delta kin2$ strains. For both fusion constructs, transformants could be identified which showed a vigorous mating reaction with compatible $\Delta kin2$ strains (data not shown). This indicates that both the N- and C-terminal GFP-Kin2 fusion proteins are biologically active. Total protein extracts of transformants were analyzed by Western blotting using the polyclonal Nkc antibody as described before. All transformants expressed levels of GFP-Kin2 which were ~5- to 20-fold higher than in haploid wild-type cells (two examples are shown in Figure 3). In fluorescence microscopy, such transformants expressing either N- or C-terminal fusion proteins showed evenly distributed slightly granular cytoplasmic staining, with many cells displaying a single intensely stained spot located at either pole of the cell (Figure 7A and B). Because of the overexpression of the fusion proteins, it is likely that these spots represent artefacts. Interestingly, upon treatment of growing cells for 30 min with 100 μ M of CCCP (carbonylcyanide-*m*-chlorophenylhydrazine), a potent uncoupler of oxidative phosphorylation, a completely different staining pattern was observed: most of the green fluorescence was found to be associated with structures characteristic of microtubules (Figure 7C). For comparison, an immunostaining of microtubules in *U. maydis* cells is shown in Figure 7D. The association of

Fig. 7. Intracellular localization of GFP-Kin2 fusion proteins. The left and right panels show DIC and epifluorescence views, respectively, of the same field. (A) FB1Δkin2/pKin2-sGFP#3 (CL53) sporidia expressing C-terminal GFP-Kin2 fusion protein. (B) FB2Δkin2/psGFP-Kin2#1 (CL42) sporidia expressing N-terminal GFP-Kin2 fusion protein. (C) The C-terminal GFP-Kin2 fusion protein expressed in CL53 is bound to microtubules after 30 min incubation with CCCP. (D) Immunostaining of microtubules in FB1Δkin2 cells. (E) Dikaryotic hyphae resulting from mating between FB1Δkin2 and FB2Δkin2/pKin2-sGFP#6 (CL47). (F) Control of untransformed FB1 cells. The bar corresponds to 10 μm.

GFP-Kin2 with microtubules is reversible, since the normal staining pattern could be restored within 3 h after removal of CCCP (data not shown). This suggests that depletion of ATP leads to tight binding of Kin2 molecules to cytoplasmic microtubules, as has been observed for conventional kinesins *in vitro* (see Bloom and Endow, 1995).

To analyze the distribution of GFP-Kin2 in hyphal cells, the transformant FB1Δkin2/pKin2-SGFP#3 was mixed with FB2Δkin2 and allowed to mate on water agar (Materials and methods). Dikaryotic hyphae were removed after 24 h and subjected to fluorescence microscopy. A cytoplasmic staining similar to that in haploid cells could be observed in the filaments (Figure 7E), and a single bright spot was sometimes visible at either the hyphal tip

or the most distal end of the cytoplasm (not shown). To demonstrate that the green fluorescence results from the GFP-Kin2 fusion proteins, an epifluorescence micrograph of untransformed cells is shown in Figure 7F. The even distribution of the GFP-Kin2 fusion proteins suggests that it is localized in the cytoplasm or bound to submicroscopic vesicles.

Electron microscopy of hyphal tips

To investigate whether the distribution of submicroscopic vesicles is altered in the Δkin2 mutant strain, CLD1121 hyphal tips were analyzed by electron microscopy and compared with hyphal tips of the wild-type strain FBD11. To preserve membranous structures, KMnO₄ was used for post-fixation. Thin sections revealed dramatic differ-

Fig. 8. Electron micrographs of hyphal tips. Overnight cultures of the diploid strains FBD11 and CLD1121 were transferred to poly-L-lysine-coated coverslips as described in Materials and methods. (A–C) FBD11 wild-type hyphae accumulate darkly stained vesicles in a less densely stained apical region. (D–F) In hyphae of the $\Delta kin2$ deletion mutant CLD1121, vesicles are distributed randomly and the organization of the apex is indistinguishable from subapical parts of the hypha. The bar corresponds to 1 μ m.

ences in the organization of the hyphal apex between wild-type and mutant cells (Figure 8). In wild-type hyphae, an apical zone, comprising ~ 1.5 μ m, appears only lightly stained and contains an accumulation of darkly stained microvesicles (Figure 8A–C). This zone is absent from mutant hyphae, instead the apical region is indistinguishable from the subapical parts and contains membraneous structures. Microvesicles can be detected in the mutant but they do not accumulate as in wild-type hyphae (Figure 8D–F).

Discussion

During its life cycle, *U. maydis* can adopt three well-defined morphologically distinct states: yeast-like growth occurs in haploid cells; upon pheromone stimulation, these cells respond by forming projections (conjugation tubes) that are characterized by polarized growth along a pheromone gradient. After tip fusion, a straight hypha develops into which the two nuclei migrate. In contrast to the short conjugation tubes, the dikaryotic hyphae show accelerated growth with no simultaneous increase in cytoplasmic content. As a consequence, the cytoplasm must be moved along with the growing hypha, as it is found only in the tip cell compartment. To elucidate the underlying molecular mechanism of hyphal tip growth, we have isolated two genes, *kin1* and *kin2*, from *U. maydis* that belong to the kinesin family of motor proteins. The products of these genes show the highest degree of similarity to the human CENP-E and the *N. crassa* Nkin protein, respectively. CENP-E is a centromere-binding protein implicated in chromosome movement during pro-

metaphase (Yen *et al.*, 1991). Since the homology between CENP-E and Kin1 is restricted to the motor domain and $\Delta kin1$ mutants are viable, we have no evidence that Kin1 has a comparable function. The *U. maydis* Kin2 protein is highly similar to the *N. crassa* Nkin protein and thus belongs to the fungal class of conventional kinesins (Steinberg and Schliwa, 1995). The similarities between Kin2 and Nkin extend across the entire amino acid sequence but are most prominent in the motor domain and the C-terminal tail region. The tail regions of kinesins have been proposed to be involved in cargo binding (Bloom and Endow, 1995).

Although a wealth of data is available on the function of conventional kinesins *in vitro*, the complex phenotype of mutants isolated in *Drosophila melanogaster* and *Caenorhabditis elegans* did not allow the unambiguous establishment of their biological function *in vivo* (see Bloom and Endow, 1995). The generation of the *kin2* null mutant demonstrates that in *U. maydis* a conventional kinesin can be deleted without obvious effects on viability and morphology of vegetatively growing cells. In this respect, it is interesting that inspection of the complete sequence of the yeast genome revealed that such a conventional kinesin does not exist in *S. cerevisiae*, indicating that budding growth in yeast and *U. maydis* does not require this type of motor molecule.

The function of Kin2 became apparent when mutants were analyzed for their potential to undergo morphological transitions. Whereas $\Delta kin2$ mutants can form normal-looking conjugation tubes (albeit somewhat delayed compared with wild-type strains), the rapid tip growth and movement of the cytoplasm into the hyphal tip compart-

ment does not occur in the *kin2* mutants. This indicates that formation of conjugation tubes and hyphal tip growth are distinct morphological transitions. We envisage that conjugation tubes arise from polarized growth of the cytoplasm, resembling the process of shmoo formation in yeast. In $\Delta kin2$ mutants, the beginnings of dikaryotic hyphae are formed, and the nuclei migrate into these structures, but rapid elongation of hyphal tips and concomitant movement of the cytoplasm is not observed. In appearance these structures resemble conjugation tubes. In addition, the two nuclei appear more closely spaced in these structure than in hyphae formed by wild-type cells. Whether the influence of Kin2 on spacing of the nuclei is a direct effect remains to be shown. The distribution of the biologically active GFP–Kin2 fusion protein in haploid cells and dikaryotic filaments does not indicate an association of Kin2 with defined organelles such as mitochondria or the nucleus. Therefore, we consider it unlikely that Kin2 plays an active role in nuclear migration. Furthermore, we have found no differences in the distribution of GFP-labeled mitochondria in wild-type and *kin2* mutant dikaryons (data not shown). This is in accordance with the observation that a recently isolated kinesin mutant of *N.crassa* shows no major alterations of microscopically visible organelle movements [see accompanying manuscript, (Seiler *et al.*, 1997)]. The uniform distribution of GFP–Kin2 fusion protein in *U.maydis* makes it unlikely that Kin2 is associated with microtubules but rather suggests an association with submicroscopical vesicles. The association of GFP–Kin2 with microtubules after depletion of ATP indicates that the interaction of GFP–Kin2 with microtubules under normal conditions is transient. The availability of a biologically active GFP–Kin2 fusion protein should facilitate the identification of the cargo that is transported by Kin2 in *U.maydis* by subcellular fractionation and biochemical characterization of vesicles that carry the green fluorescent GFP–Kin2 protein.

In several fungi, cell wall vesicles have been shown to accumulate in a zone near the tip of the hypha. However, neither the components of the cytoskeleton nor the mechanochemical force producers involved in delivering these vesicles to the hyphal tip are clearly defined (Heath, 1994). For *U.maydis*, we could demonstrate that such a vesicle-rich zone exists in wild-type hyphae but is notably absent in $\Delta kin2$ mutant hyphae. Together with the observed tip growth defect of $\Delta kin2$ mutant hyphae, this suggests that hyphal expansion requires the vectorial transport of vesicles to the tip. A possible function of the Kin2 motor protein in this process could be the long distance transport of vesicles that contain cell wall precursors and cell wall synthesizing enzymes. We cannot exclude, however, that the observed effect is indirect. For example, Kin2 could transport a component to the tip that is required for the subsequent accumulation of vesicles.

The high degree of similarity between *U.maydis* Kin2 and the *N.crassa* Nkin protein suggests that both motor proteins might have homologous functions in these two organisms. In the accompanying manuscript by Seiler *et al.* (1997) the phenotype of a *N.crassa* *nkin* mutant is described. Although this mutant is still able to grow filamentously, the rate of hyphal tip elongation is drastically reduced, deposition of cell wall material appears to be delocalized and, accordingly, filaments are highly

branched and the shape of hyphae is swollen and contorted. The accumulation of vesicles at the hyphal tip is also altered in *nkin* mutants. In addition, mutant cells show a defect in the regular distribution of nuclei. Interestingly, the movement of visible cell organelles was not impaired in mutant cells, although the transport of organelles visualized by video microscopy had been demonstrated to be microtubule dependent (Steinberg and Schliwa, 1993). This has been interpreted to indicate that Nkin is required specifically for the targeted delivery of small, secretory vesicles to the hyphal tip. Thus, in both fungi, *U.maydis* and *N.crassa*, the conventional kinesins may be essential for the rapid elongation of hyphal cells that is characteristic for fungal growth.

The pathogenicity defect of *kin2* mutants suggests that the rapid mode of hyphal growth is also crucial during the early stages of fungal development within the plant tissue. It is known that *U.maydis* requires meristematic tissue for tumor induction (see Banuett, 1995). In order to proliferate, the fungus must actively reach this tissue that might be distant from the point of entry. In this respect, it is interesting that infected plant tissue often contains collapsed fungal hyphae that correspond to the empty cell wall structures observed in mating assays outside the plant host (Snetselaar and Mims, 1992). We take this as an indication that the invading fungal cells are able to move through the plant by rapid hyphal tip extension without cytoplasmic expansion. The observation that *kin2* mutants can induce tumors in a small fraction of infected plants can then be explained by fortuitous placement of the inoculum close to meristematic tissue during hypodermic needle infection. This also implies that subsequent fungal development, including karyogamy and spore formation, is not impaired in *kin2* mutants. Fungal growth during the late stages of pathogenic development may thus occur mainly by an increase in cell mass, and apparently does not require the motor activity of Kin2. This is supported by microscopic observations that gave no hint of the presence of collapsed hyphae in tumor tissue (Snetselaar and Mims, 1994). The interesting phenotype of *kin2* mutants reinforces the intimate link between hyphal growth and pathogenic development in this system, and ultimately may help in understanding the molecular basis of fungal dimorphism.

Materials and methods

Strains, plasmids and growth conditions

For cloning purposes, the *Escherichia coli* K-12 strain DH5 α (Bethesda Research Laboratories) was used. The *U.maydis* strains 521 (*a1 b1*), FB1 (*a1 b1*), FB2 (*a2 b2*) and FBD11 (*a1 a2 b1 b2*) have been described (Banuett and Herskowitz, 1989). Plasmids pTZ18R, pTZ19R, pSL1180 (Pharmacia) and pSP72 (Promega) were used for cloning. Plasmids pCM54 (Tsukuda *et al.*, 1988) and pSMUT (Bölker *et al.*, 1995) confer hygromycin resistance, and pCBX122 (Keon *et al.*, 1991) confers resistance to the fungicide carboxin.

The *U.maydis* strains were grown in YEPS at 28°C (Tsukuda *et al.*, 1988). To test for mating and pheromone stimulation, strains were co-spotted on charcoal-containing CM plates (Holliday, 1974) and incubated at room temperature for 48 h. Agar drop matings were performed on 2% water agar overlaid with liquid paraffin according to Snetselaar *et al.* (1996). Plant infections were done as described (Gillissen *et al.*, 1992).

DNA procedures

DNA was isolated from *U.maydis* according to the protocol of Hoffman and Winston (1987). Transformation of *U.maydis* was performed as

described by Schulz *et al.* (1990). All other molecular techniques followed standard procedures (Sambrook *et al.*, 1989).

Isolation of the *kin1* and *kin2* genes

Degenerate primers K-480: 5'-CGATGGATCCGGAACACNYWYA-CNATG-3' and K-626: 5'-CCAGGGATCCKYTCNSWNCCNGCN-ARR-3' (K:T/G; N:A/T/C/G; R:A/G; S:C/G; W:A/T; Y:T/C) were used for amplification with 100 ng of *U.maydis* strain 521 DNA as template in a volume of 50 µl. PCRs contained 10 mM Tris-HCl pH 8.3, 1.5 mM MgCl₂, 50 mM KCl, 0.2 mM dNTPs, 30 pmol of primers and 5 U of *Taq* polymerase (Boehringer). The following conditions were used for amplification. After 30 cycles of amplification (1 min 94°C, 1 min 50°C and 2 min 72°C), reactions were incubated for 10 min at 72°C and the amplification products were separated on agarose gels. Two fragments (480 and 520 bp) were isolated, digested with *Bam*HI and cloned into pTZ19R for sequencing. The cloned *kin1* and *kin2* fragments were used to screen a cosmid library (Bölker *et al.*, 1995). The *kin1* gene was isolated as a 10 kb *Eco*RI fragment and cloned into pTZ19R to yield plasmid pKin1. The *kin2* gene was isolated as a 3.8 kb *Eco*RI fragment and cloned into pTZ19R to yield plasmid pKin2. The genomic sequences of *kin1* and *kin2* were derived from pKin1 and pKin2, respectively. All sequences were determined on both strands by the chain terminating method (Sanger *et al.*, 1977) using the T7 polymerase dideoxy sequencing kit (Pharmacia) or cycle sequencing and analysis on an ABI 377 DNA sequencer. Sequence comparison with the databases was done using the program BLAST (Altschul *et al.*, 1990), alignment of protein sequences was performed with the program CLUSTAL W (Thompson *et al.*, 1994).

Plasmid and strain construction

pΔKin1. The *kin1* gene was subcloned as a 3.6 kb *Hind*III fragment into pTZ18R to yield plasmid pKin1-H. Parts of the open reading frame corresponding to amino acid positions 229–526 containing the major portion of the motor domain were excised from pKin1-H with *Sal*I and *Hinc*II and replaced by the hygromycin resistance cassette derived from pCM54 as a *Bam*HI–*Nhe*I fragment.

pΔKin2. For this plasmid, 190 bp of the 5'-untranslated sequence and the major portion of the *kin2* open reading frame from amino acid 1 to 503 was excised as *Rsa*I and *Hind*III fragments and replaced by a cassette conferring carboxin resistance excised from pCBX122 as an *Eco*RV–*Sma*I fragment.

pKin2-Cbx. To generate an insertion mutant of *kin2*, the carboxin resistance cassette was isolated from pCBX122 as a *Sma*I–*Eco*RV fragment and inserted into the single *Age*I site of pKin2, thus disrupting the motor domain.

pΔKin2-Hyg, *pΔKin2-Cbx*. A deletion derivative of pKin2 was generated by PCR with outwards directing primers located 22 bp upstream of the start codon and 1284 bp downstream of the start codon. During the PCR, a single *Not*I site was introduced which was used subsequently to clone the hygromycin and carboxin resistance cassettes to yield plasmids pΔKin2-Hyg and pΔKin2-Cbx, respectively. For gene replacement, pΔKin1 was linearized with *Bgl*III, and pΔKin2 and pKin2-Cbx were cut with *Eco*RI before transformation. Gene replacements were verified by Southern analyses.

The phenotypes of the *Δkin2* and the *kin2-Cbx* insertion mutants were indistinguishable, indicating that the observed phenotype of *Δkin2* mutants was not caused by the deletion of 190 bp upstream of the start codon.

CLD1121. This stable diploid strain (*a1 a2 b1 b2 Δkin2-Cbx Δkin2-Hyg*) was generated by co-streaking of FB1Δkin2-Cbx and FB2Δkin2-Hyg on double CM medium (Holliday, 1974) and selecting for cells that are resistant to both hygromycin and carboxin. The presence of two different *a* alleles was demonstrated by assaying for pheromone production (Bölker *et al.*, 1992), the presence of two different *b* alleles was determined by PCR and subsequent restriction fragment length polymorphism analysis (R.Kahmann and M.Bölker, unpublished), and the presence of the *Δkin2-Hyg* and *Δkin2-Cbx* alleles was verified by Southern analysis.

GFP–Kin2 fusion constructs

The C-terminal SGFP–Kin2 fusion was generated by cloning the *SGFP–TYG* gene (Chiu *et al.*, 1996) as a *Xma*I–*Not*I fragment from pMFA1-SG (Spellig *et al.*, 1996) into *Xma*I and *Not*I restriction sites that had been introduced at the stop codon of *kin2* by PCR. The constitutive *otef* promoter from pOTEF-SG (Spellig *et al.*, 1996) was fused as an *Eco*RI–*Bam*HI fragment to the *Sau*3A site 54 bp upstream of the Kin2 start

codon. The integrity of the *SGFP–kin2* open reading frame was confirmed by sequencing. The hygromycin resistance cassette was introduced as a *Not*I fragment into the *Not*I site downstream of the *SGFP* gene.

For the N-terminal SGFP–Kin2 fusion, the *SGFP* gene and the *kin2* gene were linked with a short spacer coding for six amino acids (GAGAGA) that has been generated by PCR and which contains a *Ngo*MI site. The fusion gene was cloned as a *Sma*I–*Bgl*III fragment into pSP72 and sequenced. Subsequently, the *U.maydis* *hsp70* promoter (Holden *et al.*, 1989) was cloned as a *Bam*HI–*Sma*I fragment from pCM54 into the respective sites upstream of the *SGFP–kin2* gene. The hygromycin resistance cassette from pSMUT was excised as a *Bam*HI–*Bgl*III fragment and introduced into the *Bgl*III site downstream of the *kin2* gene.

Western analysis of protein extracts

Cells grown in liquid medium were precipitated and resuspended in an equal volume of yeast lysis buffer (Sambrook *et al.*, 1989). Total protein extracts were prepared by disrupting the cells in a French pressure cell twice. To remove insoluble cell debris, the extract was centrifuged twice for 10 min at 20 000 r.p.m. at 0°C. The protein concentration of the supernatant was determined and 75 µg were separated on a 7.5% SDS gel. The proteins were transferred to a nitrocellulose membrane (Schleicher and Schuell) by electroblotting. Kin2 and GFP–Kin2 fusion proteins were detected using the polyclonal Nkc antibody that was raised against a peptide derived from Nkin of *N.crassa* (Steinberg, 1997). Peroxidase-conjugated goat anti-rabbit antibody (Dianova) was used as secondary antibody.

Immunostaining

Cells were placed on coverslips treated with poly-L-lysine (1 mg/ml) and fixation and staining were performed in small droplets. Cells were fixed with PEM (30 mM PIPES, 5 mM EGTA, 5 mM MgSO₄, pH 6.7) containing 3.7% formaldehyde and 0.5% glutaraldehyde for 20 min and rinsed three times with PEM buffer. Cell walls were digested with 1 mg/ml novozyme in PEM for 50 min, rinsed twice with PEM and twice with phosphate-buffered saline (PBS) (pH 7.3). After a wash with 1% Triton X-100 in PBS, cells were rinsed four times with PBS. For microtubule staining, cells were incubated at 20°C for 35 min with mouse anti-α-tubulin monoclonal antibody (Amersham) diluted 1:80 in PBS. After rinsing six times with PBS, cells were incubated for 35 min with goat anti-mouse IgG conjugated to rhodamine (Organon Teknika Cappel) diluted 1:40 in PBS and finally rinsed six times with PBS.

Microscopy

For light and fluorescence microscopy, a ZEISS Axiophot microscope was used. Yeast-like cells were taken from liquid cultures grown in CM medium. Mating structures and hyphae were observed directly on water agar slides or after resuspension in water. For the detection of nuclei, cells were stained with 0.1 µg/ml DAPI for 2 min at 50°C. Photographic slides were digitized on a Nikon Coolscan and image contrast was enhanced using Adobe Photoshop.

Electron microscopy

Diploid sporidia were grown ON in CM medium, sedimented at 1000 g for 2 min, resuspended in an equal volume of water, and 100 µl cell of suspension was placed on a poly-L-lysine-coated coverslip. After hyphae were grown overnight in a humid chamber, the coverslips were fixed with 2% glutaraldehyde in 50 mM cacodylate buffer, pH 7.2, for 4 h at room temperature. This was followed by three rinses with buffer and a post-fixation for 1 h in 2% KMnO₄ in water. After five additional rinses with water, the cells were stained overnight in aqueous 0.5% uranyl acetate. The samples were dehydrated in a acetone/water dilution series over 1.5 h and embedded in Spurr's resin over 2 days. Sections were lead citrate stained and analyzed using a Philips CM-10 electron microscope at 80 kV.

Accession numbers

DDBJ/EMBL/GenBank accession numbers are: U92844 for the *kin1* gene and U92845 for *kin2*.

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