The Role of the Kinesin Motor KipA in Microtubule Organization and Polarized Growth of Aspergillus nidulans

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Polarized growth in filamentous fungi requires the integrity of the microtubule (MT) cytoskeleton. We found that growing MTs in Aspergillus nidulans merge at the center of fast growing tips and discovered that a kinesin motor protein, KipA, related to Tea2p of Schizosaccharomyces pombe, is required for this process. In a ΔkipA strain, MT plus ends reach the tip but show continuous lateral movement. Hyphae lose directionality and grow in curves, apparently due to mislocalization of the vesicle supply center (Spitzenkörper) in the apex. Green fluorescent protein (GFP)-KipA accumulates at MT plus ends, whereas a KipA rigor mutant protein, GFP-KipAG223E, coated MTs evenly. These findings suggest that KipA requires its intrinsic motor activity to reach the MT plus end. Using KipA as an MT plus-end marker, we found bidirectional organization of MTs and determined the locations of microtubule organizing centers at nuclei, in the cytoplasm, and at septa.

INTRODUCTION

The ability to generate cell polarity is a key feature of both prokaryotic and eukaryotic cells. In eukaryotes, it is achieved and maintained through the localized assembly of signaling complexes, the rearrangement of the cytoskeleton, the interaction of the cytoskeleton with the cortex, and the delivery of proteins to the sites of membrane growth and cell extension. One early step for the generation of asymmetry is the positioning of cell surface landmark proteins, which direct the cytoskeleton and thereby the flow of vesicles toward this site (Nelson, 2003).

The general principles of cell polarization are relatively well understood in the single-cell yeasts Saccharomyces cerevisiae and Schizosaccharomyces pombe. In S. pombe, mutants with T-shaped cells were isolated and named tip elongation aberrant (tea) (Snell and Nurse, 1994; Verde et al., 1995). Two of the corresponding genes, tea1 and tea2, were cloned and analyzed in detail (Mata and Nurse, 1997; Browning et al., 2000). Tea1p is a kelch-domain protein that localizes to the growing plus ends of MTs in the cell and is thereby delivered to the cortex (Behrens and Nurse, 2002). In addition to being transported in an MT-dependent manner, the protein affects the organization of the MT cytoskeleton: tea1 mutants have a higher frequency of cells in which MTs curl around the ends of the cell (Mata and Nurse, 1997). tea2 encodes a kinesin-like protein (Browning et al., 2000) that is responsible for the correct localization of Tea1p and localizes itself to the cell poles and the MT plus ends. One possible explanation for the Tea1p localization defect is the disturbance of the MT cytoskeleton observed in tea2 mutants. However, it was proposed that Tea2p is loaded onto MTs in the middle of the cell and then travels toward the MT plus ends by using its intrinsic motor activity (Browning et al., 2003). Thus, it could be that Tea1p is a cargo of the Tea2p motor. On the other hand, Tea2p anchorage at the cell ends depends on Tea1p (Browning et al., 2000, 2005). After being transported and delivered to the cortex, Tea1p needs to be anchored at the membrane. A candidate protein for that is Mod5p, which was discovered through an insertional mutagenesis approach (Snait and Sawin, 2003). Mod5p contains a signal for carboxy-terminal prenylation and is thus a prime candidate for membrane association. Although localization of Tea1p depends on this protein, the restriction of Mod5p localization itself to the cell tip requires the presence of Tea1p. How growth is directed to the site of Tea1p is not yet well understood.

Despite the growing body of information on cell polarity in S. pombe, little is known about the molecular mechanisms of cell polarity in filamentous fungi, in which polar growth is by far more prominent (Harris and Momany, 2004). In contrast to S. pombe, tip extension is not linked to the cell cycle, and growth direction can be changed. Fast hyphal growth in filamentous fungi requires a continuous supply of enzymes and cell wall components. This is achieved by MT-dependent vesicle transport mediated by motor proteins such as conventional kinesin (Seiler et al., 1997, 1999; Riquelme et al., 2000; Requena et al., 2001). An accumulation
of vesicles, named the vesicle supply center or “Spitzenkörper” (SPK), is located in the hyphal tip close to the cortex. Although the exact nature of the vesicles is still unknown, it is likely that the SPK serves as a transit station for secreted enzymes, e.g., for cell wall biosynthesis. The position of the SPK has been associated with the direction of hyphal growth (Girbardt, 1957; Grove and Bracker, 1970; Bartnicki-Garcia et al., 1995; Riquelme et al., 2004). Other genes and factors required for establishment and maintenance of polarity in filamentous fungi have been identified, and some of the genes have been analyzed at the molecular level (Momany et al., 1999; Shaw et al., 2002; Lin et al., 2003; Seiler and Plamann, 2003). However, it is not yet possible to develop a comprehensive model of how the components act and interact.

We analyzed the genome of Aspergillus nidulans for kinesin-like proteins and identified 11 candidates, which were phylogenetically grouped into nine of the currently 11 different kinesin families (Rischitor et al., 2004). In this study, we have characterized the kinesin-like protein KipA and found that deletion of kipA affects the maintenance of growth directionality. We propose that the polarity defect is due to a lack of temporal anchoring of MTs to the growing cortex at the cell tip.

MATERIALS AND METHODS

Strains, Plasmids, and Culture Conditions

Supplemented minimal (MM) and complete media for A. nidulans and standard strain construction procedures were used, as described by Hill and Kafer (1984). Expression of tagged genes under control of the alcA promoter was regulated by carbon source: repression on glucose, derepression on glycerol, and induction on threonine or ethanol (Waring et al., 1989). A list of A. nidulans strains used in this study is given in Table 1. Standard laboratory Escherichia coli strains (XL-1 blue, Top 10 F') with the DNeasy Plant Mini kit (QIAGEN, Hilden, Germany). Genomic DNA was extracted from A. nidulans with the DNeasy Plant Mini kit (QIAGEN, Hilden, Germany). RNA was isolated with TRIzol (Invitrogen, Paisley, Scotland, UK) for the reaction cycles. DNA sequencing was done according to the manufacturer’s protocols. DNA and RNA analyses (Southern

Table 1. A. nidulans strains used in this study

<table>
<thead>
<tr>
<th>Strain</th>
<th>Genotype*</th>
<th>Source</th>
</tr>
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<tr>
<td>SRF200</td>
<td>pyrG89, ΔargB::trpCΔB; pyroA4; veA1</td>
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<td>GR5</td>
<td>pyrG89; wA3; pyroA4; veA1</td>
<td>Waring et al., 1989</td>
</tr>
<tr>
<td>RMS011</td>
<td>pabaA1, yA2; ΔargB::trpCΔB; trpC801, veA1</td>
<td>Stringer et al., 1991</td>
</tr>
<tr>
<td>SPR26</td>
<td>pyrG89, ΔkipA::pyr4; pyroA4; veA1</td>
<td>Rischitor et al., 2004</td>
</tr>
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<td>SPR36</td>
<td>ΔkipA::pyr4; pyroA4; veA1; ΔkinA::pyr4</td>
<td>Rischitor et al., 2004</td>
</tr>
<tr>
<td>SRL1</td>
<td>GR5 transformed with pRL19</td>
<td>See text</td>
</tr>
<tr>
<td>SRS27</td>
<td>SRF200 transformed with pRS31 and pDC1 [GFP-nuclei]</td>
<td>Suelmann et al., 1997</td>
</tr>
<tr>
<td>SRS29</td>
<td>SRF200 transformed with pRS54 and pDC1 [GFP-mitochondria]</td>
<td>Suelmann and Fischer, 2000</td>
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<td>J. Warmbold, Marburg, Germany</td>
</tr>
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<td>SRL1 × RMS011</td>
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<td>SSK28</td>
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<td>SSK13 × SPR26</td>
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<td>SSK73</td>
<td>wA3; ΔkipB::argB; pyroA4, ΔkipA::pyr4; veA1</td>
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<td>XX3</td>
<td>pyrG89; nudA1, chaA1, veA1</td>
<td>Xiang et al., 1994</td>
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<td>SSK80</td>
<td>pabaA1, wA3; ΔkipA::pyr4; nudA1, veA1</td>
<td>SSK44 × XX3</td>
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<td>SSK92</td>
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<td>SSK99</td>
<td>SSK92 transformed with pJH19 and p4 [GFP-KipA and red nuclei]</td>
<td>See text</td>
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<td>SSK92 transformed with pPND1 and p4 [GFP-KipA and mRFP-1-MTs]</td>
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<td>SSK114</td>
<td>SRF200 transformed with pPR700, homologous integration [GFP-KipA*new]</td>
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<td>SSK116</td>
<td>SRF200 transformed with pPR700, ectopic integration [GFP-KipA*new (partial KipA protein)]</td>
<td>See text</td>
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* Important characteristics of the strains that might not be obvious from their genotypes are indicated in brackets.
Table 2. Plasmids used in this study

<table>
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<tr>
<th>Plasmid</th>
<th>Description</th>
<th>Source</th>
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<tr>
<td>pCR2.1-TOPO</td>
<td>TA-cloning vector [for cloning of PCR fragments]</td>
<td>Invitrogen (NV Leek, The Netherlands)</td>
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<td>pDC1</td>
<td>A. nidulans argB selectable marker plasmid</td>
<td>Aramayo et al., 1989</td>
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<tr>
<td>pl4</td>
<td>A. nidulans pyrA selectable marker plasmid</td>
<td>Osmani et al., 1999</td>
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<td>pPND1</td>
<td>kipB(N-terminus) in pCMB17apx; GFP replaced by nRFPI</td>
<td>Rischitor et al., 2004</td>
</tr>
<tr>
<td>PNRSTE1</td>
<td>1.9-kb pyr4 with flanking BonH1 and Nol site in pCR2.1-TOPO</td>
<td>Requena et al., 2001</td>
</tr>
<tr>
<td>PCMB17apx</td>
<td>alcA(p)GFP; for N-terminal fusion of GFP to proteins of interest; contains N. crassa pyr4</td>
<td>V. Efimov (Piscataway, NJ)</td>
</tr>
<tr>
<td>pjW18</td>
<td>alcA(p):staI(NLS):DsRed and argB as selectable marker in pBluescript KS-</td>
<td>Toews et al., 2004</td>
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<td>pJIH9</td>
<td>gpd(p):alkA(p):DsRed and argB as selectable marker in pBluescript KS-</td>
<td>Toews et al., 2004</td>
</tr>
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<td>pSK82</td>
<td>1-kb kipA fragment in pCMB17apx</td>
<td>See text</td>
</tr>
<tr>
<td>pPR700</td>
<td>As pSK82 except for G223E mutation yielding kipA&lt;sup&gt;Argor&lt;/sup&gt;</td>
<td>See text</td>
</tr>
</tbody>
</table>

and Northern hybridizations) were performed as described by Sambrook and Russell (1999).

**Disruption and Tagging of kipA**

Two regions of kipA were amplified by polymerase chain reaction (PCR) using genomic DNA as template and primers KipA1 (5'-CACCCAGCC-AGGATCCGACGTCGTA-H11032) and KipA2-BamHI (5'-AGGATCCGACGTCGTA-H11032-GAGTGGG) for the upstream region (positions 528 to 815 relative to the A of the start codon) and KipA3-NolI (5'-ACCCAGCCGGCTTATGGTCTGACC-3') and KipA4 (5'-GTTGTCGACGTCGTA-GACGTCGTA-H11032) for the downstream region (positions 872 to 1625 relative to the A of the start codon). The products were cloned into pCR2.1-TOPO generating pEW30 and pEW27, respectively. A 1.1-kb BamHI fragment from pEW30 was cloned into pBluescript KS- and a 750-base pair fragment from pEW27 were then cloned upstream and downstream of the pyr4 marker in PNRSTE1, generating pRL19. This plasmid was cut with KpnI and Xhol, which cut in the polylinker on either side of the insert, thus generating a fragment containing pyr4 flanked by kipA sequences. This fragment was transformed into the uracil-auxotrophic A. nidulans strain GRS5. Its homologous integration by double crossover should lead to replacement of 56 base pairs of kipA by pyr4, with consequent disruption of the motor domain. Among 60 transformants that were analyzed by PCR, five displayed the desired homologous integration at the kipA locus. Four of them had no additional integration events as shown by Southern blot analysis. One kipA strain (SRL1) was crossed to RMS011, and the resulting strain SSK13 (recognized as kipA by Southern blot) was crossed again to RMS011 to generate the kipA strain SSK44 and to SPR26 to generate the KipA<sup>alkA</sup> double-mutant strain SSK28 (identified by Southern blotting with probes for both kipA and kipB).

To create an N-terminal fusion construct, a 1-kb fragment of kipA (starting from the ATG) was amplified from genomic DNA with the primers KipA-AscI (5'-AGGATCCGACGTCGTA-H11032-GAGTGGG) and KipA-AscI (5'-GTTGTCGACGTCGTA-GACGTCGTA-H11032) and cloned into pCR2.1-TOPO, yielding pSK79. The AscI-AscI fragment from pSK79 was then subcloned into the corresponding sites of pCMB17apx (kindly provided by V. Efimov, Piscataway, NJ), yielding pSK82. Homologous recombination of this plasmid into the kipA locus should result in an N-terminal GFP fusion of the entire kipA gene under control of the alcA promoter plus a truncated 5' region under control of the kipA promoter. Among 20 transformants of strain GRS5, two displayed the kipA deletion phenotype under repressing conditions (growth on glucose) and a wild-type phenotype under inducing conditions. PCR analysis confirmed that the construct was integrated at the kipA locus in both transformants. The PCR fragments were sequenced to confirm the mutagenesis event. The low frequency of mutant colonies can be explained by the need for homologous recombination to occur downstream of the changed triplet in a 133-base pair region. Two strains (one named SSK116) with ectopic integrations of pPR700 were selected for phenotypic analyses as a control.

**Light Microscopy**

For live cell imaging, cells were grown in glass-bottom dishes (WPI, Berlin, Germany) in 2 ml of medium, either MM + 2% glyceral + pyridoxine and/or arginine or MM + 2% ethanol (or threonine) + pyridoxine and/or arginine. Cells were incubated at 30°C for 15 h, and images were captured at room temperature by using an Axiohot microscope (Carl Zeiss, Jena, Germany), a Planapochromatic 63× or 100× oil immersion objective lens, and a 50–W Hg lamp. Fluorescence was observed using standard fluorescein isothiocyanate, 4,6-diamidino-2-phenylindole (DAPI), and rhodamine filter sets. Images were collected and analyzed with a Hamamatsu Orca ER II camera system and the Vasabi software (version 1.2). Time-lapse series were obtained with an automated Vasabi program that acquires series of images with 2- or 3-s intervals, 0.5- or 0.75-s exposure time, and ~100 exposures in a sequence. Image processing and measurements were done with Image-ProPlus (Media Cybernetics, Silver Spring, MD) and Adobe Photoshop.

To determine GFP position, strains were grown on a coverslip for 24 h at room temperature in MM containing 17% gelatin, and images were captured with an Axiosvert microscope (Carl Zeiss). For motion analysis, sequences of up to 30 frames of single germline tubes were taken at 1- or 2-min intervals, and GFP position and hyphal growth were observed by phase contrast microscopy.

Indirect immuno-fluorescence of MTs and γ-tubulin was performed as described by Willins et al. (1995), with the following modifications. Cells were grown for 8 h at 37°C in MM containing appropriate supplements and fixed for 30 min at 37°C in 74% formaldehyde in PME buffer (50 mM PIPES, 5 mM EGTA, and 1 mM MgSO<sub>4</sub>, pH 6.9), followed by three washes with PME buffer. The cells were then digested with 4 mg/ml glycylsine, 11 mg/ml triselycine (both from InterSpen, San Mateo, CA), and 2 mg/ml yeast lytic enzyme (ICN, Eschwege, Germany) for 1 h at room temperature. Before adding antibodies, the cells were blocked with 3% bovine serum albumin in Tris-buffered saline (20 mM Tris and 700 mM NaCl, pH 7.6) for 1 h. Cells were then incubated with monoclonal anti-γ-tubulin antibody (Sigma-Aldrich, Taufkirchen, Germany; clone DM1A, 1:400 dilution) or a monoclonal anti-γ-tubulin antibody (Sigma-Aldrich; clone GTU88, dilution 1:400) followed by Cy3-conjugated anti-mouse-IgG secondary antibody (Molecular Probes, Leiden, The Netherlands; 1:1000 dilution). For staining of nuclei, mounting medium with DAPI (Vector Laboratories, Burlingame, CA) was used.

**RESULTS**

**Cloning of kipA**

We analyzed the genome of A. nidulans in the databases at Cereon Genomics (Cambridge, MA) and at the Whitehead Institute (http://www-genome.wi.mit.edu/annotation/fungi/aspergillus/) for kinesin-like proteins and used the nucleotide domain of A. nidulans conventional kinesin to identify 11 members of the kinesin family (Rischitor et al., 2004). In the studies reported here, we have characterized one of these proteins, KipA. The sequence of kipA was confirmed by sequencing 3.9 kb from a kipA-containing cosmid.
The rates of colony and hyphal extension of the mutants showed no difference from those of wild type (for hyphal extension, 3.8 ± 0.9 μm/min). We also did not detect any differences with respect to asexual or sexual sporulation. However, the maintenance of growth directionality was altered. Whereas wild-type hyphae extend only at their tip and grow in an approximately straight line, the mutants displayed meandering and curved hyphae (Figure 2A and B). The branching pattern was not significantly different from that of wild type. This morphological phenotype cosegregated with the knockout marker in crosses between ΔkipA and wild-type strains (our unpublished data). Because mutation of the KipA-related protein Kip2p in *S. cerevisiae* causes a misdistribution of nuclei during budding, we tested for a role of KipA in organelle movement in *A. nidulans*. Nuclei and mitochondria were visualized by GFP staining (strains SRS27, SRS29, SSK61, and SSK69) and analyzed by time-lapse videomicroscopy as described previously (Suelmann et al., 1997; Suelmann and Fischer, 2000). We could not find any difference with respect to nuclear migration (speed and directionality) or positioning. The mitochondrial network also displayed a wild-type appearance (our unpublished data). Vesicle transportation toward the hyphal tip should also not be reduced, because hyphal growth speed was not affected. Hence, the maintenance of growth directionality seems to be the main role of KipA. Another polarity phenotype became apparent when we analyzed spore germination. After the first germ tube from an *A. nidulans* conidium has reached a certain length, a second hypha emerges from the spore. In wild-type, the origin of the second hypha seems to be determined by the position of the original germ tube; thus, it emerged from the side of the spore opposite to the germ tube (bipolar) in 76% of the spores, whereas other positions were found only 24% of the time. In contrast, the second hypha emerged in seemingly random positions in 84% of the spores in the ΔkipA mutant (Figure 2D).

Determination of growth directionality in filamentous fungi involves an organelle in the center of the apical dome...
of the hyphae. This organelle, named the “vesicle supply center” or SPK, provides, for example, enzymes for cell wall biosynthesis. When the SPK moves away from the center of the cell, growth occurs in the direction of the SPK location (Figure 3A). We determined the position of the SPK by phase contrast microscopy in wild type and the ΔkipA mutant (Figure 3, B and C). Its average distance from the central axis was ~2.5-fold greater in the mutant, although the diameters of the hyphae were equivalent in mutant and wild type (2.1 ± 0.8 μm). In addition to its altered positioning, the SPK typically seemed smaller in the mutant. Because the rate of hyphal tip extension was not altered in the mutant, we presume that vesicles are simply more dispersed in the tip region.

Figure 3. Mispositioning of the SPK in a kipA mutant. (A) Correlation between SPK position and growth direction. Top, noncentral SPK (arrow) in a hypha of ΔkipA mutant strain SSK44. Bottom, 5 min later, growth has occurred in the direction of the noncentral SPK. (B) Determination of SPK position in wild-type (top, RMS011) and ΔkipA mutant (bottom, SSK44) hyphae. The center of the tip and central axis of the hypha were defined as indicated by the lines, and SPK position was determined by measuring the distance from the center of the SPK to the central axis and expressing the value as a percentage of the radius of the hypha (i.e., the distance from the central axis to the outer edge of the hypha at the position indicated by the line perpendicular to the central axis). (C, left) Examples of tracks of SPKs in wild-type (RMS011) and ΔkipA (SSK44) strains. The shaded box represents (top to bottom) half the diameter of the SPK. (C, right) Summary of data for 129 measurements (17 germlings) on strain RMS011 and 121 measurements (28 germlings) on strain SSK44. The average (shaded box) and maximum (white box) distances of the SPK from the central axis are indicated. Note that, in some cases, the SPKs moved considerably further from the central axis than seen in the typical tracks shown on the left.

Figure 4. Comparison of growth and benomyl sensitivity of wild-type and mutant strains. (A) Wild-type (RMS011) and ΔkipA (SSK44) conidia were spotted at the centers of agar plates containing different concentrations of benomyl. Colony diameters were measured after 2 d at 37°C, and the value for wild type at that concentration was used as 100% (10 colonies per strain for each concentration). (B) Wild-type and mutant strains were grown for 2 d at 30°C, and the diameters of 25 colonies per strain were measured. The strains used were RMS011, SSK44, SNR3, SSK72, SPR36, SSK73, XX3, and SSK80 (see Table 1).

Stabilization of MTs by KipA and Functional Interactions with Other Motor Proteins

Recently, it was shown that addition of low amounts of the MT-destabilizing drug benomyl caused curved growth in A. nidulans and thus that growth directionality depends on the integrity of the MT cytoskeleton (Riquelme et al., 2003). To begin investigating the potential effect of KipA on MT dynamics, we studied the sensitivity of a ΔkipA strain toward benomyl. The mutant was slightly more sensitive than wild type, suggesting a MT-stabilizing effect of KipA (Figure 4A). Because MT stability also is affected by mutations of other motor proteins, we studied genetic interaction between ΔkipA and these mutations by comparing growth at 30°C. A double mutant between conventional kinesin, ΔkinA, and ΔkipA (strain SSK72) revealed an additive phenotype in which the colonies displayed a compact appearance like ΔkinA mutants (Figure 4B) and curved hyphal growth like the ΔkipA mutant (our unpublished data). Deletion of kinA stabilizes MTs (as judged by benomyl resistance) and the additional deletion of ΔkipA did not detectably change this effect (our unpublished data). Another MT-destabilizing kinesin is the Kip3-family kinesin KipB (Rischitor et al., 2004). The ΔkipA, ΔkipB strain (SSK28) grew ~50% slower than the wild-type, although each mutation alone had no effect on hyphal extension, suggesting partially redundant functions of the two motors (Figure 4B). Benomyl resistance was not reduced in comparison with the ΔkipB strain (our unpublished data). A triple mutant, ΔkinA, ΔkipA, ΔkipB (strain SSK73) was still viable (our unpublished data). We also tested for genetic interaction between ΔkipA and a temperature-sensitive dynein mutation (nudA1). Deletion of kipA partially suppressed the growth defect of the dynein mutant
with a speed of 8–12 μm/min toward the tip (Figure 5B and Movie 2). To confirm the apparent MT association, we colocalized GFP-KipA and MTs using a red fluorescent protein (mRFP1)-KipB motor domain fusion protein, which efficiently decorates all populations of MTs (Rischitor et al., 2004). We found GFP-KipA spots at the ends of MT fibers, suggesting that KipA is a plus-end–associated protein (Figure 5C, top). In addition, we observed GFP-KipA spots at places where the MT fluorescence signal changed (Figure 5C), suggesting that the MT tracks contain overlapping MT bundles.

The expression level of GFP-KipA was increased, we observed a comet-like staining pattern. The length of the comets increased with increasing protein concentrations until the entire MT was stained (Figure 6A). To determine whether the comets were the result of one-dimensional diffusion of the protein from the plus end, we deenergized the cells by addition of 100 μM CCCP (carbonylcyanide-m-chlorophenylhydrazone) and thus presumably transformed KipA into a rigor state. After 30-min incubation, MT plus ends were still visible, but in addition filaments were rather evenly decorated, and a GFP-KipA concentration gradient toward the plus end was not obvious (Figure 6B). Thus, the comet-like appearance can be explained by hypothesizing that the motor actively moves along MTs.

To test further whether an intrinsic KipA motor activity is required for its MT plus-end localization, we created a KipA mutant version in which glycine 223 was changed to glutamate (G223E). This amino acid change is located in the predicted ATP-binding domain (P-loop) and has been

KipA Moves along MTs and Accumulates at Their Plus Ends

To examine KipA localization and movement, we created strains expressing a GFP-KipA fusion protein under control of the regulatable alcA promoter (see Materials and Methods). In strains in which GFP-KipA is the only source of KipA, growth under repressing conditions resulted in the \( \text{kipA} \) mutant phenotype, whereas wild-type hyphal growth was observed under inducing conditions, showing that the GFP-KipA fusion protein is biologically active (Figure 2C). In interphase cells, we observed a spotted distribution of the protein in the cytoplasm and labeling of the septa during growth under derepressing conditions (Figure 5A). Time-lapse analyses revealed that the spots were moving rapidly within the cell. In the hyphal tip, GFP spots were moving with a speed of 8–12 μm/min toward the tip (Figure 5B and Movie 1). When the protein arrived at the tip, the GFP fluorescence intensity decreased rapidly. The speed and direction of spot movement were similar to the dynamics of growing MTs (Figure 5B and Movie 2). To confirm the apparent MT association, we colocalized GFP-KipA and MTs using a red fluorescent protein (mRFP1)-KipB motor domain fusion protein, which efficiently decorates all populations of MTs (Rischitor et al., 2004). We found GFP-KipA spots at the ends of MT fibers, suggesting that KipA is a plus-end–associated protein (Figure 5C, top). In addition, we observed GFP-KipA spots at places where the MT fluorescence signal changed (Figure 5C), suggesting that the MT tracks contain overlapping MT bundles.

When the expression level of GFP-KipA was increased, we observed a comet-like staining pattern. The length of the comets increased with increasing protein concentrations until the entire MT was stained (Figure 6A). To determine whether the comets were the result of one-dimensional diffusion of the protein from the plus end, we deenergized the cells by addition of 100 μM CCCP (carbonylcyanide-m-chlorophenylhydrazone) and thus presumably transformed KipA into a rigor state. After 30-min incubation, MT plus ends were still visible, but in addition filaments were rather evenly decorated, and a GFP-KipA concentration gradient toward the plus end was not obvious (Figure 6B). Thus, the comet-like appearance can be explained by hypothesizing that the motor actively moves along MTs.

To test further whether an intrinsic KipA motor activity is required for its MT plus-end localization, we created a KipA mutant version in which glycine 223 was changed to glutamate (G223E). This amino acid change is located in the predicted ATP-binding domain (P-loop) and has been
shown to transform kinesin motor proteins into a “rigor” state (Nakata and Hirokawa, 1995; Browning et al., 2003). In strain SSK114, GFP-KipAG223E was the only source of KipA. Under both inducing (threonine) and repressing (glucose) conditions, we observed the kipA mutant phenotype (our unpublished data), and a GFP signal was detectable during growth either on threonine or under derepressing (glycerol) conditions. During growth on glycerol, MTs were evenly decorated, and no accumulation of the protein at the MT plus ends was detectable (Figure 6C, right). As a control, we analyzed strain SSK116, in which the plasmid had integrated ectopically. Induction of the promoter produced cytoplasmic GFP staining (Figure 6C, left), as expected given that the GFP-fusion protein contains only a truncated motor domain that lacks the MT binding regions.

In mitotic cells, GFP-KipA localized to the spindle pole bodies and the midzone of the spindle (Figure 7), as well as to the plus ends of astral MTs (our unpublished data).

**Dispersion of MTs in the Hyphal Tips of ΔkipA Mutants**

To study the behavior of MTs in detail, we compared wild-type and ΔkipA strains expressing GFP-labeled α-tubulin. We found no difference in the number and length of MTs in the cytoplasm in the two strains, and, in both cases, the MTs showed dynamic behavior and reached the tip cortex. In the mutant, the MTs paused at the cortex for an average of 7.8 ± 3.4 s, with a maximum of 15 s (43 MTs in 18 germlings), in comparison with an average of 13.5 ± 5.5 s, with a maximum of 27 s, in the wild-type (44 MTs in 16 germlings). In addition to the different times of pausing, we found a difference with respect to the arrangement of MTs in the apical dome of the tip. In wild-type cells MTs converged in the center of the tip (Figure 8A and Movie 2). This phenomenon was also evident when we examined the movement of GFP-KipA, in that the GFP signal could be tracked until it reached the center of the hyphal tip (Figure 8B). We also found that the tracks along which GFP-KipA moved were used several times (Figure 8, B and C, and Movie 3). In comparison, MTs in the ΔkipA strain did not always converge to a single point. Instead, they diverged at the tip and ended at different places in the cortex (Figure 8, D and E, and Movie 4). Another difference was observed when we analyzed the MTs after they reached the center of the tip. In wild-type cells, MTs typically remained at their position until a catastrophic event caused depolymerization. In contrast, in the ΔkipA mutant, MTs frequently changed their positions even after they reached the cortex (Figure 8, D and E, and Movie 5). These results suggest that there is a MT-cortex interaction that depends on plus-end-localized KipA.

**Microtubule Organization in A. nidulans**

MT plus-end–associated proteins have been used in various organisms to unravel the polarity and organization of the MT cytoskeleton (Straube et al., 2003; Piehl et al., 2004). Because KipA seems to be an effective marker protein for MT plus ends in A. nidulans, we used it as a tool to study MT organization and the number and location of MT-organizing centers (MTOCs). We monitored GFP-KipA traces in growing hyphal tips and in subapical compartments of interphase cells and determined the numbers of MTs growing toward the tip or oriented with their plus ends in the opposite direction (Movies 6–8). In the tip regions of 42 apical compartments, we found 285 tip-directed MTs (Figure 8B) and seven that polymerized away from the tips (Figure 10A, top).
diagram, and Movie 1). In contrast, in the area behind the tip region and in subapical compartments, the numbers of retrograde- and anterograde-growing MTs were equal (Figure 10A, top diagram). To determine whether the spindle pole bodies at the nuclei served as the only MTOCs in the cytoplasm, as in *S. cerevisiae*, we labeled nuclei with a DsRed fusion protein in a strain that also expressed GFP-KipA (SSK99). We defined an MTOC as a point from which several GFP spots originated and moved in different directions. In 42 hyphae, 118 MTs were analyzed. In young, unseptated germlings, ~50% of the MTs originated from the nucleus and ~50% from apparent MTOCs in the cytoplasm, which were often localized close to a nucleus. In some cases, an MTOC was located between the leading nucleus and the hyphal tip. In older germlings, ~40% of the MTs emanated from septa (Figure 9, A and B, and Movies 6–8). We did not observe MTs passing through the septa. MTs do not continue through the septa (C). γ-Tubulin signals are visible at the nucleus and at the septum (D).

**DISCUSSION**

Polarized growth in filamentous fungi is a fascinating process that also provides a model for the cell biology of other highly polarized cells such as neurons in higher eukaryotes. An understanding of fungal cell extension also may help to control fungal diseases of plants and animals as well as improve the use of filamentous fungi in biotechnology. In this study, we analyzed MT organization and characterized the KipA kinesin motor protein in the filamentous fungus *A. nidulans*, finding an involvement of KipA in polarized growth. We found that KipA localizes to MT plus ends, and we propose that it determines growth directionality through the control of MT cortex interactions.

**Multiple MTOCs Organize the MT Cytoskeleton in *A. nidulans***

We used GFP-KipA as a MT plus-end marker to determine the polarity and dynamic behavior of MTs in *A. nidulans*. In an earlier study, Han et al. (2001) used a strain with GFP-labeled MTs to address the polarity of MTs. They found that at the tip of the hypha, MTs are oriented almost exclusively with their plus ends toward the cortex. We confirmed this result and also observed that MTs converge in the hyphal tip and seem fixed at a central point until a catastrophe event. In addition, we noticed that several GFP-KipA signals seemed
to use the same track in the cell. This can be explained if we assume that the MT fibers as visualized are actually bundles of several MTs with individual dynamics. A catastrophe event does not lead to complete disassembly of the entire bundle but only of some MTs. New MTs can then grow along the remaining MTs.

It is well accepted that in fungi, MTs originate from the spindle pole body, a typical fungal organelle. However, it was also found in S. pombe that several MTOCs exist in the vicinity of the nucleus in interphase cells (Tran et al., 2001). Similarly, Straube et al. (2003) found in the single-cell stage of the basidiomycete U. maydis that MT formation originated from the SPB, the bud neck, and from cytoplasmic sites. The use of the different MTOCs was cell-cycle regulated. Likewise, in N. crassa, Minke et al. (1999) proposed the existence of two populations of MTs, only one of which was connected to the nuclei. Here, we analyzed for the first time the locations of MTOCs that were not connected to the nuclei in a filamentous fungus. We found MTOCs in the cytoplasm, some, but not all, of which were close to nuclei. In addition, very active MTOCs were detected at the septa. These filamentous fungus. We found MTOCs in the cytoplasm, of two populations of MTs, only one of which was connected to the nuclei. Here, we analyzed for the first time the locations of MTOCs that were not connected to the nuclei in a filamentous fungus. We found MTOCs in the cytoplasm, some, but not all, of which were close to nuclei. In addition, very active MTOCs were detected at the septa. These MTs reached the growing MT plus end.

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