Role of the spindle-pole-body protein ApsB and the cortex protein ApsA in microtubule organization and nuclear migration in *Aspergillus nidulans*

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Summary

Nuclear migration and positioning in *Aspergillus nidulans* depend on microtubules, the microtubule-dependent motor protein dynein, and auxiliary proteins, two of which are ApsA and ApsB. In *apsA* and *apsB* mutants nuclei are clustered and show various kinds of nuclear navigation defects, although nuclear migration itself is still possible. We studied the role of several components involved in nuclear migration through in vivo fluorescence microscopy using fluorescent-protein tagging. Because ApsA localizes to the cell cortex and mitotic spindles were immobile in *apsA* mutants, we suggest that astral microtubule-cortex interactions are necessary for oscillation and movement of mitotic spindles along hyphae, but not for post-mitotic nuclear migration. Mutation of *apsA* resulted in longer and curved microtubules and displayed synthetic lethality in combination with the conventional kinesin mutation *ΔkinA*. By contrast, ApsB localized to spindle-pole bodies (the fungal centrosome), to septa and to spots moving rapidly along microtubules. The number of cytoplasmic microtubules was reduced in *apsB* mutants in comparison to the wild type, indicating that cytoplasmic microtubule nucleation was affected, whereas mitotic spindle formation appeared normal. Mutation of *apsB* suppressed dynein null mutants, whereas *apsA* mutation had no effect. We suggest that nuclear positioning defects in the *apsA* and *apsB* mutants are due to different effects on microtbule organisation. A model of spindle-pole body led nuclear migration and the roles of dynein and microtubules are discussed.

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Key words: Spindle pole body, Nuclear movement, Dynein, Kinesin, Microtubules, MTOC

Introduction

Nuclear movement and positioning of the mitotic spindle is important in eukaryotic cells. In animal cells, the position of the mitotic spindle defines the cleavage plane and becomes critical when division has to occur asymmetrically (Ahringer, 2003). In plant root hairs, which elongate at the tip, nuclei move as the cells extend and thus keep a constant distance to the cortex (Ketelaar et al., 2002). Nuclear migration is also important in single cell organisms such as *Saccharomyces cerevisiae* or *Schizosaccharomyces pombe* (Adams and Cooper, 2000; Hoepfner et al., 2000; Tran et al., 2001; see movies within). In *S. cerevisiae* the nucleus moves within the mother cell by being pushed through growing and shrinking microtubules (MTs). After bud formation starts, the nucleus migrates towards the budding neck and astral MTs emanate from the spindle pole body (SPB) and sweep the cell cortex of the growing daughter bud (Adams and Cooper, 2000; Hoepfner et al., 2000; Miller et al., 1999; Shaw et al., 1998; Shaw et al., 1997; Yeh et al., 2000). They become attached to the cortex and pulling forces move the spindle into the budding neck and later one nucleus into the bud. Several proteins are essential for this interaction, among which are Kar9p, Num1p, Bud6p, Bud1p and dynein (Bloom, 2000; Bloom, 2001; Farkasovsky and Küntzel, 2001; Gundersen and Bretscher, 2003; Maekawa et al., 2003; Miller et al., 1999; Miller and Rose, 1998; Segal et al., 2002). In addition to dynein, MTs and cortical proteins, at least three kinesins, Kip2p, Kip3p and Kar3p, appear to play a role in nuclear migration in yeast (Cottingham et al., 1999; Cottingham and Hoyt, 1997; Miller et al., 1998).

Whereas *S. cerevisiae* mutants with defects in components of the nuclear distribution machinery do not display a severe growth defect, nuclear migration is essential in filamentous fungi to distribute nuclei within the extremely polarized cells (Morris, 1976; Suelmann et al., 1997). Nuclear migration is best studied in *Aspergillus nidulans* where the molecular analysis had its basis in a mutant screening (Fischer, 1999; Morris, 1976; Morris et al., 1995; Xiang and Morris, 1999). R. Morris isolated several temperature sensitive mutants (*nud*=nuclear distribution), in which nuclei did not migrate out of the conidiospores at restrictive temperature although mitosis and polar hyphal extension were not affected early after germination (Morris, 1976). Cloning of the corresponding genes as well as analysis of mutants isolated in later screenings revealed several subunits of the dynein protein complex as well

as regulatory components (Efimov, 2003; Efimov and Morris, 2000; Osmani et al., 1990; Xiang et al., 1994; Xiang and Fischer, 2004). The components are evolutionarily conserved and important in higher eukaryotes (Morris et al., 1998a; Morris et al., 1998b; Xiang et al., 1995a). For instance, malfunction of the human homolog of nudF, Lis1, causes severe brain defects and patients have only a short life expectation. However, to understand the phenomenon of nuclear distribution in *A. nidulans*, the subcellular arrangement and the interaction of the two main players, the microtubule cytoskeleton and the dynein motor protein need to be considered. Recently, MTs were visualized with GFP and their dynamics was studied (Han et al., 2001). It was found that in a growing hyphal tip MTs are generally oriented with the plus end towards the tip. Their inherent dynamic instability causes a change between elongation periods and shrinkage. However, it remains unclear how these dynamics could contribute to nuclear distribution. With regards to the motor protein dynein, it is also not clear yet how the motor can move nuclei. In early attempts, the motor was identified by immunolocalization at hyphal tips (Xiang et al., 1995b). If it were fixed there, it could attach to the MTs, which reach the cortex and subsequently start moving along them. This would cause a pulling of the MTs and could lead to a movement of connected nuclei. This model resembles the proposed mechanism of nuclear translocation in *S. cerevisiae* (see above). However, nuclei in the tip compartment of *A. nidulans* move with different velocities and stop moving at different times (Suelmann et al., 1997). It is difficult to imagine that the individual motor molecules at the tip are regulated differently while being close to each other. In addition, MT bending along the cortex of the tip or a shortening of the filaments is not detectable (our unpublished data) and (Han et al., 2001). Recently, dynein and other components of the machinery were in addition found at the growing plus end of MTs (Morris, 2003; Xiang et al., 2000; Zhang et al., 2002; Zhang et al., 2003). Analyses of the dynein distribution pattern in a conventional kinesin mutant of *A. nidulans* suggested that this motor is responsible for plus end localization (Zhang et al., 2003). The localization pattern of dynein is in agreement with the pattern in *S. cerevisiae.* In this organism, tip localization delivers the motor at the cortex and ensures the contact between the growing MT end and protein complexes in the membrane (Sheeman et al., 2003). However, for the reasons discussed above it seems unlikely that in *A. nidulans* the dynein at MT plus ends is directly involved in nuclear migration, although it could effect nuclear migration indirectly by effecting MT dynamics.

In addition to 'core'-nuclear migration components, two other *A. nidulans* genes, *apsA* and *apsB* were identified by mutagenesis and found to control nuclear positioning (Clutterbuck, 1994). The *apsA* gene encodes a 186 kDa coiled-coil protein with similarity to the cortical Num1 protein of *S. cerevisiae,* whereas ApsB is a 121 kDa coiled-coil protein and was originally localized as spots in the cytoplasm (Suelmann et al., 1998). Both *aps* mutants were long known to have nuclear migration defects (see supplementary material, Movies 1-5), but the reason was unclear (Fischer and Timberlake, 1995; Suelmann et al., 1997; Suelmann et al., 1998). In this paper, we show that the *apsA* and the *apsB* mutations have effects on the MT cytoskeleton and describe ApsB as a novel spindle-pole body associated protein. ApsB appears to be involved in MT production mainly from non-spindle-pole body centrosomes.

Materials and Methods

Strains, plasmids and culture conditions

Supplemented minimal (MM) and complete media (CM) for *A. nidulans* and standard strain construction procedures were used, as described by (Hill and Käfer, 2001). 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′) were used. Plasmids are listed in Table 2 or described below.

Molecular techniques

Standard DNA transformation procedures were used for *A. nidulans* (Yelton et al., 1984) and *E. coli* (Sambrook and Russel, 1999). For PCR experiments, standard protocols were applied using a capillary Rapid Cycler (Idaho Technology, Idaho Falls, ID, USA) for the reaction cycles. Genomic DNA was extracted from *A. nidulans* with the DNeasy Plant Mini Kit (Qiagen, Hilden, Germany). Southern blotting was performed according to published protocols (Sambrook and Russel, 1999). DNA sequencing was done commercially (MWG, Ebersberg, Germany).

N-terminal tagging of *apsB*

To facilitate making N-terminal GFP fusions, the terminator codon of the gfp2-5 gene in plasmid pMCB17 (Fernandez-Abalos et al., 1998) and the subsequent sequence up to the *Bam*HI site were replaced with the sequence GGCGCGCCGGCTTAATTAA containing *Asc*I and *Pac*I recognition sites. The *Xba*I insert in the resulting plasmid (pMCB17ap) was removed to produce plasmid pMCB17apx. The first 1469 bp of the *apsB* gene were amplifed by PCR with genomic DNA using primers 5′-TTTGGCGCGCCCGGCATGACTCTAAAAGAG-CAAAGTAGTACG-3′ and 5′-GGGTTAATTAAGCTCTTCTCCA-AAGATTCCATCTCTTC-3′. The PCR product was cut with *Asc*I and *Pac*I and cloned at the *Asc*I-*Pac*I sites of pMCB17apx to give p17apx-apsB. The PCR-derived regions of p17apx-apsB were confirmed by sequencing. GR5 strain was transformed with supercoiled p17apx-apsB. A single homologous integration of p17apx-apsB at the *apsB* locus was confirmed by PCRs and Southern blottings for five independent *pyr4*+ transformants. These strains express GFP2-5 protein sequence followed by Gly-Ala-Pro-Gly sequence and complete ApsB protein sequence. All five transformants displayed wild-type phenotypes on non-repressing medium (minimal glycerol), and *apsB*-like phenotypes (reduced conidiation and colony size) on repressing medium (minimal glucose). For co-localization experiments *apsB* was also tagged with mRFP1. mRFP1 was PCR-amplified from pDM2 using primers 5′-CGGTACCATGGC-CTCCTCCGAGG-3′ (including *Kpn*I restriction side) and 5′-CGGCGCGCCGGCGCCGGTGGAG-3′ (including *Asc*I restriction side). The PCR fragment was cloned into pCR2.1-Topo to give pDM6. Subsequently mRFP1 was cut out of pDM6 with *Kpn*I and *Asc*I and ligated into p17apx-apsB, which was linearised with *Kpn*I and *Asc*I (GFP was cut out) to give the final plasmid pDM8.

C-terminal tagging of *apsB*

The *apsB* gene and GFP were fused as described in (Suelmann et al., 1997) to give pRS48. The *gpd*-promoter was released from pJH19 with *Bam*HI and ligated into pRS48 after linearization with *Bam*HI. This resulted in the final plasmid pDM5.

Table 1. *A. nidulans* strains used in this study

Strain	Genotype[a,b]	Source
AJC1.1	*biA1; apsA1*	Clutterbuck, 1969
AJC1.5	*biA1; apsB6*	Clutterbuck, 1969
AJC1.7	*biA1; apsB10*	Clutterbuck, 1969
AJC1.8	*biA1; apsB14*	Clutterbuck, 1969
apsA5	*pyrG89, yA2; apsA5*	Efimov, 2003
apsA5/ΔA-6	apsA5 crossed to XX60 *ΔnudA::pyrG; pyrG89; apsA5*	This work
apsB14	apsB14-2 crossed to ΔF54 *apsB14*	This work
apsB14-2	AJC1.8 crossed to GR5 *pyrG89; apsB14*	Efimov, 2003
apsB14-3	AJC1.8 crossed to GR5 *pyrG89; wA3; apsB14*	This work
apsB14/ΔA-7	apsB14-3 crossed to XX60 *ΔnudA::pyrG; pyrG89;wA3; apsB14*	This work
apsB14/ΔA-9	apsB14-3 crossed to XX60 *ΔnudA::pyrG; pyrG89; apsB14*	This work
apsB14/ΔF-3	apsB14-2 crossed to ΔF54 *ΔnudF::pyr4; pyrG89; wA3*	This work
apsB14/ΔF-6	apsB14-2 crossed to ΔF54 *ΔnudF::pyr4; pyrG89*	This work
apsB14/ΔF-9	apsB14-2 crossed to ΔF54 *ΔnudF::pyr4; pyrG89; apsB14*	This work
apsB14/ΔF-11	apsB14-2 crossed to ΔF54 *ΔnudF::pyr4; pyrG89; wA3; apsB14*	This work
GR5	*pyrG89*; *wA3*; *pyroA4*	Waring et al., 1989
RMS011	*pabaA1*, *yA2*; *ΔargB::trpCΔB*; *trpC801*	Stringer et al., 1991
SDM23	SSN18 crossed to SNR3 *ΔapsA::pyr4; alcA(p)::apsA::GFP; ΔkinA::pyr4; yA2; wA3; pyroA4* (ΔapsA+ΔkinA+ApsA-GFP)	This work
SDM24	SSN18 crossed to SNR3 *ΔapsA::pyr4; ΔkinA::pyr4; yA2; wA3; pyroA4* (ΔapsA+ΔkinA)	This work
SDM40	SJW02 transformed with pDM5 and pRF280 *alcA(p)::GFP::tubA*; *gpd::apsB::GFP; gpd(p)::stuA(NLS)::GFP; wA3*; *pyroA4* (GFP-MTs+ApsB-GFP+Nuclei-GFP)	This work
SDM64	SNR3 crossed to SDM40 *ΔkinA::pyr4; gpd(p)::apsB::GFP; gpd(p)::stuA(NLS)*::GFP; *alcA(p)::GFP::tubA* (ΔkinA+ApsB-GFP+Nuclei-GFP+GFP-MTs)	This work
SDM92	XX60 crossed to SDM40 *ΔnudA::pyrG; gpd(p)::apsB::GFP; pyrG89; wA3* (ΔnudA+ApsB-GFP)	This work
SDM1000	RMS011 transformed with pJH19 and pRS50 *alcA(p)::apsB::GFP; gpd(p)::stuA(NLS)::DsRedT4; pabaA1*, *yA2* (ApsB-GFP+Nuclei-DsRed)	This work
SDV1B	SJW02 transformed with pDM8 and pDC1 *alcA(p)::GFP::tubA*; *alcA(p)::mRFP1::apsB; wA3*; *ΔargB::trpCΔB; pyroA4* (GFP-MTs+mRFP1-ApsB)	This work
SDV12	AJC1.1 crossed to SRS27 *gpd(p)::stuA(NLS)*::GFP; *apsA1* (apsA1+Nuclei-GFP)	This work
SDV16	AJC1.7 crossed to SJW02 *alcA(p)::GFP::tubA*; *apsB10* (apsB10+GFP-MTs)	This work
SDV19	SSK44 crossed to SEa3 *ΔkipA::pyr4*; *alcA(p)::GFP::apsB; wA3* (ΔkipA+GFP-ApsB)	This work
SDV24	AJC1.7 crossed to SSK92 *alcA(p)::GFP::kipA; wA3; apsB10* (apsB10+GFP-KipA)	This work
SDV25	AJC1.5 crossed to SSK92 *alcA(p)::GFP::kipA; wA3; apsB6* (apsB6+GFP-KipA)	This work
SDV26	XX3 crossed to SDM40 *alcA(p)::GFP::tubA; nudA1; wA3* (nudA^{ts} + GFP-MTs)	This work
SDV31	AJC1.1 crossed to SEa3 *alcA(p)::GFP::apsB; apsA1* (apsA1+GFP-ApsB)	This work
SEa3	GFP-apsB strain: GR5 transformed with p17apx-apsB *pyr4::alcA(p)::gfp2-5::apsB; pyrG89*; *wA3*; *pyroA*	This work
SEwild-type	apsB14-2 crossed to ΔF54, wild type selected	This work
SJW02	*alcA(p)::GFP::tubA; ΔargB::trpCΔB; wA3*; *pyroA4* (GFP-MTs)	J. Warmbold, Marburg, Germany
SJW100	SJW02 transformed with pJW18 *alcA(p)::GFP::tubA*; *gpd(p)::stuA (NLS)::DsRedT4; wA3*; *pyroA4* (GFP-MTs + Nuclei-DsRed)	J. Warmbold, Marburg, Germany
SNR3	*ΔkinA::pyr4; pyrG89*, *yA2*; *ΔargB::trpCΔB*	Requena et al., 2001
SNS9	SRF54 crossed to SPR30 *alcA(p)::GFP::tubA; ΔapsA::pyr4; biA1; pabaA1; wA1;* (ΔapsA+GFP-MTs)	This work
SPR30	*alcA(p)::GFP::tubA; ΔkipB::argB; pabaA1; wA1*	Rischitor et al., 2004
SPR50	RMS011 transformed to pRS31 and pDC1 *gpd(p)::stuA(NLS)::GFP; pabaA1*, *yA2*; *ΔargB::trpCΔB*; *trpC801* (Nuclei-GFP)	Rischitor et al., 2004
SRF30	*ΔapsA::pyr4; ΔargB::trpCΔB; pabaA1, yA2; wA3; pyroA4*	Fischer and Timberlake, 1995
SRF54	*biA1; ΔapsA::pyr4*	Suelmann et al., 1997
SRS11	*gpd(p)::stuA(NLS)::GFP; pyrG89; wA3; pyroA4; apsB6* (apsB6+Nuclei-GFP)	R. Suelman, Marburg, Germany
SRS27	*gpd(p)::stuA(NLS)::GFP; pyrG89; ΔargB::trpCΔB; pyroA4;* transformed with pDC1 and pRS31 (Nuclei-GFP)	Suelmann et al., 1998
SSK44	*ΔargB::trpCΔB*; *ΔkipA::pyr4*; *pabaA1*; *wA3*	Konzack et al., 2005
SSK92	*alcA(p)::GFP::kipA; wA3*; *pyroA4* (homologous intergration of GFP-KipA)	Konzack et al., 2005
SSN18	*alcA(p)::apsA::GFP; ΔargB::trpCΔB ΔapsA::pyr4; pabaA1, yA2; wA3; pyroA4*	Suelmann et al., 1997
XX3	*pyrG89; nudA1, chaA1* (nudA^{ts})	Xiang et al., 1994
XX60	*ΔnudA::pyrG; pyrG89*	Xiang et al., 1995b
ΔF54	*ΔnudF::pyr4; pyrG89; pyroA4; wA3*	Willins et al., 1995

[a]Important characteristics of the strains are indicated in brackets. [b]All strains carry the *veA1* mutation.

Light and fluorescence microscopy

For live-cell imaging, cells were grown in glass-bottom dishes (World Precision Instruments, Berlin, Germany) in 4 ml of minimal medium containing either 2% glycerol or 2% glucose as carbon source. Medium was supplemented with pyridoxine, p-aminobenzoic acid, biotin, arginine, uracil or uridine depending on auxotrophy of the strains. Cells were incubated at room temperature for 1-2 days and images were captured using an Axiophot microscope (Zeiss, Jena, Germany), a

Table 2. Plasmids used in this study

Plasmids	Description	Source
pCR2.1-TOPO	TA-cloning vector (for cloning of PCR fragments)	Invitrogen, NV Leek, The Netherlands
pDC1	*A. nidulans argB* selectable marker plasmid	Aramayo et al., 1989
pDM2	mRFP1 with *Not*I in pCR2.1-Topo	This work
pDM5	*gpd(p)::apsB::GFP* in pBluescript KS–	This work
pDM6	*Kpn*I-*Asc*I fragment of mRFP1 cloned into pCR2.1-Topo	This work
pDM8	GFP replaced by mRFP1 in pMCB17apx-apsB	This work
pJH19	*gpd(p)*::*stuA(NLS)*::*DsRedT4* and *argB* as selectable marker in pBluescript KS–	Toews et al., 2004
pJW18	*alcA(p)*::*stuA(NLS)*::*DsRedT4* and *argB* as selectable marker in pBluescript KS–	Toews et al., 2004
pMCB17	GFP gene (*pyr4::alcA(p)::gfp2-5*) in pUC19	Fernandez-Abalos et al., 1998
pMCB17apx	pMCB17 version for fusion of GFP to N-termini of proteins of interest	V. P. Efimov, Piscataway, USA
p17apx-apsB	1.5 kb of *apsB* in PacI/AscI restriction site of pMCB17apx	V. P. Efimov, Piscataway, USA
pRF280	*gpd(p)*::*stuA(NLS)*::GFP*; argB* in pRS31	R. Fischer, Marburg, Germany
pRS31	*gpd(p)*::*stuA(NLS)*::*GFP* in pBluescript KS–	Suelmann et al., 1997
pRS48	*apsB* tagged with GFP in *Eco*RV in pBluescript KS–	Suelmann et al., 1997
pRS50	*alcA(p)::apsB::GFP* pBluescript KS–	R. Suelman, Marburg, Germany

Plan-apochromatic 63× or 100× oil immersion objective lens, and a HBO50 Hg lamp. Fluorescence was observed using standard Zeiss filter combinations No. 09 (FITC, GFP), No. 15 (DsRed) and No. 01 (DAPI). Images were collected and analyzed with a Hamamatsu Orca ER II camera system and the Wasabi software (version 1.2). Time-lapse series were obtained with an automated Wasabi program that acquires series of images with 2- or 3-second intervals, 0.1- or 0.75-second exposure time, and about 100 exposures in a sequence. Image and video processing were done with the Wasabi software from Hamamatsu, Adobe Photoshop, ImageJ (NIH, Bethesda, Maryland, USA), and virtual dub (http://www.virtualdub.org). For benomyl studies, the drug was added 15 minutes before observation at a final concentration of 1.5 μg ml^{-1} to germlings grown for 1-2 days at room temperature and observed for 3 hours.

Results

Mutation of *apsA* and *apsB* affect MT organization

To analyze the roles of *apsA* and *apsB* in microtubule (MT) organization, we compared corresponding mutant and wild-type strains with GFP labeled MTs in interphase and during mitosis. In wild type, MTs appear as long, straight but bendable filaments. There are usually about five MT filaments in a compartment, and each filament represents a single MT or consists of a bundle of several MTs. In *apsB* mutant cells, cytoplasmic MTs were reduced to one main MT filament

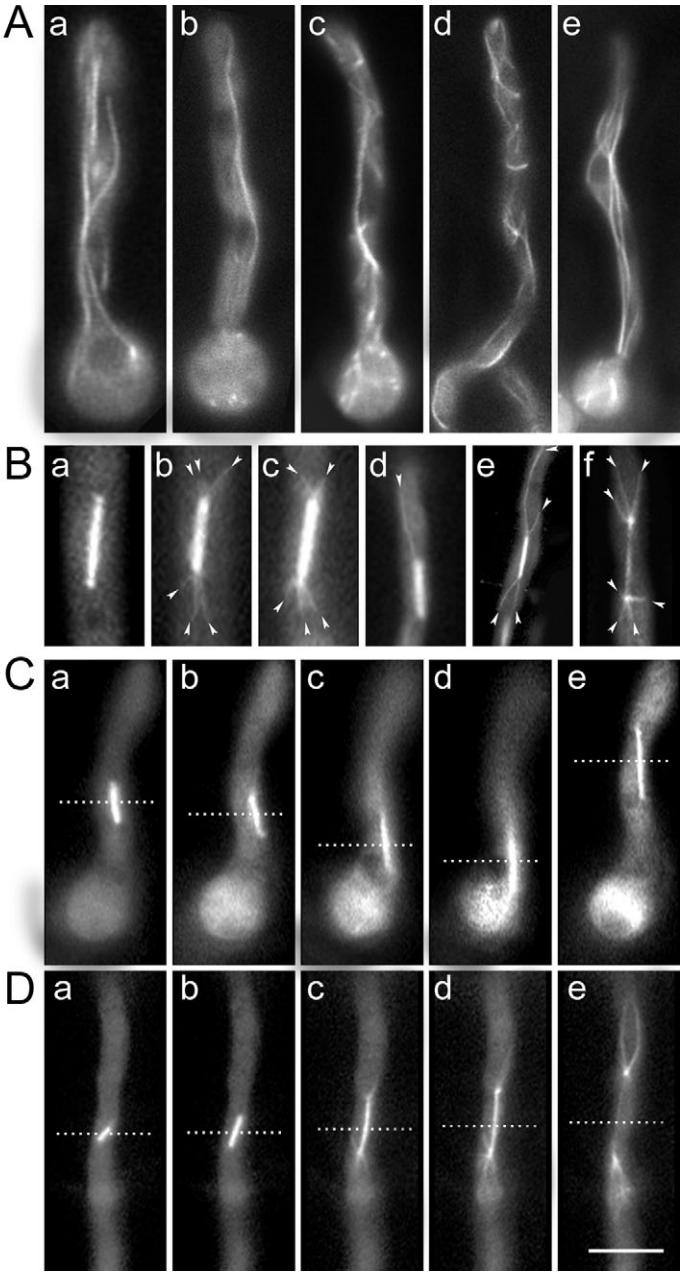

Fig. 1. Influence of *apsA* and *apsB* on cytoplasmic and mitotic MTs. (A) Compared with wild type (strain SJW02) (a), where more than three MTs can be observed at the same time, in *apsB10*-mutant cells (SDV16) (b) only one dominant cytoplasmic MT is apparent. By contrast, the number of cytoplasmic MTs in *ΔapsA* mutant cells (SNS9) (c) is increased and MTs are more curved and appear thinner. A similar phenotype was observed in a temperature-sensitive dynein mutant strain (SDV26) at restrictive temperature (42°C) (d), while MTs showed wild-type-like organization at permissive temperature (e). (B) Astral MTs (arrowheads) of the mitotic spindle are dramatically reduced in an *apsB10* strain (a,d), but not completely lost, in comparison with wild type (b,e). The difference is obvious in short and long spindles. The number of astral MTs was not reduced in *ΔapsA* strains (c,f). (C,D) Oscillation and movement of the mitotic spindle is common in wild-type strains (C,a-e), but was never observed in a *ΔapsA* strain (D,a-e). MTs were visualized with a GFP-TubA fusion protein. Dotted lines in (C,D) indicate spindle centers. Bar, 4 μm for (A), 2 μm for (B), 5 μm for (C and D) (see supplementary material Movies 6 and 7).

spanning the entire compartment. By contrast, this was not the case in *apsA* mutant strains, where MTs appeared curved and thinner and thus the number seemed higher. This curved and thinner appearance was similar to the MT organization in dynein mutants (Fig. 1A) (Xin Xiang, USU Center for Health Disparities, Bethesda, MA, personal communication). Studying mitosis, we observed a reduction of astral MTs in the case of *apsB* mutants to zero to one MT at each spindle-pole body (SPB) in comparison to up to six astrals at each pole of the spindle in wild type. Besides this, no significant change of the spindle morphology was observed (Fig. 1B). Time-lapse analyses of mitoses revealed that the duration of mitosis was unaltered in *apsB* mutants, but was twice as long in *apsA* mutants than in wild type. Whereas in wild type the spindle oscillates to a certain extent and sometimes moves along the hypha (Su et al., 2004), this movement was reduced in *apsB* and completely lost in *apsA* mutants (Fig. 1C,D) (see supplementary material, Movies 6 and 7).

ApsB is a novel spindle-pole body (SPB) associated protein

AspB was described as a cytoplasmic protein with a spot-like distribution when expressed at high levels under the control of the inducible *alcA*-promoter (Suelmann et al., 1998). We reinvestigated the distribution of ApsB at lower expression levels and compared the localization pattern of C-terminally tagged ApsB-GFP with a homologously integrated construct in which ApsB was tagged with GFP at the N-terminus (GFP-ApsB). With both constructs we observed a spot-like distribution. But in comparison to earlier studies, the number of spots was reduced and an even spacing of ApsB along hyphae became obvious. We assumed that the even distribution was due to co-localization with nuclei. We proved this co-localization through the analysis of GFP- or mRFP1-tagged ApsB in strains with GFP-, DsRedT4- or DAPI-stained nuclei. The result was further confirmed by immunostaining of ApsB-HA. Besides nuclear localized ApsB, extra ApsB spots (20-60%) were found within the cytoplasm, associated with MTs. These spots were highly mobile and moved along MTs with an average speed of 0.2-0.5 μm second^{-1} up to maximum speeds of more than 6 μm second^{-1}. The movement along a given MT occurred in both directions. Occasionally, the spots rotated around the MT axis and were able to change between different adjacent filaments without a noticeable delay. C-terminally and N-terminally tagged ApsB behaved

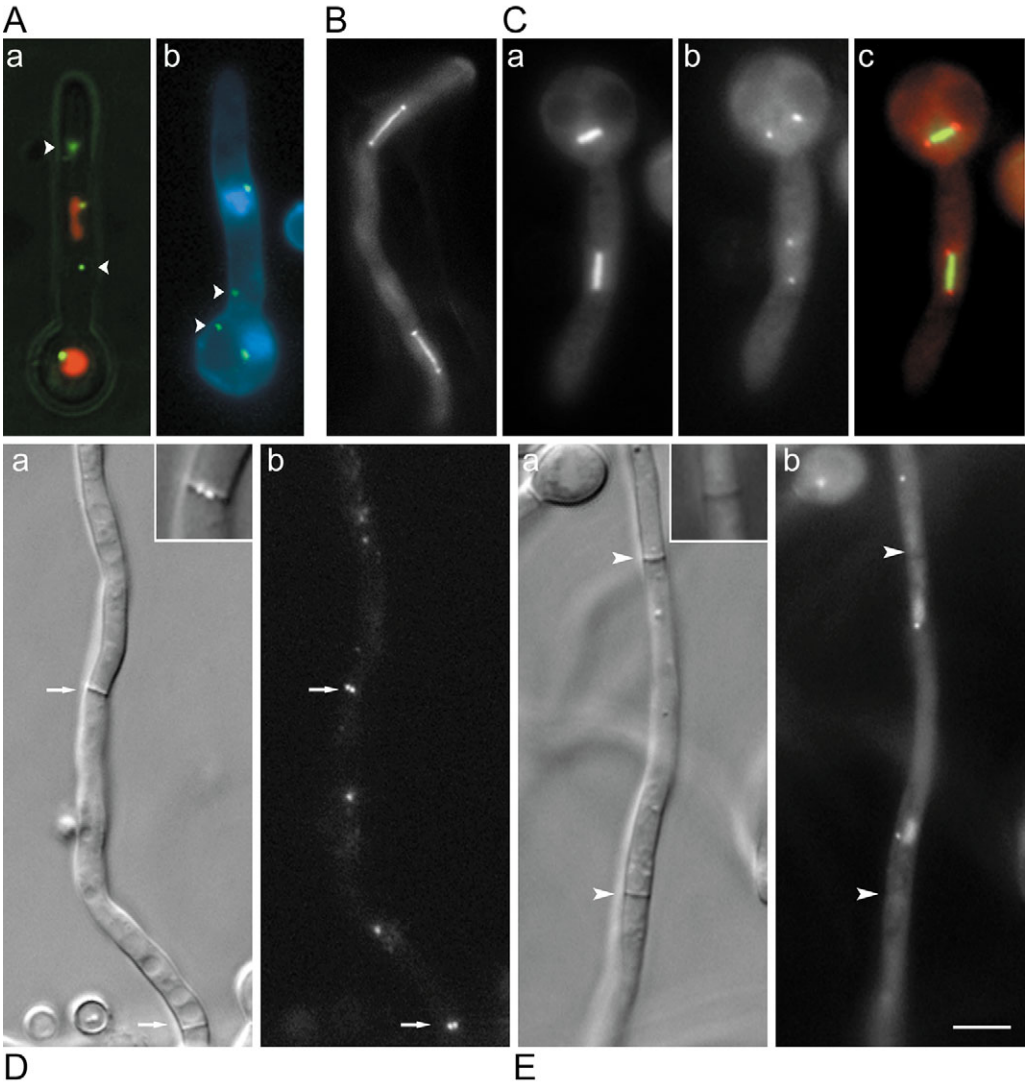

Fig. 2. Subcellular localization of ApsB. (A) Both ApsB-GFP (SDM1000) (a) and GFP-ApsB (SEa3) (b) localized to the nuclear SPB (green dots at nuclei) and to the cytoplasm (arrowheads). Cytoplasmic ApsB was always bound to MTs and moved fast up and down the filaments (see text and supplementary material, Movies 8-10a). Nuclei were stained with red fluorescent protein (DsRedT4) in (a) or with DAPI in (b). (B) C-terminally tagged ApsB as well as N-terminally tagged ApsB (C) was detected at the poles of the mitotic spindle. In (B) ApsB-GFP and GFP-TubA is visible (SDM40), while a red signal was used to set apart the mRFP1-ApsB (C,b) from the green GFP-TubA (C,a) (SDV1B). In (C,c) an overlay of (Ca,b) is shown. (D) In addition, N-terminally tagged GFP-ApsB localized to septa [arrows in (D,a,b)], while C-terminally tagged ApsB-GFP (E) did not [arrowheads in (E,a,b)]. Septa were not stained completely, but GFP-ApsB is seen as dots near the septal hole, as insert in (D, a) shows. The septa in (E) were negatively stained by weak cytoplasmic GFP background [insert in (E,a)]. Bar, 3 μm.

alike. The ApsB protein does not have any similarities to known motor proteins and thus the observed movement depends on other forces (see below).

ApsB dots, associated with the same filament, moved independent from each other (for a visual impression of ApsB dynamics see supplementary material Movies 8-10). Both nuclear and cytoplasmic localization of ApsB was independent of whether ApsB was tagged with GFP at its C- or N-terminus (Fig. 2A). Nuclear association of ApsB resembled γ-tubulin staining of the spindle-pole bodies and other SPB associated proteins (not shown) (Fox et al., 2002; Yang et al., 2004). To investigate a co-localization of ApsB with the SPB, we used a strain with GFP-labeled ApsB and GFP-labeled MTs to study mitotic spindles. The nuclear envelope of fungi remains intact during mitosis and mitotic spindles cannot be resolved as individual MT filaments and thus appear as a rod-like structure. We detected ApsB-GFP as dots at the poles of the spindles (Fig. 2B). Such dots were not seen in a control strain in which only MTs were labeled with GFP (not shown). The SPB localization of ApsB was further confirmed with a strain containing GFP-labeled MTs and ApsB N-terminally tagged with mRFP1 (Fig. 2C). Again, the localization of ApsB at the spindle poles was independent of whether the tag was N- or C-terminal. From this, we conclude that ApsB is either a component of the SPB or a closely associated protein. The SPB localization was not influenced by treatment with the MT-destabilising drug benomyl. Interestingly, whereas identical results were obtained for C- and N-terminally tagged ApsB so far, we observed that GFP-ApsB was also associated with septa, while ApsB-GFP was not (Fig. 2D,E). This was surprising, because both tagged versions of the protein rescued the *apsB* oligosporogenic mutant phenotype, and thus were biologically active (not shown). However, analysis of the MT cytoskeleton suggested a dominant-negative effect of the C-terminally tagged ApsB protein. In strains, which contained an N-terminally tagged ApsB, MT organisation was indistinguishable from wild type (Fig. 3A,B), while C-terminally tagged ApsB produced a reduction of MTs very similar to the situation found in *apsB* mutant cells (Fig. 3C,D). We next asked how the lack of ApsB or the dominant-negative influence of the C-terminally tagged ApsB protein could lead to the observed alteration of the MT cytoskeleton.

The *apsB* mutation affects MTOC activities

The MT array in *A. nidulans* is produced by the activity of MTOCs at the SPB, in the cytoplasm and at septa (Konzack et al., 2005). Because ApsB localized to the MTOC at the SPB, we anticipated that the staining pattern at the septa was also due to co-localization with the MTOC there. We used the MT plus-end localized kinesin-like motor protein KipA as plus-end marker [as described (Konzack et al., 2005)] to determine the activity of MTOCs at SPBs and septa. Comparing wild type and *apsB*-mutant strains during a 5 minute time period, we observed a reduction of newly emanating GFP-KipA signals in the mutant. At SPBs, GFP-KipA signal counts were only

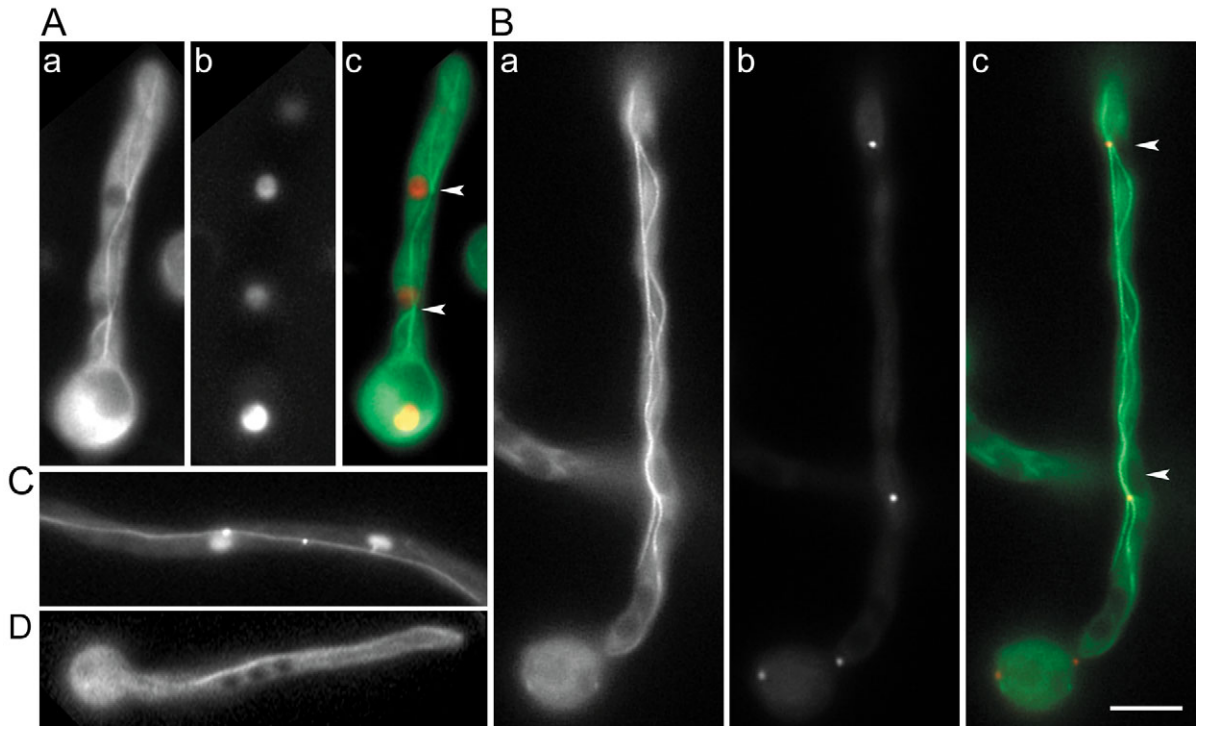

Fig. 3. Dominant-negative effect of C-terminally tagged ApsB-GFP on MT organization. (A) In wild type, MTs (GFP-TubA (a) connect adjacent nuclei [StuA(NLS)-DsRedT4 (b)] (SJW100) and several additional MTs emerge from the nuclear SPB [arrowheads in overlay (c)]. (B) In strains with N-terminally tagged ApsB, MTs were organized like in wild type (SDV1B). (b) mRFP1-ApsB dots indicate the position of the nuclear SPB of two nuclei along cytoplasmic MTs [GFP-TubA (a)] [dark areas and arrowheads in overlay (c)=nuclei]. (C) C-terminally tagged ApsB-GFP (SDM40) displayed a dominant negative phenotype reducing the number of MTs similar to the *apsB10* mutation (D) (SDV16). In (C) notice the MT-bound ApsB-GFP dot (middle) and the nuclear SPB-associated ApsB-GFP [bright, white dots connecting two nuclei to MT (oval, white areas of StuA(NLS)-GFP]. Bar, 3 μm for (A and B) and 4 μm for (C and D).

slightly reduced by about 30% in *apsB* mutants, while the situation was much more dramatic at septa, where a reduction of counts of more than 60% in *apsB* mutants compared to wild type was measured (Fig. 4) (see supplementary material, Movie 11). To test whether the presence or absence of septa influences MTOC activities, we analyzed the MT organization in a *sepA*^{ts} mutant at permissive and at a restrictive temperature (Harris et al., 1994). We could not detect any effect on the number of cytoplasmic MTs, as we did in the *apsB* mutant. This was not surprising, because cytoplasmic and SPB associated MTOC activites were unaffected in the *sepA* mutant (data not shown).

MT-dependent nuclear movement

Given that ApsA and ApsB are both involved in MT organization and mutation of either gene causes nuclear clustering, we wanted to know how MTs are involved in nuclear migration. To this end, we analyzed the MT cytoskeleton and its dynamics during nuclear movement in wild type and observed nuclei, MTs and the SPB simultaneously. We found that moving nuclei travel into the direction of the SPB and nuclei often adopt a tear-drop shape. This has been observed before in *Nectria haematococca* and *Neurospora crassa* (Inoue et al., 1998; Minke et al., 1999). The use of GFP-tagged MTs allowed us to investigate the role of MTs in this process. It appeared that the SPB was pulled by the attached MTs (Fig. 5A,B) (see supplementary material Movies 12 and 13). Berns et al. already suggested a tight connection between the SPB and MTs (Berns et al., 1992). Nuclei did not always travel individually but were sometimes connected by a MT and thus moved synchronously (Fig. 5C) (see supplementary material Movies 14 to 15). The question remains where the force-generating motors are localized. Two scenarios can be envisaged. First, MTs generated from the SPB could interact with cortical proteins such as ApsA (Num1 in *S. cerevisiae*) and MT-plus-end localized dynein could be subsequently activated at the cortex and pull the MTs with the attached nucleus. Second, MTs produced from adjacent nuclei overlap in the cytoplasm and sliding of the antiparallel filaments leads to the translocation of nuclei (Fischer, 1999; Osmani et al., 2003) (Fig. 8). In wild type it was difficult to distinguish between these two possibilities. However, we observed that nuclei still moved in strains where the dominant-negative influence of ApsB-GFP drastically reduced the number of MTs, which interact with the cortex, while overlapping MTs were still present (see supplementary material Movie 16). Therefore we assume that overlapping MTs are important for the process, because nuclei still migrated without any obvious cortical contact through MTs (Fig. 5D).

Mutation of *apsA* and *kinA* are synthetically lethal whereas mutations in *apsB* suppress dynein mutations

Nuclear distribution depends on the function of dynein (*nudA*) and, to a certain extent, on conventional kinesin (*kinA*) (Requena et al., 2001; Xiang et al., 1994). To analyze whether ApsA or ApsB functionally interact with one of these motor proteins, we created corresponding double mutants. The combination of *ΔapsA* and *ΔkinA* caused a drastic reduction of the growth rate, which is not observed in either single mutant (Fig. 6A). No obvious growth phenotype was found for the combination of *ΔapsA* with mutants of the two other *A. nidulans* kinesin motors *kipA* or *kipB* (data not shown). Similarly, the *apsA5/ΔnudA* double mutant (or the double mutant *ΔapsA/nudA1*) displayed no special phenotype and was identical to the *nudA* mutant (Fig. 6B). The *apsA5* mutation also has no effect on the *nudF* deletion, which has also a nuclear distribution defect (Efimov, 2003). In case of the *apsB6* mutation, a synthetic lethality was not observed when

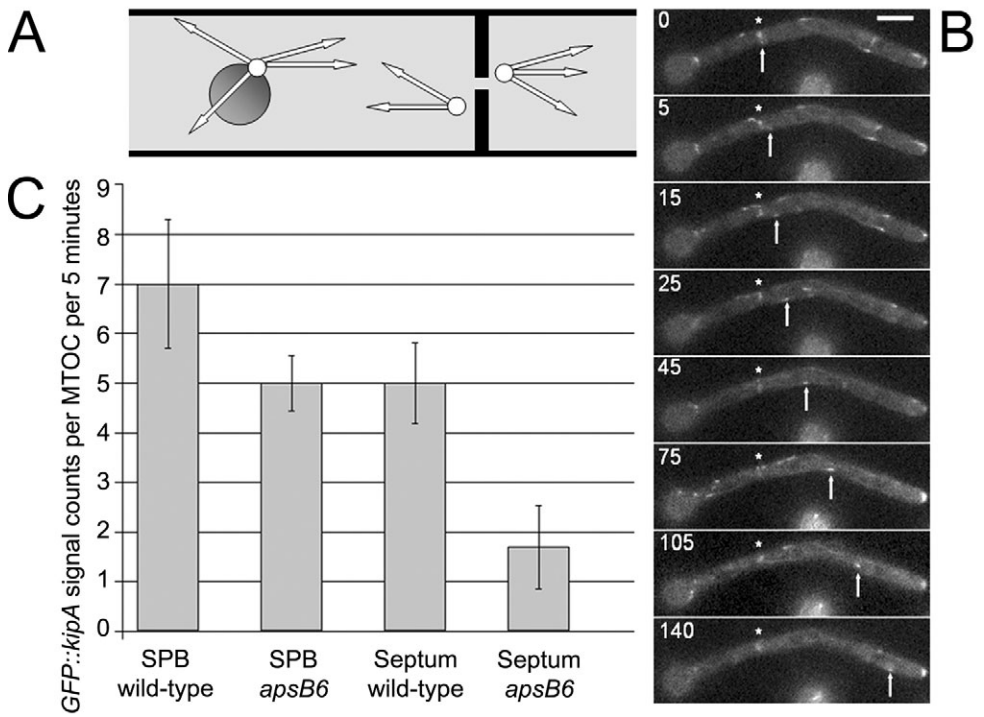

Fig. 4. Activities of nuclear and septal MTOCs in wild type and *apsB*-mutant strains. (A) Scheme of MTOC activities. Cytoplasmic MTs (white arrows) emerge from different MTOCs (small white dots), e.g. the nuclear SPB (left) and MTOCs near the hyphal septa (right) (grey ball=nucleus; vertical black line=septum). (B) KipA is a MT plus-end marker and its fluorescence signal can be followed as comets (GFP-KipA) during MT growth (Konzack et al., 2005). The white arrow follows a GFP-KipA signal emerging at a septal MTOC (asterisk) (see supplementary material, Movies 11). Time is indicated in seconds. Bar, 3 μm. (C) Quantitative analysis of GFP-KipA counts at different MTOCs. Values are the average of 100-150 MTOCs. Wild-type strain: SSK92. Mutant strains: SDV24, SDV25.

combined with the *ΔkinA* mutation. Surprisingly, the *apsB14* mutation, as well as *apsB* deletion, caused a suppression of the *ΔnudA* and *ΔnudF* growth phenotypes (Fig. 6B). DAPI staining of nuclei in germinating spores showed that nuclear migration was also slightly improved in the double mutants compared to dynein deletion strains.

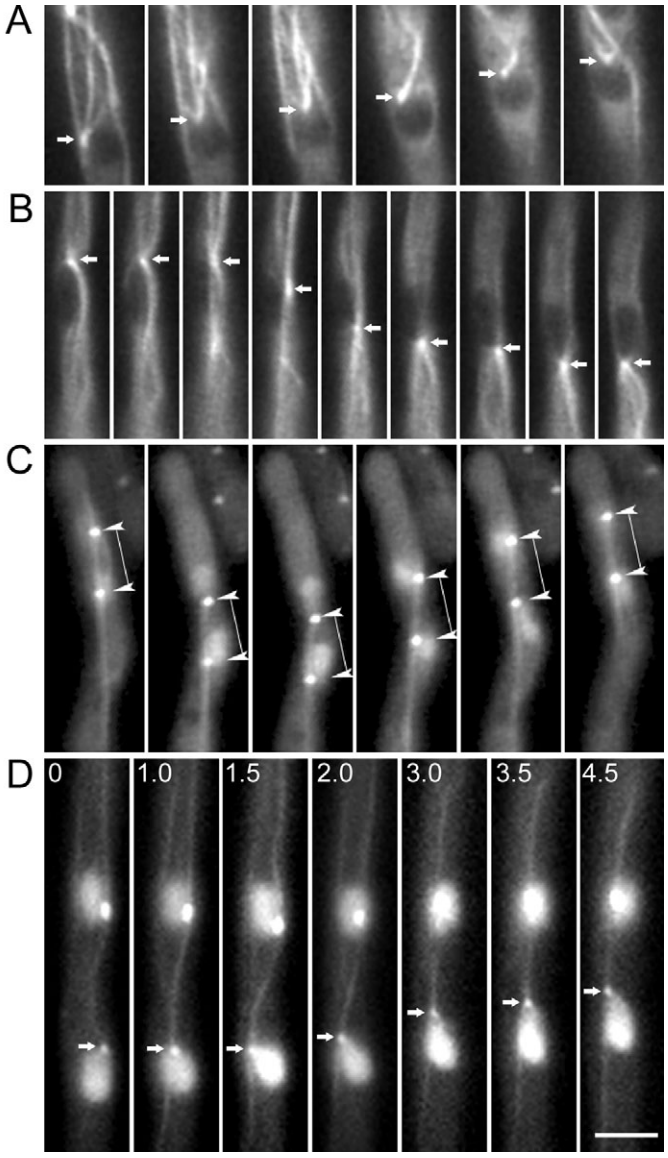

Fig. 5. Time-lapse sequences of nuclear migration and MT organization. (A,B) Interphase nuclei (dark, round or oval areas) move with the SPB (arrow) at their leading edge. The pulling force is applied to cytoplasmic MTs, which are connected with their minus ends to the SPB, thus pulling the nucleus. Notice that the nucleus in (A) migrates upwards and MTs (GFP-TubA) were located only at its front side (leading SPB). In (B) the pulling force was applied on the opposite of the former leading side, thereby moving the SPB from the upper end of the nucleus to the lower end, subsequently dragging the entire nucleus downwards. (C) If connected to the same MT, adjacent nuclei move synchronously when a pulling force is applied. (D) The lower nucleus moved upwards, although MTs coming from the SPB did not interact with the cortex, but only one central MT bundle was present. In (C,D) nuclei, microtubules and ApsB (SPB, arrows) were stained with GFP. Notice that GFP is C-terminally fused to ApsB. Bar, 2 μm for (A,D), and 3 μm for (C,D). Times between each picture is 30 seconds for (A-C) and for (D) as indicated in minutes (A,B: SJW02; C,D: SDM40). See supplementary material, Movies 12-16.

ApsB accumulates in the hyphal tip in the absence of dynein

As discussed above, ApsB moves rapidly along MTs. Such movements can be explained by an action of a processive motor protein associated with ApsB rather than by the dynamics of MTs. To address this question, we investigated ApsB movement in three different kinesin mutants (*ΔkinA, ΔkipA,* and *ΔkipB*) and in a *ΔnudA*-mutant background. Whereas the deletion of either kinesin did not affect the movement of ApsB,

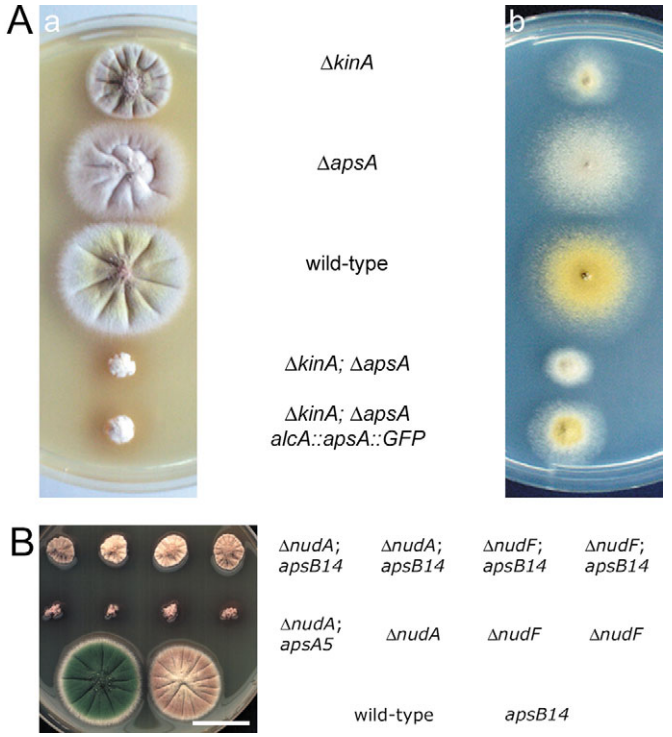

Fig. 6. Genetic interaction of *apsA* and *apsB* with molecular motors. (A) Strains were grown for three days at 37°C on glucose medium (a) to repress or on ethanol medium (b) to induce the *alcA*-promoter. A *ΔkinA* strain (SNR3) grows more compact compared to wild type, but sporulation is not affected. Hyphal growth of a *ΔapsA* strain (SRF30) is similar to wild type (RMS011), but spore production is reduced (brown colony). A double mutation of *ΔkinA* and *ΔapsA* (SDM24) results in a synthetic lethal phenotype, while strains, which contained an inducible *alcA(p)::apsA::GFP* construct in addition to the *ΔkinA* and *ΔapsA* mutation (SDM23), produces spores and shows normal growth on ethanol medium apart from the compact colony morphology due to the influence of the *ΔkinA* mutation. The cortical localization of ApsA-GFP was not influenced by the *ΔkinA* mutation (not shown). (B) *ΔnudA* and *ΔnudF* strains grow very poorly, but grow better when suppressed by the *apsB14* mutation. In comparison, *apsA5* has no effect on the dynein deletion strain. Strains were incubated at 43°C for 3 days. Strains from left to right are: apsB14/ΔA-7, apsB14/ΔA-9, apsB14/ΔF-9, apsB14/ΔF-11, apsA5/ΔA-6, XX60, apsB14/ΔF-3, apsB14/ΔF-6, SEwild-type and apsB14. Bars, 1 cm (A,B) and 1.5 cm (B).

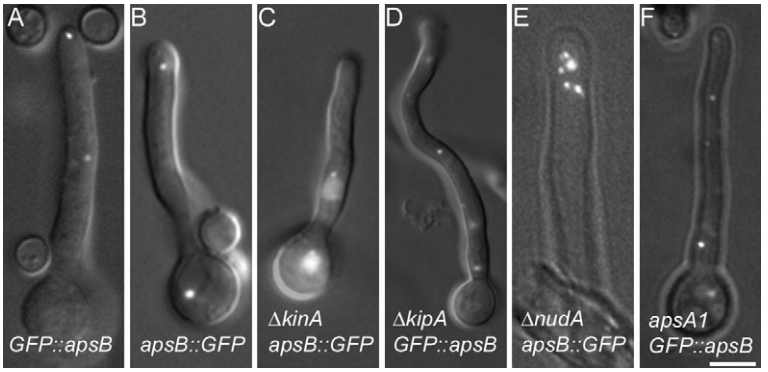

Fig. 7. ApsB accumulates at the hyphal tip in dynein-mutant cells. Cytoplasmic, MT-associated ApsB (white spots) often localizes at the hyphal tip as one single spot. This is independent of whether *apsB* is tagged N- or C-terminally with GFP (A,B) (SEa3, SDM1000). The localization pattern was not changed, neither in a *ΔkinA* (C) (SDM64) nor in a *ΔkipA* (D) (SDV19) background. However, up to 10 ApsB-GFP dots accumulated in *ΔnudA* strains (E) (SDM92). Despite their accumulation at the hyphal tip, each spot was still highly mobile (see supplementary material, Movies 17 and 18). (F) ApsB localization in *apsA1* mutant stains (SDV31). Scale bar is 3 μm for (A-D,F) and 2 μm for (E).

the lack of dynein caused an accumulation of ApsB near the apex of growing hyphae. However, the speed and bidirectionality of the ApsB movement along MTs were not affected (Fig. 7) (see supplementary material Movies 17-18). To rule out the possibility that ApsB accumulated at the tip due to the increased number of MT ends at the tip in dynein mutant strains (see Fig. 1A,d), the ApsB localization pattern was observed in *apsA1* mutant strains (Fig. 7F), which showed an increase in MT number, too (Fig. 1A,c). However, no accumulation of ApsB was detected. Therefore, ApsB accumulation appears to be the consequence of the defect in the dynein motor, but not of the MT organization itself. The localization of ApsB at the SPB was not altered in *ΔnudA*, *ΔkinA* or *ΔkipA* mutant background.

Discussion

During the past ten years GFP and other fluorescent proteins have been used extensively to study dynamic processes in pro- and eukaryotic cells. The availability of GFP-stained MTs and nuclei in *A. nidulans* allowed us to do real time studies of MT-dependent nuclear migration and to re-investigate the function of two formerly studied genes, *apsA* and *apsB*. We found that both mutations affect the MT cytoskeleton. Whereas ApsA appears to influence the length of astral and cytoplasmic MTs possibly by providing an anchoring ground for MT plus ends, ApsB appears to be involved in the regulation of the activity of MTOCs. The results will be discussed with focus on the following two points. (1) Possible roles of ApsA and ApsB in MT organization; (2) mechanism of nuclear migration and the involvement of motor proteins.

In *apsA* mutants MTs appear curved and thinner than in wild-type *A. nidulans*. This could be explained if we assume that ApsA serves a similar function as Num1 in *S. cerevisiae*, as sequence similarities suggest (Fischer and Timberlake, 1995). Lee et al. suggested that Num1 interacts with MT-plus end localized dynein at the cortex and may be involved in the offloading from the MT tip to the cortex (Lee et al., 2003). After cortical dynein is activated, it tugs at MTs and pulls attached nuclei. The situation could be similar in *A. nidulans* where ApsA could serve as a docking place for MTs. If ApsA is absent, the contact cannot be established and MTs continue to grow after reaching the cortex. That astral MTs exert a force at the elongating spindle can be concluded from the observation that mitotic spindles do not oscillate in *apsA* mutant strains and mitotic progression is delayed. Previously, Clutterbuck observed the presence of some giant nuclei in *aps* mutant hyphae, although experiments with a mitotic inhibitor or diploid stability tests did not prove a specific mitotic function of the Aps-proteins (Clutterbuck, 1994). A MT-cortex interaction appears not to be crucial for nuclear migration, because nuclei in *apsA* mutants move similarly to nuclei in *A. nidulans* wild type (Suelmann et al., 1997). The only differences are that the number of nuclei in hyphal compartments of *apsA* mutants is increased and that they are not evenly distributed. Thus MT-cortex interactions appear to be necessary for nuclear positioning or anchoring once the nuclei are distributed. Conversely, the organization of MTs could be responsible for an even spacing of nuclei in the cell. Plamann et al. suggested that nuclei are interconnected through overlapping MTs and equal forces acting on each side of a nucleus cause their even spacing (Plamann et al., 1994). If this model applies, any disturbance of the MT cytoskeleton will affect nuclear distribution (see below).

Interestingly, we found genetic interaction between *apsA* and conventional kinesin, *kinA*. KinA is not responsible for the localization of ApsA at the cortex (not shown). A link between the two proteins could be dynein, because the heavy chain of dynein, NudA, is possibly transported by KinA along MTs and accumulates at the MT-plus end (Zhang et al., 2003). However, a *nudA* nuclear distribution phenotype was not observed in the small colonies of the double mutant. Nuclei were even more clustered than in *apsA* mutants but still migrated out of the conidiospore (data not shown). This suggests that ApsA and KinA serve functions besides nuclear migration and positioning. It is also possible that synthetic inhibitory effect is caused by the stabilization of cytoplasmic MTs caused by *kinA* deletion (Requena et al., 2001).

In *apsB* mutants the number of cytoplasmic MTs was reduced due to a reduction of the MT producing activity of MTOCs. During mitosis the number of astral MTs was reduced whereas the mitotic spindle did not look altered in comparison to wild type. Mitosis itself appeared also not to be affected. In interphase cells, long MTs are oriented longitudinal and span the entire compartment. The number of those MT filaments was reduced to one or two in *apsB* mutants. It was shown recently that MT nucleation occurs at three different types of MTOCs in *A. nidulans* (Konzack et al., 2005). One important MTOC is the SPB, the *A. nidulans* centrosome equivalent, but MT nucleation also occurs at MTOCs in the cytoplasm, close to nuclei and at septa. The cytoplasmic and septal MTOCs are very poorly understood. The lack of ApsB has a more drastic

effect on the activity of septal MTOCs than on the activity of the SPB. This suggests that the nucleation centres are not identical. We found that the C-terminally GFP-tagged ApsB protein caused a dominant-negative effect with regards to MT formation. Interestingly, the protein localized still at the SPB but not at the septa anymore. This suggests that the C-terminus of ApsB is crucial for this localization and one can speculate that the C-terminus may be required for protein-protein interaction. These results are similar to results obtained recently for the *S. pombe* protein Mod20 (=Mbo1p=Mto1p). This protein was identified in a mutant screen designed to identify genes involved in cytoskeleton organization and polarity and was isolated at the same time as a component of the γ-tubulin complex (Sawin et al., 2004; Venkatram et al., 2005; Venkatram et al., 2004). Mod20 displays only a weak similarity to ApsB, but considering our findings, Mod20 probably represents a functional homologue of ApsB.

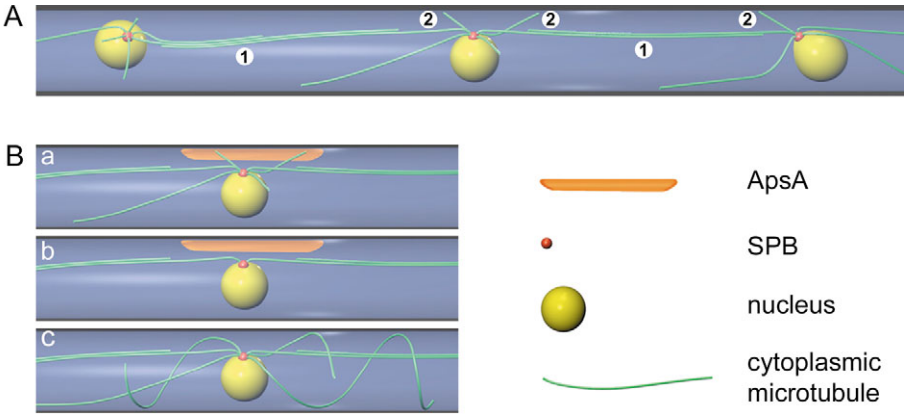

Fig. 8. Scheme of MT organization and nuclear migration in *A. nidulans*. (A) Cytoplasmic MTs are generated from SPBs. They elongate and eventually overlap to form anti-parallel bundles (1), thereby connecting adjacent nuclei. Some grow tipwards or make contact with the cortex (2). Nuclei can be moved by pulling forces of motor proteins, which could be located between overlapping filaments (1) or at the cortex (2), as the right nucleus indicates. (B) In wild-type cells, MTs contact ApsA or associated proteins (a). In *apsB* mutant cells, only a dominant, central microtubule bundle passes through the compartment, while additional MTs are absent (b). Although present, MTs cannot successfully contact the cortex in *apsA* mutant cells, hence elongating, which results in the appearance of curved MTs (c). Because certain MTs are not present in *apsB* mutants, nuclear positioning is interrupted, while MTs in *apsA* mutants miss the interaction with ApsA. Therefore, *apsA* and *apsB* mutants show very similar nuclear migration defects. Although cortical pulling forces (2) may be ineffective here, pulling forces between overlapping filaments (1) probably still work and are responsible for the observed remaining nuclear migration activity.

Given that the MTOCs at the septa are more drastically affected by the lack of ApsB and that the number of cytoplasmic MTs is largely reduced, we suggest that the normal MT array in *A. nidulans* is dependent on the activity of septal MTOCs. Whether ApsB is directly involved in MTOC function or is used to recruit proteins of the γ-tubulin complex to the MTOCs, as it was suggested recently in *S. pombe* (Sawin et al., 2004), cannot be decided yet.

Another question is how the different ApsB pools in the cell are connected. We observed that ApsB-GFP aligns and moves rapidly along MTs into both directions. ApsB, when transported to the MT minus end, would arrive at the MTOC where it could assemble into the complex. The movement of ApsB along MTs does not appear to depend on conventional kinesin, KipA or KipB. Although dynein appears to have an effect, the bidirectional transport of ApsB still occurs, which can be explained if a second motor moves along anti-parallel MT filaments. However, the nature of the movement remains to be determined.

Why does the loss of *apsB* function suppress mutations in the dynein pathway? This could be due to the effect of mutations on the MT cytoskeleton. MTs are less dynamic in the absence of dynein (Han et al., 2001) and dynein mutants can be partially rescued by the MT destabilizing drug benomyl or destabilising mutations in alpha tubulin (Willins et al., 1995). If we assume that there is a force that moves nuclei in the absence of dynein that is not MT-dependent (e.g., cytoplasmic streaming), then hyperstable MTs attached to nuclei would only hamper such movements. The reduced number of cytoplasmic MTs in the apsB mutants could facilitate residual nuclear movement in the absence of dynein by freeing nuclei from microtubules. Moreover, since ApsB localizes to septa, it could tether MTs and attached nuclei to septa. Indeed, previous studies showed that nuclei move more freely along the hyphae in *apsB* mutants (Suelmann et al., 1998) (see supplementary material Movies 2 and 3).

In this study, we attempt to document how nuclei are pulled through attached MTs. The important question is where the pulling force is localized. Several scenarios can be envisaged (Fig. 8). (1) The mechanism could be similar to *S. cerevisiae* where dynein is transported to the MT-plus end and then transfers to the cortex. Once there, it could pull the attached MT and thus translocate the nucleus. Our result that cortical protein ApsA is required for mitotic spindle oscillation indicates that MTs are indeed pulled from the cortex, at least during mitosis. Our finding that nuclei still move in *apsB* strains, in which the number of MTs is largely reduced and interactions of MTs emanating from the nuclear SPB were not observed, speaks against this model as the only mechanism. However, it could be that only very few MT-cortex interactions are sufficient and that those were overlooked in our experiments. (2) Another possibility is that nuclei move along MTs driven by dynein or kinesin attached to the SPB. Although we observed what appears as movement of SPBs along MTs (Fig. 3) (see supplementary material Movies 8 and 9), at the moment we cannot distinguish such movement from pulling of SPBs by attached MTs. (3) We observed that nuclei are sometimes connected by a MT and move synchronously (Fig. 5C) (see supplementary material Movies 14-15). Therefore overlapping MTs, could play an important role for nuclear migration as suggested already by Plamann and coworkers (Plamann et al., 1994). Although the exact mechanism still remains to be elucidated, it is possible that several or even all

three of these mechanisms contribute to nuclear distribution in filamentous fungi.

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