Aspergillus nidulans Dis1/XMAP215 Protein AlpA Localizes to Spindle Pole Bodies and Microtubule Plus Ends and Contributes to Growth Directionality

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The dynamics of cytoplasmic microtubules (MTs) is largely controlled by a protein complex at the MT plus end. In Schizosaccharomyces pombe and in filamentous fungi, MT plus end-associated proteins also determine growth directionality. We have characterized the Dis1/XMAP215 family protein AlpA from Aspergillus nidulans and show that it determines MT dynamics as well as hyphal morphology. Green fluorescent protein-tagged AlpA localized to MT-organizing centers (centrosomes) and to MT plus ends. The latter accumulation occurred independently of conventional kinesin or the Kip2-familys kinesin KipA. alpA deletion strains were viable and only slightly temperature sensitive. Mitosis, nuclear migration, and nuclear positioning were not affected, but hyphae grew in curves rather than straight, which appeared to be an effect of reduced MT growth and dynamics.

Microtubules (MTs) are hollow tubes which are generated from microtubule-organizing centers, and they perform multiple structural and dynamic functions in a cell. Although comprising an important part of the cell skeleton, MTs are very dynamic structures, which assemble at one end α,β-tubulin dimers, stop growth after some time, undergo a catastrophe event, and subsequently shrink. This dynamic instability is regulated by a number of different MT-associated proteins (MAPs), one of which was discovered in Xenopus and named XMAP215 (5). Similar proteins, which are meanwhile classified in the Dis1/XMAP215 family, exist in eukaryotes from yeast to plants and humans (17). Common to all of them is their association with MTs and the presence of TOG domains and HEAT repeats, which are responsible for interactions with many different associated proteins. One MAP can interact through its TOG domains and HEAT repeats with several other MAPs. The proteins were classified into three different groups (17). Members of the first group have four TOG domains, including one to five HEAT repeats within each of them, and a conserved C terminus. Human ch-TOG belongs to the first group together with Xenopus XMAP215, Drosophila (Mps), Dictyostelium (DdCP224), and Arabidopsis (MOR1) (Fig. 1). The second group has only one known member from Caenorhabditis elegans (ZYG-9). Members of the third group have only two TOG domains with several HEAT repeats and, in comparison to group one members, do not have a conserved C terminus. However, all of them harbor a coiled-coil region instead. XMAP215 proteins have a prominent MT-stabilizing function (12). Recently, it was shown nicely in Saccharomyces cerevisiae that the Dis1/XMAP215 protein Stu2 binds to tubulin heterodimers and associates to the MT plus end, where it appears to be responsible for the loading of α,β-tubulin dimers to the growing end (1). This activity may explain the Stu2 stabilization activity of MTs in living cells.

Besides the MT stabilization activity of Dis1/XMAP215 proteins, DdCP224, the Dictyostelium discoideum homologue, is involved in MT-cortex interactions. There is evidence that this contact is mediated by cortical dynein with which DdCP224 is able to physically interact (9).

In this paper, we have analyzed the function of the Dis1/XMAP215-like protein AlpA in Aspergillus nidulans. The protein localized at the spindle pole bodies (the fungal homologues of centromeres) and at MT plus ends. Interestingly, deletion of the gene was not lethal, although a drastic reduction of the MT array and MT dynamics was observed. Hyphae of an alpA deletion strain grew in curves, suggesting that AlpA is involved in the determination of growth directionality.

MATERIALS AND METHODS

Strains, plasmids, and culture conditions. Supplemented minimal and complete media for A. nidulans were prepared as described previously, and standard strain construction procedures were as described by Hill and Kaiser (10). A list of A. nidulans strains used in this study is given in Table 1. Standard laboratory Escherichia coli strains (XL1-Blue) were used. Plasmids are listed in Table 2.

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 ethanol) or 2% glucose as a carbon source. Medium was supplemented with pyridoxine, p-aminobenzoic acid, biotin, arginine, uracil, or uridine depending on the auxotrophy of the strains. Cells were incubated at room temperature for 1 to 2 days, and images were captured using an Axioshot microscope (Zeiss, Jena, Germany), a Plan-apochromatic 63× or 100× oil immersion objective lens, and an HBO50 Hg lamp. Alternatively, a Zeiss Axiolmager Z1 with the latest Axiosvision software...
results

Identification of a Dis1/XMAP215 family protein in *A. nidulans*. To characterize the role of the MT plus end complex for polarized growth, we searched the *A. nidulans* database with the *Schizosaccharomyces pombe* Alp14 protein sequence (4) (http://www.broad.mit.edu). The putative homologue AlpA (An5521.2) is a 96.4-kDa protein comprised of 891 amino acid residues. The open reading frame is disrupted by three short introns, 70 bp, 72 bp, and 72 bp in size. The intron-exon borders were confirmed by reverse transcription-PCR of small cDNAs, subsequent sequencing, and comparison with the sequence of genomic DNA. Protein analysis revealed eight HEAT repeats embedded in two TOG domains at the N terminus and a coiled-coil region at the C terminus (Fig. 1A). HEAT repeats of AlpA were identified using "REP" from TOG domains and heat repeats of AlpA were identified using "REP" from Protein sequences were aligned using vector NTI software (Invitrogen), MegAlign, and ClustalW software (http://www.embl-heidelberg.de). TOG domains and heat repeats of AlpA were identified using "REP" from the ExPaSy database.

**Bioinformatics.** Protein sequences were aligned using vector NTI software (Invitrogen), MegAlign, and ClustalW software (http://www.embl-heidelberg.de). TOG domains and heat repeats of AlpA were identified using "REP" from the ExPaSy database.
**TABLE 1. A. nidulans strains used in this study**

| Strain | Genotype* | Source or reference |
|--------|-----------|---------------------|
| GR5    | pyrG89 wA3 pyroA4 | 28 |
| GFP-NudA | pyrG89 wA2 pyroA4 ΔnudA::pyr4 alcA(p)::GFP::nudA::pyr4 | 6 |
| GFP-NudF | pyrG89 wA2 pyroA4 ΔnudF::pyr4 alcA(p)::GFP::nudF::pyr4 | 6 |
| RMs011 | paba1 yA2 ΔargB::trpCDΔB trpC801 | 25 |
| SCE01  | SJW02 transformed with pCE05; wA3 ΔargB::trpCDΔB pyroA4 alcA(p)::alpA::mRFP1 | This work |
|        | alcA(p)::GFP::nubA | |
| SCE05  | SRF200 transformed with pCE08; ΔargB::trpCDΔB pyroA4 alcA(p)::GFP::alpA (single homologous integration) | This work |
| SCE10  | SRF200 transformed with pCE06; pgyrG89 ΔargB::trpCDΔB pyroA4 alcA(p)::GFP::alpA::mRFP1::zipA::mRFP1::kipB695; alcA(p)::alpA::GFP alcA(p)::mRFP1::kipB695 | This work |
| SCE12  | SRF200 transformed with pCE06, pJW18, and pPND1; alcA(p)::mRFP1::zipA | This work |
| SCE35  | SSK44 transformed with pCE06; paba1 wA3 ΔargB::trpCDΔB ΔkipA::pyr4 alcA(p)::GFP (single integration) | This work |
|        | SJW02 transformed with pAT3; pyrG89 pyroA4 argB2 ΔnkuA::argB ΔalpA::pyr4 | This work |
|        | SNR1 transformed with pCE06; ΔkinA::pyr4 pyroA4 alcA(p)::GFP::alpA (single integration) | This work |
|        | SDV69f | |
|        | SDV100 | |
|        | SDV101 | |
|        | SDV102 | |
|        | SJW02  | |
| AnKin26 | pyrG89 yA2 ΔargB::trpCDΔB ΔkipA::pyr4 | 18 |
| SRF200 | paba1 yA2 pyrG89 ΔargB::trpCDΔB pyroA4 | 11 |
| SSK44  | paba1 yA2 pyrG89 ΔargB::trpCDΔB pyroA4 | 13 |
| SSK92  | paba1 yA2, pyrG89, alcA(p)::GFP::kipA::alpA::pyr4 | This work |
| TNO2A3 | TNO2A3 transformed with pPND1 and pCE08; alcA(p)::mRFP1::kipB695; alcA(p)::alpA::GFP::alpA::mRFP1::kipB695 | This work |
| SNR1   | ΔargB::trpCDΔB pyroA4 ΔkinA::pyr4 | 16 |
| ΔkipA  | pyrG89 wA3 ΔkipA::pyr4 | 18 |
| ΔalpA  | ΔalpA::pyr4 | 2 |
| ΔPCD1e | SSK44 crossed with SCS13; wA3 pyroA4 ΔkipA::pyr4 ΔalpA::pyr4 | This work |

* Only relevant genotypes are indicated. All strains carry the veA1 mutation.

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**TABLE 2. Plasmids used in this study**

| Plasmids | Construction* | Source or reference |
|----------|---------------|---------------------|
| pAT1     | 1,000 bp upstream of alpA ORF (= LB) cloned into pCR2.1 | This work |
| pAT2     | 1,001 bp downstream of alpA ORF (= RB) cloned into pCR2.1 | This work |
| pAT3     | alpA-LB::N. crassa pry-4::alpA-RB in pCS2.1 | This work |
| pCE05    | alcA::alpA::mRFP1 argB; gateway construct of pMT01 | 26; this work |
| pCE06    | alcA::alpA::GFP argB; gateway construct of pMTG-GFP | 26; this work |
| pCE08    | alcA(p)::GFP::alpA pry4; 1 kb of alpA with Ascl and PacI inserted into pMBC17apx | 27; this work |
| pCR21-TOPO | Cloning vector | Invitrogen |
| pCS1     | N. crassa pry-4 selectable marker as NotI fragment in pUMA208 | This work |
| pGFP-NudA | alcA(p)::GFP::nudA::pyr4 | 6 |
| pGFP-NudF | alcA(p)::GFP::nudF::pyr4 | 6 |
| pPND1     | Rigor mutant of KipB for staining MTs, alcA(p)::mRFP1::kipB695 | 21 |
| pMBC17apx | pMBC17 version for fusion of GFP to N termini of proteins of interest | 27 |
| pJW18     | Red nuclei, alcA(p)::mRFP1::zipA | J. Warmbold, Marburg, Germany |

* ORF, open reading frame.
**alpA deletion strains show defects in polarized growth.** The alpA gene was deleted by homologous recombination where the alpA ORF was replaced by the Neurospora crassa pyr4 gene. We used *A. nidulans* strain TNO2A3, which has a very high frequency of homologous integration (16). Homologous single integration was verified by Southern blot and PCR analysis in 5 of 7 tested strains (data not shown). Compared to the wild type, *alpA* deletion strains showed reduced colony size and compact growth, especially at higher temperatures (Fig. 3A). Although the conidiospore number was slightly reduced in *alpA* deletion strains, the morphology of conidiophores was indistinguishable from that of the wild type. Interestingly, and in contrast to the situation in *S. cerevisiae*, deletion strains were viable. This was surprising because we found only one Alp14 similar protein in the *A. nidulans* genome in comparison to two in *S. pombe*, where Dis1 can substitute for Alp14 (Fig. 1).

Hyphae of the *alpA* deletion strain did not show any difference with regard to nuclear distribution or septation, but hyphal morphology was changed. While wild-type hyphae grow relatively straight, the *alpA* deletion strain produced curly or curved hyphae (Fig. 3B, C), which were similar to hyphae of a *kipA* deletion strain (13). In addition to the curved growth phenotype, we noticed an increased number of branches in older hyphae. To show that the observed phenotypes were due to the deletion event, we constructed a plasmid where about 1 kb of the 5’ end of the *alpA* gene was fused to GFP and under the control of the inducible *alcA* promoter. The construct was integrated at the *alpA* locus (confirmed by Southern blotting), resulting in a full-length, GFP-fused version under the control of the *alcA* promoter (strain SCE05). The strain was used in the localization experiments described above. Under repressing conditions (glucose), SCE05 showed the knockout-like curved growth (Fig. 3D), whereas under inducing conditions (ethanol), wild-type-like growth was restored (Fig. 3E). This result proved that the GFP-tagged protein version was fully functional.

The curved growth phenotype in the *alpA* mutant resembled that of a *kipA* deletion strain (13). Therefore, we asked whether the lack of both genes would result in a similar or a different phenotype than that of the single mutations. The double mutant showed a more severe phenotype than the individual mutations. Hyphae appeared even more curly and similar to the *alpA* mutant, with more branches in older hyphae. Colonies were much smaller than the colonies of the parent strains, indicating an additive effect of *ΔkipA* and *ΔalpA* (Fig. 4A).

Because the growth direction of hyphae depends on the localization of the Spitzenkörper in the apex, we analyzed the position of this organelle in the wild type and compared it to the one in the *alpA*, *kipA*, and *alpA kipA* double deletion strains. Whereas in the wild type, the Spitzenkörper was found in the center of the hyphae in 70% and noncentral in 30% of the cases (*n* = 50), in the *alpA* deletion strain, only 22% showed the central position and 78% the noncentral one (*n* = 50). In comparison, in the *kipA* deletion strain, the percentages were 28% (central) and 72% (noncentral) (*n* = 50), and in the *alpA kipA* double deletion strain, the percentages were 52% (central) and 48% (noncentral) (*n* = 64) (Fig. 4B, C). It was surprising that the number of central and noncentral positioning of the Spitzenkörper was almost even in the double mutant.
In addition, we noticed that in 18% of the cases two Spitzenkörper were observed in the hyphal tip. In comparison, this number was only 5% in the wild type, 10% in the alpA mutant, and 5% in the kipA mutant. If there were two Spitzenkörper in the apex, we counted them as one event of non-central organelles in the quantification shown in Fig. 4A.

To test whether AlpA might play a role in the initiation of polarized growth, we analyzed the germination pattern of conidiospores (Fig. 4D). Wild-type conidiospores produce a second germ tube after the first germ tube has reached a certain length, and this second hypha emerges from a place opposite the first hypha. In contrast, the alpA deletion strain produced the second germ tube normally in angles smaller than 180° from the first hypha (Fig. 4D). This germination pattern resembled the one from the kipA mutant strain (13).

**AlpA determines cytoplasmic MT dynamics.** To further unravel the function of alpA in *A. nidulans*, we studied the effect of the alpA deletion on the MT cytoskeleton. MTs were visualized in the alpA deletion strain by GFP staining (6) (strain SDV86). Compared to the wild type (SJW02), the number of MTs was reduced in the alpA strain. Basically, only one thick MT bundle (according to Veith et al. [27]), connecting adjacent nuclei, was visible, in addition to some shorter MTs emerging from the nuclear spindle pole bodies, while in the wild-type strain several single and bundled MTs were present (Fig. 5A, B). In addition, the normally highly dynamic MTs appeared more stable and less dynamic. Whereas wild-type MTs polymerize at a rate of 14 μm per min (6), the extension rate in the alpA mutant was only 6 μm per minute. It has to be noted that growth of MTs only occurred occasionally. Most MTs did not elongate nor shrink. After MTs have reached the hyphal tip, they normally disassemble (MT catastrophe) within 20 s (see Movie S02 in the supplemental material) (13). In the ΔalpA background, fewer MTs reached the tip (4 in 5 min, compared to 20 in the wild type [36 hyphae analyzed]), and disassembly did not occur within minutes (see Movie S03 in the supplemental material). In addition, the number of emerging MTs in the mutant was reduced by 85% (25 hyphae analyzed). The mitotic spindle and mitosis itself were indistinguishable from that of the wild type (see Movie S04 in the supplemental material).

To analyze whether AlpA influences the stability of MTs, we tested the sensitivity of an alpA deletion strain (SDV83b) toward the microtubule-destabilizing agent benomyl. Whereas the wild type was able to produce colonies up to a concentration of 0.8 g/ml, the alpA mutant was unable to grow at concentrations higher than 0.6 g/ml (Fig. 6). This result suggests that AlpA stabilizes MTs in *A. nidulans*.

**Interdependence of AlpA with other MT plus end-associated proteins.** The fact that AlpA localized to the MT plus end in interphase cells raised the question of how it reaches the destination and whether this localization depends on the presence or activity of other MT plus end-associated proteins. To this end, we studied interactions between AlpA and the kinesins KinA and KipA, the dynein pathway components NudA and NudF, and the Clip170 homologue ClipA.

We analyzed AlpA MT plus end localization in ΔkipA and ΔkinA mutant backgrounds (strains SCE35 and SDV69f). The situation for GFP-AlpA in the ΔkipA and ΔkinA backgrounds was wild-type-like (not shown). Both KipA and KinA have been shown to be involved in MT plus end accumulation of ClipA and NudA, respectively (2, 31), but neither of those...
two kinesins was responsible for AlpA plus end localization. These results are in agreement with recent findings in *S. cerevisiae*, where Al-Bassam et al. (1) showed for the AlpA homologue Stu2 that it localizes to MT plus ends independently of any motor protein. Localization was dependent on the second TOG domain of Stu2, whereas the first TOG domain promotes the addition of α/β-tubulin dimers to the growing MT end.

To analyze the role of AlpA at the MT plus end and in polarized growth, we sought to determine whether AlpA is required for the recruitment of other proteins, such as the kinesin-like protein KipA, ClipA, the dynein motor NudA, or one of its regulators (NudF) to this place. Therefore, we constructed *alpA* deletion strains in which KipA or ClipA were labeled with GFP. Normally, both proteins accumulate at the MT plus end and hitchhike with the growing MT end. The visible movement of the KipA- or ClipA-GFP spots were described as comets (see Movie S05 in the supplemental material) (2, 13). KipA movement in an *alpA* deletion strain was reduced, and GFP-KipA partly decorated cytoplasmic MTs behind the plus end instead of moving with the MT plus end (Fig. 5C, D; see also Movie 06 in the supplemental material). An accumulation of the GFP fusion protein was still visible at the MT plus end. Because MTs did not extend as fast as in the wild type (see above), KipA-GFP comets were not observed. Similar results were obtained for ClipA (our own results and L. Zhuang and X. Xiang [Bethesda, MD], personal communication), dynein (NudA), and its regulator NudF. In strains with fusion proteins of GFP-NudA and GFP-NudF in a *ΔalpA* background (strains SDV100 and SDV101), MTs were similarly GFP decorated (Fig. 5E, F). However, in comparison to KipA, longer stretches of MTs were decorated with either NudA or NudF. Further experiments should address the question of whether the slight differences in localization are of functional importance or due to, e.g., different protein amounts of NudA, NudF, and KipA. The fact that the localization of components of the dynein pathway appears to be affected in *alpA* mutants does not cause nuclear distribution defects (see above) suggests that even in the absence of AlpA sufficient...
amounts of, e.g., dynein reach their normal place in the cell and serve the wild-type function.

**DISCUSSION**

In this paper, we characterized the Dis1/XMAP215 family protein AlpA from *A. nidulans* and found that it is associated with the MT plus end during mitosis and in interphase. AlpA plays a role in controlling MT dynamics and is important for the determination of growth polarity. Whereas the mechanism of MT stabilization was recently shown in *S. cerevisiae* (1), a role in polarized growth has not been described before. Polarized growth of filamentous fungi depends on the continuous delivery of secretory vesicles (7, 20). These vesicles provide new membranes and deliver, e.g., enzymes for cell wall biosynthesis. Because the vesicles are generated some distance away from the growing tip, they need to be transported long distances. It is assumed that MTs and conventional kinesin provide the basis for this long-distance transportation (18, 23). The first destination of the vesicles is an organelle close to the apex named the vesicle supply center or Spitzenkörper (8). The location of this organelle determines growth direction. For the last few micrometers between the Spitzenkörper and the cell membrane, fungi probably employ the actin cytoskeleton and its associated motors. According to this model, MTs contribute to polarized growth as tracks for the transportation of vesicles. Surprisingly, deletion of *alpA* seems not to affect long-distance vesicle transportation and accumulation of the vesicles in the Spitzenkörper significantly, despite the dramatic effects on MT organization. This result suggests that only few MTs are sufficient for efficient vesicle transportation. This is in agreement with observations that the growth rate of hyphae does not change during mitosis, although most of the cytoplasmic MTs are degraded during nuclear division (19). Another explanation for the observed growth in the *alpA* deletion strain could be the vesicle transport activity of the actin cytoskeleton. However, the fact that mutations in tubulin-encoding genes or in MT-dependent motor protein encoding-genes affect hyphal extension highly suggests an important role of MTs in polarized growth (18, 23). Although the Spitzenkörper was not obviously reduced in size in the *alpA* mutant, the position appears to be dependent on AlpA, as it was shown before for KipA (Fig. 5). An open question remains, however, why the number of centrally localized Spitzenkörpers increased again in the *alpA kipA* double mutant. Perhaps this is linked to the observation that the number of hyphae with two Spitzenkörpers in the apex was increased.

Results with *S. pombe* and *A. nidulans* suggest a second role for MTs in the determination of growth direction, and this feature is obviously affected in *alpA* deletion strains (13–15). According to the model of *S. pombe*, so called cell-end factors

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**FIG. 5.** *alpA* affects MTs and MT plus end-localized proteins. (A) Several MTs and bundles thereof stained with GFP are obvious in the wild type (SJW02). (B) The number and dynamics (see Movies S02 and S03 in the supplemental material) of MTs in an *alpA* deletion strain (SDV86) were reduced compared to the wild type. Arrowheads point to nuclei and arrows to spindle pole bodies. (C) While GFP-KipA localized to MT plus ends and moved as comets in the wild type (SSK92) (see Movie S05 in the supplemental material), (D) GFP-KipA decorated short fragments of MTs in the ∆*alpA* background (SDV87) (see Movie S06 in the supplemental material). (E) Fragments of MTs were also GFP decorated in ∆*alpA* strains with GFP-NudA (E) and GFP-NudF (F) fusion proteins (strains SDV100 and SDV101). Arrows in panels D to F point to MT plus ends as determined by the growth at this end. Bars in panel B, 5 μm (A, C, and D), 6 μm (B), and 4 μm (E and F).

**FIG. 6.** Benomyl sensitivity of the wild-type (upper row of colonies) and *alpA* deletion (lower row) strains. Benomyl was added in concentrations from 0 to 0.8 μg/ml, and colonies were grown for 2 days at 37°C.
are transported towards the MT plus end and hitchhike with the growing MTs towards the cell cortex. A cell end factor is, for instance, the membrane-associated protein Mod5, which was suggested to act as an anchor for Tea1 and Tea4. The latter protein in turn binds the formin For3, which catalyzes actin polymerization (15, 24). Although we were not able to identify a Mod5 homologue in A. nidulans or other aspergilli yet, the presence of the kinesin KipA (Tea2) and TeaA (Tea1) as well as a curved growth phenotype upon deletion of either of them (13) (results for TeaA are unpublished) suggests at least partial conservation of the mechanism. If this is the case and if deletion of alp4 caused a phenotype similar to that of deletion of kip4, the question is how AlpA is involved in polarity determination. It was shown in D. discoideum that DdCpd224 interacts with cortical dynein and thereby could mediate the contact between MT plus ends and the cortex (9). In A. nidulans, the situation could be similar, and a missing cortical contact could lead to the curved hyphal growth. However, it has to be noted that dynein mutants of A. nidulans do not display the same hyphal growth phenotype. In addition, dynein-mediated MT-cortical interactions are required for nuclear migration and nuclear positioning (27). Both phenomena were not affected in alp4 mutants. Therefore, it seems likely that the lack of AlpA drastically reduces MT dynamics and that this leads to a reduction of specific cell end marker delivery. One of the key challenges is therefore to identify such cell end marker proteins in filamentous fungi. The fact that the alp4 kip4 double mutant displayed a more severe phenotype with regard to hyphal extension in comparison to the strains with only one mutation suggests that the two genes also act in different pathways.

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