Interaction of the Aspergillus nidulans Microtubule-Organizing Center (MTOC) Component ApsB with Gamma-Tubulin and Evidence for a Role of a Subclass of Peroxisomes in the Formation of Septal MTOCs

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Peroxisomes are a diverse class of organelles involved in different physiological processes in eukaryotic cells. Although proteins imported into peroxisomes carry a peroxisomal targeting sequence at the C terminus (PTS1) or an alternative one close to the N terminus (PTS2), the protein content of peroxisomes varies drastically. Here we suggest a new class of peroxisomes involved in microtubule (MT) formation. Eukaryotic cells assemble MTs from distinct points in the cell. In the fungus Aspergillus nidulans, septum-associated microtubule-organizing centers (sMTOCs) are very active in addition to the spindle pole bodies (SPBs). Previously, we identified a novel MTOC-associated protein, ApsB (Schizosaccharomyces pombe mto1), whose absence affected MT formation from sMTOCs more than from SPBs, suggesting that the two protein complexes are organized differently. We show here that sMTOCs share at least two further components, gamma-tubulin and GcpC (S. pombe Alp6) with SPBs and found that ApsB interacts with gamma-tubulin. In addition, we discovered that ApsB interacts with the Woronin body protein HexA and is targeted to a subclass of peroxisomes via a PTS2 peroxisomal targeting sequence. The PTS2 motif was necessary for function but could be replaced with a PTS1 motif at the C terminus of ApsB. These results suggest a novel function for a subclass of peroxisomes in cytoskeletal organization.
hybridization (Roche Applied Science, Technical Resources, Roche Diagnostics GmbH, Mannheim, Germany).

**Bioluminescence fluorescence complementation assay (BIFC).** The enhanced yellow fluorescent protein (eYFP)-tagged N-terminal half (YFPF) was amplified from MM containing 2% glucose and 2% threonine to induce the cloned gene **apdB**. Similarly, hex4 was amplified from MM containing 2% glucose and 2% threonine to induce the cloned gene **apdB**. 3A2 (hex4::pVietag18, 19). Given that MTs are generally composed of a large protein complex with gamma-tubulin as one characteristic member, we anticipate that they are involved in septal MTOC formation.

**MATERIALS AND METHODS**

Strains, plasmids, and culture conditions. The preparation of the supplement/2/h of 2A6b and cloned into the BamHI-SalI restriction fragments. Image and video processing was done using the Zeiss Cell Observer SD, which combines the high-end Cell Observer software. Image and video processing was done using the Zeiss Axiocam and AxioVision software. Image and video processing was done using the Zeiss Axiocam and AxioVision software. Image and video processing was done using the Zeiss Axiocam and AxioVision software.

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The yeast strains used for transformation were AH109, Y187, and PJ69-4A (Clontech).

**Site-directed mutagenesis.** The peroxisomal target sequence of *apsB* was mutated using pDV21a as the template and the QuikChange XL site-directed mutagenesis kit from Stratagene. The last two amino acids were mutated using primer 5'GGGTTTGGGAAAGCTACGTGAACGAGTCCGAGTAAAGGAGG-3' and the corresponding antiparallel primer, giving pDV43. Successful mutagenesis was confirmed by commercial sequencing (MWG Biotech, Ebersberg, Germany).

**GFP or mRFP1 tagging of proteins.** pMCI71a7px was used as the basic vector for the tagging of *apsB* or *hexA* with GFP, and pDM8s was used for tagging with mRFP1 (see reference 35). Full-length *hexA* was amplified from genomic DNA using primers 5'GGTATGCCCGGCCCCTGCTGTTACTACGACGACGACG-3' and 5'CCTTATATTTATTAGACGGGAAGTGAGTAGATGC-3'. To obtain in vivo protein expression levels, we expressed the proteins under the control of the corresponding natural promoters. The *apsB* promoter (1.33 kb) was amplified from genomic DNA using primers 5'GGCCGCGGCGCGCGCTGTTACGTACGACGAGAAGG-3' and 5'CCTGATCCGGGCGCTGTTACGTACGACGAGAAGG-3'.

### Table 1. *A. nidulans*, *E. coli*, and *S. cerevisiae* strains used in this study

| Strain   | Genotype                                                                 | Source                  |
|----------|--------------------------------------------------------------------------|-------------------------|
| AJC1.5   | *biA1 apsB6*                                                             | J. Clutterbuck (1969)   |
| AJC1.7   | *biA1 apsB10*                                                            | J. Clutterbuck (1969)   |
| FGSC89   | *biA1 argB2*                                                             | FGSC                    |
| GJA28    | *biA1 ΔhexA::argB (FGSC89 transformed with ΔhexA::argB deletion cassette)* | G. Jedd, Singapore      |
| GR5      | ppyG89 wA3 pyroA4                                                        | 38                      |
| MH11269  | *biA1 niiA4 pyroA4 niiC::bar*                                             | 12                      |
| SDV38    | *alcA*; pYFP::apsB1.2; *alcA*; pYFP::hexA680 wA3 pyroA4 (GR5 transformed with pDV15 and pDV22b) | This work               |
| SDV42    | *alcA*; pYFP::hexA1.0; *alcA*; pYFP::apsB1.2 wA3 pyroA4 (GR5 transformed with pDV19a and pDV23a) | This work               |
| SDV49-4  | *alcA*; mRFP1::apsB1.3; *alcA*; pGFP::hexA4.7; ppyroA4 ΔalkA::argB (TN02A3 transformed with pDV15 and pDM8s) | This work               |
| SDV70b   | yA1 pyroA4 ribO2 areA102 gpd(p)::GFP::acaE; *alcA*; mRFP1::apsB; (TALX207-10 transformed with pDV42a) | This work               |
| SDV73    | *alcA*; pGFP::apsB3.2; *alcA*; mRFP1::hexA4.680; ppyroA4 ΔalkA::argB [TN02A3 transformed with pDV39 and pMC171a7px (apsB)] | This work               |
| SDV77    | *alcA*; pGFP::apsB::PTS2.1; ppyroA4 ΔalkA::argB (TN02A3 transformed with pDV43) | This work               |
| SDV78c   | *alcA*; mRFP1::PTS2.2; gpd(p)::GFP::acaE; yA1 ribo2 areA102 (TALX207-10 transformed with pDV39 and pTNN1) | This work               |
| SDV79    | ΔhexA; δalkA(p)::GFP::apsBPTS2.1.3; (GJA28 crossed with SDV77a) | This work               |
| SDV80    | *apsB6; *alcA*; pGFP::PTS2.1 (AJC1.5 crossed with SDV77) | This work               |
| SDV88    | *apsB6; *alcA*; pGFP::apsB1.5; (AJC1.5 crossed with SEA3) | This work               |
| SDV95    | ΔhexA; δalkA(p); pGFP::apsBPTS2.1.3; (SDV82 crossed with SRS25) | This work               |
| SDV98    | *apsB10; *alcA*; pGFP::PTS2.1; (AJC1.7 crossed with SNEW02) | This work               |
| SDV99    | *apsB10; *alcA*; pGFP::PTS2.1.3; (SDV98 transformed with pDV43a) | This work               |
| SEA3     | *alcA*; pGFP::apsB; wA3 pyroA4 | 35                      |
| SJW02    | *alcA*; pGFP::PTS2.1; ΔalkA::PTS2.4; | 35                      |
| SNZ11    | *alcA*; pYFP::apsB1.7; *alcA*; pYFP::ΔalkA::PTS2.4; | This work               |
| SNZ16    | *alcA*; pGFP::PTS2.1; ΔalkA::PTS2.4; | This work               |
| SNZ22    | *alcA*; pGFP::PTS2.1; gpd(p)::DrsRed::strA(NLS) (SNZ16 transformed with pJH19 and pTNN1) | This work               |
| SNZ34    | *apsB10; *alcA*; pGFP::apsBPTS2.1; (AJC1.7 transformed with pNZ16) | This work               |
| SNZ37    | *alcA*; pGFP::PTS2.1; ΔalkA::PTS2.4; (SNZ16 transformed with pNZ25 and pTN1) | This work               |
| SNZ59    | *apsB10; *alcA*; pGFP::PTS2.1; (TN02A3 transformed with pNZ-S137) | This work               |
| SNZ61    | *ptsB; *ptsA; (TN02A3 transformed with pNZ-S136) | This work               |
| SNZ94    | *ptsB; *ptsA; (TN02A3 transformed with pNZ-S136) | This work               |
| SNZ-S80  | *ptsB; *ptsA; (SNZ-S80 transformed with gpd(p)::GFP::acaE and riboB+ plasmid) | This work               |
| TNLX207-10 | *yA1 pyroA4 areA102 transformed with gpd(p)::GFP::acaE and riboB+ plasmid | This work               |
| TNLX207  | *yA1 pyroA4 areA102 transformed with gpd(p)::GFP::acaE and riboB+ plasmid | This work               |
| TN02A3   | ppyG89 pyroA4 ΔalkA::argB | This work               |
| PJ69-4A  | MATa trp1-901 leu2-3 ura3-52 his3-200 galΔ gal80Δ GAL2-ADE-LYS::GAL1-HIS3 met2::GAL7-lacZ | S. Osmani               |
| AH109    | MATa trp1-901 leu2-3,112 ura3-52 his3-200 galΔ gal80Δ LYS2::GAL1UAS-GAL1TATA-HIS3 GAL2UAS-GAL2TATA-ADE2 UR43::MEL1UAS-MEL1TATA-lacZ GAL2-ADE-LYS::GAL1-HIS3 met2::GAL7-lacZ | 13                      |
| Y187     | MATa ura3-52 his3-200 ade2-101 trp1-901 leu2-3,112 galΔ mer::gal80Δ | This work               |
| FGSC     | ppyG89 wA3 pyroA4 ΔalkA::argB | This work               |
| SO451    | ppyG89 wA3 pyroA4 ΔalkA::argB | FGSC                    |
| SRS24    | gpd(p)::GFP::stru(NLS) pabA1 ΔalkA::argB::GFP::GFP::GAL7-lacZ | 31                      |
| TALX207-10 | *yA1 pyroA4 areA102 transformed with gpd(p)::GFP::acaE and riboB+ plasmid | M. Hynes and A. Andrianopoulos, Melbourne, Australia |

* FGSC, Fungal Genetics Stock Center.
TABLE 2. Plasmids used in this study

| Plasmid                  | Construction                                                                 | Source          |
|--------------------------|------------------------------------------------------------------------------|-----------------|
| pCR2.1-TOPO              | Cloning vector                                                               | Invitrogen      |
| pDM8a                    | GFP replaced with mRFP1 in pMCB17apx-apsB                                     | This work       |
| pDV7                     | alcA(p): YFP::apsB3::pyr4 GFP of pMCB17apx-apsB replaced with YFP           | This work       |
| pDV8                     | alcA(p): YFP::apsB3::pyr4 GFP of pMCB17apx-apsB replaced with YFP           | This work       |
| pDV15                    | alcA(p): GFP::hecA3::pyr4 pMCB17apx with full-length hecA                   | This work       |
| pDV17                    | alcA(p): YFP::hecA4::pyr4 apsB of pDV8 replaced with hecA                | This work       |
| pDV39                    | alcA(p): YFP::hecA4::pyr4 apsB of pDV7 replaced with full-length hecA        | This work       |
| pDV21a                   | pMCB17-apx containing full-length apsB of 3.2 kb between Ascl and Pac restriction sites; alcA(p): GFP::apsB3::pyr4 | This work; 35 |
| pDV22b                   | alcA(p): YFP::apsB3::pyr4 apsB of pDV7 replaced with full-length apsB         | This work       |
| pDV23                    | alcA(p): YFP::apsB3::pyr4 apsB of pDV8 replaced with full-length apsB         | This work       |
| pDV39                    | alcA(p): mRFP1::hecA4::pyr4 apsB of pDM8a changed with hecA                | This work       |
| pDV42a                   | alcA(p): mRFP1::apsB3::pyr4 pDM8a with full-length apsB                     | 35             |
| pDV43                    | PTS2 of apsB in pDV21a is mutated alcA(p): GFP::apsB3::SRL::pyr4             | This work       |
| pDV50                    | alcA(p): YFP::γtubulin1.8::pyr4 apsB of pDV8 replaced with full-length γtubulin1.8 | This work       |
| pENTRMT-D-Topo           | Cloning vector                                                               | Invitrogen      |
| pJH19                    | gpd(p): struct(NLS):DsRed argB                                              | 34             |
| pMCB17apx(-apsB)         | pMCB17 version for fusion of GFP to N termini of proteins of interest (with 1.5 kb of apsB) | 35; V. Efimov  |
| pMT-3 × HA              | Gateway destination vector                                                  | 34             |
| pNZ16                    | PTS1 (SRL) added before stop codon of apsB::PTS2::SRL in pDV43;             | This work       |
|                         | alcA(p): GFP::apsB3::SRL::pyr4                                               |                 |
| pNZ17                    | pMCB17-apx containing full-length γtubulin of 1 kb between Ascl and Pac restriction sites; alcA(p): GFP::γtubulin1.8::pyr4 | This work       |
| pNZ21                    | apsB::SRL without stop codon in pENTRMT-D-Topo                              |                 |
| pNZS23                   | apsB::SRL from pNZ21 cloned into pMT-3 × HA                                 | This work       |
| pNZ-S136                 | alcA(p) of pNZ17 replaced with 1.16-kb γtubulin(p) EcoRI and BssW restriction sites; γtubulin1.8::pyr4 | This work       |
|                         | alcA(p): GFP::γtubulin1.8::pyr4                                              |                 |
|                         | pNZ-S137 alcA(p) of pDV21 Replaced with 1.33-kb apsB(p) ArgII and KpnI restriction sites; | This work       |
|                         | apsB::SRL:GFP::γtubulin1.8::pyr4                                             |                 |
| pRS88                    | apsB in BamHI-Sall sites of pGAD424                                          | This work       |
| pRS89                    | apsB in BamHI-Sall sites of pGBT9                                            | This work       |
| pRS91                    | cDNA clone of hecA in pGAD424                                                | This work       |
| pS1-N4                   | pSM14 containing full-length apsB of 3.2 kb between Ascl and Pac restriction sites; alcA(p):3×HA::apsB3::pyr4 | This work       |
|                         | alcA(p):3×HA::apsB3::pyr4                                                    |                 |
| pSM14                    | GFP of pMCB17apx replaced with 3×HA between KpnI and Ascl restriction sites | This work       |
|                         | pTNI: pyr4 from A. fumigatus                                                | 25             |

CCACTGCG-3’ (apsB_nat(p)); KpnI_revet (the AvrII and KpnI restriction sites are in italics), cloned instead of alcA(p) into pDV21; giving plasmid pNZ-S17 [apsB(p):GFP::γtubulin1.8::pyr4], and transformed into TN02A3, giving SNZ9. The gamma-tubulin promoter (1.6 kb) was cloned from genomic DNA using primers 5’-GAATTCCATTACCCAGATTAACCTCG-3’ (Gamma_tub_nat(p)_EcoRI fwd) and 5’-GGCGCGCCACTCCCCGACGATTTTCG-3’ (Gamma_tub_nat(p)_Bswl_revet) (EcoRI and BssW restriction sites are in italics), cloned instead of alcA(p) into pNZ17 giving plasmid pNZ-S16 (GFP::γtubulin1.8::pyr4), and transformed into TN02A3, giving SNZ61. AlpB AN4867 (S. pombe Alp6) was amplified via fusion PCR using primers 5’-GGGAGGACAAATACAAACTCG-3’ (Alp6_mite_fwd) and 5’-ctcaggccggaacaccgtagCTCCAGGCAATTGCTTTGC-3’ (Alp6_linker_revet) to amplify the C-terminal fragment of AlpB without the stop codon and primers 5’-ctcaggccggaacaccgtagCTCCAGGCAATTGCTTTGC-3’ (Alp6_RB_link_fwd) (linkers in lower case letters) and 5’-ACGGGATCAAGCCGAAAGCAAGG-3’ (Alp6_RB_revet) to amplify the right border of AlpB. The two PCR products were fused to a GFP::pyrG PCR cassette (kindly provided by S. Osmani, Ohio State University) to generate a 5.5 fusion PCR product using primers 5’-CAGCTCCTGAGACCCTAATTG-3’ (Alp6_Nprimer_fwd) and 5’-CTTATCACTGTGCTTTGACG-3’ (Alp6_Nprimer_rever). The fusion PCR product was transformed into A. nidulans strain SO451, giving SNZ-SH80 [apsB(p):GFP::γtubulin1.8::pyr4].

**Generation of the apsB3.2::PTS2::SRL (PTS1) construct.** A PTS1 targeting sequence (SRL) was added to the C terminus of AlpB by amplifying the full-length mutated gene apsB3.2::PTS2::SRL in pDV43 using primers 5’-TTTGGCAGCGCGCCGCGTTGTATTACAGCG-3’ (apsB_Asc_fwd) and 5’-CTTATCACTGTGCTTTGACG-3’ (SRL_PTS1_Pacl_revet) (PTS1 is in lowercase letters). The PCR product was cloned between the Ascl and Pac restriction sites in the vector pMCB17apx and confirmed via sequencing, giving plasmid pNZ16, which was transformed into apsB10 mutant strain AJC1.7, generating strain SNZ34 [apsB10, alcA(p):GFP::γtubulin1.8::pyr4::apxB::PTS2::SRL]. Ectopic integration of the construct and the presence of the mutated endogenous apsB locus were confirmed by PCR, Southern blotting, and sequencing of the PCR products. Likewise, transformation of the apsB construct was done with pNZ16 into apsB deletion strain SRS24, generating SNZ94 with the same rescue phenotype as in the case of AJC1.7.

**Immunostaining.** Spores (10⁷/ml) were incubated with 0.5 ml MM on sterile covergrips for 12 to 24 h at room temperature (RT). Cells were fixed for 30 min with formaldehyde and digested for 1 h using digestion solution (Gluconex, β-1,3-glucanase, lyticase, and Driselase in Na-phosphate buffer with 50% egg white), washed with PBS, incubated in −20°C methanol for 10 min, and blocked with Tris-buffered saline–Tween 20 (TBST) plus 5% skim milk before incubation with the first monoclonal antibody (anti-gamma-tubulin T6657 at 1:500; Sigma-Aldrich) in TBST overnight at 4°C. Next, cells were washed and incubated with the Alexa Fluor647-labeled goat anti-mouse secondary antibody (A11003 at 1:200 in TBST; Molecular Probes) for 1 h at RT. Cells were washed and mounted on microscope slides (with VECTASHIELD mounting medium with DAPI [4',6-diamidino-2-phenylindole]), sealed with nail polish, and stored at 4°C overnight in the dark before microscopy.

**RESULTS**

Identification of gamma-tubulin and GcpCAlp6 at septal MTOCs and interaction of ApsB with gamma-tubulin. A. nidulans ApsB has been localized at SPBs and at septa, suggesting the presence of MTOCs at septa (Fig. 1) (35). MTOCs are large protein complexes which consist of several proteins, gamma-tubulin, and associated gamma-tubulin complex proteins, which are mostly conserved from yeast to humans (26).
also share this protein (Fig. 1). During the course of our experiments, this gene was analyzed in the laboratory of B. Oakley and was named gcpC (40).

In order to demonstrate that ApsB and gamma-tubulin colocalize at MTOCs, we visualized gamma-tubulin in a GFP-ApsB-expressing strain (Fig. 2A). Next, we showed that ApsB not only colocalizes but also interacts with gamma-tubulin. To this end, we applied the BiFC assay system and fused full-length ApsB with the N-terminal part of YFP and full-length gamma-tubulin with the C-terminal part of YFP. Corresponding A. nidulans strains showed a YFP signal at nuclei and at septa (Fig. 2B). Interestingly, we also found a fluorescence signal at the tips of all actively growing hyphae (Fig. 2C). Previously, ApsB had already been found at the hyphal tip and growing MTs were also reported to originate from the hyphal tip in some cases (16, 35). Gamma-tubulin alone was not visible at the hyphal tip, probably due to the high cytoplasmic background. Some cytoplasmic spots were also observed, as shown before for ApsB alone (35). Control experiments with ApsB or gamma-tubulin alone did not result in any fluorescence.

The ApsB–gamma-tubulin interaction result was confirmed by coimmunoprecipitation using hemagglutinin (HA)-ApsB and GFP–gamma-tubulin tagged proteins. Gamma-tubulin was detected in the precipitate obtained with anti-HA antibodies (Fig. 2D).

**ApsB is associated with peroxisomes.** To further analyze the role of ApsB, we employed a yeast two-hybrid analysis. The cDNA of apsB was cloned into pGBT7 (pRS89) and transformed into PJ69-4A. This strain was used as a recipient strain for a yeast two-hybrid gene bank kindly provided by S. Osmani (24). Besides ApsB itself, we identified five putative interacting clones (three unknown proteins, one Zn²⁺ finger protein, and one putative nucleoside transporter). The translation product of one of the clones displayed sequence identity to A. nidulans HexA, the homologue of the N. crassa Hex-1 protein (15). To prove the interaction between ApsB and HexA, we cloned the full-length hexA gene and tested it in the interaction screen. A yeast strain of mating type a (AH109) containing the GAL4 binding domain with apsB (Y2HapsB-AD) was crossed to a yeast strain of mating type α (Y187) containing the GAL4 activation domain with hexA (Y2HhexA-AD), or just the empty vector (pGAD424) as a control. Diploids were identified by selective growth on YEPD agar medium lacking leucine and tryptophan (YEPLD⁻) (Fig. 3A), as described by Cagney et al. (2). To look for positive protein-protein interactions, colonies were subsequently inoculated onto YEPD medium lacking leucine, tryptophan, and histidine (YEPLD⁻/H⁻) (Fig. 3B). This medium allows growth only if the strains produce their own histidine due to a positive interaction of the respective proteins. Colonies with apsB/hexA and apsB/ hexA grew well in comparison to the apsB/empty-vector combination. The weak background growth was reduced by the addition of 3 mM 3-amino-1,2,4-triazole (3-AT) (Fig. 3C). The positive interaction was confirmed with the β-Gal assay on membranes. ApsB interacted with itself, as well as with HexA, while in combination with the empty vector no reaction occurred (Fig. 3D). To exclude the possibility that neither HexA nor ApsB has an intrinsic affinity for other proteins and gives false positives in the yeast two-hybrid assay, we tested both of

![FIG. 1. Gamma-tubulin and AlpB localize to septal MTOCs.](image)

(A) GFP-ApsB, GFP–gamma-tubulin, and AlpBΔNls-GFP localize to the SPBs. Nuclei are stained with DsRed-StuA(NLS) (SNZ22) or DAPI. (B) Localization of the same GFP-tagged proteins to the septal pore (two spots in the center). Fluorescence (left), differential interference contrast (DIC, middle), and merged (right) microscopic images are shown. The strains used were SNZ59 (GFP-ApsB), SNZ61 (GFP–gamma-tubulin), and SNZ-SH80 (AlpBΔNls-GFP). All proteins were expressed from their natural promoters.

fortunately, the most important protein of MTOCs, gamma-tubulin, has not been identified at septa of A. nidulans before. In our own experiments, we were able to detect a very weak signal at septa when gamma-tubulin was expressed from its own promoter and fused to GFP. In S. pombe, it has also been reported that gamma-tubulin was present at nonnuclear MTOCs in very small amounts and thus was also not easy to detect (29). To further elucidate the composition of septal MTOCs, we searched the A. nidulans genome for a homologue of S. pombe Alp6 (S. cerevisiae Spe98, human Gcp3) and identified the open reading frame AN4867 (968 amino acids in length) with 35% identity to Alp6. In order to localize the corresponding protein, we constructed a C-terminal GFP fusion protein expressed from the native promoter and transformed it into A. nidulans (SO451). The protein localized to MTOCs at nuclei and at septa, indicating that the two MTOCs
them and the empty vector against a library of *Treponema pallidum* with 73 different proteins (kindly provided by P. Uetz) (not shown). Growing on YPED LTH/H11002, the empty vector produced three strains (4%) with a false-positive reaction, which were also seen for *apsB* and *hexA*. Despite these, no positive interaction with any of the remaining 70 *T. pallidum* proteins was found, indicating that neither ApsB nor HexA interacts randomly with given proteins and confirming that the HexA/ApsB interaction was specific. Sequence inspection of ApsB revealed a putative peroxisomal targeting sequence (PTS2), KIRDLEKQL, at amino acid positions 66 to 74. Likewise, proteins with sequence similarity to ApsB (29), such as mto1 (formerly known as mod20 or mbo1) and pep1 (*S. pombe*), NCU02332.1 and NCU02411.1 (*N. crassa*), and AAH46878 (*Drosophila melanogaster*) and CDK5RAP2 (*Homo sapiens*) all have a possible PTS2, as identified with the software program psort (http://psort.hgc.jp/).

ApsB localizes to a subclass of peroxisomes. To obtain further proof of the peroxisomal localization of ApsB, we compared its localization with the localization of the peroxisomal enzyme AcuE (acetate-malate synthase). This protein was tagged with GFP (strain TALX207-10, kindly provided by M. Hynes and A. Andrianopoulos, Melbourne, Australia) and co-expressed in a strain with mRFP1-ApsB (strain SDV70b). Fourteen percent of the spots showed green and red fluorescence (Fig. 4A). In addition, we analyzed GFP-AcuE and mRFP1-tagged HexA (SDV78c), which confirmed the localization of mRFP1-HexA to peroxisomes (Fig. 4B). However, one important difference between ApsB and HexA was the frequency of colocalization with AcuE. While HexA and AcuE...
had about 95% hits, ApsB and AcuE showed only 14% colocalization, indicating that ApsB was transported only to a subclass of peroxisomes. In addition, we determined the frequency of mRFP1-ApsB and GFP-HexA colocalization to 10% (Fig. 4C). Similar results were obtained with *S. macrospora*, where Pro40 also colocalized only partially with HexA (5). An interaction between ApsB and HexA in *A. nidulans* was also shown in vivo using the BiFC assay system in strains coexpressing the N-terminal half of YFP (YN) fused to *hexA* and the C-terminal half of YFP (YC) fused to *apsB* or the other way around (Fig. 4D). ApsB-HexA colocalizing spots were found in the cytoplasm and at some septa (10%). To obtain a clearer picture of the ApsB and HexA structures at septa, we used deconvolution and laser-scanning spinning-disc microscopy. ApsB appeared normally as two spots in the center of the septal pore, whereas HexA localized normally on each side of the pore (Fig. 5). In three-dimensional (3D) reconstruction pictures, the spots appeared with a longer shape along the rim of the septum. Time course experiments revealed that ApsB colocalized with the constricting ring during septation (Fig. 6). These data show that at septa ApsB does not localize to peroxisomes or the Woronin body but rather the putative MTOC is embedded in the membrane of the septal pore.

**Mutation of the peroxisomal target sequence in ApsB leads to HexA-like localization at septa.** In order to test the functionality of the PTS2 sequence in ApsB, we mutated the consensus sequence (Q73L and L74R) and fused the modified ApsB protein with GFP (pDV43). The construct (GFP-FIG. 3. Interaction of HexA and ApsB in the yeast two-hybrid system. (A) After crossing of Y2HapsB-BD with pGAD424 (empty vector), Y2HapsB-AD (*apsB*), or Y2HhexA-AD (*hexA*), diploids were grown on YEPD agar lacking leucine and tryptophan. Four colonies of each strain are shown for each combination. From here, colonies were inoculated onto selective YEPD medium lacking leucine, tryptophan, and histidine, which supports growth only in the case of interaction (B). (C) The same as in panel B but with the addition of 3 mM 3-AT to reduce background growth. (D) β-Gal assay of the colonies shown in panel A.

ApsB/empty  ApsB/apsB  ApsB/hexA

B

C

D

FIG. 4. ApsB localizes to a subclass of peroxisomes. (A) mRFP1-ApsB colocalized with GFP-AcuE, a peroxisomal enzyme, at a frequency of 14%, while the remaining 86% did not colocalize (arrowheads). The strain is SDV70b. (B) mRFP1-tagged HexA colocalized with GFP-tagged AcuE (SDV78c) in 95%. Only 5% of the spots were either GFP or mRFP1 labeled (arrowheads). (C) Colocalization of GFP-HexA and mRFP1-ApsB (SDV49-4). The frequency of colocalization was about 10%. (D) Bimolecular fluorescence complementation assay of HexA and ApsB (strain SDV42). Identical results were obtained with strain SDV43.
ApsB PTS2mut was first transformed into wild-type A. nidulans strain TN02A3 (resulting in SDV77) and then introduced into strains in which either hexA was deleted (SDV79) or apsB was mutated (SDV80) or both hexA and apsB were deleted (SDV95). In strain SDV77, the localization pattern of GFP-ApsB PTS2mut at SPBs and at cytoplasmic spots looked like that of wild-type GFP-ApsB (Fig. 7A). However, it was surprising to find that the localization of GFP-ApsB PTS2mut at septa (Fig. 7C) did not show the normal localization of GFP-ApsB (Fig. 7B) but resembled in 70% of the cases the pattern of GFP-HexA (Fig. 7D), whereas in 22% of the cases it was similar to the GFP-ApsB localization. These localization patterns could be achieved through a piggyback import mechanism of GFP-ApsB PTS2mut along with HexA or ApsB, which were still present as fully functional proteins in SDV77. Only in a strain lacking both ApsB and HexA (SDV95), the specific localization of the mutated ApsB protein was lost at septa (Fig. 7E). The results obtained with the last strain clearly argue for a role for peroxisomes in the transport of ApsB to septal MTOCs. It remains to be elucidated how the assembly of the MTOC at septa occurs.

The PTS2 motif of ApsB is important for asexual spore formation. To test whether the altered localization pattern of GFP-ApsB PTS2mut at septa prevents its biological function, we analyzed if the mutated ApsB protein is able to complement the oligosporogenic phenotype produced by an apsB mutation. Therefore, we transformed ApsB alcA(p)::GFP::apsB and the PTS2-mutated ApsB protein (GFP-ApsB PTS2mut) into strain AJC1.5 (apsB6). Under repressing conditions (glucose), all three strains showed brown colonies, due to the reduced numbers of spores, which is typical for apsB mutant strains (4, 32). Under inducing conditions (glycerol or sorbitol), however, wild-type ApsB protein was able to complement the oligosporogenic phenotype (spores were produced), while PTS2-mutated ApsB did not complement it (Fig. 8A and B).

As we previously described, ApsB is important for the production of MTs at sMTOCs (35). Therefore, we wanted to know if the failure of PTS2-mutated ApsB to complement the oligosporogenic phenotype was due to an inability to restore the MTOC activity at septa. In a GFP-ApsB PTS2mut strain with GFP-labeled MTs and an apsB6 background, the number of MTs was similar to the number of MTs in apsB mutant strains (data not shown). Therefore,
we assume that PTS2 of ApsB is important for its function at septa.

Next we tested if the function of PTS2-mutated ApsB can be restored by adding a PTS1 targeting sequence (SRL) at the C terminus of ApsB. We transformed PTS2-mutated ApsB with the PTS1 signal fused to the C-terminal part of the protein (GFP-ApsB_PTS2mut_SRL) expressed from the alcA promoter into strain AJC1.7 (apsB10), resulting in SNZ34, and into apsB deletion strain (SRS24), resulting in SNZ94. The apsB10 mutation converts codon 83 into a stop codon, and thus the mutant lacks most of the 1,052-amino-acid-long ApsB protein (data not shown). The transformed plasmids were integrated ectopically. Transformants of both strains (SNZ34 and SNZ94) appeared with the brown apsB mutant-like phenotype under repressing conditions (glucose) and a wild-type-like, spore-producing phenotype under inducing conditions (sorbitol). These results suggest that the ApsB-PTS1 protein was able to complement the developmental phenotype (Fig. 8).

DISCUSSION

In this paper, we show that ApsB interacts with gamma-tubulin at SPBs, at septa, at the tips of growing hyphae, and in spot-like structures in the cytoplasm. This is the first evidence for the presence of gamma-tubulin at septa and in the hyphal tip region. We had evidence before that MTOCs exist at septa, but the nature of these MTOCs remained elusive (35). Our new results show that at least two other proteins associated with nuclear MTOCs exist in septal MTOCs, GcpC and the crucial protein gamma-tubulin. These findings are in agreement with the recent localization of GcpC (40). However, it is still unclear if sMTOCs share more or all proteins with nuclear MTOCs or whether specific proteins exist only at one or the other place. The biggest unsolved question is still the anchorage of sMTOCs. Structurally, the nuclear MTOC of S. cerevisiae has been studied the best and recently similar results were obtained with A. gossypii (14, 18, 19). It is likely that the situation is similar in A. nidulans and that nuclear MTOCs are embedded in the nuclear envelope. However, structural information about sMTOCs is still missing. Our fluorescence microscopy studies indicate that the MTOC appears as two dots inside the septal rim. The structure is clearly different from that of Woronin bodies at septa. Sometimes the two ApsB dots appeared to be connected through a third small dot. This has been described before in S. pombe for the equatorial MTOCs (eMTOCs), which are also characterized by the ApsB-homologous protein mto1 (formerly named mod20 or mbo1) (10, 29). In this yeasts species, MTs are generated from nuclear MTOCs, eMTOCs, and interphase MTOCs (10, 28). The importance of
non-nucleus-associated MTOCs was nicely demonstrated in enucleate cells (3).

We also identified the ApsB–gamma-tubulin interaction in the tips of growing hyphae. This is also the first evidence for gamma-tubulin in the hyphal tip. In comparison, in the chytridiomycete Allomyces macrogynus, gamma-tubulin has been identified as a component of the Spitzenkörper (21). Further evidence that gamma-tubulin may be functional in the hyphal tip comes from our observation that some MTs emanate from the hyphal tip and grow into the cytoplasm (16). We speculated at the time that either MTs which did not stop growth after reaching the hyphal tip or MT fragments close to the hyphal tip could be the origin of polymerization. However, our new results point to the possibility that MTOCs exist in the apical region of the hypha.

Several lines of evidence show that the spot-like appearance of ApsB and the ApsB–gamma-tubulin interaction are due to peroxisomal localization: colocalization with AcuE and HexA and the drastic reduction of the number of cytoplasmic spots in a pexC mutant. One very strong argument is the importance of the PTS2 sequence and the rescue of the PTS2 mutation by the addition of a PTS1 sequence to the C terminus. The nonfunctionality of ApsB with a mutated PTS2 sequence could still be explained by the fact that this region appears to be evolutionarily conserved from yeast to humans (29), but the rescue of the mutation by the addition of the PTS1 sequence speaks clearly against this possibility. We envisage three possible explanations for the role of the peroxisomal localization. (i) Peroxisomes serve as hosts for sMTOCs. (ii) Peroxisomes catalyze a reaction that is required for MTOC function at the septum and is ApsB dependent. (iii) Peroxisomes serve as transport vehicles for sMTOC-associated proteins. Our results point to a transport function for peroxisomes. In agreement with such a role is the observation of fast-moving mto1 (ApsB) spots in S. pombe (29). These structures could represent peroxisomes. However, many open questions remain to be solved, e.g., how the proteins are further recruited from the peroxisomes to the sMTOCs. Against all three possibilities speaks the observation that the septal localization of ApsB and sMTOC function in mutants with defects in PTS1 or PTS2 peroxisomal protein import or in pexC mutants lacking peroxisomes appeared sim-

![Fig. 8. The peroxisomal target sequence of ApsB is important for complementation of the oligosporogenic phenotype of apsB mutants.](image-url)
ilar to the situation in the wild type (results not shown). How-
never, it has to be considered that the pexC mutant strain dis-
plays pleiotropic phenotypes and that the possibility of a
piggyback import mechanism might mask the possible effects of
PTSI or PTSS defects (12).

From our results we conclude that ApsB defines a new class
of peroxisomes that is—besides the Woronin bodies—the sec-
ond example of peroxisomes as organelles with a function
beyond metabolic pathways (30).

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