The Cell End Marker Protein TeaC Is Involved in Growth Directionality and Septation in Aspergillus nidulans

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Received 25 July 2008/Accepted 30 April 2009

Polarized growth in filamentous fungi depends on the correct spatial organization of the microtubule (MT) and actin cytoskeleton. In Schizosaccharomyces pombe it was shown that the MT cytoskeleton is required for the delivery of so-called cell end marker proteins, e.g., Tea1 and Tea4, to the cell poles. Subsequently, these markers recruit several proteins required for polarized growth, e.g., a formin, which catalyzes actin cable formation. The latest results suggest that this machinery is conserved from fission yeast to Aspergillus nidulans. Here, we have characterized TeaC, a putative homologue of Tea4. Sequence identity between TeaC and Tea4 is only 12.5%, but they both share an SH3 domain in the N-terminal region. Deletion of teaC affected polarized growth and hyphal directionality. Whereas wild-type hyphae grow straight, hyphae of the mutant grow in a zig-zag way, similar to the hyphae of teaA deletion (tea1) strains. Some small, anucleate compartments were observed. Overexpression of teaC repressed septation and caused abnormal swelling of germinating conidia. In agreement with the two roles in polarized growth and in septation, TeaC localized to hyphal tips and to septa. TeaC interacted with the cell end marker protein TeaA at hyphal tips and with the formin SepA at hyphal tips and at septa.

Filamentous fungi represent fascinating model organisms for studying the establishment and maintenance of cell polarity, because cell growth takes place at the tip of the extremely elongated hyphae. Hyphal extension requires the continuous expansion of the membrane and the cell wall and is driven by continuous fusion of secretion vesicles at the tip (8, 12). The transportation of vesicles is probably achieved by the coordinated action of the MT and the actin cytoskeleton. According to one model, vesicles first travel along MTs, are unloaded close to the hyphal tip, where they form a microscopically visible structure the “Spitzenkörper,” which is also called the “vesicle supply center,” referring to the assumed function (24, 25). For the last step, vesicle transportation from the Spitzenkörper to the apical membrane, actin-myosin-dependent movement is used. Anti-cytoskeletal drug experiments have shown that hyphae can grow for some time in the absence of MTs but not in the absence of the actin cytoskeleton (14, 27, 30a).

In Schizosaccharomyces pombe it was shown clearly that the polarization of the actin cytoskeleton depends on the MT cytoskeleton (2, 7). In 1994, polarity mutants of S. pombe were isolated and subsequent cloning of one of the genes identified the polarity determinant Tea1 (19, 29). Because this protein labels the growing cell end, this and other subsequently isolated proteins of this class were named cell end markers. It was shown that cell end localization of Tea1 requires the activity of a kinesin motor protein, Tea2, which transports the protein to the MT plus end (3). Together with the growing MT, Tea1 reaches the cortex, where it is unloaded and binds to a prenylated and membrane-anchored receptor protein, Mod5 (28). The formin For3, which catalyzes actin cable formation, is recruited to the tip through binding to another cell end marker protein, Tea4, which confers tethering to Tea1 (7, 18, 33). Tea4 is required for For3 localization at the cell tip, specifically during initiation of bipolar growth (18).

Recently, it was shown that components of this polarity determination machinery are conserved in the filamentous fungus A. nidulans (8). The first component identified was the Tea2 homologue, KipA, a kinesin-7 motor protein (16). Deletion of the gene did not affect hyphal tip extension but polarity determination. Instead of growing straight, hyphae grew in curves. KipA moves along MTs and accumulates at the MT plus end. The identification of Tea1 and a Mod5 homologue was more difficult, because the primary structure of these cell end marker proteins is not well conserved in filamentous fungi. A Tea1 homologue, TeaA, only displayed 27% sequence identity. However, the presence of Kelch repeats in both proteins suggested conserved functions (31). A Mod5 homologue was identified by a conserved CAAX prenylation motif at the C terminus. Systematic analyses of proteins with such a motif in the A. nidulans genome led to the identification of TeaR. Like Tea1 and Mod5, TeaA and TeaR localize at or close to the hyphal membrane at the growing cell end (31). However, correct localization of TeaR requires TeaA. In addition, sterol-rich membrane domains define the place of TeaR attachment to the hyphal tip. In contrast to S. pombe, TeaA and TeaR are still transported to the hyphal tip in the absence of the motor protein KipA, but their localization is disturbed in comparison to wild type. This suggests that other proteins are necessary for exact TeaA positioning, whose localization depends on KipA.

We characterized a homologue of the S. pombe cell end
Genomic DNA and the primers TeaC-left-for (5'-H11032 clones, and downregulation of the gene through the inducible alcA deletion event was confirmed by crosses, recomplementation with studies and named SYH21. Coupling of the observed phenotypes with the gene formed as described previously (26).

Genomic DNA was extracted from sequencing was done commercially (MWG Biotech, Ebersberg, Germany). were applied using a Biometra TRIO Thermoblock for the reaction cycles. DNA were amplified by PCR using A. nidulans (MM) and complete medium for 1. Standard laboratory Escherichia coli (37) and Molecular techniques. Standard DNA transformation procedures were used with the DNeasy plant minikit for the upstream region of teaC-PR(5'-CGG TAC CAT GGT GAG CA A GA GGA GGT ACC A GTT GGT GGT ATT GTA GGT TT 3') and teaC-PR(5'-CGG TAC CAT GGT GAG CA A GA GGA GGT ACC A GTT GGT GGT ATT GTA GGT TT 3') into the corresponding sites of pCMB17apx, yielding pYH01 and pYH09 (39). This study

Deletion of teaC. Flanking regions of teaC were amplified by PCR using genomic DNA and the primers TeaC-left-for (5'-GAA CAG TTG CCT TTC AGG CCG TAG CAG GAT GTT CAA AGG AGG AGG TAC CGC TAG 3') and TeaC-left-rev-SfiI (5'-TGG TGG CCA TCT AGG CCG TAG 3') for the upstream region of teaC and the primers TeaC-right-for-SfiI (5'-AAT AGG CCT GAT TGG CCA CCT CGC CCT GCA CCA CTA C-3') and TeaC-right-rev (5'-AGA GGC TGG ATT CCT TCT 3') for the downstream region and cloned into pCR2.1-TOPO to generate plasmids pYH32 and pYH33, respectively. The SfiI restriction sites are underlined. In a three-fragment ligation, the N. crassa pry-4 gene was released from plasmid pS11 with SfiI and ligated between the two teaC-flanking regions, resulting in the vector pYH14.

Transformants of strain TN02A3 were screened by PCR for the homologous integration event. Single integration of the construct was confirmed by Southern blotting (see Fig. S1 in the supplemental material). A total of 20 strains were analyzed, of which 8 showed the teaC deletion pattern. All eight deletion strains displayed the same phenotypes. One teaC deletion strain was selected for further studies and named SYH22. Coupling of the observed phenotypes with the gene deletion event was confirmed by crosses, recombination with teaC-derived clones, and downregulation of the gene through the inducible alcA promoter.

Tagging of proteins with GFP and mRFP1. To create an N-terminal green fluorescent protein (GFP) fusion construct of TeaC, a 1.2-kb N-terminal fragment of teaC (starting from ATG) was amplified from genomic DNA with the primers teaC-AR(5'-TAG GCG CCG CCG TGG CTA GAC CTA GAA TGG 3') and teaC-PR(5'-CGG TAC CAT GGT GAG CA A GA GGA GGT ACC A GTT GGT GGT ATT GTA GGT TT 3') and teaC-PR(5'-CGG TAC CAT GGT GAG CA A GA GGA GGT ACC A GTT GGT GGT ATT GTA GGT TT 3') digested with AvrI and KpnI, and ligated with AvrI-KpnI-digested YH24. Using the same approach as for TeaC, N-terminal GFP fusion constructs of TeaC were created. All plasmids were transformed into the uracil-auxotrophic strain TN02A3 (Δnek4a). The integration events were confirmed by PCR and Southern blotting (results not shown).

For bimolecular fluorescence complementation (BiFC) analyses, the N-terminal half of GFP (YFPN) was fused to the N terminus of the protein of interest. YFPN (154 amino acids of YFP and 5 amino acids linker) was amplified with the primers fwd-Kpn-YFPN-P (5'-CGG TAC CAT GGT GAG CA A GA GGA GGT ACC A GTT GGT GGT ATT GTA GGT TT 3') and reverse-YFPN-N-Li-Asc (5'-CGG CCG GCC GCG GTG GAT GGA GGA GGT ACC A GTT GGT GGT ATT GTA GGT TT 3') digested with AvrI and KpnI, and ligated with AvrI-KpnI-digested YH24, yielding YHP06 (alcA promoter replaced with the native promoter in YH24). Using the same approach as for TeaC, N-terminal GFP fusion constructs of TeaC were created. All plasmids were transformed into the uracil-auxotrophic strain TN02A3 (Δnek4a). The integration events were confirmed by PCR and Southern blotting (results not shown).

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

| Strain | Genotype | Source or reference |
|--------|----------|---------------------|
| TN02A3 | pygG89; argB2; ΔnkaA::argB; pyroA4 | 22 |
| RMS011 | pabaA1 yA2; ΔargB::trpcCΔB; pyroA4 | 30 |
| SRL1  | ΔkipA::pyr4; pygG89; pyroA4 (ΔkipA) | 16 |
| SFW02 | wa3; ΔargB::trpcCΔB; pyroA4; alcA(Δ::GFP::nubA) (GFP-MTs) | 6 |
| SSSK1 | SRF200 transformed with pSK76, ΔteaA::argB; pygG89; ΔargB::trpcCΔB; pyroA4 (ΔteaA) | 16 |
| SNT17 | ppyG89; wA3; alcA(Δ::GFP::kipA::rigoG (GFP::kipA::rigoG) | 31 |
| SNT19 | TN02A3 transformed with pNT28 [teaA(Δ::mRFP1-teaA)] | 31 |
| SNT28 | TN02A3 transformed with pNT9 (GFP-sepA) | 31 |
| SNT52 | SNT49 crossed to RMS011 [teaA(Δ::mRFP1-teaA)] | 31 |
| SYH03 | TN02A3 with pYH06 [alcA(Δ::GFP::teaA)] | This study |
| SYH05 | TN02A3 transformed with pYH03 and pYH09 (YFPN-sepA and YFPN-teaC) | This study |
| SYH06 | TN02A3 transformed with pYH01 and pYH09 (YFPN-teaA and YFPN-teaC) | This study |
| SYH13 | SYH03 crossed to SNT52 [GFP::teaC, teaA(Δ::mRFP1-teaA)] | This study |
| SYH17 | TN02A3 transformed with pYH30 [teaC(Δ::mRFP1-teaC)] | This study |
| SYH18 | SYH20 crossed to SNT28 [teaC(Δ::mRFP1-teaC) and GFP-SepA] | This study |
| SYH19 | SYH20 crossed to SSK91 [teaC(Δ::mRFP1-teaC) and ΔteaA] | This study |
| SYH20 | SYH20 crossed to SSK91 [teaC(Δ::mRFP1-teaC) and ΔteaA] | This study |
| SYH21 | TN02A3 transformed with pYH14 (ΔteaC) | This study |
| SYH22 | SYH20 crossed to SFW02 [GFP-MT and teaC(Δ::mRFP1-teaC)] | This study |
| SYH23 | SYH20 crossed to SNT17 [GFP-kipA4 and teaC(Δ::mRFP1-teaC)] | This study |
| SYH24 | SYH21 crossed to SNT52 [teaA(Δ::mRFP1-teaA) and ΔteaA] | This study |
| SYH25 | SYH17 crossed to SRL1 [ΔkipA and teaC(Δ::mRFP1-teaC)] | This study |
| SYH26 | SYH28 crossed to SYH27 (GFP-MTs and GFP-SepA) | This study |
| SYH27 | TN02A3 transformed with pYH24 (mRFP1-TeaC) | This study |
| SYH28 | RMS011 crossed to SNT28 (GFP-sepA) | This study |

a All strains are veA1.
(356 amino acids) with the primers TeaC-EMF (5′-GGC CGA ATC CAT GCC TAG ACC TAG AAT GG-3′) and TeaC-BMR (5′-GGA TCC TTA AGC TCG TCG TCA AG-3′) or corresponding to the C-terminal half of TeaC (428 amino acids) with the primers TeaC-EMF (5′-GAA TTC GAA AAG CCG CGC TTA AGC ATT CAT GGC-3′) and TeaC-BMR (5′-GGA TCC TTA AGC TCG TCG TCA AG-3′) or corresponding to the C-terminal half of TeaC from pYH17 and pYH19 were amplified and cloned into the pGBK7 vector, which contains the Gal4 DNA-BD and the TRP1 marker, yielding pYH17 and pYH19 (BD Clontech). The fragments of TeaC cDNA corresponding to the N-terminal half and C-terminal half of TeaC from pYN18 and pYN19 and amplified and cloned into the pGal4T7 vector, which contains the GAL4 DNA-AD and the LEU2 marker (BD Clontech), yielding pYN18 and pYN19. The fragments from pYH06 in pDV8 and pYN19 from pYN18 were ligated with SfiI sites 16 kb from pCS1 and pCMB17apx. The system utilizes two reporter genes (his3 and lacZ) under the control of the GAL4-responsive UAS. The β-galactosidase activity was analyzed by liquid culture assays using ONPG (o-nitrophenyl-β-D-galactopyranoside; Sigma) as a substrate. The activity was calculated in Miller units (20). Experiments were repeated three times.

**Light and fluorescence microscopy.** For live-cell imaging of germlings and young hyphae, cells were grown on coverslips in 0.5 mL of MM plus 2% glycerol (derepression of the alc4 promoter and thus moderate expression of the gene), MM plus 2% threonine (activation of the gene thus high expression levels), or MM plus 2% glycerol. The mycelium was harvested by filtration through Miracloth (Calbiochem, Heidelberg, Germany), dried by pressing between paper towels, and immediately frozen in liquid nitrogen. After the mycelium was ground in liquid nitrogen, the material was resuspended in protein extraction buffer (50 mM Tris-HCl [pH 8], 150 mM NaCl) supplemented with protease inhibitors (1 mM phenylmethylsulfonyl fluoride, 10 μM leupeptin, 1 μM pepstatin). Protein extracts were clarified twice by centrifugation (centrifuge 5403; Eppendorf, Hamburg, Germany) at 13,000 rpm at 4°C for 10 min. The protein concentration was quantified with a Roti-Quant kit (Roth, Karlsruhe, Germany). After denaturation of the samples, protein extracts were loaded on a 7.5% sodium dodecyl sulfate-polyacrylamide gel. For blotting, nitrocellulose membranes from Schleicher & Schuell (Dassel, Germany) were used. For Western blotting, we used rabbit polyclonal anti-GFP (Sigma-Aldrich, Munich, Germany) at a dilution of 1:4,000 and polyclonal anti-rabbit antibodies coupled to peroxidase (product A0545; Sigma-Aldrich) at a dilution of 1:4,000.

**RESULTS**

**Isolation and deletion of teaC.** We searched the A. nidulans genome at the Broad Institute (Cambridge, MA) (http://www.broad.mit.edu/annotation/genome/aspergillus_group/MultiHome.html) for proteins with similarity to the cell end marker protein Tea4 from S. pombe and identified one open reading frame (AN1099.3) (11). DNA and cDNA sequences were confirmed, and five introns with lengths of 66, 60, 66, 46, and 45 bp were determined. We named the gene teaC, although sequence similarity between the 687-amino-acid A. nidulans protein and the 810-amino-acid S. pombe protein concentration of 60 μg/ml in the medium. Hoescht 33342 (Molecular Probes) was used at a final concentration of 10 μg/ml in the medium.

**Protein extracts and Western blotting.** For induction of the alc4 promoter, A. nidulans cultures were shaken in MM containing 2% threonine or 2% glycerol for 48 h. The mycelium was harvested by filtration through Miracloth (Calbiochem, Heidelberg, Germany), dried by pressing between paper towels, and immediately frozen in liquid nitrogen. After the mycelium was ground in liquid nitrogen, the material was resuspended in protein extraction buffer (50 mM Tris-HCl [pH 8], 150 mM NaCl) supplemented with protease inhibitors (1 mM phenylmethylsulfonyl fluoride, 10 μM leupeptin, 1 μM pepstatin). Protein extracts were clarified twice by centrifugation (centrifuge 5403; Eppendorf, Hamburg, Germany) at 13,000 rpm at 4°C for 10 min. The protein concentration was quantified with a Roti-Quant kit (Roth, Karlsruhe, Germany). After denaturation of the samples, protein extracts were loaded on a 7.5% sodium dodecyl sulfate-polyacrylamide gel. For blotting, nitrocellulose membranes from Schleicher & Schuell (Dassel, Germany) were used. For Western blotting, we used rabbit polyclonal anti-GFP (Sigma-Aldrich, Munich, Germany) at a dilution of 1:4,000 and polyclonal anti-rabbit antibodies coupled to peroxidase (product A0545; Sigma-Aldrich) at a dilution of 1:4,000.
was restricted to a conserved SH3 domain (Fig. 1A). Within this 86-amino-acid domain the identity was 63% with an e-value of $6e^{-27}$. Outside this region, the homology was very low, and the overall identity was only 12.5%. The sequence similarity to the *S. cerevisiae* Bud14 protein, which shares some functional relationship with Tea4, was even lower. In other aspergilli, proteins with high identities to *A. nidulans* TeaC were found (Fig. 1B). The identity to corresponding proteins from other ascomycetous fungi was again much lower, e.g., *Penicillium chrysogenum* (43%) and *Magnaporthe grisea* (only 22%). Sequence identity to basidiomycetous species was less than 10%. These results indicate that TeaC sequences are only poorly conserved within fungi.

To analyze the function of teaC in *A. nidulans*, we constructed a teaC deletion strain (SYH21; see Materials and Methods) and compared it to a ΔteaA deletion strain constructed previously (31). Both strains produced compact and small colonies (75 to 85% of the diameter) compared to the wild type. The growth defect was slightly more severe in the ΔteaA deletion strain (Fig. 2A). In both strains hyphae displayed a zig-zag growth phenotype compared to straight hyphae in the wild type (Fig. 2B). The effect on the maintenance...
of growth directionality was also stronger in the ΔteaA strain. The mutant phenotype was mainly seen in germlings.

The ΔteaC mutants also showed an increased number of septa and branches, probably due to the reduced extension rate of the hyphae and not specific for the teaC deletion (Fig. 2C and see Fig. S2 in the supplemental material). In addition, we noticed some small compartments (5 μm; 8% [n = 100]), which did not contain any nucleus (Fig. 2D). This did not occur in the wild type or in slower-growing A. nidulans strains.

Another polarity defect has been detected in the way the second germ tube emerges from a conidiospore. In the wild type, the second hypha emerges opposite to the first germ tube (bipolar) in 83% of the spores. In contrast, the second hyphae emerged at random positions in 56% of the spores in the ΔteaC mutant strain. A similar defect was observed in 55% of the spores in the ΔteaA mutant (n = 100) (Fig. 2E). This suggests that teaA and teaC are required for the selection of the site of polarity initiation.

**Overexpression of TeaC inhibits septum formation.** We next tested whether overexpression of TeaC would also cause any morphological phenotype. We expressed teaC using the control of the alcA promoter under inducing conditions (threonine; see Materials and Methods). When the GFP-TeaC strain was grown on threonine MM colonies appeared smaller than the ones from wild type (Fig. 3A). The high expression level of TeaC was shown by Western blotting and an increase in GFP fluorescence (Fig. 3B and C; see also below). Under inducing conditions, we often observed lysis at the hyphal tips (Fig. 3D) and rarely branching of hyphae. Furthermore, the number of conidia that produced a second germ tube was reduced from 66% to 17% (n = 100) (Fig. 3E). Some conidia without a second germ tube grew continuously isotropic up to 20 μm in diameter after 2 days. Some hyphae appeared to be empty, probably because of leakage of the cytoplasm. This was visualized by calcofluor white staining of the cell wall and GFP staining of the cytoplasm. Whereas the spore on the right side in Fig. 3F showed fluorescence in both channels, the left spore showed only fluorescence of the cell wall, indicating an empty spore.

When we analyzed the number of septa, we found that teaC overexpression repressed almost completely septation (Fig. 4A). Because TeaC interacts with SepA and possibly regulates the localization and/or the activity (see below), we sought to determine which phenotype would be caused by overexpression of TeaC, together with the overexpression of SepA. The displayed phenotype was similar to the phenotype of teaC overexpression, namely, very few septa (Fig. 4B and C). In addition, conidia were swollen, hyphae had an irregular shape, and hyphal tips appeared very large. To quantify this effect, we counted the number of septa in germlings, which were between 100 and 300 μm in length (n = 50) (Fig. 4C). Overexpression of SepA alone did not cause a reduction of the number of septa, and the morphology was less severely affected (data not shown).

**Localization of TeaC at the hyphal tip depends on microtubules (MTs) but not on the kinesin KipA.** To investigate the subcellular localization of TeaC, we constructed an A. nidulans strain expressing a GFP-TeaC fusion protein under the control of the alcA promoter (see above and Materials and Methods). The construct was transformed into strain TN02A3, and a transformant was selected in which the construct was integrated into the teaC locus (SYH03). This leads to duplication of the 5′ end of the gene under the control of the endogenous promoter and the GFP-teaC fusion construct under the control of the alcA promoter. Under repression conditions, the strain displayed the ΔteaC deletion phenotype (see above). Under derepressed conditions, wild-type hyphal morphology was obtained. This experiment showed that the observed phenotypes in the ΔteaC deletion strain were indeed caused by the deletion and not by another mutation, and it showed that the GFP-TeaC fusion protein was functional. GFP-TeaC localized to
one bright point at all hyphal tips and at newly forming septa (Fig. 5A). In addition, some weaker points on the side of the tip were observed. The GFP-TeaC points always localized close to the plasma membrane or appeared attached to it. Furthermore, some spots appeared in the cytoplasm, which could represent the MT plus ends (see also Fig. 6A).

In growing tips, the Spitzenkörper has an important role as vesicle supply center. To compare the localization of TeaC with the Spitzenkörper, we stained hyphae with FM4-64, which has been used in several fungi to stain the Spitzenkörper (9, 23, 31). The Spitzenkörper appeared to colocalize with GFP-TeaC (Fig. 5B), although from these pictures it was not unambiguously clear whether TeaC is part of the Spitzenkörper or only attached to the membrane and therefore very close to the Spitzenkörper.

To confirm TeaC localization and to prove that the observed localization was not an artifact of the expression under alcA promoter control, we constructed a strain (SYH17) producing mRFP1-TeaC under the control of the native promoter. SYH17, in which mRFP1-TeaC is the only source of TeaC, did not show the phenotype observed in the ΔteaC mutant, indicating that mRFP1-TeaC at this expression level was biologically functional. Although signals of mRFP1 appeared much weaker, mRFP1-TeaC still localized to one point, and weaker signals were observed along the apex and at forming septa (Fig. 5C). The single spot of mRFP1-TeaC normally localized at the center of the hyphal apex (Table 3). At a small number of tips (13%, n = 100), mRFP1-TeaC localized along the tip membrane. For further experiments, we normally used strains producing mRFP1-TeaC under the native promoter, when we analyzed the localization of TeaC.

To test whether TeaC localization at the hyphal tip depends on MTs, we studied a strain with GFP-labeled α-tubulin and mRFP1-TeaC (SYH22) and found that TeaC localized to the plus end of the MTs, although the signal intensity was close to the detection limit (Fig. 6A). Next, we used the MT-destabilizing drug benomyl. After this treatment (2.5 μg of benomyl/ml), almost all fluorescence of GFP-MTs was diffused into the cytoplasm within 5 min (data not shown), and the mRFP1-TeaC point at the tips was sometimes divided into several points and disappeared after 30 to 40 min from >80% of the tips (n = 100) (Fig. 6B and Table 3). The delay between the disappearance of the TeaC spot and the disassembly of the MTs suggests that TeaC is rather stable at the cortex.
To analyze whether TeaC is transported in a kinesin-7-dependent way to the MT plus end, we studied TeaC localization in a ΔkipA mutant. mRFP1-TeaC still localized to one point at nearly 80% of the tips (n = 100), indicating KipA-independent polarization. However, the mRFP1-TeaC point did not localize to the center of the apex and moved away to the side of the hypha (Fig. 6C and Table 3). We further studied this effect by localization of TeaC in a KipA<sup>igoA</sup> mutant, in which KipA (fused to GFP) harbors a point mutation in the ATP-binding domain (16, 38). GFP-KipA<sup>igoA</sup> binds but does not move along MTs, and thus decorates them. In this strain (SYH23) mRFP1-TeaC still localized at 90% of the tips, but the mRFP1-TeaA point often moved away to the side of the apex and sometimes divided into two points (Table 3). These results indicate that TeaC can be transported independently of KipA to the tip and that other KipA-transported proteins are probably required for exact positioning of TeaC. We are assuming that the key for further understanding of the cell end marker protein complex lies in the characterization of cargoes of the KipA motor protein.

We investigated also the effect of cytochalasin A (2 μg/ml), an inhibitor of actin polymerization, on TeaC localization. mRFP1-TeaC dispersed around the tips within 50% of the tips (n = 100) by 2 to 10 min after the treatment (Fig. 6D and Table 3) compared to the control strain only treated with 0.02% dimethyl sulfoxide. Taken together, our results suggest that TeaC localization depends on the MT and the actin cytoskeleton.

**TeaC connects TeaA and SepA.** To investigate whether *A. nidulans* TeaC colocalizes with TeaA, we constructed a strain (SYH13) expressing mRFP1-TeaA and GFP-TeaC and compared their localization patterns. The mRFP1-TeaA point at the apex colocalized with that of GFP-TeaC. If mRFP1-TeaA localized at two points (10%, n = 100) TeaC also colocalized to the same spots (Fig. 7A). Likewise, we tested colocalization of TeaC with SepA. We constructed a strain (SYH18) expressing GFP-SepA and <i>teaC(p)-mRFP1-teaC</i>. TeaC and SepA did not obviously colocalize at the hyphal apex. When SepA localized at the hyphal apex, TeaC appeared adjacent to the SepA signal (11%, n = 100). When TeaC localized at the hyphal apex at one point, SepA did not localize at the hyphal apex (89%, n = 100) (Fig. 7B).

We also found no obvious colocalization at septa (Fig. 7C). It appeared that TeaC surrounded SepA (Fig. 7C). While doing time course experiments, we found that SepA and TeaC follow a constricting ring but that TeaC constriction was slightly delayed in comparison to SepA (Fig. 7D and E).

Next, we sought to determine whether TeaA/TeaC and TeaC/SepA would interact. This was studied by BiFC. The N-terminal half of YFP (YFP<sup>N</sup>) was fused to SepA and TeaA, and the C-terminal half of YFP (YFP<sup>C</sup>) was tagged with TeaC. Strains expressing only YFP<sup>N</sup>-SepA, YFP<sup>N</sup>-TeaA, or YFP<sup>C</sup>-TeaC showed no YFP fluorescence. In contrast, in the strain expressing both YFP<sup>N</sup>-SepA and YFP<sup>C</sup>-TeaC produced YFP signals as a single point and along the apex and at hyphal septa signals (11%, n = 100) (Fig. 7B).

| Category          | mRFP1-TeaC localization pattern (%) |
|-------------------|-------------------------------------|
| Wild type         | ![Wild type](image)                  |
| Wild type         | ![Wild type with benomyl](image)    |
| ΔkipA<sup>mutant</sup> | ![ΔkipA<sup>mutant</sup>](image)   |
| KipA<sup>igoA</sup> mutant | ![KipA<sup>igoA</sup> mutant](image) |
| Wild type with cytochalasin A | ![Wild type with cytochalasin A](image) |
| ΔteaA<sup>mutant</sup> | ![ΔteaA<sup>mutant</sup>](image)   |

*The localization pattern of mRFP1-TeaC of 100 to 200 hyphal tips was analyzed and grouped into six different categories. The construct was expressed from the <i>teaC</i> promoter. The numbers indicate the percentages of the hyphal tips of these groups. Hyphae were grown on minimal medium with glycerol as a carbon source for 1 day. For drug treatment, hyphae were incubated in the presence of 2.5 μg of benomyl/ml for 30 min or 2 μg of cytochalasin A/ml for 2 to 10 min.*
In the case of the combination of YFP N-TeaA and YFPC-TeaC, YFP signals were detected only at the hyphal tip (Fig. 7G). We also tested YFP N-TeaR (the membrane anchor protein) and YFPC-TeaC interaction, but no YFP fluorescence was observed (data not shown). The observed interactions were analyzed with the yeast-two hybrid system. TeaA showed self-interaction and interaction with TeaC. In both cases, the C termini of TeaA and TeaC were important for the interaction (Fig. 8). Interaction of TeaC and SepA could not be detected in this assay, because SepA alone already induced the expression of the yeast two hybrid reporters (data not shown).

**Localization dependency of TeaC and TeaA.** We analyzed TeaC localization in a ΔTeaA mutant and found that the correct positioning of TeaC depends on TeaA (Fig. 9A and Table 3). In the absence of TeaA, TeaC appeared dispersed in the cytoplasm. We also analyzed TeaA localization in a ΔTeaC mutant. mRFP1-tagged TeaA did not concentrate in one point in the apex but appeared as several points along the membrane (Fig. 9B).

**DISCUSSION**

Polarized growth of *A. nidulans* is achieved through a collaborative action of the MT and the actin cytoskeleton, in which cell end marker proteins regulate polarity maintenance through the putative conserved mechanism of *S. pombe* (8). In the present study, we analyzed another component of the *S. pombe* machinery, the SH3-domain protein Tea4, which is involved in the transmission of positional information through the interaction with both Tea1 and the formin For3 (18). We show here that in *A. nidulans* TeaC serves functions in polarized growth and in septation. The role of TeaC will be discussed in the light of recent findings for *S. pombe* Tea4 and the closest *S. cerevisiae* homologue Bud14 (4, 15).

Deletion of teaC caused a growth defect and zig-zag hyphal phenotype, similar to but not identical to the one of the teaA deletion mutant. Interaction studies revealed that TeaC interacted with TeaA and SepA at hyphal tips. These results suggest that the function of TeaC at the hyphal tips is comparable to the function of Tea4 in *S. pombe*, namely, the recruitment of SepA to the growth zone and hence the polarization of the actin cytoskeleton. However, TeaC and SepA did not obviously colocalize at the hyphal tips, although protein-protein interaction was found. This may be explained by the mechanism of actin polymerization by formin. In *S. pombe*, it was suggested that For3 is activated and promotes actin filament assembly at the cell cortex for only a few seconds, and then For3 is inactivated and released from the cortex by retrograde flow along actin filaments (17). In *S. cerevisiae* it was shown recently that Bud14 displaces the formin from growing barbed ends of actin cables (4). On the other hand, it can also be that SepA is very dynamic at the tip and the interaction with TeaC is only weak and transient.

The zig-zag hyphal phenotype in the teaC-deletion mutant was weaker than that in the teaA deletion mutant, suggesting that the contribution of TeaC to the function for polarity maintenance is lower than that of TeaA. TeaA probably serves additional functions. In fact, in *S. pombe*, Tea1 is necessary for the localization of Pom1, a DYRK-family protein kinase (33). Pom1 kinase interacts with Rga4, which plays a role as GAP for Cdc42, a regulator of F-actin (32).

Besides the action of TeaC at the hyphal tip, we discovered that the protein localized at the septa. Similar to the situation at the hyphal tip, BiFC analysis showed the interaction between TeaC and SepA at septa, but TeaC did not obviously

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**FIG. 7.** Interaction of TeaC with TeaA and SepA. (A) Colocalization of TeaC and TeaA in strain SYH18; (B) visualization of TeaC and SepA in strain SYH13 at the hyphal tip; (C) partially colocalization of TeaC and SepA in strain SYH13 at the septa; (D) localization of SepA in strain SNT28; (E) localization of TeaC in strain SYH03; (F) interaction of TeaC with SepA (strain SYH05) shown in the bimolecular fluorescence complementation assay; (G) interaction of TeaC with TeaA (strain SYH06) shown in the bimolecular fluorescence complementation assay. Hyphae are 3 to 4 μm in diameter.
colocalize with SepA. This may be explained again by the release of inactive formin from the plasma membrane at septation sites (17). The importance of TeaC at septa is also shown by our finding that overexpression of TeaC inhibits septation. However, in contrast to the role of SepA, TeaC appears not to be essential for septation, because deletion of teaC still allows normal septation (12a, 12b, 27a). There are several possibilities for how the inhibition effect can be explained. (i) Recently, it was shown that S. cerevisiae Bud14 negatively controls the activity of the formin Bni1 (4). If TeaC negatively controls SepA at septa, then overexpression would cause a reduction of the number of septa. Contradicting this idea is the finding that in S. pombe overexpression of Tea4 led to long actin cables (18). (ii) A large amount of TeaC protein in the cytoplasm could trap SepA in the cytoplasm and prevent specific localization at septation sites. In agreement with this hypothesis is the fact that we did not see any SepA-GFP rings when TeaC or TeaC and SepA were overexpressed and the germlings displayed in Fig. 4C observed in the GFP channel (data not shown). (iii) In S. pombe, the position of interphase nuclei determines the cleavage site through a protein called Mid1. Mid1 is a nuclear protein, which moves out of the nucleus upon phosphorylation and marks the cell cortex in the vicinity of the nucleus for septum formation (15, 21). Likewise, septation in A. nidulans depends on mitosis and the position of the nucleus (35, 36). However, as a difference to S. pombe,
septation does not follow every mitosis, with the result of Mid1 for septum formation in septation does not follow every mitosis, with the result of mid1 homologue along the hyphae in addition to tea4. Such a function for tea4 has been shown recently as the mechanism for tip occlusion of septation in a. nidulans (16). tea4 deletion would thus impair site selection of septation. Indeed, we observed some short and empty compartments. Nevertheless, we did not find more septa close to the hyphal tip in tea4 deletion strains, indicating that as-yet-unknown factors need to locally control the activity of a putative Mid1 homologue along the hyphae in addition to tea4.

Another possibility to explain the role of tea4 in polarized growth and septation is the recruitment of other proteins involved in both processes. One candidate is the protein phosphatase 1 BimG, which is an a. nidulans homologue of S. pombe Dis2 and the s. cerevisiae homologue Glc7 (1, 10). This phosphatase localizes to different places in A. nidulans, among which the membrane at growing tips and septa. The involvement in different processes, such as mitosis and hyphal growth, probably depends on several pathway-specific targets. Although there is evidence in S. pombe that tea4 interacts with Dis2, and the localization pattern of tea4 at the septum resembles the pattern of BimG, the localization pattern of the two proteins at the tip looks different. Whereas BimG localizes along the membrane and excludes the very tip, Tea4 appears to be restricted to the very tip. Therefore, a direct link to Tea4 remains to be shown.

ACKNOWLEDGMENTS

This study was supported by the priority program “Lebensmittel und Gesundheit” of the Landesstiftung Baden Württemberg, by the Center for Functional Nanostructures, and by the DFG. N.T. was a fellow of the Humboldt Foundation.

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