F-box protein Rcy1 regulates the endosome-Golgi transport by ubiquitination of Snc1 in rcy1Δ mutants and an accumulation of (nonubiquitinated) Snc1–K63R in early endosomes, supporting the idea that Rcy1/Skp1 is part of a functional ubiquitin-ligase complex (10).

growth in filamentous fungi depends on the continuous flow of vesicles, which deliver enzymes for cell wall biosynthesis to the growing tip (11). In addition, subapical endocytosis is required for recycling of excess membrane and membrane-bound proteins (12–15). The direction of vesicle flow is strictly regulated through the orientation and polarization of the microtubule and the actin cytoskeletons. Vesicles can be transported along either cytoskeleton for specific purposes. Whereas microtubules are necessary for long-distance transportation of exocytic vesicles and endosomes, actin serves as track for short-distance transportation of secretory vesicles. Deletion of rcy1 leads to an accumulation of Snc1 at early endosomes and causes a cold-sensitive growth defect. Rcy1 is an effector of the Rab GTPase Ypt31/32 and is also necessary for the recycling of the subtilisin-like protease Kex2 and the phospholipid translocase Cdc50/Drs2 (7, 8). In contrast, the Rcy1 orthologue in Schizosaccharomyces pombe, Pof6, is involved in cell separation and is essential for viability (9). The role of Pof6 and Skp1 in cell separation is unclear, but it was speculated that it could involve the exocyst complex. Moreover, it was proposed that Rcy1/Pof6 forms a non-SCF complex with Skp1 during v-SNARE recycling, but more recent studies have shown reduced ubiquitination of Snc1 in rcy1Δ mutants and an accumulation of (nonubiquitinated) Snc1–K63R in early endosomes, supporting the idea that Rcy1/Skp1 is part of a functional ubiquitin-ligase complex (10).

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 grew for at least 4 days, and the samples were then collected and stained with a 1% silver nitrate solution. The stained samples were observed under a light microscope.

The results obtained from the silver staining analysis showed that the filamentous growth in the medium with and without Rcy1 was compared. The filamentous growth in the medium without Rcy1 was significantly shorter and thinner than that in the medium with Rcy1. This indicates that Rcy1 plays an important role in the filamentous growth of A. nidulans.

It is well known that the filamentous growth of filamentous fungi is regulated by various factors including signal transduction, gene expression, and membrane transport. The role of Rcy1 in filamentous growth suggests that it is involved in the regulation of these processes. Further studies are needed to elucidate the molecular mechanism of Rcy1 in filamentous growth.
vesicles prior to fusion with the membrane. In *Aspergillus nidu-
lans*, close to the growing tip, almost all microtubules are oriented
with their plus ends toward the tip, whereas actin filaments have
their origin at the tip membrane. This organization is achieved
through interplay between the two cytoskeletons through the ac-

dovement of v-SNARE, which is necessary for the positioning of cell

**MATERIALS AND METHODS**

**Strains, plasmids, and culture conditions.** Supplemented minimal me-
dia (MM) for *A. nidulans* were prepared as described, and standard strain
construction procedures were performed as previously described (21). A
list of *A. nidulans* strains used in this study is given in Table 1. Standard
laboratory *Escherichia coli* strains (XL-1 blue and Top 10 F') were used.
Plasmids are listed in Table 1.

**Molecular techniques.** Standard DNA transformation procedures
were used for *A. nidulans* (31) and *E. coli* (32). For PCR experiments,
standard protocols were applied using a Biometra Personal cycler (Biome-

tra, Göttingen) for the reaction cycles. DNA sequencing was done com-

dercially (eurofins-MWG-operon, Ebersberg, Germany). Genomic DNA
was extracted from *A. nidulans* with an innuPREP Plant DNA kit (analytikjena,
Jena, Germany). DNA analyses (Southern hybridizations) were performed
as described in reference 32.

**Deletion of **rcyA.** rcyA flanking regions were amplified by PCR using
genoic DNA and primers rcyA-p3 (5’-AGCTGCGGCAAGGCCAGCGAA-3’
and rcyA-p1 (5’-GAAGGATGTTTGGCAATGCTTCTGTTCAAGGA
TTG-3’) for the upstream region of rcyA and rcyA-p5 (5’-ATCAGTGCTC
CTTGAGACATGTCAGCAACCGTAAATG-3’) and rcyA-p8 (5’-CG
TAGCAAGTGTCTTCACCT-3’) for the downstream region. The **pyr**G gene
from plasmid pFNO3 (S. Osmani) was amplified by PCR and used as the
template together with rcyA-flanking regions for the fusion-PCR. The dele-
tation cassette was amplified using the fusion-PCR method (33) and primers
rcyA-p2 (5’-AGCTGCGGCAAGGCCAGCGAA-3’) and rcyA-p7 (5’-AGCT
TGAGACATGTCAGCAACCGTAAATG-3’) and rcyA-p8 (5’-CG
TAGCAAGTGTCTTCACCT-3’) for the downstream region. The **pyr**G gene
from plasmid pFNO3 (S. Osmani) was amplified by PCR and used as the
template together with rcyA-flanking regions for the fusion-PCR. The dele-
tation cassette was amplified using the fusion-PCR method (33) and primers
rcyA-p2 (5’-AGCTGCGGCAAGGCCAGCGAA-3’) and rcyA-p7 (5’-AGCT
TGAGACATGTCAGCAACCGTAAATG-3’) and rcyA-p8 (5’-CG
TAGCAAGTGTCTTCACCT-3’) for the downstream region. The **pyr**G gene

**Tagging of proteins with GFP.** For fluorescence microscopy, a 0.7-kb
fragment of the **rcyA** gene was cloned in the pCMB17apx plasmid (28),
where N-terminal fusions of green fluorescent protein (GFP) to proteins
of interest are expressed under the control of the alcA promoter, containing
Neurospora crassa **pyr**-4 as a selection marker, yielding pSH13. GFP and
**pyr**-4 were replaced with mRFP1 and pycA, yielding pSH30. The resulting
plasmids are listed in Table 2. The primer set used for **rcyA** was
AN10061-AscI (5’-GGGCAGGGCGCTATGGTCAAAAGGCAGGGAAGTGCC-
3’) and AN10061-PacI (5’-TTATAATTGGTATGAGTTCGACCCAT

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

| Strain | Genotype | Reference or source |
|--------|----------|---------------------|
| TN02A3 | pyrG98 argR2 Δnkua::argB pyroA4 | 22 |
| RMS011 | pabaA yA2 ΔargB::ptrpCΔB trpC801 veA1 | 23 |
| SJW02  | wa3 pyrA4 alcA(p)-GFP-tubA ΔargB::ptrpCΔB | J. Warmbold, Marburg, Germany |
| SSK44  | pabaA wa3 ΔargB::ptrpCΔB ΔkipA::pyr4 veA1 | 24 |
| SSK92  | wa3 pyrA4 alcA(p)-GFP-kiptA | 24 |
| SSK114 | pyrG98 ΔargB::trpCΔB pyroA4 veA1 alcA(p)-GFP-kiptG::pyr-4 | 24 |
| LO1535 | fwa1 nicA2 pyrG98 pyroA4 Δbka::argB synA(p)-GFP-synA | 25 |
| SNT56  | pabaA tea(p)-mRFP1-teaA teaR(p)-GFP-teaR | 19 |
| SNZ14  | alcA(p)-GFP-uncA::pyrA4 | 26 |
| SSH01  | TN02A3 transformed with pSH13 [alcA(p)-GFP-rcyA] | This study |
| SSH08  | SSK44 crossed with SSH01 [ΔkipA alcA(p)-GFP-rcyA] | This study |
| SSH14  | TN02A3 transformed with pSH30 [alcA(p)-mRFP1-rcyA pyrG98] | 27 |
| SSH27  | wa3 yA2 ΔargB::ptrpCΔB trpC801 [alcA(p)-GFP-kiptA] | This study |
| SSH36  | ΔrcyA::pyrG**Δ** in TN02A3 pyrA4 | This study |
| SSH37  | SSK92 crossed with SSH14 [alcA(p)-GFP-kiptA alcA(p)-mRFP1-rcyA] | This study |
| SSH43  | SSH27 crossed with SSH36 [alcA(p)-GFP-kiptA ΔrcyA] | This study |
| SSH44  | SNT56 crossed with SSH36 [ΔrcyA teaA(p)-mRFP1-teaA teaR(p)-GFP-teaR] | This study |
| SSH55  | SNZ14 crossed with SSH14 [alcA(p)-gfp-uncA::pyrA4 alcA(p)-mRFP1-rcyA] | This study |
| SSH56  | LO1535 crossed with SSH14 [synA(p)-GFP-synA alcA(p)-mRFP1-rcyA] | This study |
| SSH66  | SSK114 crossed with SSH14 [alcA(p)-GFP-kiptG::pyrA4 alcA(p)-mRFP1-rcyA] | This study |
| SSH67  | TN02A3 transformed with pSH46 [alcA(p)-mRFP1-rcyA ΔΔF-box pyrG98] | This study |
| SSH69  | SSH67 crossed with SSH27 [alcA(p)-mRFP1-rcyA ΔΔF-box alcA(p)-GFP-kiptA] | This study |
| SSH73  | TN02A3 transformed with pSH47 [rcy(p)-GFP-rcyA pyroA4] | This study |
| SSH89  | SSH36 crossed with LO1535 [ΔrcyA synA(p)-GFP-synA] | This study |
| SSH91  | SSK114 crossed with SSH14 [alcA(p)-GFP-kinA::pyrA4 alcA(p)-mRFP1-rcyA] | This study |
| SCoS135| TN02A3 transformed with p1789 [alcA(p)-GFP-naba pyrG98] | C. Seidel, Karlsruhe, Germany |
| SNG67  | SCoS135 crossed with RMS011 [alcA(p)-GFP-naba pabaA1] | This study |
| SSH93  | SNG67 crossed with SSH14 [alcA(p)-GFP-naba alcA(p)-mRFP1-rcyA] | This study |

* All strains harbored the veA1 mutation in addition.
C-3') (cloning sites are underlined). The ΔF box-deletion mutant was generated with the primers Delta-Fbox (5'-GGGGCGGCTTAGATAGGTTCTGTGGGATAGAAGG-3') and AN10061-Pacl, and the PCR fragment was cloned into Ascl-Pacl-digested pSH30. All of these plasmids were transformed into the uracil-auxotrophic TN02A3 (ΔnkuA) strain (Table 1). The integration events were confirmed by PCR and Southern blotting.

Light/fluorescence microscopy. For live-cell imaging of germlings and young hyphae, cells were grown on coverslips in 0.4 ml MM plus 2% glycerol (derepression of the alcA promoter), MM plus 2% glucose (repression of the alcA promoter), or MM plus 2% threonine (induction of the alcA promoter) (35). Cells were incubated at room temperature overnight. Images were captured at room temperature using an Axiopt microscope (Zeiss, Jena, Germany). Images were collected and analyzed with the Axiovision system (Zeiss).

For FM-64 staining, germlings were grown in MM plus 2% threonine medium overnight and stained with 0.3 ml medium containing 10 μM FM-64 (from a stock solution in dimethyl sulfoxide [DMSO]), kept for 15 min, washed in 2.5 ml of medium, and transferred to 2.5 ml of fresh medium (36).

Real-time PCR (RT-PCR). For RNA isolation, mycelium was collected, shock-frozen in liquid nitrogen, and lyophilized. RNA was extracted with a Fungal RNA kit from Omega Bio-Tek following the manufacturer's protocol. RNA samples were obtained from TN02A3 (wild-type strain) and SSH36 (ΔrcyA) strain. For RNA digestion, an Ambion Turbo DNA Free kit was used. For real-time PCR, a Bioline Sensifast SYBR kit and a Fluorescein One Step kit were used according to the manufacturer's protocol. RNA samples were obtained from TN02A3 (wild-type strain) and SSH36 (ΔrcyA) strain. For normalization of the kipA transcript levels, histone 2B primers H2B-RT fwd (5'-GGAGAGTAGGTCTCCTTCCGTGTC-3') and H2B-RT rev (5'-GAAGAGTAGGTCTCCTTCCGTGTC-3') were used.

Western blotting. A. nidulans strains SSH27 (GFP-KipA) and SSH43 (GFP-KipA, ΔrcyA) were cultured in MM plus 2% glucose and strain SSH69 (GFP-KipA, mRFP1-RcyA-ΔFbox) in MM plus 2% threonine and 0.2% glucose for 24 h. The mycelium was ground in liquid nitrogen, resuspended in protein extraction buffer (50 mM Tris-HCl [pH 7.4], 150 mM NaCl, 5 mM EDTA), supplemented with a cocktail of protease inhibitors (Sigma), and centrifuged at 10,000 × g for 15 min. The supernatant (clarified cell lysate) and a MiniProtein system (Bio-Rad) were used for Western blotting following the manufacturer's instructions and a polyclonal anti-GFP antibody (Sigma) and a monoclonal anti-gamma-tubulin antibody (Sigma) as internal controls for the basal protein amount. The signal intensities of the Western blotting bands were measured with a Chemi-Smart-5100 gelpod device (Peqlab) and quantified with the ChemiCap program (peqlab). The wild-type value was set to 100%. The experiment was repeated twice with three different amounts of total protein.

**RESULTS**

Identification of the RcyA F-box protein in a yeast two-hybrid screening with KipA as bait. In a yeast two-hybrid screening with the KipA kinesin-7 motor protein of *A. nidulans*, several interacting proteins were identified (27). One of the candidates characterized here was AN10061. The yeast two-hybrid clone spans 1.4 kb of a cDNA corresponding to a region close to the 3' end of the gene (Fig. 1A). The complete cDNA comprises 2.6 kb and encodes a polypeptide of 880 amino acids, which shares 35% amino acid identity with Sl2 of *Yarrowia lipolytica* (37), 29% identity with Rcy1 of *S. cerevisiae* (6, 38), and 32.5% identity with Pof6 of *S. pombe* (9). In accordance with the budding yeast's name, we used the name rcyA for the *A. nidulans* gene. The *A. nidulans* RcyA polypeptide of between 100 to 850 amino acids also shares 25% identity and 40% similarity with exocyst complex component Sec10. In addition, RcyA contains an F-box domain at the N terminus. Sec10 is conserved in all eukaryotes, and the *A. nidulans* orthologue is AN8879 (39, 40). This *A. nidulans* Sec10 orthologue shares 24% identity and 39% similarity with the human Sec10 protein. At the extreme C terminus of RcyA, a putative prenylation motif (CaaX) was detected using the PrePS program (http://mendel.imp.ac.at/sat/PrePS/index.html). Proteins with these three features (similarity to Sec10, F-box proteins at the N terminus, and a CaaX motif at the C terminus) were found only in fungi, while Sec10 orthologues are present in all eukaryotes (Fig. 1B).

Localization of GFP-RcyA. In order to unravel the molecular function of RcyA, we studied the subcellular distribution of the protein. RcyA was fused to GFP at the N terminus and expressed from the endogenous promoter or the inducible promoter of the alcohol dehydrogenase (*alcA*) (35). With glucose as the carbon source, the promoter is repressed; with glycerol as the carbon source, it is derepressed and expressed at a low level; and with threonine or ethanol as the carbon source, high expression levels can be obtained. The localization pattern of GFP-RcyA expressed under the control of the endogenous promoter was similar to that of GFP-RcyA expressed under control of the *alcA* promoter derepressed condition with glycerol as the carbon source. GFP-RcyA accumulated at the tip in a dynamic manner (Fig. 2A; see also Movie S1 in the supplemental material). The fluorescence signal sometimes moved back from the tip to a subapical region (Fig. 2A and B, arrows). Along hyphae, especially in regions further back, GFP-RcyA moved as small spots bidirectionally (Fig. 2C, arrowheads) and localized in larger accumulations close to the nuclei (Fig. 2B and C, arrows). The small spots moved at 2.0 μm/s on average (1.8 μm/s ± 0.5 μm/s standard deviation [SD]; n = 10) and at a maximum of 3.0 μm/s (Fig. 2C). GFP-RcyA also localized at formin septa (Fig. 2D, arrow) but not at mature septa (Fig. 2D, arrowhead).

**RcyA transport.** Because RcyA was identified as a kinesin-7-interacting protein by yeast two-hybrid screening (27), we anticipated that the dynamics of RcyA would depend on KipA. To test this hypothesis, we studied the localization of RcyA in the absence of KipA. However, no obvious difference from the wild type was observed. GFP-RcyA still accumulated at hyphal tips in the *kipA* deletion strain and moved bidirectionally on small spots at a similar speed (Fig. 3A and data not shown). In addition, the signal of GFP-KipA at microtubule plus ends did not colocalize with that of mRFP1-RcyA (data not shown). In the case of KipA, a rigor version of the motor was used. Such rigor variants of kinesin bind
irreversibly to microtubules (26, 41, 42). In a strain expressing GFP-KipA\textsuperscript{mtn}, mRFP-RcyA did not colocalize along the microtubules decorated with GFP-KipA\textsuperscript{mtn} (Fig. 3B). These results indicate that KipA is not required for RcyA movement and distribution in the hyphae.

The dynamic behavior of GFP-RcyA around the hyphal tip resembled the movement of secretory vesicles. Therefore, we compared the localization of mRFP-1-RcyA with that of GFP-SynA (v-SNARE) as a marker for such vesicles (25; see also Movie S2 in the supplemental material). Since the fluorescent signal of secretory vesicles appeared to be very weak and since the vesicles moved very quickly, it was hard to document exact colocalization in movies, but their dynamic behaviors looked very similar. Besides, SynA and RcyA colocalized at small dots at subapical regions, which might represent the late Golgi or trans-Golgi network (Fig. 3C, arrows) (43). Because conventional kinesin (kinesin-1) is involved in protein secretion, we hypothesized that KipA could be involved in RcyA movement (44–46). Indeed, mRFP-RcyA colocalized with GFP-KipA\textsuperscript{mtn} (Fig. 3D) (47). These results further suggest that RcyA is transported to the hyphal tip on secretory vesicles.

Because the bidirectional movement of RcyA at backward regions resembled the movement of early endosomes (26, 30), we compared the localization of mRFP-1-RcyA with that of GFP-RabA (Rab GTPase) as a marker for early endosomes (Fig. 3E and F; see also Movie S2 in the supplemental material). However, colocalization of RabA and RcyA was hardly observed. Likewise, mRFP-RcyA colocalized only partially along microtubules decorated with GFP-Unca\textsuperscript{mtn} (Fig. 3G). UncA is involved in early endosome movement (15, 26). These results suggest that only a fraction of RcyA is transported along microtubules on early endosomes.

**Deletion of rcyA.** In order to determine the function of RcyA in A. nidulans, we created an rcyA-deletion strain. The rcyA-deletion strain grew as well as the wild type on agar plates, and no effect on conidiation or conidium density was observed (Fig. 4A). However, hyphae of the rcyA-deletion strain sometimes showed abnormal swellings, branch formation close to the tip, and split tips (Fig. 4B). The abnormal swellings around the tip were observed in 5% of the hyphae, and the tip split phenotype was observed in 1% of the hyphal tips (n = 400), but this phenotype was not observed in the wild type.

Because Rcy1 in S. cerevisiae is thought to be involved in membrane recycling but not in endocytosis, we tested whether the absence of RcyA in A. nidulans would affect membrane recycling. To this end, the membrane was stained with the fluorescent dye FM4-64 (36). Hyphae were incubated 15 min in the presence of the dye and then, after washing with media, immediately analyzed in the microscope at room temperature. The rcyA-deletion strain did not show obvious differences from the wild type in the internalization of FM4-64 (data not shown). When we treated the hyphae with 0.1% Triton X-100, the dye was released from the vacuole (Fig. 4C). Therefore, the rcyA-deletion strain showed a moderate decrease in the percentage of hyphae with the dye in the cytoplasm (Fig. 4D). These results suggest that RcyA might be involved in membrane recycling.

**FIG 1** (A) Scheme of the RcyA protein. The F-box domain, the region with similarity to Sec10, and the CAAX motif are labeled. The original yeast two-hybrid (Y2H) clone is indicated above the scheme. (B) Relatedness analysis of RcyA. Fungal orthologues of RcyA and of Sec10 were aligned by tCoffee, and the tree was plotted at the EBI website (http://www.ebi.ac.uk/Tools/msa/tcoffee/). For the alignment, we used the Rcy1 orthologues of the following organisms: Saccharomyces cerevisiae (Rcy1p) (YIL204C), Saccharomyces paradoxus (spar343-g75.1), Saccharomyces mikatae (smk835-g3.1), Saccharomyces bayanus (sabayc610-g6.1), Candida glabrata (CAGLOF02497g), Saccharomyces castellii (ScasS31.3), Kluveromyces waltii (Kwal33.13585), Kluveromyces lactis (KLLA0C03300g), Saccharomyces kluveri (SAKL0C04004g), Ashbya gossypii (AFR644C), Candida lusitaniae (CLUG04919), Debaryomyces hansenii (DEHA2E19052g), Candida guilliermondii (PGUG03406.1), Candida tropicalis (CTR05296.3), Candida tropicalis2 (CTR06241.3), Candida albicans (orf19.3203), Candida parapsilosis (CPAG03322), Lodderomyces elongisporus (LELG03751), Yarrowia lipolytica (Sls2) (YALI0B19074g), Aspergillus nidulans (RcyA) (AN10061), Neurospora crassa (NCU03658), Schizosaccharomyces japonicus (SJAG02753), Schizosaccharomyces octopus (SOCG02810), and Schizosaccharomyces pombe (Pof6) (SPCC18.04). In addition, we included the Sec10 orthologues of the following organisms (boxed): Schizosaccharomyces cerevisiae (YLR166C), Aspergillus nidulans (AN8879), Schizosaccharomyces pombe (SEC10) (SPAC13FS.06c), and Homo sapiens (hSec10) gi|24418661|np|O00471.1.
phae with the dye on ice for 15 min before microscopic analysis, the internalization of FM4-64 was partially delayed in the rcyA-deletion strain (data not shown). However, this could have been an indirect effect, given that the rcy1 deletion caused a cold-sensitive phenotype in S. cerevisiae (10). Next, we tried to analyze membrane recycling, which means the transport of internalized dye from early endosomes back to the plasma membrane. It was hard to clearly visualize membrane recycling using this method, and no clear difference was observed in the numbers and sizes of early endosomes between the wild-type strain and the rcyA-deletion strain (data not shown).

Because S. cerevisiae Rcy1 is involved in v-SNARE Snc1 recycling, the localization of GFP-SynA (v-SNARE) was investigated in the rcyA-deletion strain (Fig. 4C). GFP-SynA localized at most hyphal tips without any obvious differences from the wild type (Fig. 4C, lower panel). Since the hyphae of the rcyA-deletion strain sometimes showed abnormal swellings or branch formation close to the tip, we investigated the localization of the two cell end markers TeaA and TeaR. Whereas both proteins were restricted to an area along the cytoplasmic membrane at the tip in the wild type, both proteins appeared less concentrated at the plasma membrane and localized at subapical swellings and also in the cytoplasm in the rcyA-deletion strain (data not shown).

Overexpression of rcyA. If RcyA is involved in membrane recycling, we anticipated not only that downregulation of rcyA would disturb hyphal morphology but also that an increase of the concentration could affect hyphal growth and morphology. To test this hypothesis, the alcA(p)-GFP-rcyA construct was induced with threonine as the carbon source. The first obvious phenotypic changes were the much slower colony growth compared to the wild type and the lack of conidia (Fig. 5A). Under repressed conditions (with glucose as the carbon source), colonies grew as fast as the wild type. The expression level of GFP-RcyA under induced and repressed conditions was visible as the intensity of the GFP signal (Fig. 5B). Another phenotype concerned spore germination. A. nidulans conidia normally form a second germ tube at the side opposite the first one (Fig. 5C, left) (48). However, conidia of the rcyA-overexpression strain sometimes did not form second germ tubes (35%; n = 100) or formed a second germ tube at a random position (10%; n = 100). If there was a second germ tube, occasionally it appeared swollen (Fig. 5C, right).

FIG 2 Localization of RcyA in A. nidulans. (A) GFP-RcyA was expressed under the control of the native promoter (SSH73). The elapsed time is given in seconds. (B) Time-lapse fluorescence microscopy images and the corresponding kymograph of the strain (SSH01). The three images correspond to three consecutive frames (300 ms each). The complete sequence spans 10 s. (C) A single picture of a time-lapse sequence of GFP-RcyA in strain SSH01 with the corresponding kymograph. The arrowheads indicate rapid moving RcyA spots. (D) Localization of mFRP1-RcyA at a forming septum (arrow) in the SSH54 strain. Strains with the RcyA construct under the control of the alcA promoter were grown with MM plus 2% glycerol in the medium. Scale bars represent 5 μm.
RcyA controls the concentration of KipA. As described above, RcyA localization was independent of KipA. On the other hand, RcyA was isolated as a KipA-interacting protein. Therefore, we studied the role of this interaction. First, we confirmed the interaction using the bimolecular fluorescence complementation (BiFC) method. A bright signal close to the hyphal tip indicated that this interaction test result was positive (Fig. 6A).

In order to characterize the role of the two important motifs of RcyA, the CaaX motif and the F-box domain, we created strains in which the corresponding regions were deleted. When the CaaX motif was missing, no mRFP1 signal was observed at hyphal tips and forming seta but very weak staining of the cytoplasm was observed (Fig. 6B, data not shown). In contrast, deletion of the F-box domain of RcyA showed signal accumulation around the tips of hyphae (Fig. 6C). Other morphological features resembled the ones observed for the rcyA deletion.

*S. cerevisiae* Rcy1 was shown to control the turnover of several proteins (9). We hypothesized that *A. nidulans* RcyA could be involved in the turnover of KipA and thus that kinesin-7 could be a novel target for the F-box protein. In order to test this hypothesis, we compared the KipA concentration, as a GFP-KipA fusion protein, in an *rcyA*-deletion strain with the concentration in the wild type. GFP-KipA associates with the microtubule plus ends and appears as comet-like moving structures in the wild type (Fig. 6D, left) (24). In contrast, in the absence of RcyA, microtubules were evenly decorated (Fig. 6D, right). Such a decoration of the entire microtubule was also observed when KipA was overexpressed (24). This result suggested that the KipA protein concentration was higher in the *rcyA*-deletion strain than in the wild type. To further prove this hypothesis, the protein amount of GFP-KipA was determined by Western blot analysis (Fig. 6E). As a control, gamma-tubulin was chosen. Indeed, the KipA concentration was increased by about 50% in the *rcyA*-deletion strain in comparison to the wild type (Fig. 6E and F). Likewise, the KipA concentration was increased when only the F-box domain of RcyA was deleted (Fig. 6F). Because the GFP-*kipA* construct was expressed from the alcA promoter in both the wild-type strain and the *ΔrcyA* strain, an effect of RcyA on the transcription of *kipA* was unlikely. Indeed, the mRNA levels of *kipA* revealed no difference in the two strains (Fig. 6G). These results suggest that the RcyA interaction is necessary for the control of the KipA turnover and that RcyA is the specific adaptor for KipA in the ubiquitination and subsequent proteasome-degradation pathway.

**DISCUSSION**

The growth form of filamentous fungi requires massive membrane flow for the continuous extension of the plasma membrane at hyphal tips and the delivery of enzymes required for cell wall biosynthesis. Both are achieved through the fusion of secretory vesicles with the membrane at the growing tip. However, there is excellent evidence that endocytosis is also important for polar growth (12, 49). On the one hand, excess membrane can be removed through endocytosis. Likewise, a slaB mutant in which membrane internalization is inhibited shows massive invaginations of the membrane (50). SlaB is a key regulator of F-actin and the endocytic internalization machinery. Interestingly, deletion of the gene is lethal, showing the importance of endocytosis for polar growth. However, deletion of *rcyA* did not show any obvious severe morphological phenotypes or defects in the uptake of FM4-64. This is in agreement with the findings in *S. cerevisiae*, where only membrane recycling but not membrane internalization is disturbed in the *rcy1*-deletion strain (10).
On the other hand, endocytosis actively occurs at a subapical ring of the hyphae and could contribute to the maintenance of polarity by recycling necessary components, such as cell end marker proteins (50). We found that the distribution of cell end marker proteins such as TeaR and TeaA in *A. nidulans* is impaired in the absence of RcyA. Since cell end marker proteins define the growth direction and are involved in branch formation, the misdistribution could be the reason for the observed changes in polar growth and branching in some minor fraction of the hyphae. Rcy1 in *S. cerevisiae* is thought to be involved in membrane recycling through the recycling of the v-SNARE Snc1 (8, 10). The v-SNARE SynA in *A. nidulans* occasionally accumulated at subapical regions in abnormally swollen tips (Fig. 4C, lower panel). This result supports the idea of a conserved function of RcyA with respect to SynA recycling; however, the defect of SynA recycling in the rcyA-deletion strain was only partial and appeared weaker than that of *S. cerevisiae*.

In comparison to the described role of *S. cerevisiae* Rcy1, Pof6 in *S. pombe* plays a critical role in cell separation, and gene deletion is lethal (9). We did not find any evidence for such a role in *A. nidulans*, although RcyA was found at septa. Of course, *A. nidulans* does not require cell separation and thus one would not expect a phenotype during vegetative growth. Since conidia are formed in a budding-like process, one would also not expect a phenotype corresponding to sporulation. However, one would expect a role of RcyA in filamentous fungi with a dimorphic switch between the filamentous form and a fission-yeast form, such as *Penicillium marneffei*.

Additionally, we identified KipA as a novel putative target for RcyA. The cellular concentration of KipA protein was increased upon deletion of the gene, while the gene expression level was comparable. We propose that RcyA is the E3 ubiquitin ligase adaptor responsible for the specific degradation of KipA in a SCF complex and that RcyA is necessary for the control of the KipA turnover. Here we cannot exclude the possibility that the increase of KipA protein levels in the *rcyA*-deletion strain is due to translational control. To exclude this possibility, the half-life time of KipA needs to be investigated. The overexpression of *rcyA* did not reduce the KipA concentration significantly and did not phenopy the *kipA*-deletion phenotype (data not shown), suggesting that the residual KipA amount is sufficient. However, there is some similarity between the *kipA*-deletion phenotype and the *rcyA*-deletion phenotype, because both deletions disturb cell end marker organization. Nevertheless, TeaR was scattered along the plasma membrane in the *rcyA*-deletion strain, whereas it was organized in a compact structure in the *kipA*-deletion strain. This
difference might be explained if we assume that membrane recycling is unaffected in the kipA-deletion strain but disturbed in the absence of RcyA. Taken together, our results are further evidence for a role of the endocytic ring in polarity maintenance and the importance of endocytosis in polar growth.

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