Endocytosis Is Crucial for Cell Polarity and Apical Membrane Recycling in the Filamentous Fungus *Aspergillus oryzae*

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Establishing the occurrence of endocytosis in filamentous fungi was elusive in the past mainly due to the lack of reliable indicators of endocytosis. Recently, however, it was shown that the fluorescent dye N-(3-triethylammoniumpropyl)-4-(p-diethyl-aminophenyl-hexatrienyl)pyridinium dibromide (FM4-64) and the plasma membrane protein AoUapC (*Aspergillus oryzae* UapC) fused to enhanced green fluorescent protein (EGFP) were internalized from the plasma membrane by endocytosis. Although the occurrence of endocytosis was clearly demonstrated, its physiological importance in filamentous fungi still remains largely unaddressed. We generated a strain in which *A. oryzae* end4 (*Aoen4*), the *A. oryzae* homolog of *Saccharomyces cerevisiae END4/SLA2*, was expressed from the *Aoen4* locus under the control of a regulatable thiA promoter. The growth of this strain was severely impaired, and its hyphal morphology was altered in the *Aoen4*-repressed condition. Moreover, in the *Aoen4*-repressed condition, neither FM4-64 nor AoUapC-EGFP was internalized, indicating defective endocytosis. Furthermore, the localization of a secretory soluble N-ethylmaleimide-sensitive factor attachment protein receptor (SNARE) was abnormal in the *Aoen4*-repressed condition. Aberrant accumulation of cell wall components was also observed by calcofluor white staining and transmission electron microscopy analysis, and several genes that encode cell wall-building enzymes were upregulated, indicating that the regulation of cell wall synthesis is abnormal in the *Aoen4*-repressed condition, whereas *Aop11* disruptants do not display the phenotype exhibited in the *Aoen4*-repressed condition. Our results strongly suggest that endocytosis is crucial for the hyphal tip growth in filamentous fungi.

The filamentous fungus *Aspergillus oryzae* has been used in industrial fermentation processes and is regarded to be safe for humans. *A. oryzae* can secrete several proteins, such as alpha-amylase, into the medium. Thus, *A. oryzae* is a potential host for heterologous protein production. Since the completion of *A. oryzae* genome sequencing (18) in recent years, many applied and basic studies have been conducted on *A. oryzae* using its genome sequencing data. In particular, studies on vesicular trafficking, including the secretory pathway, are of increasing importance because they are closely related to protein production. For example, endoplasmic reticulum and vacuole dynamics and systematic soluble *N*-ethylmaleimide-sensitive factor attachment protein receptor (SNARE) protein analyses have been performed in *A. oryzae* (16, 19, 23, 30, 31, 32). However, endocytosis, an intracellular trafficking pathway, has not been studied as well in *A. oryzae* as in other filamentous fungi.

Endocytosis is an important cellular process that occurs, for example, in signal transduction and reconstruction of cell polarity and is conserved in eukaryotic cells. The detailed mechanism of endocytosis has been well studied in model organisms such as yeasts. Many proteins are involved in the endocytic process, which is regulated spatiotemporally (12). *Saccharomyces cerevisiae END4/SLA2* (synthetic lethal with *ABP1*) is an endocytosis-associated gene that has been studied in detail (3, 6, 22, 27, 35, 43, 44). End4p/Sla2p is essential for fluid-phase and receptor-mediated endocytosis and actin assembly and polarization (27). The protein has the epsin N-terminal homology (ENTH) and the AP180 N-terminal homology (ANTH) domains, which bind to phosphatidylinositol-4,5-bisphosphate in the plasma membrane in the N-terminal region, and the I/LWEQ domain, which is proposed to be the actin-binding domain in the C-terminal region; it also functions as an adaptor that connects the invaginated plasma membrane and actin cytoskeleton, which plays an important role in endocytosis, to generate force for invaginating the plasma membrane into the intracellular space and forming endocytic pits (13, 33). Abp1p (*actin-binding protein*) forms actin patches by polymerization of the actin cytoskeleton. It is suggested that endocytosis occurs at the sites in which Abp1p localizes, i.e., cortical actin patches (21, 22). Hence, Abp1p has been used as a tool to investigate the subcellular space in which endocytosis occurs (21).

Establishing the existence of endocytosis in filamentous fungi was elusive in the past mainly due to the lack of reliable indicators of endocytosis (28). However, it has been confirmed that the fluorescent dye N-(3-triethylammoniumpropyl)-4-(p-diethyl-aminophenyl-hexatrienyl)pyridinium dibromide (FM4-64) and the plasma membrane protein AoUapC (*Aspergillus oryzae* UapC [uric acid-xanthine permease]) fused to enhanced green fluorescent protein (EGFP) were internalized from the plasma membrane by endocytosis (8, 25). Moreover, recently, in *Aspergillus nidulans*, the localization of components required for endocytosis has been analyzed in living hyphae (1, 37, 41). ActA and FimA, which are actin and fimbrin, respectively, are mostly localized in the hyphal tip region (41). Furthermore,
Aopil1, an actin-binding protein, is primarily localized in the apical region and is used as an endocytic site marker. AmpA, the amphiphysin homolog in A. nidulans, and SlaB, the End4p/Sla2p homolog, are also localized in sites in which AbpA is localized (1). These endocytic components are localized near the hyphal tip regions but slightly away from the apex where exocytosis preferentially occurs (37). Although the occurrence of endocytosis was clearly demonstrated and the localization of endocytic components was analyzed, the physiological importance of endocytosis in filamentous fungi still remains largely unaddressed.

In this report, we analyzed the physiological significance of endocytosis by generating strains that conditionally express A. oryzae end4 (Aoen4), the A. oryzae homolog of S. cerevisiae ENDO4/SLA2. Hyphae grown in the Aoen4-depressed condition displayed aberrant morphology: endocytic defects in Aopil1-EGFP and FM4-64; abnormal apical recycling of EGFP-fused AoAbp1; and abnormal cell wall synthesis. These results suggest that endocytosis plays crucial roles in the physiology of hyphal growth.

### MATERIALS AND METHODS

**A. oryzae strains and plasmids.** The *A. oryzae* strains used in this study are listed in Table 1. A. oryzae RIB40 is the wild-type strain that was used as the DNA donor. The cDNA was prepared as follows. Total RNA (1 μg) was treated with DNase (Clontech) and used as the template. The cDNA was amplified using oligo(dT)12-18 primers (Invitrogen, Tokyo, Japan) and Prime Script reverse transcriptase (TaKaRa, Kyoto, Japan). For the rapid amplification of cDNA (AE117-end analysis of Aoen4, we used the Gene Racer kit (Invitrogen) according to the manufacturer’s instructions. For DNA or cDNA cloning, the PstI-DNA polymerase (TaKaRa) was used. For Aoen4 cDNA cloning, the Aoen4 cDNA-F (5'-ATGACCTTGGACGCAGG-3') and Aoen4 cDNA-R (5'-GTCTTCATTGAGAGGCTGT-3') primers were used. To detect the Aoen4 expression in the Aoen4-disrupted strain, a DNA fragment amplified by PCR using the Aopil1 up-F (5'-GGCTTGAACCTGGCTACGAAAGACGACG-3') and Aopil1 up-R (5'-GACTACCTGAGTTTATGGCTGCAG-3') primers was introduced into the pg3EH vector digested with Smal. The resultant plasmid, named pgEP1, was digested with Smal; subsequently, the adeA sequence was amplified from pAdeA that had been digested with KpnI and PstI; the resultant plasmid was named pgEAPl. The Aoen4-F (5'-ATGACCTTGGACGCAGG-3') and Aoen4-R (5'-TTCCGTAGAAGTACGACGGCA-3') primers were introduced into pg5P2 digested with Smal, and a DNA fragment amplified by PCR using the Aopil1 down-F (5'-GCCAATGCGCCACCAACAACGC-3') and Aopil1 down-R (5'-CGCATGCTCTCAAGCGAGGGG-3') primers was introduced into the pg5PF2 vector digested with Smal; this DNA fragment was digested with KpnI and PstI and was exploited as the specific probe for confirming the Aopil1 disruptions.

| Strain(s) | Relevant genotype | Reference or source |
|-----------|-------------------|---------------------|
| RIB40     | Wild type         |                     |
| niaD300   | niaD              | 20                  |
| NSR13     | niaD s' a' adeA'  | 11                  |
| NSRNA70-1-1| niaD s' a' adeA' | 38                  |
| NSRNA70-1-1| niaD niaD         | This study          |
| NSRNA70-1-1A| niaD s' adeA' adeA| This study          |
| TE4-1 and TE4-2 | niaD s' adeA' | This study          |
| AAD1      | niaD (PamB-Naoap1-mdasd niaD) | This study          |
| TEUE3     | niaD s' (PamB-Aoaac-C-epgf s') adeA' | This study          |
| TEUA1     | niaD (PamB-Naoap1-mdasd niaD) s'C (PamB-Aoaac-C-epgf s') adeA | This study          |
| TESn1     | niaD (PamB-Aoaac-C-epgf niaD) s'C adeA' | This study          |
| TEN1      | niaD niaD s' adeA' adeA2 | This study          |
| TEAE1     | niaD (PamB-Aoaac-C-epgf niaD) s'C adeA' | This study          |
| DP1, DP2, and DP3 | niaD s' adeA' Aopil1 adeA | This study          |

### Fluorescence microscopy, culture media, and staining.

For fluorescence microscopy, we used an Olympus System microscope model BX52 (Olympus, Tokyo, Japan) equipped with a UPlanApo 100 x objective lens (1.35 numerical aperture) (Olympus). A GFP filter (495/520-nm excitation, 510-nm dichroic, 530/535-nm emission) (Chroma Technologies, Brattleboro, VT) was used for detecting GFP fluorescence. A DsRed filter (560/620-nm excitation, 590-nm dichroic, 630/660-nm emission) (Chroma Technologies) was used to detect DsRed fluorescence. A DAPI filter (330- to 385-nm excitation, 400-nm dichroic, >420-nm emission) and UV excitation cube (Olympus) was used to observe the fluorescence of calcifluor white. The images were analyzed by using MetaMorph software (Molecular Devices Co., Sunnyvale, CA). Confocal microscopy was performed with an IX71 inverted microscope (Olympus) equipped with 100 x and 40 x objectives (1.00 numerical aperture); 488-nm (Furukawa Electric, Japan) and 561-nm (Chroma) semiconductor lasers were used to excite GFP, DsRed, and DualView filters (Nikon Roper, Chiba, Japan); a CSU22 confocal scanning system (Yokogawa Electronics, Tokyo, Japan); and an Andor iXon cooled digital charge-coupled-device camera (Andor Technology PLC, Belfast, United Kingdom). Images were analyzed with the Andor iQ software.
Approximately 10<sup>6</sup> conidia were inoculated in 100 µl liquid medium and incubated on cover glasses for fluorescence microscopy of calcofluor white staining or in glass-based dishes (Asahi Techno Glass, Chiba, Japan) for other microscopic observations using confocal laser microscopy. They were grown at 30°C for approximately 20 h, and indirect immunofluorescence microscopy was performed as described previously. Czapek-Dox (CD) medium (0.3% NaNO<sub>3</sub>, 0.2% KCl, 0.1% KH<sub>2</sub>PO<sub>4</sub>, 0.05% MgSO<sub>4</sub>·7H<sub>2</sub>O, 0.002% FeSO<sub>4</sub>·7H<sub>2</sub>O, 2% glucose (pH 5.5)), CDM medium with 0.015% methionine. M medium [0.2% NH<sub>4</sub>Cl, 0.1% (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>, 0.05% KCl, 0.1% KH<sub>2</sub>PO<sub>4</sub>, 0.05% MgSO<sub>4</sub>·7H<sub>2</sub>O, 0.002% FeSO<sub>4</sub>·7H<sub>2</sub>O, 2% glucose (pH 5.5)], and MM medium (M medium with 0.15% methionine) were used for cultivation to suit the autophagy of each strain. To lower the expression of fusion genes under PmmyB, we used each medium containing glycerol (2% glucose was replaced with 1.95% glycerol and 0.05% glucose) as the carbon source (36).

The induction of AoUapC-EGFP internalization was performed as described previously (8). FM4-64 (Molecular Probes, Eugene, OR) was prepared as a 1.6 mM solution in dimethyl sulfoxide. Approximately 20 h after inoculation, the cultures were transferred into a medium containing 8 µM FM4-64 and incubated for 2 min at room temperature. After incubation, FM4-64-containing medium was replaced with fresh dye-free medium, and the samples were examined. For calcofluor white (Sigma) staining, the cultures were fixed with 0.5% glutaraldehyde, 3.7% formaldehyde, and 50 mM phosphate-buffered saline (PBS) (8 g of NaCl, 1.2 g of KCl, 1.44 g of Na<sub>2</sub>HPO<sub>4</sub>, 0.24 g of KH<sub>2</sub>PO<sub>4</sub> in 1 liter [pH 7.5]) for 10 min, then washed with PBS, and incubated in medium containing 0.1 mg/ml calcofluor white for 3 min at room temperature. After two washes with PBS, the samples were observed by fluorescence microscopy. For indirect immunofluorescence microscopy, the cultures had been incubated for approximately 12 h were fixed with 3.7% formaldehyde, 5 mM MgSO<sub>4</sub>, and 2.5 mM EGTA in PBS for 15 min. The cultures were washed twice with PBS containing 0.05% Tween 20 (PBST) after each operation. After fixation, the cultures were digested with 3 mg/ml Yatalase (TaKaRa), 1 mg/ml lyzing enzyme (Sigma), and 10 mg/ml egg white (Sigma) in PBST and thereafter incubated in methanol for 10 min at −20°C. Primary and secondary antibody reactions were performed for 1 h. The primary antibody was the rabbit anti-actin antibody (1:500 dilution; Sigma), while the secondary antibody was the fluorescein isothiocyanate-conjugated immunoglobulin G antibody (1:200 dilution; Sigma); both antibodies were diluted in PBS containing 0.1 mg/ml bovine serum albumin. The cultures were mounted in PBST and examined by microscopy.

**TEM analysis.** Transmission electron microscopy (TEM) analysis was performed as described previously (10). After incubation for 27 h in a submerged culture, the mycelia were harvested and fixed for 4 h in 4% glutaraldehyde with 0.1 M phosphate buffer and thereafter for 3 h in 1% osmium tetroxide at 4°C. Ultrathin sections were stained with uranyl acetate for 30 min and then with lead citrate for 5 min, and these sections were observed with a JEOL transmission electron microscope (JEM-1010).

**Real-time RT-PCR analysis.** Real-time reverse transcription-PCR (RT-PCR) analysis was performed as described previously (14). Template cDNA from A. oryzae TEUE3 cultured in AoM medium with 0.15% methionine, and AoM medium repressed conditions was prepared as described above. The specific primers used for real-time RT-PCR analysis in this study are listed in Table 2. The expression of each gene was normalized to that of gpdA.

**Western blot analysis.** Mycelia grown in submerged culture at 30°C for 24 h were harvested, ground to a powder by liquid nitrogen, and suspended in a buffer (10 mM MgSO<sub>4</sub>, 100 mM Tris-HCl [pH 7.5], 1 mM phenylmethylsulfonyl fluoride, and 1,100 protease inhibitor cocktail [Sigma]). After 1 h at 4°C, the samples were centrifuged at 500 × g for 5 min to remove the debris, and the supernatant was removed and boiled with 4% sample buffer. After sodium dodecyl sulfate-polyacrylamide gel electrophoresis on a 6% acrylamide gel, the samples were transferred to a nitrocellulose membrane. Blotting was performed using 5% skim milk in TBS (2.42 g of Tris, 29.22 g NaCl, and 1 g of Tween 20 [all in 1 liter; pH 7.5]) for 30 min. The anti-GFP mouse monoclonal antibody (1:5,000 dilution, Clontech) was used as the primary antibody, while the peroxidase-labeled mouse immunoglobulin G antibody (1:500 dilution; Vector) was used as the secondary antibody. Both antibody reactions were performed for 1 h. Detection was carried out using the ECL detection reagents (Pierce) and a luminescent image analyzer LAS-4000miniEPUV (Fujifilm, Japan).

| TABLE 2. Primers for real-time RT-PCR analysis |
|---|---|---|
| Gene | Gene ID<sup>a</sup> | Sequence<sup>b</sup> |
| chsA | AO090012000084 | 5′-CGATCAACATCCGTGATGTG-3′ 5′-CCCCACAGAATGACTCGGA-3′ |
| chsB | AO090701000589 | 5′-TACCTGGACCTTGGGACCATAT-3′ 5′-ACTGTCATCAAATGCTACTG-3′ |
| chsC | AO090011000449 | 5′-TCACGGATCTGTCACCAAC-3′ 5′-CTTGTAGTTAATCGGCCCTGTC-3′ |
| chsY | AO090026000323 | 5′-CGAAAAGAGATGGGAGGAGG-3′ 5′-CTTCGTTGCTGATGTTGATA-3′ |
| chsZ | AO090026000324 | 5′-CTGTCACGTTATGCTCGGA-3′ 5′-GCCATCTTCTGGTCATCTG-3′ |

<sup>a</sup> Gene ID, gene identification.  
<sup>b</sup> For each gene, the top sequence shows the sequence of the forward primer, and the bottom sequence is the sequence of the reverse primer.

**RESULTS**

**Generation of strains that conditionally express Aoend4.** To study the physiological importance of endocytosis in fungal cells, we attempted to generate strains that had endocytic defects. S. cerevisiae cells lacking END4/SLA2 exhibited endocytic defects. Hence, we used the A. oryzae genome database (http://www.bio.nite.go.jp/dogan/MicroTop?GENOME_ID=ao) to clone the cDNA of Aoend4 (DDB accession no. AB430739), which is the A. oryzae homolog of S. cerevisiae END4/SLA2, from the A. oryzae RIB40 wild-type strain. We performed rapid amplification of cDNA 5′-end analysis and determined the Aoend4 start codon. The deduced Aoend4 sequence consists of 1,043 amino acids and displays 34.6% amino acid identity to End4p/Sla2p (Fig. 1A). Based on Pfam (http://pfam.sanger.ac.uk/) motif analysis, Aoend4 has the ENTH and ANTH domains in the N-terminal region and the I/LWEO domain in the C-terminal region. Moreover, according to COILS program (http://www.ch.embnet.org/software/COILS_form.html) analysis, Aoend4 has two coiled-coil regions, which may allow for interactions with other endocytic proteins. These sequence analyses suggested that Aoend4 is similar to End4p/Sla2p in terms of endocytic functions. Aoend4 is also similar to A. nidulans slaB (84.6% amino acid identity) (1).

Next, we tried to generate an Aoend4 disruptant. We obtained three heterokaryon strains; however, the Aoend4 disruptant was not obtained, probably because Aoend4 is essential for hyphal growth (data not shown). This result is consistent with that obtained from the A. nidulans slaB mutant (1). Thus, we generated strains in which Aoend4 was expressed under the thi4 promoter (Pthi4) from the Aoend4 locus. In this construct, Pthi4 was located just upstream of Aoend4, whereas the endogenous promoter region of Aoend4 was located upstream of Pthi4. Thus, Aoend4 was regulated by Pthi4 and not by the endogenous Aoend4 promoter (Fig. 1B). Two strains were obtained, and these were named the TE4 strains (TE4-1 and TE4-2). For a control, NS13 strain was generated, in which...
Aoend4 is regulated under its own promoter, and has the same auxotrophy as TE4 strains. TE4 strains were confirmed to be homokaryon strains by Southern blot analysis (Fig. 1B). When cultured in the absence of thiamine, P\textit{thiA} drives the downstream gene, whereas in the presence of thiamine, P\textit{thiA} represses it. When cultured with more than 10 μM thiamine, P\textit{thiA} almost completely represses the downstream gene (29).

In the following experiments, we used 10 μM thiamine to shut off Aoend4 expression.

**Growth defect in the Aoend4-repressed condition.** The TE4 strains displayed a remarkable growth defect and formed irregular-shaped colonies on the culture plates as a result of apical growth defects that occurred when thiamine was added to the medium by inoculation with conidia (Fig. 2A). We confirmed that the TE4 strains in the Aoend4-repressed condition also showed a similar growth defect in liquid culture (Fig. 2B). Moreover, more severe growth defect was observed when the TE4 strains were cultured on plates containing thiamine and 1 M NaCl or 1.2 M sorbitol, suggesting that the TE4 strains in the Aoend4-repressed condition exhibited higher salt and osmotic stress sensitivities (Fig. 2A).

Next, we studied the hyphal morphology of the TE4 strains by microscopy. The hyphal morphology in the Aoend4-repressed condition was abnormal in comparison with that in the Aoend4-expressed condition (Fig. 3A). Moreover, the hyphal diameter in the Aoend4-repressed condition was larger than that in the Aoend4-expressed condition, suggesting defective apical cell polarity in the Aoend4-repressed condition (Fig. 3B).

**Endocytic defects in the Aoend4-repressed condition.** To test whether the hyphae grown in the Aoend4-repressed condition exhibited endocytic defects, the TEUE3 strain, which expresses the AouapC-egfp fusion gene in the conditional Aoend4 background, was generated. AouapC is a putative plasma mem-


**FIG. 3.** Hyphae in the *Aoen4d*-repressed condition display abnormal morphology. (A) Hyphae that were grown in the MM liquid medium in the absence (−) or presence (+) of thiamine at 30°C for 18 h were observed by microscopy. Bar, 10 μm. (B) After 20 h of incubation at 30°C, the maximum width of hyphae within 10 μm from the hyphal tip was measured using the Andor iQ software. For each strain and condition, 20 hyphal tips were measured, and the average diameters are shown. The gray and black bars indicate the hyphal diameters (in microns) cultured in the absence or presence of thiamine, respectively. Four independent experiments were performed. The error bars represent standard deviations. Values that were significantly different (*P* < 0.01) by Student’s *t* test are indicated (**).
and Spitzenkörper-like structure but accumulated throughout the plasma membrane. This suggested that EGFP-AoSnc1 is not recycled from the plasma membrane to the tip region by apical recycling of endocytosis and that the defects in SNARE protein recycling result in abnormal secretion to the tip, leading to defective apical polarity (Fig. 6A, right). Moreover, in the Aoend4-repressed condition, FM4-64 accumulated in the aberrant invagination structures on the plasma membrane that were labeled with EGFP-AoSnc1 (Fig. 6A, right, arrows).

To investigate the dynamics of EGFP-AoSnc1 in the hyphal tip in the Aoend4-repressed condition, we performed FRAP analysis. In the Aoend4-expressed condition, EGFP-AoSnc1 fluorescence was recovered in the Spitzenkörper-like structure in the tip region approximately 1 min after photobleaching (Fig. 6B, top). However, in the Aoend4-repressed condition, EGFP-AoSnc1 fluorescence was not recovered in the tip region at more than 1 min after photobleaching, possibly as a result of membrane flux (Fig. 6B, bottom, right). These FRAP analyses demonstrated that in the Aoend4-repressed condition, secretion in the tip region is abnormal.

Regulation of cell wall components is abnormal in the Aoend4-repressed condition. The TEM analysis showed that the cell wall of Δend4/sla2 mutants is thicker than that of the wild-type strains of S. cerevisiae and Schizosaccharomyces pombe (5, 6). Thus, we stained hyphae grown in the Aoend4-repressed condition with calcofluor white, which stains chitin, the major cell wall component in filamentous fungi (Fig. 7A). In the Aoend4-repressed condition, abnormal accumulation of the cell wall labeled with calcofluor white was observed, indicating that the cell wall in the Aoend4-repressed condition was abnormal in comparison with that in the Aoend4-expressed condition. However, in contrast to budding and fission yeasts, the cell wall in the Aoend4-repressed condition was not uniformly thicker at the cell surface but was thicker at specific locations.
sites on the cell surface. Moreover, the accumulation of the cell wall coincided with the presence of large invagination structures labeled with AoUapC-EGFP (Fig. 7A, right, arrows).

For a more precise analysis of the sites where the cell wall was thicker, we performed TEM analysis (Fig. 7B). In the Aoend4-expressed condition, the width of the cell wall was uniform (Fig. 7B, left). In the Aoend4-repressed condition, it was confirmed that the cell wall was thicker, and a non-cell wall component was observed in the large invagination structures (Fig. 7B, right, arrows), suggesting that cell wall regulation is abnormal in this condition.

On the basis of our observation that the cell wall is abnormal in the Aoend4-repressed condition, we further analyzed the expression of cell wall synthases by real-time RT-PCR in the Aoend4-expressed and Aoend4-repressed conditions (Fig. 7C). Five chitin synthases (chsA, chsB, chsC, chsY, and chsZ) and one 1,3-beta-glucan synthase (Aoβks1), which display comparatively high expression under normal culture conditions, were selected for expression analyses. These six cell wall synthases tended to be expressed at higher levels in the Aoend4-repressed condition than in the Aoend4-expressed condition, suggesting that the upregulation of cell wall synthases is due to endocytic defects.

Complementation analysis of Aoend4. To complement the phenotype in the Aoend4-repressed condition and analyze AoEnd4 localization, we generated a strain that expressed AoEnd4-EGFP in the conditional Aoend4 background. When the control strain TEN1 was cultured in the endogenous Aoend4-repressed condition, mycelial growth was more severely inhibited than in the host strain, presumably due to differences in auxotrophy (Fig. 8A). In contrast, the Aoend4-repressed condition and analyze AoEnd4 localization, we generated a strain that expressed the Aoend4-egfp fusion gene under PamyB at the niaD locus in the conditional Aoend4 background. When the control strain TEN1 was cultured in the endogenous Aoend4-repressed condition, mycelial growth was more severely inhibited than in the host strain, presumably due to differences in auxotrophy (Fig. 8A). In contrast, TEAEN1, a strain that expressed AoEnd4-EGFP in the conditional Aoend4 background, formed normal-sized and round colonies in the endogenous Aoend4-repressed condition; these colonies were similar to those formed in the endogenous Aoend4-expressed condition, even when Aoend4-egfp expression was low (Fig. 8A). This indicated that TEAEN1 growth is complemented...
in the endogenous Aoend4-repressed condition, and the AoEnd4-EGFP fusion protein is functional.

We next analyzed AoEnd4-EGFP localization by using confocal microscopy. When hyphae were cultured in the endogenous Aoend4-expressed condition, AoEnd4-EGFP was localized not at specific sites but in the cytoplasm, suggesting that AoEnd4-EGFP could not localize to the appropriate region by competing with endogenous AoEnd4. On the other hand, in the endogenous Aoend4-repressed condition, AoEnd4-EGFP was mainly localized on the sides of the hyphal tip and partly in patches of the plasma membrane (Fig. 8B), which is consistent with A. nidulans SlaB localization (1). Western blot analysis was performed to confirm the expression of the AoEnd4-EGFP fusion protein in the endogenous Aoend4-expressed or Aoend4-repressed condition (Fig. 8C).

To verify the rescue of endocytosis in the endogenous Aoend4-repressed condition, we performed FM4-64 staining (Fig. 8D). When the TEN1 strain was cultured in the endogenous Aoend4-repressed condition, FM4-64 was not internalized, and instead, it accumulated in the plasma membrane. In contrast, when the TEAEN1 strain was cultured in both the endogenous Aoend4-expressed and Aoend4-repressed conditions, FM4-64 was internalized from the plasma membrane, and Spitzenkörper-like structures and endocytic compartments, including the vacuolar membrane, were stained. These results demonstrated that Aoend4 repression caused the phenotypes described above.

S. cerevisiae Pil1p functions as a component of the eisosome, which is a plasma membrane domain in which endocytosis
occurs, and disruption of its gene results in the formation of aberrant invagination structures (42). To investigate whether AoPIL1 (DDJB accession no. AB430741), the sole A. oryzae homolog of PIL1, functions in endocytosis, we generated DP strains, AoPIL1 disruptants. To investigate whether endocytosis occurred in the DP strains, we stained the samples with FM4-64. In the control and DP strains, FM4-64 was internalized, and Spitzenkörper-like structures and endocytic compartments were stained, indicating that normal endocytosis occurs in the DP strains (Fig. 8E). This result suggested that unlike AoEnd4, AoPil1 does not play a crucial role in endocytosis.

**DISCUSSION**

Recently, in filamentous fungi, the localization of endocytic components, such as A. nidulans AbpA, AmpA, and SlAB, was analyzed in living hyphae (1). However, there are few reports on the physiological importance of endocytosis in filamentous fungi, mainly due to the lack of reliable indicators of endocytosis. In this study, we investigated the physiological roles of endocytosis in filamentous fungi by generating strains that conditionally express Aoend4 and analyzed their phenotypes in the Aoend4-repressed condition.

On the basis of motif analysis, AoEnd4 is the homolog of S. cerevisiae End4p/SlA2p, suggesting that the function of AoenD4 is similar to that of End4p/SlA2p in endocytosis. The A. nidulans fimA disruptants, fimA being the homolog of S. cerevisiae SAC6, also displayed deficiency in FM4-64 internalization, which was similar to that observed in the Aoend4-repressed condition (41). In the Aoend4-repressed condition, AoAbp1 was not localized at the hyphal tip but was dispersed in the plasma membrane as cortical patches. AoAbp1 was capable of localizing in the plasma membrane but not only in the tip region, indicating that AoEnd4 was not required for the formation of cortical actin patches and AoAbp1 localization was dependent on AoEnd4. In S. cerevisiae ∆end4/sla2 cells, actin comet tails are observed, and the amount of actin increases, suggesting that End4p/SlA2p negatively regulates actin polymerization (9, 13). In the Aoend4-repressed condition, the number of AoAbp1 patches in the plasma membrane increased, which is consistent with the result from S. cerevisiae ∆end4/sla2 cells. However, the AoAbp1 patches were hardly localized in the invagination structures. This result suggests that the components required for AoAbp1 recruitment do not localize in the invagination structures. However, further analyses of other endocytic components in filamentous fungi are required.

S. cerevisiae and C. albicans ∆end4/sla2 cells display defects in filamentous growth (2, 44). In A. nidulans, SlAB is not required for hyphal germination but is essential for hyphal growth (1); this is consistent with the results obtained in the Aoend4-repressed condition in this study, which suggest that endocytosis is not required for hyphal germination but is required for polarized growth. Hyphae that showed the endocytic defect displayed aberrant hyphal morphology, probably due to the lack of endocytosis, which is the counterpart of exocytosis, which is required for hyphal growth in the tip region. In the Aoend4-repressed condition, high sensitivity to salt and osmotic stress was probably caused by endocytic defects in channels or receptors that sense the outer environment. In A. nidulans, ambient pH signaling might be regulated by the endocytosis of a seven-transmembrane protein PalH (7, 26).

Due to continuous tip elongation in filamentous fungi, it is thought that these organisms need to recycle certain components, such as cell wall-building enzymes, to the tip region (34, 41). Calcofluor white staining revealed that chitin, a major cell wall component in filamentous fungi, is accumulated in aberrant invagination structures in the Aoend4-repressed condition. In addition, TEM analysis revealed the presence of non-cell wall components in the aberrant invagination structures. These results predict that proteins involved in cell wall synthesis, such as chitin synthases, probably could not be recycled to the tip region and therefore accumulated in the aberrant invagination structures. Moreover, we found that the expression of cell wall synthases increased in the Aoend4-repressed condition. One possible explanation for the high expression of these genes is that regulatory component(s) of these cell wall synthases cannot be internalized by endocytosis. Of the cell wall synthases analyzed in this study, A. oryzae ChsY and ChsZ are the homologs of A. nidulans CsmA and CsmB, respectively (4, 39, 40). These chitin synthases have a myosin motor-like domain (MMD) and directly interact with actin, which has its most important functions in endocytosis. Thus, chitin synthases with MMD are thought to be possibly associated with endocytic recycling. Although MMD does not function like the myosin motor, which uses ATP, but interacts only with actin (39, 40), there is a possibility that chitin synthases with MMD bind to actin through this domain and undergo endocytic recycling to the tip region.

Filamentous fungi are important and have been examined in both basic and applied studies. Filamentous fungi, including A. oryzae, are potential hosts for heterologous protein production. However, there are many obstacles for protein production in these organisms. Further clarification and a better understanding of intracellular trafficking involving the endocytic pathway are required because endocytosis and exocytosis are closely related (34, 37). Further, filamentous fungi are now regarded as model organisms in studies on apical growth, and their machinery in the apical region, in which both exocytosis and endocytosis are vital, is being investigated (1, 34, 37, 41). Our results strongly indicate that endocytosis has a crucial role in hyphal growth by recycling of secretory SNARE and components of the plasma membrane, such as cell wall synthases in the tip region. However, endocytosis in filamentous fungi has not been investigated in detail so far. Endocytosis is a part of several physiological processes in many eukaryotic cells, and better understanding of filamentous fungi requires elucidation of its physiological roles in the organisms.

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