Depletion of ε-COP in the COPI Vesicular Coat Reduces Cleistothecium Production in Aspergillus nidulans

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Abstract

We have previously isolated ε-COP, the α-COP interactor in COPI of Aspergillus nidulans, by yeast two-hybrid screening. To understand the function of ε-COP, the aneA⁺ gene for ε-COP/AneA was deleted by homologous recombination using a gene-specific disruption cassette. Deletion of the ε-COP gene showed no detectable changes in vegetative growth or assexual development, but resulted in decrease in the production of the fruiting body, cleistothecium, under conditions favorable for sexual development. Unlike in the budding yeast Saccharomyces cerevisiae, in A. nidulans, over-expression of ε-COP did not rescue the thermo-sensitive growth defect of the α-COP mutant at 42°C. Together, these data show that ε-COP is not essential for viability, but it plays a role in fruit body formation in A. nidulans.

Keywords  α-COP, Aspergillus nidulans, Cleistothecium, ε-COP, Sexual development

Eukaryotic cells contain a collection of spatially separated internal organelles embedded in the cytoplasm. Communication between these internal organelles is mediated by trafficking events that are mainly accomplished by vesicular transport. These trafficking events facilitate the targeted delivery of newly synthesized proteins and lipids, uptake of extracellular cargo, and also play a role in regulatory processes [1]. The cargo recognized by coat proteins is stabilized, carried in the vesicle, and delivered to the target membrane. Among the various coat proteins identified to date, coatomer, the coat complex of COPI-coated vesicles composed of seven coat proteins [ε-, β-, β', γ, δ, ε, and ζ-COP] and the small ras-like GTPase, ADP ribosylation factor, mediates recycling of proteins in Golgi-to-endoplasmic reticulum (ER) interactions and within the Golgi complex [2, 3].

In yeast, all COPs, except ε-COP, are essential for cell viability. ε-COP stabilizes α-COP, and thus coatomer in the elevated temperatures [4]. In addition, α-, γ-, δ-, and ζ-COP mutants, referred to as ret mutants, show inability to retrieve proteins with a di-lysine motif (KKXX) [5-7]. α- and β'-COPs are involved in the recruitment of di-lysine-tagged proteins [6]. Ret1/α-COP of Saccharomyces cerevisiae is known to contribute to the maintenance of cell wall integrity and biogenesis by influencing glycosylation and localization of the secretome including cell wall proteins [8-12]. Quantitative proteomic analysis has revealed an association between calcineurin, a calcium-calmodulin-associated protein phosphatase, and ε-COP during high temperature stress in the human fungal pathogen Cryptococcus neoformans [13]. In Aspergillus nidulans, α-COP is essential for viability. However, like in yeast, a mutation in A. nidulans α-COP gene results in a temperature-sensitive defect in growth, cell-wall construction, and protein secretion [14, 15]. Interestingly, the temperature dependent-osmo-sensitive phenotype of the S. cerevisiae α-COP mutant is suppressed by introduction of the A. nidulans sod³/C (stabilization of disomy) gene for α-COP [15]. In addition, the C-terminus of the A. nidulans ε-COP, AneA, including tetra-trico peptide repeat (TPR: W₇G₇Y₂G₇Y₂A₇Y₇A₇P₇) interacts with the C-terminus of α-COP [16]. These findings suggest that the action of the coatomer in filamentous fungi may be similar to that in yeast, but the function of ε-COP in conjunction with α-COP needs to be elucidated in A. nidulans.

In this study, to understand the function of ε-COP in A.
K. nidulans during development, we performed phenotypic analyses of the aneA deletion mutant and α-COP mutant with over-expression of the gene encoding ε-COP, aneA^+. 

**MATERIALS AND METHODS**

**Strains and growth conditions.** *Aspergillus nidulans* strains and plasmids used in this study are listed in Table 1. Plasmid amplification and transformation of *Escherichia coli* have been described previously [16]. Previously described growth conditions including those for inducing development were used for *A. nidulans* [17, 18]. Expression of aneA via niiA promoter is induced by 0.6% sodium nitrate and repressed by 0.2% ammonium tartrate.

**Construction of a disruption cassette.** An aneA^+ disruption cassette (DC) was constructed according to methods described by Yu et al. [19]. Using genomic DNA prepared from *A. nidulans* FGSC A4, DNA fragments for the DC were amplified by PCR by using appropriate primer sets (AneA-A1/-A2, AneA-B1/-B2, and argB-For/-Rev). The complete DC was amplified by a nested-PCR primer set (AneA-C1/-C2), purified, and then used to transform the TJ1 strain. The primers used in this study are listed in Table 2.

**Nucleic acid preparation, Northern blotting, and Southern blotting.** Nucleic acid preparation and northern blot analysis were performed as described previously [17]. For Southern blotting, genomic DNA was treated with appropriate restriction enzymes and then separated on 1% agarose gels. The gels were washed with distilled water (DW) and soaked twice in a depurination solution (250 mM HCl) for 15 min. After the reaction, the gel was

**Table 1.** List of *Aspergillus nidulans* strains and plasmids used in this study

| Strains       | Genotypes            | Sources               |
|---------------|----------------------|-----------------------|
| FGSC A4       | veA+                 | FGSC                  |
| TJ1           | yA2; argB2; pyroA4; veA+ | S. K. Chae            |
| SK880         | yA2; argB2; pyroA4; veA+; pIL16 (argB') | S. K. Chae            |
| DEC           | yA2; argB2; pyroA4; veA+; aneA::argB | This study            |
| PBR2          | pyrG89; yA2          | This Lab              |
| B120          | pyrG89; yA2; sod^c^C1 | S. J. Assinder        |
| B120A         | pyrG89; yA2; sod^c^C1;pRG3-AMA1-nii(p)::Pyr4 | This study            |
| B120AE        | pyrG89; yA2; sod^c^C1;pRG3-AMA1-nii(p)::aneA::Pyr4 | This study            |

**Plasmids**

| pRG3-AMA1     | Amp::AMA1;Pyr4::niiA | S. K. Chae            |
| pAME          | Amp::AMA1;Pyr4::nii::aneA | This study            |

FGSC, Fungal Genetics Stock Center.

**Table 2.** List of oligonucleotides used in this study

| Oligo           | Sequence (5'-3')        |
|-----------------|-------------------------|
| aneA^+ disruption | GGC CAC GTT CAC CGG TAA |
| AneA-A1         | AGT CAA ATG AGG CCT CTA AAC TGG TCA TGA CAA TGG GCT GTT TAG |
| AneA-A2         | AGC CAA GGT AGA TCC AGG CCT AAC ACA AAT ATG ATT AGT CCT GTC |
| AneA-B1         | GAC ACT CAA TCA CGG GGC |
| AneA-B2         | GAC CAG TTT AGA GGC CTC |
| argB-For        | GTG TTA GGC CTG GAT CTA |
| argB-Rev        | AGG AAC CCA TTT CTG TTC GC |
| AneA-C1         | GAG ATC CTC AGC GAG CTC AA |
| AneA-C2         | CCC CGG GGG GAA TGG ATC CAT TCT CTG CAG |
| AneA-D1         | TG TGC GGG CAA TGA AGA AAC CTT GGC GGA GTA |
| AneA-D2         | GGG AGC TCC GCC ATT TTC TT |
| Southern probe  | CCC CAG AGC CCT CAA TAA AT |
| Northern probe  | AneA-E1                 | CCG AGC TCC GCC ATT TTC TT |
| Northern probe  | AneA-E2                 | CCC CAG AGC CCT CAA TAA AT |
| AneA-F1         | GAA TTC ATG GAT CCA TCT TCT GCA G |
| AneA-F2         | CTC GAG ATC GAA AAA GTC ACT CTT CT |

Bold and underlined letters indicate argB complement sequence.

Bold letters indicate restriction enzyme linker.
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Briefly, the genomic DNA contained in the processed gel was transferred onto a Hybond-N+ membrane (Amersham Biosciences, Pittsburg, PA, USA). Gene-specific probes were prepared from the PCR-generated fragments and were labeled using the ECL direct nucleic acid labeling system (Amersham Biosciences). The signals were visualized by exposing the membrane to an X-ray film.

**RESULTS AND DISCUSSION**

Effect of ε-COP depletion on growth and stress response. The DC for deletion of the *aneA*+ gene was constructed and used to transform the recipient strain. Southern blot analysis of *Pst I*-digested genomic DNA from candidate strains with a PCR-amplified probe specific for the 5'-flanking region of *aneA*+ confirmed the deletion of the chromosomal *aneA* gene by revealing an expected band of 5.7 kb (Fig. 1). To investigate the function of *aneA* in development of *A. nidulans*, the pattern of hyphal growth including radial growth, septation, and asexual sporulation were examined in one of the deletion mutant strains. However, we observed no differences between the wild-type and deletion mutant (data not shown). These results indicated that ε-COP is not essential for the viability of *A. nidulans*; these results are similar to those obtained in yeasts.

Recently, COPI proteins were reported to play roles in responses to ER stress and thermal stress in yeast [11, 13]. Here, we tested the effect of ε-COP deletion on sensitivity to environmental stresses caused by treatment of the fungi with several drugs that are known to exert adverse effects on cellular processes related to the stress response. No detectable changes were observed in the ε-COP-defective strain by treatment with drugs including inhibitors of cell-wall biosynthesis (calcofluor white, congo red, and caspofungin), N-glycosylation (tunicamycin), ergosterol biosynthesis (terbinafine), and glycerol biosynthesis (fludioxonil), an inducer of apoptosis (farnesol), and a Ca++

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**Fig. 1.** Southern blot analysis of *aneA* deletion strains. The left panel shows the results from the recipient strain (lane 1) and *aneA*-deletion strains (lanes 2 and 3). The schematic diagrams in the right panel display the chromosome structures of the wild-type strain (upper) and the constructed *aneA*-deletion strain (lower). A cassette containing *argB* with a 5'-flanking region and a 3'-flanking region was integrated into the *aneA*+ locus of the genome of *Aspergillus nidulans* by homologous recombination. The probe used for Southern blotting was located at the 5'-flanking region of *aneA*+.

**Fig. 2.** Formation of the sexual reproductive organ (cleistothecium) of the isogenic wild-type (SK880) and *aneA*-deletion strain. Sexual reproduction was induced by incubating the cells on plates containing 1% glucose and 0.1% sodium nitrate under hypoxic and dark conditions. The cleistothecia are indicated by white arrows. WT and Δ*aneA* stand for wild-type and *aneA*-deletion strain, respectively.
chelator (EGTA). Thermal stress, which makes the ε-COP-dependent yeast strain unviable [4], showed no effect on viability and vegetative growth in the ε-COP deletion strains (data not shown). Taken together, these results show that ε-COP in A. nidulans is neither essential for viability nor involved in stress responses unlike in yeast.

**Effect of ε-COP depletion on development.** In A. nidulans, a mutation in the sod"C" gene encoding α-COP is responsible for non-disjunction of the chromosome during cell division [20]. COPI depletion also results in the failure of cytokinesis and a reduction in the number of overlapping central spindle microtubules during meiotic divisions for spermatogenesis in Drosophila [21]. In the ε-COP-depleted (ΔaneA) A. nidulans strain, we observed no defects in asexual development (data not shown), but significant defects in sexual development (Fig. 2). Even after culturing in conditions favorable for cleistothecium development, with hypoxic treatment with 1% glucose in the dark [22], the ΔaneA strain showed more than 50% decrease of fruiting body formation (Table 3). In addition, in 1% glucose without hypoxic treatment, fruiting body formation was almost completely abolished by ε-COP depletion. In 2% lactose without hypoxic treatment, which predominantly induces the formation of fruiting bodies, ε-COP depletion showed no deleterious effect on fruiting body formation (Table 3). Because the effect of C-sources and hypoxic treatment was tested in the presence of 0.1% sodium nitrate as a nitrogen source, we tested 0.2% yeast extract, which preferentially induces sexual development, and found that the defect in fruiting body formation on 1% glucose without hypoxic treatment was recovered in ΔaneA deletion strain (data not shown).

Although further experiments are required to determine the underlying mechanisms, our results suggested that ε-COP plays a role in sexual development in A. nidulans under certain environmental conditions, possibly by affecting cytokinesis and/or construction of ER-based spindle envelopes, as observed in fruit fly spermatogenesis [21].

**Suppression of sod"C1" mutation by forced expression of aneA**. Our previous study showed that the C-terminal domains of both α-COP and ε-COP are essential for their interaction, and that the N-terminal WD40 motif of α-COP and the TPR region of ε-COP are involved in controlling the interaction between these two COPs in A. nidulans [16]. We also reported that A. nidulans α-COP can substitute for S. cerevisiae α-COP functions in vivo [15]. In yeast, ε-COP is known to stabilize the thermo-sensitive α-COP mutation. Thus, over-expression of ε-COP confers viability to the α-COP mutant at elevated temperatures [4]. Therefore, we investigated whether the phenotype of the sod"C1" mutant was suppressed by over-expression of aneA" in A. nidulans. When a pRG3-AMA1 plasmid containing the aneA" gene encoding ε-COP was introduced into the sod"C1" mutant, over-expression of aneA" was confirmed by northern blot analysis (Fig. 3A). However, over-expression of ε-COP could not rescue the thermo-sensitive phenotype of the sod"C1" mutant at a non-permissive temperature (42°C)

### Table 3. Effect of carbon sources on cleistothecium production

| Carbon source       | No. of cleistothecia/cm² | WT    | ΔaneA |
|---------------------|--------------------------|-------|-------|
| 1% Glucose + sealing| +++                      | +     |       |
| 1% Glucose          | +++                      | –     |       |
| 2% Lactose          | +++                      | +++   |       |

Average number of mature cleistothecia per cm² of 3 different area of a plate was calculated: –, < 1; +, 1–50; ++, 50–100; ++++, > 100.

In all cases, plates were incubated under dark condition to enhance cleistothecium development.

![Fig. 3. Suppression of the temperature-dependent osmo-sensitive phenotype in the sod"C1" mutant. A. Expression of aneA" was confirmed by northern blot analysis; B. Growth patterns of wild type (PBR2) and the sod"C1" mutant (B120) with an empty vector (B120A) or the aneA" over-expression vector (B120AE) were observed at permissive (30°C) and non-permissive (42°C) temperatures.](image-url)
(Fig. 3B). These results indicate that ε-COP is able to interact with α-COP [16], but does not stabilize α-COP at elevated temperatures unlike in yeast [4].

In summary, our results indicate that ε-COP in A. nidulans is not essential but related to formation of the sexual reproductive organ in response to particular environmental factor(s). It is worth highlighting that recent reports indicate that the function of the COPI complex (and its subunits) is not confined to intracellular vesicular trafficking. The COPI complex is also involved in other cellular events such as chromosome disjunction in A. nidulans [20], male development in chicken embryos [23], ER stress responses in yeast [11], association with calcineurin during heat stress [13], meiotic divisions for spermatogenesis in Drosophila [21], induction of productive autophagy and cellular survival [24], intercompartmental trafficking of specific RNAs in neuronal cells [25], and neurite outgrowth [26]. Although further studies are necessary to confirm the actual role of ε-COP (and/or COPI) in sexual development of fungi, our results open up interesting avenues for further studies on the function of the COPI complex.

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