

Secreted Acb1 Contributes to the Yeast-to-Hypha Transition in *Cryptococcus neoformans*

Xinping Xu, Youbao Zhao, Elyssa Kirkman, Xiaorong Lin

Department of Biology, Texas A&M University, College Station, Texas, USA

Adaptation to stress by eukaryotic pathogens is often accompanied by a transition in cellular morphology. The human fungal pathogen *Cryptococcus neoformans* is known to switch between the yeast and the filamentous form in response to amoebic predation or during mating. As in the classic dimorphic fungal pathogens, the morphotype is associated with the ability of cryptococci to infect various hosts. Many cryptococcal factors and environmental stimuli, including pheromones (small peptides) and nutrient limitation, are known to induce the yeast-to-hypha transition. We recently discovered that secreted matricellular proteins could also act as intercellular signals to promote the yeast-to-hypha transition. Here we show that the secreted acyl coenzyme A (acyl-CoA)-binding protein Acb1 plays an important role in enhancing this morphotype transition. Acb1 does not possess a signal peptide. Its extracellular secretion and, consequently, its function in filamentation are dependent on an unconventional GRASP (Golgi reassembly stacking protein)-dependent secretion pathway. Surprisingly, intracellular recruitment of Acb1 to the secretory vesicles is independent of Grasp. In addition to Acb1, Grasp possibly controls the secretion of other cargos, because the *graspΔ* mutant, but not the *acb1Δ* mutant, is defective in capsule production and macrophage phagocytosis. Nonetheless, Acb1 is likely the major or the sole effector of Grasp in terms of filamentation. Furthermore, we found that the key residue of Acb1 for acyl binding, Y80, is critical for the proper subcellular localization and secretion of Acb1 and for cryptococcal morphogenesis.

Adaptation to a changing environment by eukaryotic microbes is often accompanied by a transition in cellular morphology. The human fungal pathogen *Cryptococcus neoformans* causes devastating cryptococcal meningitis, which claims the lives of hundreds of thousands of people each year (1). Late diagnosis, limited options for antifungals, and the lack of vaccines to prevent cryptococcosis all contribute to the high mortality rate of this disease (2). *C. neoformans* typically grows as yeasts but can switch from yeasts to filaments (hyphae or pseudohyphae) in response to predation (e.g., by amoebae) or during sexual reproduction (3–7). As with many other fungal pathogens, the morphotype of *C. neoformans* shapes its interactions with various hosts (8). As we demonstrated recently, the hyphal form is associated with the attenuation of virulence in mouse models of cryptococcosis, because the hyphal morphotype elicits strong and protective host immune responses (9, 10). On the other hand, the hyphal morphotype assists the fungus in resisting predation from soil amoebae (8), increases its ability to explore the environment (11), and is linked to its unisexual and bisexual reproduction (3, 12–14). Thus, it is important to understand the factors that promote cryptococcal hyphal growth.

Many environmental stimuli and a few cryptococcal factors that promote hyphal growth in *C. neoformans* have been identified (12, 15–18). Pheromones are the most prominent cryptococcal molecules that stimulate mating and filamentation. We recently discovered that the matricellular and hypha-specific protein Cfl1, when released from the cell wall, can also act as an intercellular communication signal to stimulate the yeast-to-hypha transition (19, 20). Here we decided to investigate the potential role of the secreted protein Acb1 in filamentation and sexual reproduction in *C. neoformans*.

The acyl coenzyme A (acyl-CoA)-binding protein Acbp was first identified in mammals because its processed peptide inhibited the binding of diazepam to the gamma-aminobutyric acid

(GABA) receptor, which gave rise to its name, diazepam binding inhibitor (DBI) (21). The homolog of DBI in *Dictyostelium discoideum*, called peptide signal spore differentiation factor 2 (SDF2), activates sporulation within the fruiting body (22). Similarly, SDF2 is processed from *Dictyostelium* AcbA (23, 24). In the absence of AcbA, *D. discoideum* fruiting bodies generate about 10% as many viable spores as the wild type (WT). Interestingly, coin-cubation of the *acbAΔ* mutant with wild-type cells restored the level of sporulation to that of the wild type (23). It was proposed that the AcbA secreted from the wild type was sufficient to complement the sporulation defect of the *Dictyostelium acbAΔ* mutant.

Acbps were later found to be widely distributed in the eukaryotic domain, and they play important roles in a wide range of biological processes (25–27). Among higher eukaryotes, there are multiple copies of Acbp-encoding genes in one genome, and these proteins differ in size and subcellular localization (28–34). Nonetheless, all Acbps are conserved in the acyl-CoA-binding domain (34). Not surprisingly, Acb1 in *Saccharomyces cerevisiae* helps transport newly synthesized acyl-CoA esters from the fatty acid synthase to acyl-CoA-consuming processes (35). Acb1 plays an

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Address correspondence to Xiaorong Lin, xlin@bio.tamu.edu.

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important role in fatty acid elongation, membrane assembly, and protein trafficking in *S. cerevisiae* (36, 37).

Despite the predicted cytosolic localization of AcbA, due to the absence of a classical secretion signal or any transmembrane domain (38), it has been found to be located in puncta or vesicles in the cortical region in *D. discoideum* (39). The vast majority of AcbA proteins are intracellular; <5% are secreted extracellularly (39). However, extracellular secretion and postsecretion processing are critical for the signaling function of *Dictyostelium* AcbA in promoting spore generation (23, 39). The extracellular secretion of AcbA requires an unconventional pathway that is dependent on the Golgi apparatus-associated protein Grasp (Golgi reassembly stacking protein) in *D. discoideum* (38).

In the present study, we set out to investigate the role of secreted Acb1 in the yeast-to-hypha transition and sporulation in *C. neoformans*. We found that Acb1 contributes to the cryptococcal yeast-to-hypha transition. Interestingly, the secretion of Acb1 is dependent on its acyl-CoA-binding ability and on the Grasp protein in *C. neoformans*. Accordingly, mutation of the acyl-CoA-binding domain of Acb1 or deletion of the *GRASP* gene impairs cryptococcal hyphal growth.

MATERIALS AND METHODS

Media, strains, and *in vitro* phenotypic assay. YPD medium (2% Bacto peptone, 1% yeast extract, and 2% glucose) was used for routine culture. For phenotypic assays, YNB medium (6.7 g/liter of yeast nitrogen base without amino acids or ammonium) without glucose was used as the base medium for testing the utilization of a specific nutrient source added to a final concentration of 2%, as indicated in the text and figures. For the phenotypic assays, wild-type and mutant cells were suspended at the same cell density. Cell suspensions with serial dilutions were spotted onto the relevant medium and were incubated for 2 to 3 days before photographs were taken. For the filamentation assay, we used V8 juice agar (50 ml V8 juice–0.5 g KH_2PO_4 , in 1 liter [pH 5 or 7; adjusted with KOH]), YNB, or YPD medium. V8 juice medium is a commonly used mating medium in laboratory research (40). YNB is a minimal medium, and YPD is a nutrient-rich medium. All mutant strains were generated in the backgrounds of reference strains XL280 (serotype D) and H99 (serotype A). Both XL280 and H99 have publicly available genomes and congenic pairs (41, 42). The strains used in this study are listed in Table S1 in the supplemental material.

For bisexual mating, parental strains (mating types α and **a**) with equal number of cells were cocultured together on the YNB, V8 juice, or YPD medium in the dark at 22°C. Mating was examined microscopically for the formation of mating hyphae and spores. For unisexual mating (self-filamentation without a partner of an opposite mating type), individual isolates at the same cell density were dropped onto the YNB or V8 juice medium alone. Self-filamentation and sporulation were examined microscopically as described previously (14). XL280 is a hyperfilamentous strain, and it filaments robustly on V8 medium, which renders the reduction in the filamentation of the *acb1* Δ mutant less obvious. The reduction in the filamentation of the *acb1* Δ mutant is much more pronounced on the suboptimal YNB medium. In comparison, H99 strains filament poorly during bisexual mating on all these media, and the reduction in the filamentation of the *acb1* Δ mutant is evident irrespective of the medium used.

Confrontation assay. The confrontation assay was used to test whether secreted products from the wild type could restore the bisexual mating defect of the *acb1* Δ mutant. The procedure was performed as described by us previously (19). Briefly, cocultured α and **a** cells of either the wild-type or the mutant strains at a 1:1 ratio were spotted onto the relevant medium (YNB or YPD medium) as donor strains. After the donor strains were incubated for 3 days, the recipient α -a cocultures (wild-

type or mutant strains) were spotted onto the medium in close proximity to the donor cells (distance, <5 mm). After an additional 48 h of incubation, the cells were photographed in order to enable examination of colony morphology and the formation of hyphae in the recipients.

Gene deletion and complement. The knockout and complementation constructs were generated as described previously (14, 43). To disrupt the *ACB1* or *GRASP* gene, we amplified the 1-kb 5' and 3' flanking sequences of the coding region by using the genomic DNA isolated from strain XL280 α or H99 α as the template and the NEO or NAT dominant drug marker amplified from plasmid pAII or pJAF1, respectively. The knockout constructs with 5' and 3' flanking sequences bordering the selective marker gene were generated by overlap PCR as described by us previously (43). The knockout constructs were introduced into strains XL280 α , XL280a, H99 α , and KN99a by biolistic transformation as described previously (44). The resulting transformants were screened for gene replacement via homologous recombination events by PCR. The genetic linkage between the phenotype and the gene deletion was confirmed by analyzing the segregation pattern of the meiotic progeny generated from a bisexual cross between the mutant and a wild-type mating partner (43). For complementation, the wild-type genes with 1 to 1.5 kb upstream of their open reading frames (ORFs) were amplified by PCR, digested with proper restrictive digestion enzymes, and introduced into the pXL1-mCherry plasmid (9). The resulting plasmid, pXL1-*ACB1*-mCherry, was confirmed by enzyme digestion and gel electrophoresis. The plasmids were then linearized and were transformed into the relevant *Cryptococcus* strains through biolistic transformation or electroporation as described by us previously (43). The primers and plasmids used for this study are listed in Table S2 in the supplemental material.

Target site-directed mutagenesis. To mutate the acyl-CoA-binding site, the key residue of Acb1, Y80, was mutated to A (Y80A) by using a site-directed mutagenesis kit (QuikChange II; Agilent Technologies) according to the manufacturer's instructions. The fragment with the mutated allele of *ACB1* and the 1-kb sequences upstream of the *ACB1* ORF was ligated into plasmid PXL1-mCherry (9). The resulting plasmid, PXL1-Acb1(Y80A)-mCherry, was linearized and was transformed into XL280 or H99 as described above.

Microscopic examination. To examine the subcellular localization of Acb1::mCherry or Acb1(Y80A)::mCherry, the relevant strains were cultured on YPD or YNB agar medium at 30°C for 24 h. Images were acquired and processed with a Zeiss M2 imaging system with the AxioCam MRm camera and Zen 11 software (Carl Zeiss Microscopy).

RNA extraction and qPCR. RNA extraction and quantitative PCR (qPCR) were performed as described previously (9). Briefly, strains with opposite mating types were cocultured on YNB agar medium for the indicated durations (see Fig. 1C). Cells were harvested, washed with cold water, immediately frozen in liquid nitrogen, and then lyophilized. Cells were broken into a fine powder with glass beads, and total RNA was extracted with the PureLink RNA minikit (Life Technologies) according to the manufacturer's instructions. First-strand cDNA was synthesized with a SuperScript III cDNA synthesis kit (Invitrogen) according to the manufacturer's instructions. The housekeeping gene *TEF1* was used as the endogenous control. The relative transcript levels were determined by using the comparative $\Delta\Delta C_T$ method as described previously (9). Three biological replicates were performed for each sample, and their values were used to calculate the mean value and standard error.

Protein extraction and Western blotting. Strains carrying Acb1-mCherry or Acb1(Y80A)-mCherry in the wild-type or *grasp* Δ mutant background were cultured in YNB liquid medium with the proteinase inhibitors PMSF (phenylmethylsulfonyl fluoride) and protease inhibitor cocktail (Roche Inc.) for 48 h. The culture supernatant was separated from the cell pellet by centrifugation. The supernatant was concentrated with an Amicon Ultra-15 centrifugal filter (EMD Millipore) and was denatured with an SDS-containing loading buffer before electrophoresis in an SDS gel. The cell pellet was washed twice with cold phosphate-buffered saline (PBS) and was then lyophilized. The dried cells were disrupted by a cell

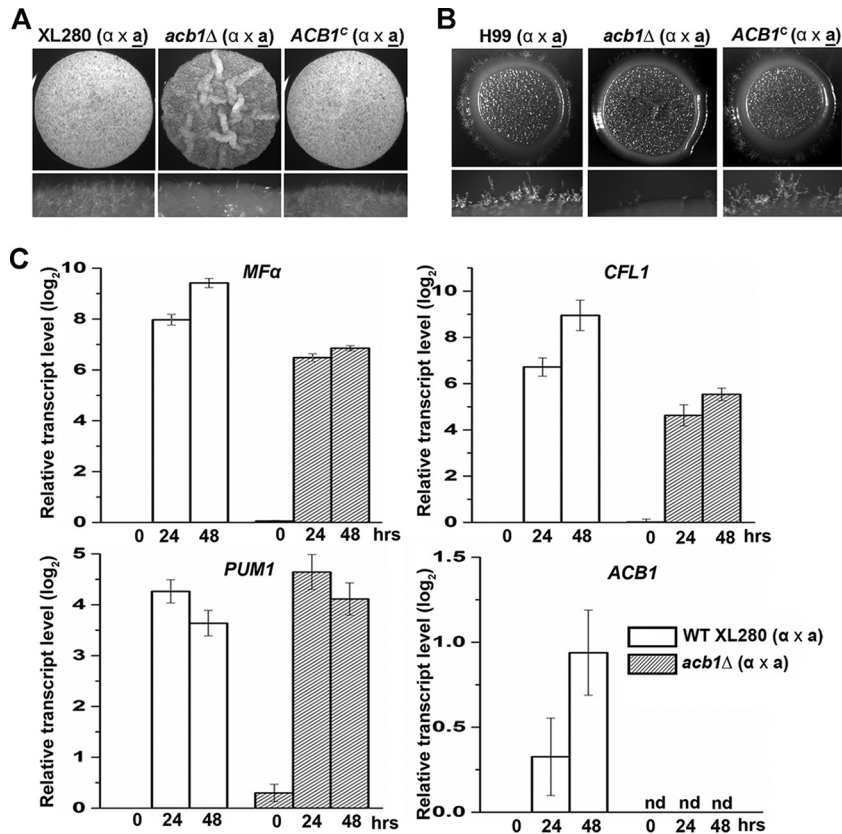


FIG 1 Deletion of *ACB1* impairs filamentation during bisexual mating in *Cryptococcus neoformans*. (A) The α - α mating pairs of the WT XL280 strains, the *acb1* Δ mutants, and the *ACB1*^c-complemented strains were cultured on YNB medium for 48 h. (B) The α - α mating pairs of the WT H99 strains, the *acb1* Δ mutants, and the *ACB1*-complemented strains were cultured on YNB medium for 9 days. (C) Measurement of the transcript levels of *MF α* , *CFL1*, *PUM1*, and *ACB1* by reverse transcription-quantitative PCR. The transcript level of each gene in the wild type at the 0-h time point was set at 1 (log₂ value, 0) for comparison. RNA samples were extracted at 0 h, 24 h, and 48 h after the bisexual mating of WT XL280 and the *acb1* Δ mutants on YNB medium. nd, not detected.

disruptor (Next Advance) with glass beads. The total proteins were extracted with a lysis buffer (25 mM HEPES [pH 7.5], 300 mM NaCl, 2 mM EDTA, plus a proteinase inhibitor cocktail) and were then denatured with the SDS-containing loading buffer before electrophoresis in an SDS gel. Western blotting was carried out as described previously (19, 45). Briefly, the samples were separated on an SDS–12% PAGE gel and were transferred to a polyvinylidene difluoride (PVDF) membrane (Millipore) for 1 h at 30 V in a TE 70 ECL semidry transfer unit (GE Healthcare). The blots were incubated with an anti-mCherry primary antibody (dilution, 1/2,000), washed, and then incubated with a rabbit anti-mouse secondary antibody (dilution, 1/10,000) (Clontech Inc.). Signals were detected by using the enhanced chemiluminescence (ECL) system according to the instructions provided by the manufacturer (Pierce).

Phagocytosis assay. The phagocytosis assay was performed as described by us previously (8). Briefly, the J774A.1 macrophage cell line (ATCC TIB-67) was cultured in Dulbecco's modified Eagle's medium (DMEM; catalog no. 30-2002) with 10% fetal bovine serum (FBS). Three hundred microliters of culture with 2.5×10^5 freshly grown J774A.1 cells was seeded into each well of a 24-well microtiter plate. The macrophages were cultured at 37°C under 5% CO₂ overnight, and the medium was then replaced with fresh medium. Each well was inoculated with *Cryptococcus* cells to achieve a multiplicity of infection (MOI) of 3. After 30 s of mixing in a rocker, the cocultures were incubated at 37°C under 5% CO₂ for an additional 3 h. The cocultures were then washed three times with warm PBS (500 μ l/well) to remove the medium and nonadherent cells. Then PBS plus 0.1% Tween 20 was added to the culture, which was incubated for 10 min at 37°C to lyse the macrophages. The supernatant was then

harvested, serially diluted, and spread onto YNB agar plates. The cryptococcal CFU was counted after 2 days of incubation at 30°C.

RESULTS

Deletion of the *ACB1* gene reduced hyphal growth. Based on our transcriptome-sequencing (RNA-seq) data, *ACB1* is one of the abundantly expressed genes (46). Since secreted and processed Acb1 is involved in sexual reproduction as a signal molecule in other species (23, 39), we decided to test whether Acb1 is also important for *Cryptococcus* sexual reproduction. We first examined the transcript level of *ACB1* during bisexual mating. We found that the expression of *ACB1* was modestly increased during mating (Fig. 1C), suggesting a possible role for Acb1 in this biological process.

To examine the role of Acb1 in sexual reproduction and hypha formation in *C. neoformans*, the *ACB1* gene (CNM01420) was deleted in the hyperfilamentous serotype D strain XL280 α and its congenic strain XL280a (3, 47). No *ACB1* transcript could be detected in the *acb1* Δ mutants (Fig. 1C), as expected. Under mating-inducing conditions on V8 juice agar medium, the α - α mating pair of the *acb1* Δ mutant showed dramatically reduced filamentation relative to that of the wild type, as reflected in the less white and fluffy mutant colony (Fig. 1A). Ectopic introduction of a wild-type allele of *ACB1* into the *acb1* Δ mutant restored the defect (Fig. 1A), supporting the role of Acb1 in

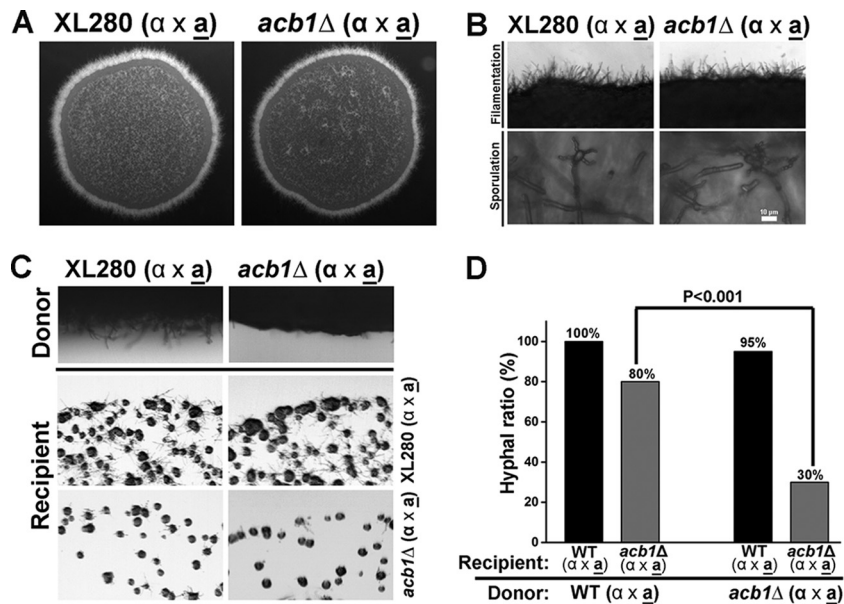


FIG 2 Secreted products from the wild type, but not from the *acb1Δ* mutant, enhanced filamentation in neighboring cells. (A) Images of colonies formed by α - α bisexual mating of WT XL280 and the corresponding *acb1Δ* mutant on V8 medium (pH 7). (B) Images of the colony edge (top) and basidiospores (bottom) generated by the bisexual mating of the WT strain or the *acb1Δ* mutant on V8 medium. (C) Confrontation assay using WT and mutant mating pairs as the donor or recipient. (D) Frequency of filament formation by recipient colonies at 48 h postinoculation. Eighty percent of the *acb1Δ* mutant recipient colonies formed hyphae when the donor was WT. In contrast, 30% of the *acb1Δ* mutant recipient colonies formed hyphae when confronted with an *acb1Δ* mutant donor.

filamentation. Under mating-suppressing conditions on YPD agar medium, the colony derived from the wild-type α - α mating pair was wrinkled, with some degree of filamentation at the colony edge (see Fig. S1A in the supplemental material). In contrast, the colony derived from the *acb1Δ* α - α mating pair was smooth and almost barren at the edge (see Fig. S1A). During unisexual mating with only α cells, wild-type XL280 α cells cultured on YNB medium produced a wrinkled colony with hyphae at the colony edge (see Fig. S1B). The *acb1Δ* mutant generated a smooth colony with very few hyphae at the colony edge (see Fig. S1B). Ectopic introduction of the wild-type *ACB1* gene into the *acb1Δ* mutant restored self-filamentation to the wild-type level. These observations indicate that Acb1 also enhances hyphal growth during self-filamentation.

To test whether the role of Acb1 in filamentation is conserved in other *Cryptococcus* subspecies, we deleted the *ACB1* gene (CNAG_06140) in the serotype A reference strain H99 α and the mating type *a* strain KN99*a*. As with XL280, the *acb1Δ* α - α mating pair in the H99 background showed a drastic reduction in filamentation when cultured on V8 juice agar medium, and robust filamentation could be restored by the introduction of a wild-type copy of *ACB1* (Fig. 1B). Taken together, the results indicate that Acb1 has a conserved role in enhancing filamentation during bisexual mating in *C. neoformans*. Because sporulation is preceded by a yeast-to-hypha transition in *C. neoformans* (48), and because Acb1 in *Dictyostelium discoideum* is known to trigger sporulation within fruiting bodies (23), we decided to test if Acb1 is required for sporulation in *C. neoformans*. We examined spore production by α - α bisexual mating of the wild-type strain XL280 and the corresponding *acb1Δ* mutant on V8 juice medium. Although the level of filamentation was reduced in the *acb1Δ* mutant, both mating pairs produced 4 chains of basidiospores (Fig. 2A and B), and

we found no apparent defect or drastic reduction in spore production.

Deletion of *ACB1* reduced the transcript level of the pheromone gene *MF α* and the hypha-specific gene *CFL1*. *C. neoformans* undergoes a yeast-to-hypha transition during both unisexual and bisexual mating. The pheromone signaling pathway initiates the process under mating-inducing conditions (14), and the activation of the filamentation pathway eventually leads to hyphal growth (9, 14). To understand how the loss of *ACB1* affects filamentation, we decided to measure the impact of *ACB1* deletion on the transcript levels of *MF α* , *CFL1*, and *PUM1* during bisexual mating. The pheromone *MF α* is the initial signaling factor initiating mating (49). The secreted protein Cfl1 is a specific marker for filamentation (19). *Pum1* is a genetic linker between filamentation and sporulation (48). The basal levels of all three transcripts at 0 h were similar for the wild type and the *acb1Δ* mutant (Fig. 1C). The transcript levels of both *MF α* and *CFL1* were induced in the wild type as well as in the *acb1Δ* mutant during mating (Fig. 1C). However, the degrees of induction for both *MF α* and *CFL1* were lower in the *acb1Δ* mutant than in the wild type (Fig. 1C). This is consistent with the reduced filamentation observed in the *acb1Δ* mutant. Interestingly, the transcript levels of *PUM1*, a gene that connects filamentation with sporulation in *C. neoformans* (48), were comparable for the wild type and the *acb1Δ* mutant at all three time points examined (Fig. 1C). Given that deletion of *PUM1* causes the formation of barren basidial heads without spores (48), the finding that *PUM1* expression is unaltered in the *acb1Δ* mutant is consistent with the observation that the *acb1Δ* mutant displays no specific defects in sporulation (Fig. 2B). Taken together, these observations indicate that secreted Acb1 contributes to the cryptococcal morphotype transition at least partly through its effects on the pheromone signaling and filamentation pathways.

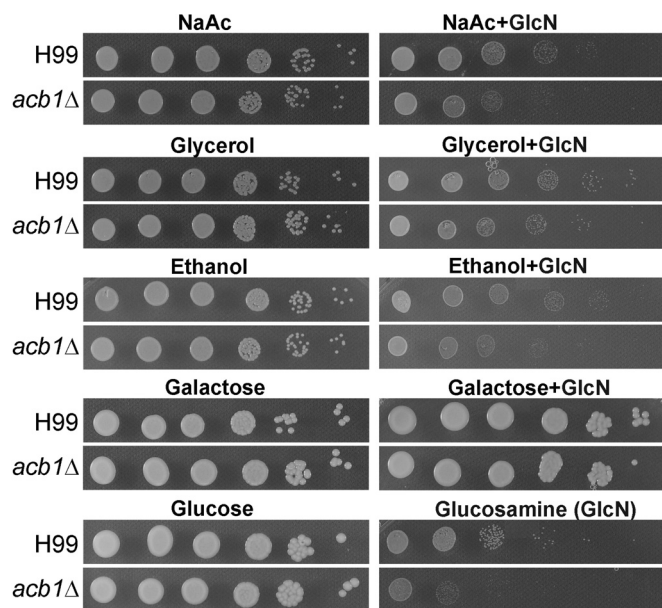


FIG 3 Growth of the *acb1Δ* mutant and the corresponding wild-type strain on different carbon sources in the absence and presence of glucosamine (GlcN).

Secreted products from the wild type, but not the *acb1Δ* mutant, could enhance hyphal formation in a nearby *acb1Δ* recipient strain. In *Dictyostelium discoideum*, extracellular Acb1 secreted from wild-type cells acts as a signal and can compensate for the loss of *ACB1* in nearby mutant cells in terms of sporulation (23). Since Acb1 is important for filamentation in *C. neoformans* and is highly expressed, we hypothesize that secreted Acb1 from the wild type may also act as a signal in promoting filamentation in the *Cryptococcus acb1Δ* mutant. To test this hypothesis, we performed confrontation assays, in which the donor and the recipient were placed in close proximity but not physically touching each other. The wild-type recipient filamented well regardless of whether the donor was the wild type or the *acb1Δ* mutant (Fig. 2C and D; see also Fig. S2 in the supplemental material). However, more *acb1Δ* recipient colonies formed filaments when the donor was the wild type rather than the *acb1Δ* mutant. Despite the increased frequency of hypha formation by *acb1Δ* recipient colonies with a wild-type donor, the hyphae formed by the mutant were rudimentary at the time point examined (see Fig. S2). Nonetheless, the evidence suggests that products secreted from the wild-type donor, but not from the *acb1Δ* mutant donor, enhanced the frequency of filamentation by the nearby *acb1Δ* recipient cells.

Acb1 promotes the utilization of alternative carbon sources. As an acyl-CoA-binding protein, Acb1 regulates growth in different media and under different conditions, as demonstrated in *S. cerevisiae* (35–37, 50, 51). To our surprise, we found no apparent growth defect in the *Cryptococcus acb1Δ* mutants in either the rich YPD medium or the minimum YNB medium (Fig. 3; see also Fig. S3 and S4 in the supplemental material). The mutants also showed no difference from the corresponding wild-type strains in their tolerance of SDS and antifungal drugs such as caspofungin, polymyxin B, and fluconazole (not shown). In yeast and mammalian cells, the *acb1Δ* mutant showed a severe defect in long-chain fatty acid metabolism (35, 52). However, in *C. neoformans*, the *acb1Δ* mutant grew well on lipids as the sole carbon source, just like the wild type (see Fig. S5 in the supplemental material).

More surprisingly, the *acb1Δ* mutant in either the XL280 or the H99 background grew equally well as the corresponding wild-type strains on media with different carbon sources (glucose, galactose, glycerol, sodium acetate [NaAc], or ethanol) or different nitrogen sources [(NH₄)₂SO₄, NaNO₃, glycine, aspartic acid, or thiamine] (Fig. 3; see also Fig. S3 and S4 in the supplemental material). This is, again, different from what was observed for the *S. cerevisiae acb1Δ* mutant, which showed growth defects with different carbon sources (35, 37, 50).

The presence of the preferred carbon source (usually glucose) represses the utilization of other carbon sources (53, 54). This is called catabolite repression. Catabolite repression can be observed with the addition of glucosamine, a glucose mimic (55, 56). The idea is that the presence of glucosamine (GlcN) suppresses the catabolism of other carbon sources and thus inhibits growth even when other carbon sources are available. Indeed, we found that the addition of glucosamine inhibited the utilization of NaAc, glycerol, and ethanol by the wild type (Fig. 3; see also Fig. S4 in the supplemental material). The *acb1Δ* mutant showed more-severe growth deficiency than the wild type in using NaAc or ethanol in the presence of glucosamine (Fig. 3; see also Fig. S4). This suggests that Acb1 in the wild type might be involved in relaxing catabolite repression, which could be useful for an organism found in soil and decaying vegetation, where complex carbon sources other than glucose are likely to be present.

The Y80 residue in the acyl-CoA-binding domain is critical for the function and subcellular localization of Acb1. The acyl-CoA-binding domain is highly conserved among Acb proteins (Fig. 4A), suggesting the importance of this domain to the function of Acb1. In this region, Y80 has been shown to be a conserved and important residue for binding acyl-CoA: a mutation of this residue can decrease a protein's acyl-binding ability 1,000-fold (28, 57–59).

To examine if the acyl-CoA-binding domain is critical for the function of *Cryptococcus* Acb1 in filamentation, we made a mutated (Y80A) allele of Acb1 through site-directed mutagenesis. The Acb1(Y80A) mutated allele, when introduced into the *acb1Δ* mutant, could not restore the mutant's filamentation defect, in contrast to the wild-type allele (Fig. 4B). This result suggests that acyl-CoA-binding ability is critical for the function of Acb1 in filamentation.

Acb1, despite its predicted cytosolic location, is known to be recruited to the secretory pathway in other organisms (28, 39). Under a wide-field epifluorescence microscope, the mCherry-labeled Acb1 in *C. neoformans* was located in intracellular puncta (Fig. 4C) that are consistent with secretory vesicles. In *Dictyostelium discoideum*, Acbp is also localized to intracellular puncta (39). Acb1(Y80A)-mCherry, however, showed a diffused cytoplasmic localization (Fig. 4C). The cytosolic localization of Acb1(Y80A) in *C. neoformans* is consistent with previous observations with *S. cerevisiae* and *D. discoideum*, where a decreased or abolished ability of Acbp to bind to acyl-CoA is associated with increased cytosolic localization (28, 39). Thus, previously published literature and our observation, taken together, indicate that the recruitment of Acb1 to the secretory pathway requires its acyl-CoA-binding capability. It is tempting to speculate that the binding partner of Acb1 might help bring this otherwise cytosolic protein to the secretory pathway.

In *D. discoideum*, a minor proportion of AcbA proteins was secreted extracellularly (39). This is also true in *C. neoformans*,

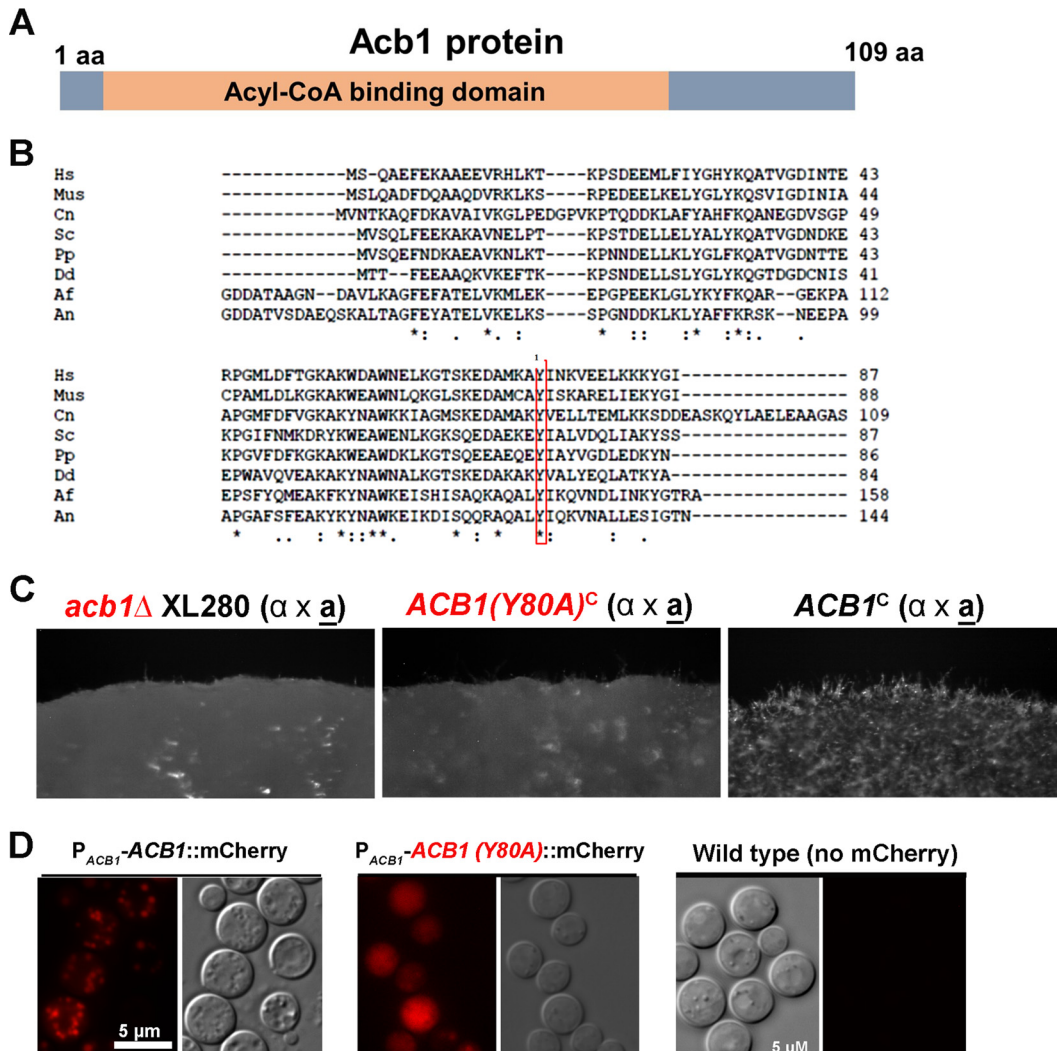


FIG 4 Mutation of the key residue Y80 affects the function and subcellular localization of Acb1. (A) Diagram of the Acb1 protein. (B) Acb1 is a highly conserved protein among different species. Shown is a multiple alignment of proteins from the following species: Hs, *Homo sapiens*; Mus, mouse species; Cn, *Cryptococcus neoformans*; Sc, *Saccharomyces cerevisiae*; Pp, *Pichia pastoris*; Dd, *Dictyostelium discoideum*; Af, *Aspergillus fumigatus*; An, *Aspergillus nidulans*. (C) The mutated Acb1(Y80A) strain could not restore the filamentation defect of the *acb1*Δ mutant. The *acb1*Δ mutant, the *acb1*Δ mutant transformed with the wild-type allele [*ACB1^c*], and the *acb1*Δ mutant transformed with the Y80A allele [*ACB1(Y80A)^c*] were cultured on YNB medium for 48 h. (D) Subcellular localization of Acb1-mCherry and Acb1(Y80A)-mCherry. The fluorescent image of nontransformed wild-type cells was used as the negative control.

where we found most of the Acb1 proteins in the total-cell lysate and some in the culture supernatant (Fig. 5B). Because Acb1(Y80A) is localized to the cytosol, we speculated that extracellular secretion of this mutated protein would be abolished. Indeed, we could not detect any Acb1(Y80A) in the culture supernatant, although we could easily detect the protein in the cell lysate. This suggests that the mutated Acb1(Y80A) protein was not released to the environment. Thus, the alteration of this key residue affects the function of Acb1 as well as its subcellular localization (Fig. 5B).

The extracellular secretion of Acb1, but not its recruitment to the secretory pathway, is dependent on Grasp. Acb1 lacks a signal peptide and is not a typical secretory protein that uses the conventional or the general secretion pathway. In *S. cerevisiae* and *D. discoideum*, the secretion of Acb1 was shown to be dependent on GRASPs (Golgi reassembly stacking proteins) (38, 60–62),

which were originally identified as factors required for the stacking of Golgi cisternae and the tethering of vesicles destined to fuse with the Golgi apparatus (63, 64). In addition to Acb1, the secretion of other factors, such as integrin and CFTR (cystic fibrosis transmembrane conductance regulator), also depends on the unconventional Grasp-mediated pathway in other organisms (38, 65–68). Mammalian genomes encode two orthologs of *GRASP* genes (66), whereas only one *GRASP* gene has been found in *C. neoformans* and yeast previously (61, 69). It is noteworthy that in both fungal species, Grasp was shown not to be involved into Golgi stacking but to be important for molecular secretion (61, 69).

To test if Grasp in *C. neoformans* is involved in the recruitment of Acb1 to the secretory pathway and/or in its extracellular secretion, we deleted the *GRASP* gene. We then examined the localization and extracellular secretion of Acb1-mCherry in the *grasp*Δ

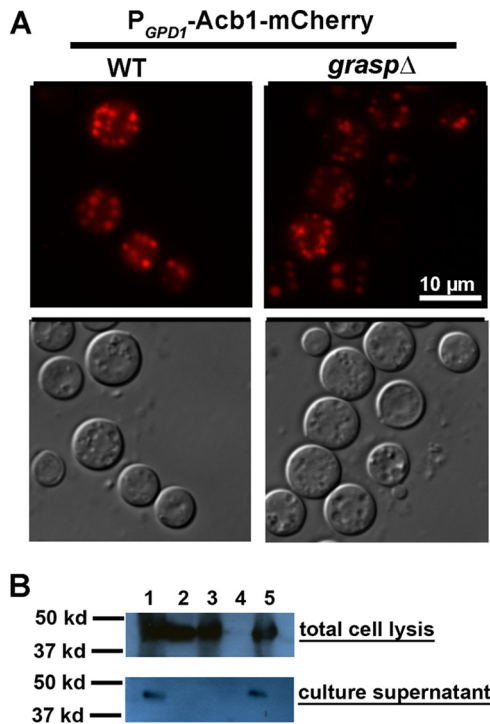


FIG 5 The subcellular localization of Acb1 is independent of Grasp, but the secretion of Acb1 requires Grasp. (A) Intracellular localization of Acb1-mCherry in the wild-type or *graspΔ* mutant background. (B) Western blots of supernatants from different strains. Lanes: 1, Acb1-mCherry in the *acb1Δ* mutant; 2, Acb1-mCherry in the *graspΔ* mutant; 3, Acb1(Y80A)-mCherry in the *acb1Δ* mutant; 4, XL280 without any mCherry, used as a negative control; 5, Acb1-mCherry in WT XL280.

mutant background. Interestingly, we found that Acb1-mCherry was localized to vesicles in the *graspΔ* mutant, as observed in the wild type (Fig. 5A). This suggests that the recruitment of Acb1 to the secretory pathway is independent of Grasp.

Next, we tested whether the absence of Grasp affects the extracellular secretion of Acb1 in *C. neoformans* by using P_{ACB1} -ACB1-mCherry as the reporter. We detected a strong Acb1-mCherry signal from the cell lysate of the *graspΔ* mutant (Fig. 5B). This is consistent with our microscopic observation of Acb1-mCherry in intracellular vesicles in the wild type as well as in the *graspΔ* mutant. However, no Acb1-mCherry was detected in the supernatant derived from the *graspΔ* mutant (Fig. 5B), in contrast to its presence in the supernatant derived from the wild-type background. This suggests that extracellular release of Acb1-mCherry is abolished in the *graspΔ* mutant. Taking these findings together, we conclude that the recruitment of Acb1 to the secretory pathway is independent of Grasp but that its extracellular secretion requires Grasp.

The *graspΔ* mutant recapitulated the *acb1Δ* mutant phenotype in terms of filamentation. The evidence presented above indicates that Acb1 proteins are predominantly localized intracellularly, with some being secreted extracellularly. Since Acb1 is important for filamentation, and since secreted products from the wild-type donor, but not the *acb1Δ* mutant donor, can enhance filamentation in nearby *acb1Δ* cells, we hypothesize that released extracellular Acb1 is important for filamentation. Since Grasp is required for the extracellular secretion of Acb1, but not for its

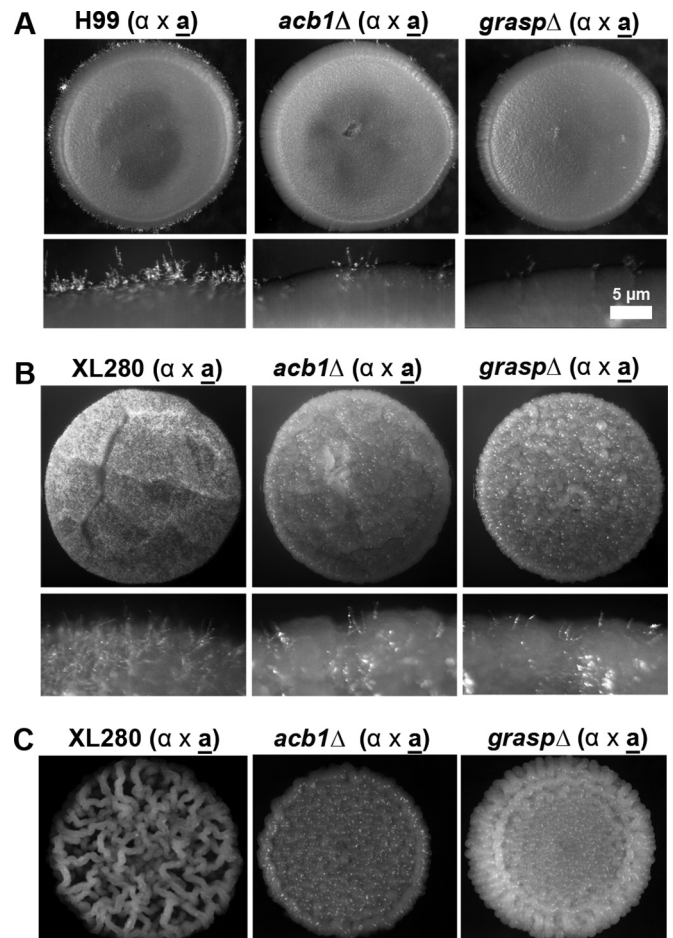


FIG 6 The *graspΔ* mutant recapitulates the phenotype of the *acb1Δ* mutant in terms of colony morphology and filamentation. Shown are a-α cocultures of the wild type, the *acb1Δ* mutant, and the *graspΔ* mutant in the H99 background on V8 juice medium (pH 5) (A), in the XL280 background on YNB medium (B), and in the XL280 background on YPD medium (C). Panels A and B include images of the entire colonies and the colony edges. Panel C shows images of the entire colonies.

production or intracellular localization, we decided to test our hypothesis using the *graspΔ* mutant. The *graspΔ* mutant in the H99 background showed reduced filamentation during bisexual mating on V8 medium, as observed for the *acb1Δ* mutant (Fig. 6A). Similarly, the *graspΔ* mutant in the XL280 background yielded a colony with smoother morphology and showed less-robust hyphal production than the wild type during bisexual mating on YNB medium, resembling the *acb1Δ* mutant (Fig. 6B). Consistently, the *graspΔ* a-α mixed culture gave rise to a smooth colony on YPD medium, in contrast to the wrinkled colony generated by the wild-type a-α mixed culture (Fig. 6C). Thus, the *graspΔ* mutant displayed the same colony morphology and filamentation phenotypes as the *acb1Δ* mutant. This suggests that Grasp regulates cryptococcal morphogenesis mainly through extracellularly secreted Acb1.

Deletion of GRASP, but not deletion of ACB1, affects capsule production and phagocytosis by macrophages. We showed in the preceding section that the *graspΔ* mutant recapitulates the same colony morphology and filamentation phenotypes as the *acb1Δ* mutant. It was shown previously that the *graspΔ* mutant in

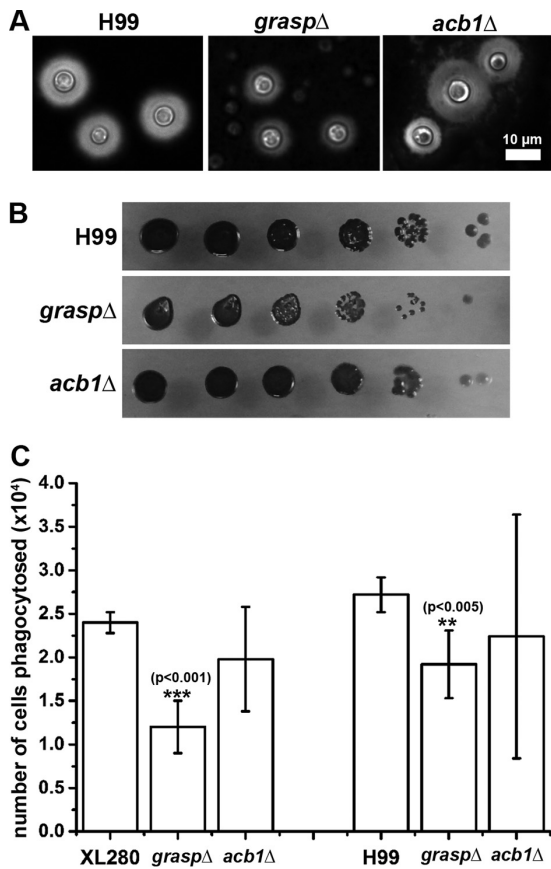


FIG 7 The *graspΔ* mutant showed slight deficiencies in multiple classic cryptococcal virulence traits. (A) The WT, the *graspΔ* mutant, and the *acb1Δ* mutant after culture on RPMI medium for 2 days. The capsule (halo surrounding the yeast cells) was visualized by negative staining with India ink. (B) The WT, the *graspΔ* mutant, and the *acb1Δ* mutant were cultured on L-3,4-dihydroxyphenylalanine medium for 3 days in order to test melanin formation. The dark pigment indicates melanin. (C) Macrophages were inoculated with the WT, the *graspΔ* mutant, and the *acb1Δ* mutant on RPMI medium and were cocultured for 3 h. The numbers of phagocytosed *Cryptococcus* cells were determined by CFU counting and were graphed.

C. neoformans is defective in capsule production and macrophage phagocytosis (69). To test if such defects in the *graspΔ* mutant are caused by its defect in the extracellular secretion of Acb1, we first examined the production of capsule and melanin in the *acb1Δ* mutant. To our surprise, deletion of *ACB1* had no apparent impact on capsule production and melanization. In contrast, deletion of *GRASP* greatly reduced capsule size (Fig. 7A), a finding consistent with the previous report (69). There might be a slight reduction in the melanization of the *graspΔ* mutant (Fig. 7B). Next, we tested the phagocytosis of the *acb1Δ* mutant in both the XL280 and H99 backgrounds by J774A.1 murine macrophages. Although the levels of phagocytosis of the *acb1Δ* mutants might be slightly lower than those of the corresponding wild-type strains (Fig. 7C), the differences were not statistically significant. In contrast, the levels of phagocytosis of the *graspΔ* mutants were significantly lower than those of wild-type cells in both the XL280 and H99 backgrounds (Fig. 7C), as demonstrated previously for the *graspΔ* mutant in the H99 background (69). These results indicate that Acb1 is not the only effector of Grasp in *C. neoformans* but is

likely the major, or the sole, effector of Grasp in terms of filamentation.

DISCUSSION

We demonstrated previously that the secreted matricellular protein Cfl1, a downstream target of the global regulator Znf2 (9, 14), plays important roles in cellular and colony morphogenesis in the environmental fungal pathogen *C. neoformans* (19, 20). In this study, we investigated the role of the secretory protein Acb1 in the cryptococcal yeast-to-hypha morphological transition and in cryptococcal sporulation, given the importance of its ortholog in sporulation in *Dictyostelium discoideum* and *Pichia pastoris* (23, 62). In contrast to Acbp in *D. discoideum* (23), we found that Acb1 in *Cryptococcus neoformans* is not critical for sporulation *per se* but that secreted Acb1 is important for hyphal growth, which precedes sporulation during both unisexual and bisexual reproduction. The function of Acb1 in cellular and colony morphology is conserved in both serotype A and serotype D, two subspecies of the *C. neoformans* species complex (70). However, in contrast to *CFL1*, *ACB1* is unlikely to be controlled by Znf2 at the transcript level, despite the modest increase in the number of *ACB1* transcripts during bisexual mating. First, *ACB1* is not among the genes in the *ZNF2^{oe}* strain or the *znf2Δ* strain that are differentially expressed relative to the wild-type control (9, 14). Second, Znf2-controlled genes are typically expressed at low levels during yeast growth and are highly induced during filamentous growth (9, 19). This is not the case for *ACB1*. The transcript level of the *ACB1* gene is high even during yeast growth in YPD medium. In fact, on the basis of our recent RNA-seq data analyses, *ACB1* ranks in the top ~5% among all cryptococcal genes expressed (46). However, whether Znf2 directly or indirectly affects the activity of Acb1 at other regulatory levels (e.g., translation, protein localization, secretion, or modification) has yet to be investigated.

The cryptococcal genome carries two genes that encode proteins with an acyl-CoA-binding domain. One is CNAG_06140, which we named *ACB1* in this study; it is predicted to encode a protein of a little over 100 amino acids (Fig. 4A). The other is CNAG_01191, which is predicted to encode a long-chain fatty acid transporter of 458 amino acids. Given that Acbp in *D. discoideum* and Acb1 in *S. cerevisiae* are composed of 84 and 87 amino acids, respectively (23, 26, 35, 71), we considered CNAG_06140/Acb1 in *C. neoformans* more likely to be an ortholog of Acb proteins. Furthermore, based on our RNA-seq data, the transcript level of *ACB1* is about 10-fold higher than that of CNAG_01191 (46). Thus, we focused on *ACB1* in this study. However, it is likely that the lack of any defect of the *Cryptococcus acb1Δ* mutant in utilizing various carbon sources could be due to functional redundancy of Acb1 and the protein encoded by CNAG_01191 in fatty acid metabolism.

The observation that secreted products from the wild type, but not the *acb1Δ* mutant, can partially restore the filamentation defect of a nearby *acb1Δ* mutant suggests that secreted Acb1 proteins can act intercellularly. Given that the filamentation of the *acb1Δ* mutant is not as robust as that of the wild type, even when it is confronted by the wild-type donor, Acb1 likely functions in a paracrine fashion. We hypothesize that it is the extracellular Acb1 proteins, and not the intracellular Acb1 proteins, that are critical for filamentation. This hypothesis is consistent with the predicted paracrine signaling function of Acb1 and is also corroborated by the finding that the *graspΔ* mutant, which is defective in secreting

Acb1 to the environment but not in recruiting Acb1 to the secretory pathway, displays a drastic reduction in filamentation, similar to that of the *acb1Δ* mutant. The hypothesis is further supported by the observation that mislocalization of Acb1(Y80) to the cytosol and the consequent lack of protein secretion render the protein nonfunctional.

One interesting aspect of Acb1 is its unconventional recruitment to the secretory pathway. Although Grasp is critical for the extracellular secretion of Acb1, Grasp is not involved in recruiting Acb1 to the secretory vesicles intracellularly. Since the Y80 mutation is known to disrupt the acyl-binding property of Acb1 (58, 59, 72), and since Acb1(Y80A) showed diffused localization in the cytosol, it is tempting to speculate that the binding partner of Acb1, be it a lipid or a protein, might help recruit Acb1 to the vesicles through interaction with its acyl-binding domain. How Grasp recognizes Acb1 after Acb1 is recruited to secretory vesicles and how it assists in the extracellular secretion of Acb1 are unknown. It is clear, on the basis of this study and a previous study (69), that Grasp is involved in the secretion of Acb1 and additional factors in *C. neoformans*. In agreement with this idea, the secretion of integrin in *Drosophila melanogaster* and of CFTR in mammalian cells also depends on an unconventional Grasp-mediated mechanism (67, 68, 73). Given that many proteins that are found to be secreted extracellularly are atypical proteins that possess no signal peptide in fungi (74–78), it is important to continue the investigation into these atypical proteins and the corresponding unconventional secretory pathways.

In accord with the idea that Grasp is responsible for the secretion of factors in addition to Acb1, the *graspΔ* mutant showed drastically decreased capsule production (69; this study), while the *acb1Δ* mutant showed normal capsule production. Similarly, in contrast to the severe phagocytosis defect of the *graspΔ* mutant, the *acb1Δ* mutant behaves similarly to the wild type in the phagocytosis assay. Thus, Grasp likely affects the secretion of various molecules (e.g., capsule and possibly some cell wall proteins) that contribute to the phenotypic defects of the *graspΔ* mutant in various assays. Nonetheless, in terms of the effect of Grasp on hyphal growth, Acb1 appears to be the major factor, if not the sole factor.

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