A Possible Role for Exocytosis in Aflatoxin Export in *Aspergillus parasiticus* \(^\dagger\)

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Received 17 May 2010/Accepted 14 September 2010

Filamentous fungi synthesize bioactive secondary metabolites with major human health and economic impacts. Little is known about the mechanisms that mediate the export of these metabolites to the cell exterior. *Aspergillus parasiticus* synthesizes aflatoxin, a secondary metabolite that is one of the most potent naturally occurring carcinogens known. We previously demonstrated that aflatoxin is synthesized and compartmentalized in specialized vesicles called aflatoxisomes and that these subcellular organelles also play a role in the export process. In the current study, we tested the hypothesis that aflatoxisomes fuse with the cytoplasmic membrane to facilitate the release of aflatoxin into the growth environment. Microscopic analysis of *A. parasiticus* grown under aflatoxin-inducing and non-aflatoxin-inducing conditions generated several lines of experimental evidence that supported the hypothesis. On the basis of the evidence, we propose that export of the mycotoxin aflatoxin in *Aspergillus parasiticus* occurs by exocytosis, and we present a model to illustrate this export mechanism.

Secondary metabolites are chemically diverse natural products synthesized by plants, fungi, bacteria, algae, and animals. Secondary metabolites have an enormous impact on humans. Antibiotics, for example, are essential elements of the multi-billion-dollar pharmaceutical industry, whereas mycotoxins cause hundreds of millions of dollars in damage to agriculture annually (11, 15). These chemicals help the producing organism to survive nutrient limitation (16). They also contribute to cellular defense mechanisms and development (11, 12), reduce cellular oxidative stress (10), and help maintain cellular homeostasis by regulating carbon flow in the cell (17).

Many fungal secondary metabolites are exported outside the cell; examples include antibiotics and mycotoxins (3, 14). We and others conducted extensive studies on the regulation of fungal secondary metabolism at the molecular (11, 15) and cellular (3, 7) levels. However, little is known about the mechanisms that mediate secondary metabolite export or why export occurs.

The filamentous fungus *Aspergillus parasiticus* produces aflatoxin, a secondary metabolite and the most potent naturally occurring carcinogen known. More than 90% of aflatoxin is exported to the cell exterior (3), making *A. parasiticus* an excellent model for studying secondary metabolite export. We recently demonstrated that specialized trafficking vesicles called aflatoxisomes play a key role in aflatoxin synthesis and export (3). As synthesis initiates, vesicle-vacuole fusion is downregulated by the global regulator *Velvet*, resulting in the accumulation of aflatoxisomes which contain at least the last two functional enzymes in the aflatoxin pathway and sequester aflatoxin (3). Treatments that block vesicle-vacuole fusion increase the number of aflatoxisomes, increase the quantity of aflatoxin accumulated in aflatoxisomes, and increase aflatoxin export to the cell exterior (3). On the basis of these previous observations, we hypothesized that aflatoxisomes play a direct role in aflatoxin export.

Vesicle-mediated export could theoretically occur by one (or more) of at least three mechanisms (Fig. 1). (i) Vesicles pass across the cytoplasmic membrane intact and “shuttle” their contents into the external environment. This proposed mechanism mediates virulence factor release in *Cryptococcus neoformans* and *Histoplasma capsulatum* (1) during pathogenesis. (ii) Vesicles fuse to the cytoplasmic membrane and “pump” vesicle contents to the exterior using transporter proteins similar to those that mediate resistance to antifungal agents (4, 5). (iii) Vesicles fuse with the cytoplasmic membrane, which evacuates, bursts, and “blasts” vesicle contents to the exterior. This process is similar to exocytosis, a proposed secretory mechanism for specific proteins in filamentous fungi (18). We conducted the current study to determine which, if any, of these possible mechanisms most accurately reflects the process of aflatoxin export in *A. parasiticus*.

**MATERIALS AND METHODS**

Strains, media, and growth conditions. *Aspergillus parasiticus* strain SU-1 (ATCC 56775) was the wild-type aflatoxin producer used in this study. *A. parasiticus* strain AF1 (affR), derived from SU-1, was used as a non-aflatoxin-producing control strain. *A. parasiticus* strain B62 (nor-1 brn-1; ATCC 24690) was derived from SU-1 and carries a genetic block in the aflatoxin biosynthetic pathway (9). Strain B62 accumulates large quantities of the bright red aflatoxin intermediate norsolorinic acid (NA) as well as significantly reduced quantities of aflatoxin compared to SU-1 (9). YES liquid medium (2% yeast extract and 6% sucrose; pH 5.8) was used as an aflatoxin-inducing growth
medium. YEP liquid medium (2% yeast extract and 6% peptone, pH 5.8) was used as a non-aflatoxin-inducing medium. Conidiospores (spores) from frozen stocks of \textit{A. parasiticus} were inoculated into liquid growth medium at 10^6 spores per ml and incubated for appropriate time periods at 30°C with shaking at 150 rpm in the dark (standard growth conditions). Sortin1 treatment of \textit{A. parasiticus} strain V-86 (synthesizes the aflatoxin enzyme Ver-1 fused with enhanced green fluorescent protein [EGFP] at the C-terminal end [13]) was conducted with established dosages (3).

Protein analysis. Protein concentration measurements and Western blot analysis were performed as described previously (3).

Microscopy. FUN-1 stain (Invitrogen, Carlsbad, CA) was used according to the manufacturer’s instructions, as described previously (6). For fluorescence microscopy, microscope slides carrying samples were observed under a Labophot fluorescence microscope (Nikon, Inc., Melville, NY). For bright-field microscopy, microscope slides carrying samples were observed under a Labophot fluorescence microscope (Nikon, Inc., Melville, NY). For confocal laser scanning microscopy, MDY-64 (a vacuole and vesicle membrane dye) and Cell Tracker Blue CMAC (a vacuolar lumen stain based on peptidase activity) (Molecular Probes, Invitrogen, Carlsbad, CA) were used to visualize vacuoles and vesicles. These dyes were utilized according to the manufacturer’s protocols, with modifications, as described previously (2). Images were acquired using an Olympus Fluoview 1000 confocal laser scanning microscope (CLSM) (Olympus, Center Valley, PA) using a 60×/1.42-numerical-aperture oil objective and a 430–470-band-pass (BP) emission filter set under excitation with the 405-nm diode laser line for CMAC fluorescence (355-nm excitation/466-nm emission) and a 505–525-BP emission filter set under excitation with the 488-nm diode laser line for MDY-64 fluorescence (451-nm excitation/497-nm emission).

Surface immunofluorescence. Rabbit antibodies against aflatoxin B$_1$ (Sigma, St. Louis, MO) were used as primary antibodies to detect aflatoxin on the hyphal surface. Fluorescein isothiocyanate (FITC)-labeled anti-rabbit IgG (Sigma) was used as a secondary antibody to detect the binding pattern of the primary antibody. Rabbit antibodies against satratoxin (kindly provided by James J. Pestka) were used as a control to demonstrate immunoassay specificity. To conduct the assay, a mycelial pellet harvested from liquid growth medium wasuntangled with sterile forceps until single hyphae were obtained. Several hyphae were added to 50 μl of fresh YES with anti-AFB$_1$ (α-AFB$_1$) antibodies (diluted 1:10) and incubated for 30 min at 30°C. Labeled mycelia were centrifuged at 13,000 rpm at 4°C, washed three times with fresh YES medium at room temperature (RT), and treated for 15 min with FITC-labeled goat anti-rabbit IgG (secondary antibody; diluted 1:50 in fresh YES) at RT. Finally, mycelia were centrifuged and washed three times with fresh YES medium at RT before being observed under a Labophot fluorescence microscope (Nikon, Inc., Melville, NY).

RESULTS

To shed light on possible export mechanisms in \textit{A. parasiticus}, the fungus was grown for 36 h in an aflatoxin-inducing liquid medium (yeast extract-sucrose [YES] medium) under standard conditions (30°C, with shaking at 150 rpm, in the dark) (3). Samples of mycelia were treated with a vesicle-vacuole membrane stain (MDY64) (Invitrogen, Carlsbad, CA) and a vesicle-vacuole lumen stain (CMAC) (Invitrogen, Carlsbad, CA) (Fig. 2a) and then analyzed by confocal laser scanning microscopy (z series). We observed a shift of vesicles (green membrane with blue lumen) from the cytoplasm to the inner surface of the cytoplasmic membrane (Fig. 2a, yellow arrows) in \textit{A. parasiticus} at the onset of peak levels of aflatoxin synthesis. At this time (36 h), a large number of vesicles fused with the cytoplasmic membrane (Fig. 2a, red arrows), and some of these appeared to evaginate into the external growth environment (white arrows). Our previous data (3) strongly suggest that these vesicles are aflatoxisomes that carry aflatoxin and functional aflatoxin enzymes. In support of this idea, we previously observed that aflatoxin synthesis increases during a transition from active growth to stationary phase (24 to 36 h) (8). During this transition, the number of vesicles carrying the EGFP-tagged aflatoxin enzyme Ver-1 increased significantly (8) and these adhered to the inner surface of the cytoplasmic membrane. Under these conditions, intact vesicles were not...
detected in the growth medium either by light microscopy or by fluorescence microscopy.

Interestingly, treatment of the fungus with Sortin1 (19), a low-molecular-weight protein sorting inhibitor, induced a transmembrane vesicle shuttle in *A. parasiticus* (see Fig. S1 in the supplemental material). This observation strongly suggests that transmembrane shuttle machinery exists in *A. parasiticus* but is likely not involved in aflatoxin export. However, Sortin1 treatment also inhibited aflatoxin production and growth severely (data not shown), suggesting that the vesicles observed in the medium under Sortin1 treatment may contribute to primary metabolism.

FUN-1 (Invitrogen, Carlsbad, CA), a fluorescent vital dye, accumulates in vacuoles via endocytosis and appears as bright red cylindrical intravacuolar structures (CIVS) (6). As expected, CIVS were observed in vacuoles during the early stages of aflatoxin synthesis (up to 24 h of growth) (Fig. 2b). However, later in stationary phase, when aflatoxin synthesis and aflatoxin accumulation in vacuoles via endocytosis and appears as bright red-orange CIVS within vacuoles (white arrows). Instead, diffuse patches of red/orange fluorescence are observed in protrusions near the hyphal surface (white arrows). (b) Fluorescence image showing aflatoxin in the cell exterior. (a) Model explaining the specificity of antibody binding shown in Fig. 2e and the fluorescence pattern shown in panel b. (b) Fluorescence image of *A. parasiticus* B62 grown for 72 h in YES. Norsolorinic acid accumulation on the cell surface enhances the intensity of red fluorescent patches in vesicles near the hyphal surface (white arrows). Nuclei (green) were stained with SYTOX green. (c) Western blot analysis. *A. parasiticus* SU-1 (wild type) and an *avaA* mutant (AC11) derived from SU-1 were grown in YES medium for 72 h. AC11 produces significantly higher numbers of aflatoxisomes and larger quantities of aflatoxin than SU-1 (1). Aflatoxin enzymes Vers-1, Vbs, and OmtA in the growth medium were detected by Western blot analysis.

Non-aflatoxin-inducing medium (yeast extract-peptone [YEP] growth medium) or on the surface of AFS10, a regulatory mutant that does not make aflatoxin (data not shown) or pathway intermediates. These data strongly support the involvement of the blast mechanism in aflatoxin export.

On the basis of these observations, we hypothesize (Fig. 1) that initially, aflatoxisomes carrying aflatoxin and aflatoxin enzymes are transported to the inner surface of the cytoplasmic membrane, where they fuse with this structure. The cytoplasmic membrane at this site then evaginates (protrudes) outward, bursts, and blasts vesicle contents into the growth medium; this series of events is similar to exocytosis (18). As a result of the blast, a “crater” forms on the exterior hyphal surface as illustrated in Fig. 3a. In support of this proposed series of events, we observed that while most aflatoxin is blasted into the growth medium, some aflatoxin remains bound to the crater surface and this bound toxin provides binding sites for anti-AFB₁ antibodies (Fig. 2e).

To begin to test the details of this model, we analyzed toxin export in *A. parasiticus* B62 grown under standard conditions (30°C, with shaking at 150 rpm, in the dark). *A. parasiticus* B62 carries a nonfunctional Nor-1 enzyme and accumulates norsolorinic acid (NA), a bright red visible pigment and the first stable intermediate in aflatoxin synthesis (9). NA exhibits poor solubility in aqueous solution; hence, NA binds to the mycelium and is not detected in the growth medium. The accumulation of NA is a useful method for visualizing the accumulation of aflatoxin, which is not visible under light in the visible wavelength. After 72 h of growth in YES growth medium, we stained *A. parasiticus* B62 with FUN-1 and observed the mycelium by use of a fluorescence microscope. At 72 h, *A. para-
situs B62 accumulated CIVS in patches on (or within) the exterior surface of the mycelium (Fig. 3b), and these patches appeared similar to patches detected by anti-AFB3 antibodies in A. parasiticus SU-1 (wild type) (Fig. 2e). This observation tends to support the exocytosis (blast) model. Because NA is reported to be synthesized in peroxisomes (13), we hypothesize that NA in A. parasiticus B62 is transported by aflatoxosomes to the cytoplasmic membrane for export. Because of its low-level solubility in aqueous solution, we also propose that NA accumulates on the mycelial surface and blocks the ability of CIVS to exit cells; the localized increases in NA and CIVS result in the more-intense red staining observed at the cell surface in A. parasiticus B62 (compare Fig. 3b with 2d and e).

We previously demonstrated that aflatoxosomes in A. parasiticus SU-1 (wild type) carry at least three aflatoxin enzymes (OmtA, Ver-1, and Vbs) in aflatoxosomes (3). The current study demonstrates that these proteins can be detected in the growth medium at 72 h of growth after the rate of aflatoxin synthesis declines (Fig. 3c). We previously disrupted ava4 (which encodes one protein in the tethering complex that drives vesicle vacuole fusion) in A. parasiticus AC-11 (Δava4) (3); this strain accumulates higher numbers of aflatoxosomes and exports aflatoxin at significantly higher levels than SU-1. In the current study, we demonstrated a 2-fold increase in aflatoxin enzymes present in the growth medium (Fig. 3c). In contrast to aflatoxin, the aflatoxin enzymes could not be detected on the exterior cell surface in the current study using enzyme-specific antibodies (data not shown). These observations also tend to support the exocytosis (blast) mechanism.

**DISCUSSION**

In contrast to the significant quantity of data in support of the proposed exocytosis (blast) mechanism presented here, we have yet to obtain any direct evidence supporting or disproving a “pump” export mechanism in A. parasiticus. We recently completed proteomic profile analysis (MudPIT) of a pure vesicle-vacuole fraction containing aflatoxosomes (unpublished data), and the data suggest that AtT, an MFS-like protein, is present in this fraction. However, Chang et al. disrupted afIT in A. parasiticus and reported no effect on aflatoxin secretion (4). Although these observations tend to support our model, we cannot rule out the possibility that transporter proteins might play a role in secondary metabolite export in the initial stages of aflatoxin secretion. It is possible that removal of an MFS transporter by gene disruption would not block export completely (for example, see penicillin and cephalosporin secretion [14]), since the blast mechanism would still theoretically operate and compensate for the impaired pump. This alternative export pathway (pump/blast) is depicted in Fig. 1.

This work may have important practical implications. We demonstrated that we can increase or decrease aflatoxin export by modifying the growth medium composition or by introducing a specific mutation into the transport machinery. These observations suggest that control of the export pathway provides a great opportunity to manipulate fungal secondary metabolism to increase production of beneficial secondary metabolites or to reduce production of detrimental ones. Future research will now focus on the export mechanism in greater detail to test our hypotheses and the accuracy of our model. In addition, the methods that we developed for detection of surface immunofluorescence in our current work may serve as a useful diagnostic tool to monitor the level of vesicle-mediated secondary metabolite export in filamentous fungi.

**ACKNOWLEDGMENTS**

This work was supported by NIH grant R01 52003-19 and the Michigan Agricultural Experiment Station.

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