Calcineurin Localizes to the Hyphal Septum in *Aspergillus fumigatus*: Implications for Septum Formation and Conidiophore Development

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Calcineurin, a Ca\(^{2+}\)-calmodulin-dependent protein phosphatase (PP2B), regulates diverse processes, including morphogenesis, ion homeostasis, virulence, and stress responses in fungi (1, 3–5, 9, 12, 13, 16–18, 28). In *Aspergillus fumigatus*, calcineurin is required for hyphal growth, cell wall integrity, conidial morphology, PO\(_4\)\(^{3-}\) transport, and pathogenicity via its downstream target, CrzA (2, 6, 20–24). However, the specific molecular mechanisms by which the calcineurin signal transduction pathway regulates hyphal extension or cell wall biosynthesis remain largely unknown.

To elucidate calcineurin roles in hyphal growth, its localization pattern was analyzed during growth by fusion to enhanced green fluorescent protein (EGFP), CnaA-EGFP, which was expressed in the *Aspergillus fumigatus* ΔcnaA mutant. CnaA-EGFP localized in actively growing hyphal tips, at the septa, and at junctions between the vesicle and phialides in an actin-dependent manner. This is the first study to implicate calcineurin in septum formation and conidiophore development of a filamentous fungus.

A functional calcineurin A fusion to enhanced green fluorescent protein (EGFP), CnaA-EGFP, was expressed in the *Aspergillus fumigatus* ΔcnaA mutant. CnaA-EGFP localized in actively growing hyphal tips, at the septa, and at junctions between the vesicle and phialides in an actin-dependent manner. This is the first study to implicate calcineurin in septum formation and conidiophore development of a filamentous fungus.

Because the ΔcnaA mutant exhibits stunted hyphal growth (6, 21), we next examined whether the cnaA-egfp expression strain returned to wild-type growth. As shown in Fig. 1B, radial growth, quantified as previously described (21), indicated that cnaA-egfp expression complemented the cnaA deletion and restored the wild-type phenotype. The cnaA-egfp expression strain was also grown in the presence of FK506, a specific inhibitor of calcineurin, to reconfirm that hyphal growth was stunted after FK506 treatment in a similar manner to that of the wild-type strain (Fig. 1C, plate lane 4).

Microscopic examination of the wild-type and cnaA-egfp expression strains grown in the presence of FK506 also revealed a highly branched phenotype similar to the ΔcnaA mutant (Fig. 1D, lower panel). Taken together, these results indicate that the cnaA-egfp construct is functional after placement in the ΔcnaA mutant.

Next, in order to observe the localization patterns of CnaA-EGFP, the cnaA-egfp expression strain was grown on slide cultures for 24 h. While fluorescence microscopy revealed a general distribution of the CnaA-EGFP fusion protein in the cytoplasm, dot-like structures were observed closer to the cell wall in conidia (Fig. 2A and B) and at the hyphal tips (Fig. 2D). Very interestingly, the CnaA-EGFP fusion protein highly concentrated and localized on either side of the septa (Fig. 2G [see inset image]). The fusion protein localized both at the newly formed septa and the septa of the older hyphal compartments (Fig. 2C, E, F, and G). We noted that the CnaA-EGFP dot-like structures moved and concentrated in the middle of the septum (Fig. 2H) and at the septa of the older hyphal compartments (Fig. 2C, E, F, and G).

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Three-dimensional imaging of the septum showed a disk-like appearance of CnaA-EGFP localization around the septal pore (see figure in the supplemental material). In addition, the CnaA-EGFP fusion protein localized to dot-like structures (resembling cortical patches) during the formation of the conidiophore. As shown in Fig. 3A and B, the dot-like structures seemed to move into the vesicle and localize at junctions between the vesicle and phialides in a mature conidiophore (Fig. 3D). The CnaA-EGFP spots outlined the points of formation of a new septum as well (Fig. 3B, arrowheads). Transformation of A. fumigatus with the pUCGH plasmid alone showed only cytoplasmic localization of EGFP (14).

Because the CnaA-EGFP localized to points of phialide formation, we next examined whether conidiophore development was impaired in the ΔcnaA mutant and, as well, upon treatment of the wild-type and cnaA-egfp expression strains with FK506. In contrast to conidiophores observed in the cnaA-egfp expression and wild-type strains (Fig. 1D, upper panel), ΔcnaA mutant or FK506 treated cells showed a complete absence of conidiophores, indicating calcineurin is required for conidiophore formation (Fig. 1D, lower panel). In addition, hyphae treated with FK506 showed several septa dividing smaller compartments, in comparison to the untreated wild-type strain that showed septa at regular intervals dividing large compartments (data not shown), indicating that calcineurin may be required for regular hyphal extension and septation and a possible reason for the overall blunted growth following calcineurin inhibition. In Schizosaccharomyces pombe it was reported that calcineurin localized at the septa, and its deletion resulted in thick and incomplete septation (15). Interestingly, a recent study in Aspergillus nidulans has implicated a role for the protein phosphatase 1 (BIMG) in septum formation (10). However, in contrast to our results, BIMG-GFP only transiently localized to the septum.
Microtubules and actin filaments are involved in vesicle transport in filamentous fungi (8). The presence of calcineurin in dot-like structures at the hyphal tips, as cortical patches during conidiophore formation, at junctions of phialide formation, and at the septa, prompted us to examine whether the microtubular network or actin filaments were contributing to calcineurin localization. To test this hypothesis, the CnaA-EGFP expression strain was grown for 16 h and then treated with nocodazole (to inhibit microtubules) or cytochalasin A (to inhibit actin polymerization) for an additional 8 h. While the nocodazole-treated sample did not show any mislocalization of CnaA-EGFP (data not shown), the cytochalasin A-treated sample showed a complete cytosolic redistribution of CnaA-EGFP (Fig. 4C and D), indicating that the transport of calcineurin to the septum is an actin-dependent process. Treatment of hyphae grown for 16, 20, and 22 h with cytochalasin A for additional time periods of 1, 4, and 2 h, respectively, also mislocalized CnaA-EGFP from the septa (data not shown). These data also revealed that actin may be required for not only formation of septa but also the maintenance of CnaA-EGFP at completed septa (septa that were formed before the addition of cytochalasin A).

In addition to reports on localization of chitin synthases at the septum (11, 25), two recent reports on A. nidulans PkcA::GFP (26) and SwoM::GFP (27), also involved in the cell wall integrity pathway, showed localization patterns similar to that observed in our study. Interestingly, calmodulin, which binds and activates calcineurin, has also been implicated in the organization of actin cytoskeleton (7, 19, 29). Future directions will include an examination of the interaction of calcineurin with other proteins mediating cell wall formation.
biosynthesis. It would be premature at this stage of analysis to ascribe a detailed mechanism for calcineurin localization and functions at the septum and in conidiophore development. Our future studies will address the questions of how and why calcineurin localizes at the septum, since it is currently unclear whether cnaA mRNA localizes at the septum and is translated locally or is transported to the septum via its interacting proteins.

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