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

Praveen Rao Juvvadi, Jarrod R. Fortwendel, Nadthanan Pinchai, B. Zachary Perfect, Joseph Heitman, and William J. Steinbach

Department of Pediatrics, Duke University Medical Center, Durham, North Carolina; Department of Molecular Genetics and Microbiology, Duke University Medical Center, Durham, North Carolina; Department of Medicine, Duke University Medical Center, Durham, North Carolina; and Department of Pharmacology and Cancer Biology, Duke University Medical Center, Durham, North Carolina

Received 18 June 2008/Accepted 23 June 2008

Calcineurin, a Ca²⁺-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, PO₄⁻ 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, 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.
Three-dimensional imaging of the septum showed a disk-like appearance of CnaA-EGFP localization around the septal pore (see the 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

FIG. 2. Localization of CnaA-EGFP during growth of A. fumigatus. The cnaA-egfp strain was grown in 100 μl of GMM liquid medium on coverslips for 24 h and observed by fluorescence microscopy. (A) Distribution of CnaA-EGFP in dormant conidia at 0 h of growth. (B) Swollen conidium at 3 h of growth. CnaA-EGFP is localized in small vesicular structures at the region of germ tube formation. (C) Germling at 6 h of growth showing a newly formed septum to which CnaA-EGFP is localized. (D) The CnaA-EGFP is localized to the hyphal tip in dot-like structures. (E) Middle regions of hypha showing mature septa with CnaA-EGFP. (F) CnaA-EGFP fusion protein in dot-like structures seen closely associated with the central region of the septum. (G) Vesicle showing the localization of CnaA-EGFP at its septum. Scale bar, 10 μm.
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 \textit{cnaA} mRNA localizes at the septum and is translated locally or is transported to the septum via its interacting proteins.

W.J.S. is supported by an NIH/NIAID K08 A1061149 award, a Basic Science Faculty Development grant from the American Society for Transplantation, and a Children’s Miracle Network grant.

REFERENCES

1. Bader, T., K. Schroppel, S. Bentink, N. Agabian, G. Kohler, and J. Morshchauser. 2006. Role of calcineurin in stress resistance, morphogenesis, and virulence of a \textit{Candida albicans} wild-type strain. Infect. Immun. 74:4366–4369.
2. Cramer, R. A., Jr., R. Z. Perfect, N. Pinchay, S. Park, D. S. Perlis, Y. G. Asfaw, J. Heitman, J. R. Perfect, and W. J. Steinbach. 2008. Calcineurin target CrzA regulates conidial germination, hyphal growth, and pathogenesis of Aspergillus fumigatus. Eukaryot. Cell 7:1083–1097.

3. Cruz, M. C., D. S. Fox, and J. Heitman. 2001. Calcineurin is required for hyphal elongation during mating and haploid fruiting in Cryptococcus neoformans. EMBO J. 20:1020–1032.

4. Cruz, M. C., A. L. Goldstein, J. R. Blunkenship, M. Del Poeta, D. Davis, M. E. Cardenas, J. R. Perfect, J. H. McCusker, and J. Heitman. 2002. Calcineurin is essential for survival during membrane stress in Candida albicans. EMBO J. 21:546–559.

5. Cyert, M. S. 2003. Calcineurin signaling in Saccharomyces cerevisiae: how yeast go crazy in response to stress. Biochem. Biophys. Res. Commun. 311:1143–1150.

6. da Silva Ferreira, M. E., T. Heinekamp, A. Härts, A. A. Brakhage, C. P. Semighini, S. D. Harris, M. Savoldi, P. F. de Gouveia, M. H. de Souza Goldman, and G. H. Harris. 2007. Functional characterization of the Aspergillus fumigatus calcineurin. Fungal Genet. Biol. 44:219–230.

7. Desrivieres, S., F. T. Cooke, H. Morales-Johansson, P. J. Parker, and M. N. Hall. 2002. Calmodulin controls organization of the actin cytoskeleton via regulation of phosphatidylinositol (4,5)-bisphosphate synthesis in Saccharomyces cerevisiae. Biochem. J. 366:945–951.

8. Fischer, R., N. Zekert, and N. Takeshita. 2008. Polarized growth in fungal-interplay between the cytoskeleton, positional markers and membrane domains. Mol. Microbiol. 68:813–826.

9. Fox, D. S., and J. Heitman. 2002. Good fungi gone bad: the corruption of calcineurin. Bioessays 24:894–903.

10. Fox, H., P. C. Hickey, J. M. Fernández-Abalos, P. Lanness, N. D. Read, and J. H. Doonan. 2002. Dynamic distribution of BIMoPP1 in living hyphae of Aspergillus indicates a novel role in septum formation. Mol. Microbiol. 45:1219–1230.

11. Ichinomiya, M., E. Yamada, S. Yamashita, A. Ohta, and H. Horinouchi. 2005. Class I and class II chitin synthases are involved in septum formation in the filamentous fungus Aspergillus nidulans. Eukaryot. Cell 4:1125–1136.

12. Juvadi, P. R., Y. Kuroki, M. Arioka, H. Nakajima, and K. Kitamoto. 2003. Functional analysis of the calcineurin-encoding gene cnaA from Aspergillus oryzae: evidence for its putative role in stress adaptation. Arch. Microbiol. 179:16–422.

13. Kraus, P. R., and J. Heitman. 2003. Coping with stress: calmodulin and calcineurin in model and pathogenic fungi. Biochem. Biophys. Res. Commun. 311:1151–1157.

14. Langfelder, K., R. Philippe, B. Jahn, J. P. Latgé, and A. A. Brakhage. 2001. Differential expression of the Aspergillus fumigatus pckP gene detected in vitro and in vivo with green fluorescent protein. Infect. Immun. 69:6411–6418.

15. Lu, Y.-R. Sugiuira, T. Yada, H. Cheng, S. O. Sio, H. Shuntoh, and T. Kuno. 2002. Calcineurin is implicated in the regulation of the septation initiation network in fission yeast. Genes Cells 7:1009–1019.

16. Mendoza, L., F. J. Quintero, R. A. Bressan, P. M. Hasegawa, and J. M. Pardo. 1996. Activated calcineurin confers high tolerance to ion stress and alters the budding pattern and cell morphology of yeast cells. J. Biol. Chem. 271:23861–23867.

17. Miyakawa, T., and M. Mizunuma. 2007. Physiological roles of calcineurin in Saccharomyces cerevisiae with special emphasis on its roles in G2/M cell-cycle regulation. Biosci. Biotechnol. Biochem. 71:633–645.

18. Odom, A., S. Muir, E. Lim, D. L. Toffaletti, J. Perfect, and J. Heitman. 1997. Calcineurin is required for virulence of Cryptococcus neoformans. EMBO J. 16:2576–2589.

19. Sekiya-Kawasaki, M., D. Botstein, and Y. Ohy. 1998. Identification of functional connections between calmodulin and the yeast actin skeleton. Genetics 148:43–58.

20. Sarti, F. M., I. Malavazi, M. E. da Silva Ferreira, M. Savoldi, M. R. Von Zeska Kress, M. H. de Souza Goldman, O. Loss, E. Bignell, and G. H. Goldman. 2008. Functional characterization of the Aspergillus fumigatus CRZ1 homologue, CrzA. Mol. Microbiol. 67:1274–1291.

21. Steinbach, W. J., R. A. Cramer, B. Z. Perfect, Y. G. Asfaw, T. C. Sauer, L. K. Najvar, W. R. Kirkpatrick, T. F. Patterson, D. K. Benjamin, J. Heitman, and J. R. Perfect. 2006. Calcineurin controls growth, morphology, and pathogenicity in Aspergillus fumigatus. Eukaryot. Cell 5:1091–1103.

22. Steinbach, W. J., R. A. Cramer, B. Z. Perfect, C. Henn, K. Nielsen, J. Heitman, and J. R. Perfect. 2007. Calcineurin inhibition or mutation enhances cell wall inhibitors against Aspergillus fumigatus. Antimicrob. Agents Chemother. 51:2979–2981.

23. Steinbach, W. J., J. L. Reedy, R. A. Cramer, Jr., J. R. Perfect, and J. Heitman. 2007. Harnessing calcineurin as a novel anti-infective agent against invasive fungal infections. Nat. Rev. Microbiol. 5:418–430.

24. Stie, J., and D. Fox. 2008. Calcineurin regulation in fungi and beyond. Eukaryot. Cell 7:177–186.

25. Takeshita, N., S. Yamashita, A. Ohta, and H. Horinouchi. 2006. Aspergillus nidulans class V and VI chitin synthases ComA and ComB, each with a myosin motor-like domain, perform compensatory functions that are essential for hyphal tip growth. Mol. Microbiol. 59:1380–1394.

26. Teepe, A. G., D. M. Loprete, Z. He, T. A. Hoggard, and T. W. Hill. 2007. The protein kinase C orthologue PkcA plays a role in cell wall integrity and polarized growth in Aspergillus nidulans. Fungal Genet. Biol. 44:554–562.

27. Upadhyay, S., and B. D. Shaw. 2006. A phosphoglucone isomerase mutant in Aspergillus nidulans is defective in hyphal polarity and conidiation. Fungal Genet. Biol. 43:739–751.

28. Yoshida, T., T. Toda, and M. Yanagida. 1994. A calcinuric-like gene ppk1+ in fission yeast: mutant defects in cytokinesis, cell polarity, mating and spindle pole body positioning. J. Cell Sci. 107:1725–1735.

29. Yuan, J., G. X. Shi, Y. Shao, G. Dai, J. N. Wei, D. C. Chang, and C. J. Li. 2008. Calmodulin bound to stress fibers but not microtubules involves regulation of cell morphology and motility. Int. J. Biochem. Cell Biol. 40:284–293.