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Elucidating the *Candida albicans* calcineurin signaling cascade controlling stress response and virulence

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ABSTRACT

The protein phosphatase calcineurin is a key mediator of virulence and antifungal susceptibility of multiple fungal pathogens including *Candida albicans*, *Cryptococcus neoformans*, and *Aspergillus fumigatus*, and has clinical potential as a therapeutic target to increase the efficacy of the current antifungal armamentarium. Despite the importance of this signaling pathway, few components of the calcineurin-signaling pathway are known in *C. albicans*. Here we identified and analyzed additional components of the *C. albicans* calcineurin cascade, including the *RCN1* (regulator of calcineurin1), *MID1*, and *CCH1* genes, which mediate calcineurin functions in other species. When heterologously expressed in *Saccharomyces cerevisiae*, *C. albicans* Rcn1 inhibited calcineurin function. Although *rcn1*/*rcn1*, *mid1*/*mid1*, and *cch1*/*cch1* mutant strains share some phenotypes with calcineurin mutants, they do not completely recapitulate the phenotypes of a calcineurin mutant strain. These studies extend our understanding of the *C. albicans* calcineurin signaling cascade and its host-niche specific role in virulence.

1. Introduction

Calcineurin is a calcium, calmodulin-dependent serine–threonine specific protein phosphatase that is highly conserved from yeast to humans and mediates many important cellular processes (Hemenway and Heitman, 1999). In mammalian cells, calcineurin is involved in cardiac muscle differentiation (Chin et al., 1998; Kramer et al., 2003; Parsons et al., 2007), memory (Mansuy et al., 1998; Weitlauf and Winder, 2001), T-cell activation (Clipstone and Crabtree, 1992), and apoptosis (Krebs, 1998; Saito et al., 2000; Shibasaki and McKeon, 1995; Wang et al., 1999). The immunosuppressants FK506 and Cyclosporin A (CsA) exert their effect by entering cells and binding to an immunophilin protein partner (FKBP12 for FK506, and Cyclophilin A for CsA) (Cardenas et al., 1994, 1995; Clipstone et al., 1994; Ho et al., 1996). This protein–drug complex subsequently binds calcineurin and inhibits its activity and functions. In human T-cells, calcineurin activates a transcription factor (NF-AT), which promotes the expression of cytokines and T-cell proliferation (Crabtree, 1999). Due to the highly conserved nature of calcineurin, it was subsequently found that FK506 and CsA can inhibit not only mammalian calcineurin, but also fungal calcineurin (Blankenship et al., 2003a; Breuder et al., 1994; Foor et al., 1992; Nakamura et al., 1993; Steinbach et al., 2007).

Candida spp. are normal components of the human microbiota; however, under conditions of immunosuppression or altered host defenses these commensals have the ability to cause serious mucocutaneous and systemic disease (Odds, 1988). Diseases due to *Candida* spp. manifest a variety of clinical manifestations, ranging from mucocutaneous infections of the mouth (thrush), esophagus, and vagina to life-threatening systemic infections, where *Candida* spp. enter the bloodstream and disseminate throughout the body to infiltrate target organs (Edwards, 1991). Although *Candida albicans* has historically accounted for the majority of candidal infections, following the introduction of the antifungal fluconazole numerous other species have increased in prevalence including *Candida glabrata*, *Candida parapsilosis*, *Candida tropicalis*, and *Candida krusei* (Hazen, 1995; Krcmery and Barnes, 2002; Merz et al., 1986; Nguyen et al., 1996; Pfaller et al., 1998). Despite intensive drug discovery efforts, there are still only three classes of antifungal drugs that are available to treat serious fungal infections, Amphotericin B, azoles and echinocandins. Additionally, despite their widespread usage the azoles are fungistatic rather than fungicidal. Thus, there is need for new strategies and therapeutics to combat fungal infections.

Interestingly, the combination of calcineurin inhibitors and the normally fungistatic antifungal fluconazole result in potent killing of *C. albicans*, as well as other more drug resistant species such as *C. glabrata* (Cruz et al., 2002; Marchetti et al., 2000; Onyewu et al.,

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2003). Additionally, *C. albicans* calcineurin mutants have attenuated virulence in murine models of systemic infection (Bader et al., 2003; Blankenship et al., 2003b), and have faster rates of disease resolution in murine keratitis models (Onyewu et al., 2006). The attenuated virulence of *C. albicans* calcineurin mutants in systemic disease is attributable to the inability of these strains to withstand the calcium stress imposed by serum and thus survive transit through the bloodstream (Blankenship and Heitman, 2005). However, the direct role of calcineurin in *C. albicans* virulence appears to be host-niche-specific as there was no virulence defect seen in either a vaginal or a pulmonary model of infection (Bader et al., 2006). Thus, calcineurin inhibitors have two potential mechanisms of action in the clinic: (1) as single agents in cases of disseminated disease or ocular infections to directly impair survival of the yeasts or (2) as combination therapy to enhance the efficacy of current antifungal therapies. However, the immunosuppressive nature of calcineurin inhibitors limits their use in systemic therapy. Therefore, we were interested in further characterizing the calcineurin signaling cascade to learn more about this important stress response pathway. We also wanted to elucidate other components that could serve as alternative drug targets that would circumvent the immunosuppressive effects of inhibiting calcineurin.

In *C. albicans*, calcineurin is required for cells to survive stressors such as high cations (Li^+, Na^+, and Ca^{2+}), antifungal drug treatment (azoles), and the host bloodstream (Bader et al., 2003; Blankenship and Heitman, 2005; Blankenship et al., 2003b; Sanglard et al., 2003). Based upon homology with *Saccharomyces cerevisiae* Crz1, the downstream transcription factor Crz1 (Cyert, 2003) was previously identified in *C. albicans*, and shown to shuttle into the nucleus in a calcineurin-dependent manner (Karababa et al., 2006). However, phenotypic analysis of *crz1*/*crz1* strains only partially recapitulated a calcineurin mutant phenotype. Although *crz1*/*crz1* mutant strains are sensitive to cations and membrane stresses, they exhibited an intermediate phenotype compared with calcineurin mutants (Karababa et al., 2006; Onyewu et al., 2004; Santos and de Larrinoa, 2005). Microarray studies have suggested that Crz1 is the primary mediator of the calcineurin-dependent transcriptional response (Karababa et al., 2006). As a first step towards elucidating other potential genes in the *C. albicans* calcineurin pathway, we took a candidate gene approach based on analogous signaling pathways in *S. cerevisiae*.

Few proteins are known that are direct binding partners of calcineurin; these include calmodulin, transcription factors (Crz1, *C. albicans*; Crz1/Tcn1 *S. cerevisiae*; NF-AT, mammalian cells), and the RCAN family of proteins (Beals et al., 1997; Cyert, 2003; Davies et al., 2007; Hilioti and Cunningham, 2003; Karababa et al., 2006; Kingsbury and Cunningham, 2000; Klee et al., 1979; Matheos et al., 1997; Onyewu et al., 2004; Santos and de Larrinoa, 2005; Stathopoulos and Cyert, 1997). Members of the RCAN family have been identified in species including *S. cerevisiae* (Rcn1), *Cryptococcus neoformans* (Cbp1), and humans (DSCR1/MCIP1) based upon a conserved FLISPPxSP domain (Davies et al., 2007; Gorlach et al., 2000; Hilioti and Cunningham, 2003; Strippoli et al., 2000a). The function of these proteins has been best explored in *S. cerevisiae* where they exert both positive and negative effects on calcineurin function. Rcn1 binds calcineurin and inhibits its function. However, upon phosphorylation by a GSK3 kinase Rcn1 is degraded thereby relieving calcineurin inhibition (Hilioti et al., 2004). In *S. cerevisiae*, Rcn1 expression is induced in a calcineurin-dependent manner, and the phosphorylated protein is itself a substrate for calcineurin (Gorlach et al., 2000; Hilioti and Cunningham, 2003; Hilioti et al., 2004; Kishi et al., 2007). Overexpression of RCAN family members (or their calcineurin binding domain) inhibits calcineurin function in both *S. cerevisiae* and in mammalian cells (Fuentes et al., 2000; Gorlach et al., 2000; Hilioti and Cunningham, 2003; Hilioti et al., 2004; Vega et al., 2002). Thus, RCANs serve as important control elements of the calcineurin cascade that could potentially be manipulated to therapeutically inhibit calcineurin function.

Another key aspect of calcineurin signaling is regulation of cellular calcium homeostasis and signaling. Direct targets of calcineurin include calcium channels (Vcx1, Mid1/Cch1) (Bonilla et al., 2002; Cunningham and Fink, 1996). In *S. cerevisiae*, endoplasmic reticulum stress activates the Mpk1 pathway, which activates a plasma membrane calcium channel composed of Cch1 and Mid1 (Bonilla and Cunningham, 2003; Bonilla et al., 2002). Activation of the channel results in calcium influx and activation of calcineurin, which subsequently feedback inhibits the channel through dephosphorylation (Cunningham and Fink, 1994a). Thus, Mid1 and Cch1 control calcineurin activation. Previous studies in *C. albicans* characterized Mid1 and Cch1 roles in galvano- and thigmotropism (Brand et al., 2007). Deletion of either or both calcium channels significantly decreased calcium accumulation. However, the mutants differed in their response to various stimuli: Cch1 appears to play a greater role in hyphal orientation in response to electric fields, while loss of Mid1 had a more significant impact on hyphal tip reorientation in response to physical contract. Interestingly, calcineurin was required for the reorientation of hyphae in an electric field, but not involved in thigmotropism; however, Crz1 was required for both processes (Brand et al., 2007).

In this study, we used mutant analysis to investigate the roles *C. albicans* Rcn1, Mid1, and Cch1 homologs play in the response of *C. albicans* to stress. We found that while each of these proteins is required for the maximal resistance to some stressors, none of them is as important as calcineurin itself. Therefore, each of these proteins likely functions in only part of the calcineurin-signaling pathway.

2. Materials and methods

2.1. Strains and media

All strains were routinely propagated on YPD medium (1% yeast extract, 2% bacto peptone, 2% dextrose, and 2% bacto agar (DIFCO)). YPD + 300 mM $CaCl_2$ medium was made similarly to YPD except that the media was adjusted to pH 5 prior to autoclaving. The $CaCl_2$ solution was sterilized separately and the two solutions were mixed after autoclaving. All strains used in this study are listed in Table 1.

2.2. Gene disruptions

All deletion strains were generated in the SC5314 background. All primers used in strain construction are listed in Table 2. For disruption of the *RCN1* gene, two ~500 bp regions with homology to the 5′ promoter and 3′ terminator region of *RCN1* were PCR amplified, and cloned into plasmid pSFS2A (Reuss et al., 2004) with KpnI/XhoI, and NotI/SacI, respectively, generating plasmid pJLR1. Plasmid pJLR1 was digested with KpnI/SacI and the disruption cassette consisting of the *SAT1* flipper cassette surrounded by ~500 bp of homology flanking *RCN1* was gel purified. SC5314 was transformed with approximately 1 μg of DNA as previously described (Reuss et al., 2004). For all deletion strains, at least three independent transformations were performed at each step of disruption and an independent transformant was selected from each transformation for further analysis. Nourseothricin resistant isolates were selected on YPD + 200 μg/ml NAT (Werner). Correct integrants (*RCN1*/*rcn1::SAT1*) were confirmed by colony PCR and then by Southern blot. At least three independent strains that had correctly integrated the disruption cassette were grown overnight in YPM (1% yeast extract, 2% bacto peptone, 2% maltose, and 2% Bactoagar (Dif-