MINIREVIEW

Calcineurin Regulation in Fungi and Beyond

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Calcineurin (PP3/PP2B) is a serine-threonine-specific phosphatase of the protein phosphatase (PP) family (EC 3.1.3.16), which also includes PP1, PP2A, and PP4 to -7 (25). Phosphatases share a highly conserved primary structure in eukaryotes and were originally grouped based on their preferential dephosphorylation of the alpha subunit (PP2) or beta subunit (PP1) of phosphorylase kinase and the sensitivity (PP1) or insensitivity (PP2) of phosphatase activity to heat-stable cytosolic proteins known as inhibitor 1 and inhibitor 2. Calcineurin differs from other phosphatases in metal ion requirements, drug sensitivity, range of substrate specificity, and cellular regulation. These differential properties arise out of the unique structural aspects of calcineurin that confer its complex regulation in eukaryotic cells. Calcineurin exists as a heterodimer of 57- to 71 kDa catalytic subunits (CnA) and 18- to 20-kDa regulatory subunits (CnB), in which primary sequence and higher-order structural features define the relatively narrow substrate range characteristic of this enzyme and facilitate key regulatory aspects of its cellular function (Fig. 1) (18, 40, 104). Additionally, the exclusive bimetallic Fe²⁺/Zn²⁺ metal center of calcineurin (Fig. 1) catalyzes phosphoester hydrolysis by a two-step metal-activated process requiring reduced iron, linking the functional activation of calcineurin with changes in cellular redox chemistry (74, 87).

The CnA subunit contains the catalytic domain and three regulatory elements, which include the CnB-binding, the calmodulin-binding, and the autoinhibitory domains, located toward the carboxyl terminus (60, 76). In resting cells, the autoinhibitory domain blocks the catalytic center and thus provides an intrinsic mechanism for the coordination of calcineurin phosphatase activity with changes in the cellular activation state. Intrinsic structural features that confer the self-regulation of calcineurin are not found in PP1 and PP2A, which exist as free catalytic subunits that are constitutively active in the absence of allosteric inhibitors (25, 69). The CnB- and calmodulin-binding domains of calcineurin work in concert with autoregulatory mechanisms to coordinate phosphatase activity with the modulation of intracellular calcium ion homeostasis and associated changes in the cellular activation state. CnB is an essential structural component of calcineurin that is required for both CnA stabilization and Ca²⁺/calmodulin activation of calcineurin (61, 91, 93, 109, 119). Calmodulin and CnB, though functionally dissimilar, are structurally conserved and 35% identical in primary sequence (3, 72). In each, Ca²⁺-binding activity is mediated through four EF-hand Ca²⁺-binding loops similarly positioned at opposite ends of a symmetrical dumbbell-shaped tertiary structure (3). The appropriate cellular functions of calmodulin and CnB depend on their relative affinity for Ca²⁺, which allows for the structural stability of CnB at basal Ca²⁺ levels present in resting cells and facilitates the inducible activation of calmodulin at higher intracellular Ca²⁺ concentrations. Ca²⁺-bound calmodulin associates with the CnA subunit of calcineurin and displaces the autoinhibitory domain to allow substrate access to the catalytic site.

Ca²⁺ and calmodulin cooperatively interact with calcineurin to further modulate the activity of this phosphatase in response to different types of Ca²⁺ signals and spatiotemporal changes in intracellular Ca²⁺ concentration. For example, activation of calcineurin pathways regulating lymphocyte immune function requires sustained increases in intracellular Ca²⁺, whereas in some neuronal cells limited windows of intracellular Ca²⁺ increase afforded by brief Ca²⁺ spikes are sufficient to elicit activation of this phosphatase (39, 59). Molecular clues into how cells use intracellular Ca²⁺ gradients to modulate calcineurin signaling have recently been discovered. These studies, using the model yeast Saccharomyces cerevisiae, revealed that the relative affinity of the calcineurin-substrate association can determine the calcium threshold of calcineurin signaling and affect both the specificity of calcineurin signal transduction and subsequent nature of calcineurin-dependent cellular responses (103). Thus, the activation and substrate recognition properties of calcineurin establish a stable but flexible framework that can be rapidly tailored to meet the signaling requirements of eukaryotic cells.

The heterodimeric structure of calcineurin not only autoregulates enzyme activity and provides a basis for cellular modulation of calcineurin signal transduction but also defines its characteristic sensitivity to microbiologically derived immunomodulators. Calcineurin is resistant to the toxins okadaic acid, calcineurin, and microcystin, which serve as potent inhibitors of PP1 and PP2A activity, but its enzymatic function is uniquely sensitive to the macrolides cyclosporine (CsA) and FK506 (tacrolimus). These agents interact with calcineurin through the immunophilins cyclophilin A and FK506-binding protein 12 (FKBP12), respectively (8). CsA-cyclophilin A and FK506-FKBP12 are structurally unrelated complexes that interact at distinct but overlapping sites on calcineurin (84, 93). This re-

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² Published ahead of print on 7 December 2007.
results in competitive binding between the inhibitory complexes, which, when bound to calcineurin, indirectly inhibit phosphatase function by sterically interfering with the binding of phosphoprotein substrates (84).

CsA- and FK506-immunophilin inhibitory complexes, together with genetic approaches, have been applied in numerous studies and experimental contexts to elucidate the central role of calcineurin in stress signaling, cell cycle regulation, and sporulation in fungi (Table 1) and the physiological function of higher eukaryotes, as well as evolutionary aspects of metazoan development, including cell commitment, tissue development, and organogenesis. The involvement of calcineurin in many essential aspects of cell- and organ-specific processes in humans underscores the clinical significance of this pathway as a therapeutic target in a diverse array of human disorders, including several autoimmune and inflammatory conditions, and the widespread use of FK506 and CsA as immunosuppressants.

The role of the immunophilins cyclophilin A and FKBP12 in calcineurin regulation may not be strictly limited to immunosuppressant-mediated interactions, as both proteins are capable of interacting with calcineurin and modulating phosphatase activity in the absence of CsA and FK506. These regulators, along with a growing number of other calcineurin modulators, including the regulators of calcineurin (RCAN) family, supplement autoregulation and other intrinsic features of enzyme control to coordinate the diverse functional roles of calcineurin in biological and pathological processes. This review focuses on the role of conserved calcineurin regulators in fungi and fungal pathogenesis and, where applicable, draws parallels to recent findings in higher eukaryotes.

**IMMUNOPHILINS**

A growing number of protein families, many of which are conserved in higher and lower eukaryotes, that are capable of directly interacting with calcineurin and either modifying or inhibiting its activity are being identified. Such interactions and their associated cellular effects were first described for members of the immunophilin superfamily. Immunophilins are named for the drug-dependent functions of both the cyclophilins and the FKBPs, which form high-affinity bimolecular complexes that subsequently bind to and inactivate calcineurin (53). This drug-immunophilin interaction, because of its ability to inhibit calcineurin-dependent T-cell function, has led to the

![FIG. 1. Three-dimensional ribbon diagram of the calcineurin heterodimer. The image was generated in Swiss-PdbViewer version 3.9b1 from the protein data bank file 1TCO. The catalytic (CnA) subunit is shown in blue, the regulatory (CnB) subunit is yellow, the calcium ions are depicted as gray spheres, and the ions of the Fe$^{2+}$/Zn$^{2+}$ cluster are represented as magenta spheres.](image-url)

**TABLE 1. Calcineurin function in fungal species**

| Organism                | Gene(s) for: | Functions                                                                 | References     |
|-------------------------|--------------|---------------------------------------------------------------------------|----------------|
|                         | Calcineurin catalytic subunit | Regulatory subunit | Target | Cofactor |                                                                 |                            |
| *S. cerevisiae*         | CNA1, CNA2   | CNB1 | CRZ1, HPH1 | RCN1 | Ion stress, wall integrity, pheromone response, cell cycle regulation | 21, 35, 54, 89, 94, 96, 99 |
| *S. pombe*              | ppb1         | cnb1 | prz1        |     | Ion homeostasis, wall integrity, cell morphology, hyphal growth, morphogenesis, membrane trafficking | 68, 85, 126 |
| *A. fumigatus*          | CnaA         | CnaB |             |     | Wall integrity, conidial morphology, hyphal growth, PO$_4$ transport, survival in sera, invasive growth | 36, 110, 111 |
| *C. albicans*           | CNA1         | CNB1 | CRZ1        |     | Ion stress, wall integrity, morphogenesis, survival in sera, virulence | 13, 14, 16, 17, 33, 106 |
| *C. neoformans*         | CNA1         | CNB1 |             | RCN1 | Ion stress, wall integrity, morphogenesis, 37°C growth, virulence | 31, 34, 47, 48, 78, 98 |
widespread therapeutic use of CsA and FK506 as suppressants in graft rejection and in inflammatory and autoimmune disorders (12, 62, 125). CsA- and FK506-targeted immunophilins, along with other, relatively minor immunophilin classes, comprise this superfamily. Immunophilins can vary significantly in relative size, primary structure, and tertiary structure (53, 73) and are differentially, but widely, expressed in eukaryotes, with a more limited expression in bacteria and archaea (77, 88, 114, 116). Among the minor immunophilin classes is a recently identified group, the FK506- and CsA-binding proteins, found in several species of protozoa and bacteria, that uniquely express CsA- and FK506-binding domains in tandem within a single polypeptide chain (2).

Despite the sequence and structural diversity that exists between families, most immunophilins possess a functionally conserved, largely noninducible cis/trans isomerase activity. This enzymatic activity catalytically converts side chains that are N terminal to proline residues from the naturally occurring trans configuration present in nascent polypeptides to the cis isomeric form. This process is rate limiting and characterized by the transition of peptidyl-prolyl bonds from low- to high-energy states. The essential nature of this enzymatic function in eukaryotes is controversial, but it has been shown to facilitate many biosynthetic and repair processes, including the proper folding of nascent proline-containing polypeptides, the assembly of oligomeric protein complexes, and the refolding of recycled or damaged proteins (53, 64, 65, 108, 118). Structurally, immunophilins consist of a catalytic domain with or without other functionally independent domains, such as tetratricopeptide repeat, signal peptides, and/or a conserved WD40 domain that contains a C-terminal Trp-Asp dipeptide (2, 15, 53). These and other domains define a modular nature of immunophilin polypeptide structure that functions in allosteric control of isomerase activity, provides additional contact sites for signaling proteins, and allows for structural flexibility, subcellular localization, and other properties of this superfamily that explain the specialized diverse functions at all eukaryotic levels (57, 82, 95, 105, 107, 127).

Thirteen immunophilin genes have been identified in the yeast S. cerevisiae, including eight cyclophilin genes, the ESS1 gene, and four genes that encode FKBP family members (8). S. cerevisiae mutants lacking one, several, or all 12 cyclophilin/FKBP genes are unaffected in viability or the ability to grow on enriched medium, thus indicating the nonessential nature of these two gene families under normal growth conditions on complex media (38). Cyclophilin and FKBP gene products are also dispensable for cell growth during stress conditions, as a mutant lacking all 12 immunophilin genes exhibited wild-type growth in the presence of toxic amino acid derivatives, i.e., canavanine (0.6 μg/ml) or l-azetidine-2-carboxylic acid (20 μg/ml), and grew normally under conditions of cation stress induced by elevated free calcium (100 mM) or lithium ions (200 mM). Furthermore, no phenotype was discernible during mating or sporulation of immunophilin-deficient yeast after cell growth at different temperatures (16° to 37°C) using carbon sources such as glucose, glycerol, galactose, and sucrose or in response to nitrogen starvation (38).

Important functions have been identified in yeast for specific FKBP s during amino acid biosynthesis (7), as well as for cyclophilins in meiotic cell division (6, 8, 101). The human pathogen Cryptococcus neoformans carries four immunophilin genes, including two related cyclophilin genes and another gene encoding a 12-kDa FKBP gene product (FKBP12). Though FKBP12 can mediate the toxic effects of FKBP-targeted macrolides, i.e., FK506 and rapamycin, in this fungus, the loss of FKBP12 expression does not impair growth, differentiation, or virulence (30, 32). By contrast, the cyclophilin genes encode two 18-kDa homologs, Cpa1 and Cpa2 that, in addition to mediating CsA cytotoxicity, have distinct but overlapping roles in cell growth, mating, and virulence (117). For example, cpa2 mutants grew normally at elevated temperatures in vitro, displayed no defects in mating, and were unaffected in virulence capacity in rabbit models of cryptococcosis relative to wild-type organisms. Interestingly, cpa1 mutants were similar to cpa2 mutants in mating ability but were compromised in both high-temperature growth in vitro and animal pathogenesis, whereas cpa1 cpa2 double mutants are severely affected in all three parameters (117).

One potential regulatory target of immunophilins in budding yeast is calcineurin. Direct physical interaction between either cyclophilin A or FKBP12 and the catalytic subunit of calcineurin was shown by a yeast two-hybrid assay and further verified by the purification of recombinant His6cyclophilin A- and His6FKBP12-calcineurin complexes from cell lysates (23). This interaction was specific for the CNA1 isoform but did not involve the autoinhibitory and calmodulin-binding domains, and it could be distinguished from drug-induced immunophilin-calcineurin interactions by the contingency of the latter on the expression of both the catalytic and regulatory subunits of calcineurin. Consistent with these findings, drug-independent and -dependent immunophilin-calcineurin interactions involve distinct surface residues on FKBP12. Unlike drug-dependent interactions that prevent calcineurin function, drug-independent immunophilin interactions may physiologically modulate calcineurin function. For example, S. cerevisiae mutants lacking FKBP12 expression demonstrated enhanced calcineurin-dependent growth recovery after pheromone-induced cell cycle arrest, relative to isogenic controls (23). The apparent regulatory effect of FKBP12 on calcineurin signal transduction was further evident in the potentiated growth of an FKBP12-null mutant on 500 mM lithium chloride (23).

Similar drug-independent regulatory interactions were also observed between calcineurin and the FKBP family paralog, FKBP35, of Plasmodium falciparum (80). In that study, a recombinant FKBP35 substantially inhibited calcineurin activity in vitro, and this association was implicated in modulating the infectivity of this obligate parasite in human erythrocytes (80). Additional evidence for immunophilin regulation of calcineurin comes from studies correlating the pathological, growth factor-independent proliferation of megakaryocytes in idiopathic myelofibrosis with the overexpression of FKBP51 (56). The use of an idiopathic myelofibrosis model cell line, together with normal hematopoietic (CD34+) control cells, demonstrated that FKBP51 overexpression was associated with the marked inhibition of calcineurin signal transduction (56). In this system, inhibitory FKBP51-calcineurin interactions conferred cytokine-independent cell survival and apoptosis-resistant cell growth properties that could be reproduced by the pharmacologic inhibition of calcineurin activity with CsA. These studies further implicate defective immunophilin
gene expression as an additional contributing factor in the calcineurin-mediated pathology of congenic neurological disorders such as Down syndrome. The relevance of immunophilins in neurological disorders is further indicated by their high expression in neurons and in observed dosage-related effects on neurodegenerative disease (10, 11, 26, 45, 57, 90, 92).

The therapeutic implications of cyclophilins and FKBP5 extend well beyond their current drug-dependent roles in calcineurin targeting. The diverse functions of specific immunophilins in physiological and pathological processes, including inflammation, adaptive immunity, steroid receptor regulation, gene transcription, and the transmission and progression of blood-borne disease, together with their modular structure, underscore their potential therapeutic importance (20, 86, 120). The potential involvement of immunophilins as cofactors in the allosteric regulation of calcineurin activity and other primary signaling effectors could allow for targeted modulation of specific immunophilin transduction pathways or effector interactions. Approaches such as the use of synthetic peptides to target specific domains or protein interaction sites may eventually offer an effective alternative to therapeutically intractable disorders relating to immune dysfunction, neurodegenerative and metabolic disease, and infectious and/or communicable disease by allowing for the precise targeting of affected organs or cell types.

**RCANs: REGULATORS OF CALCINEURIN**

Significant insight into the complexity of the calcineurin signaling network in fungi and its regulation has come through the characterization of the calcineurin regulator Rcn1 (previously named Cbp1) in *S. cerevisiae* and *C. neoformans*. In both organisms, Rcn1 uniquely serves as both a required cofactor and a regulator of calcineurin phosphatase activity through direct binding of calcineurin (48, 58, 67, 75). Moreover, Rcn1 is the first conserved physiologic regulator of calcineurin to be identified in fungi. The widespread importance of this molecule in the regulation of calcineurin signal transduction is indicated by its conservation in higher eukaryotes, including nematodes (DSCR1 and RCN-1 [81, 112]), indicated by its conservation in higher eukaryotes, including cule in the regulation of calcineurin signal transduction is in-identified in fungi. The widespread importance of this mole-

deral domains; is sensitive to calcineurin-FK506/FKBP12 inter-

actions; and occurs independently of calcineurin activation by Ca²⁺/calmodulin (50, 58, 73, 102, 115). Several C-terminal motifs have been identified in the mammalian Rcn1 functional homolog, RCAN1, including a PxIxT motif, similar to the calcineurin-specific PxIxT docking site of the NFATc family of transcriptional regulators, and an ELHA motif (4, 9). Both domains bind calcineurin independently and differentially affect the functional association of Ca²⁺/calmodulin-activated calcineurin with NFATc and subsequent calcineurin/NFATc-directed gene expression (9). Rcn1 has a central 22-amino-acid serine-proline (SP) repeat region that is conserved in RCAN1 of higher eukaryotes and is functionally analogous to the conserved SP repeat of the calcineurin target and effector protein Crz1p in budding yeast (66, 67, 75).

This conserved region is essential for Rcn1 modulation of calcineurin function in vivo, as well as for the integration of calcineurin signal transduction pathways with other stress-activated signaling networks. Calcineurin activity is modulated by changes in the phosphorylation state of the two serine residues of the SP repeat, which are tandemly targeted for phosphory-

lation by p42/44 mitogen-activated protein kinase (MAPK) and the glycogen synthase kinase (GSK3) family member Mck1, respectively (66, 67). The phosphorylation state of the SP repeat does not significantly influence calcineurin associa-

tion (48) but may modulate the function of Rcn1 as a targeting subunit or specificity modulator, depending on the relative stoichiometry of calcineurin regulatory components (58, 66, 75, 115). In view of the critical importance of the SP repeat phosphory-

lation to the regulatory function of Rcn1, the studies described above give insight into the mechanisms underlying the selective integration of Ca²⁺/calcineurin signaling networks with the stimulus-specific, Ca²⁺-independent signaling systems, as well as the significance of Rcn1 as a conduit for the precise control of calcineurin activity.

Rcn1 and its functional homologs are subject to additional, possibly species-specific, forms of posttranslational control that regulate protein stability through changes in phosphorylation state or by the formation of multimolecular complexes. Studies examining the regulated expression of recombinant epitope (hemagglutinin [HA])-tagged Rcn1-HA in *S. cerevisiae* cmd1-6 (expressing nonfunctional calmodulin) and cnb1 (expressing no calcineurin B) mutants, in or wild-type organisms under nonsignaling conditions, indicated that calcineurin expression, regardless of its activation state, was required for the cellular accumulation of Rcn1-HA, thus suggesting the primary role of constitutive Rcn1-calcineurin complexes in regulating Rcn1 half-life (75). Although the Rcn1 mammalian homolog RCAN1 is also associated with calcineurin in unstimulated cells under physiological conditions, this constitutive interaction has no discernible effect on molecular stability (55).

Studies have shown that coexpression of calcineurin and a recombinant HA-RCAN1 construct have no effect on the basal accumulation of RCAN1 (55). Instead, the SP repeat region of HA-RCAN1 constructs was hyperphosphorylated in COS-7 or human neuroblastoma (SH-SY5Y) transfectants under basal conditions, and this hyperphosphorylation resulted in rapid RCAN1 degradation through proteasome targeting (55). Furthermore, mutation of the specific serine residues within the SP repeat targeted by effector kinases dramatically increased the half-lives of exogenously expressed HA-RCAN1 constructs (55). These results are of particular interest in view of the association of calcineurin function with the phosphorylation
state of the SP repeat region of RCAN1 and suggest that basal expression levels of RCAN1 are determined through the calcineurin-independent modulation of RCAN1 phosphorylation (55, 115). Other mechanisms, including genetic, posttranscriptional, and kinase-independent posttranslational mechanisms, are also implicated in the control of RCAN1 protein levels in higher eukaryotes. For example, the pronounced effect of cycloheximide pretreatment on the RCAN1 recovery from HA-RCAN1 transfectants suggests the importance of genetic control and/or posttranscriptional mechanisms in the maintenance of cellular RCAN1 (55). In addition, direct association of phosphorylated RCAN1 with 14-3-3 family members, which are proposed to regulate molecular stability, has been observed in Chinese hamster ovary cells transfected with the human AT1 receptor (27, 70, 97, 123).

Considered together, these findings suggest a possible divergence in the mechanisms underlying the maintenance of Rcn1/RCAN expression between lower and higher eukaryotes. As Rcn1 expression determines the positive or negative modulation of calcineurin function in the model yeast S. cerevisiae and the human pathogen C. neoformans, cellular control of Rcn1 stability may be fundamental to the temporal coordination and/or fine-tuning of stress-induced calcineurin signal transduction, with coincident physiological changes in cellular metabolism and activation states.

The functional properties of Rcn1 and its regulatory interactions with calcineurin in S. cerevisiae have been previously described in a proposed model based on the regulation of the scerine/threonine phosphatase PP1 by inhibitor 2 (S, 66, 67). In this model, Rcn1 interaction with calcineurin results in allosteric changes in calcineurin conformation that either stimulate or suppress the phosphatase function in a manner dependent on the phosphorylation state of Rcn1. According to this model, Rcn1-mediated regulation of calcineurin function by the concomitant action of priming (p44 MAPK) and effector (GSK3) kinases is determined by stress-induced changes in the expression of calcineurin, Rcn1, and priming/effector kinases. The primary role of Rcn1 and GSK3 in calcineurin regulation would suggest a rapid, transient mechanism for the efficient integration and coordinate regulation of calcineurin signal transduction in eukaryotic cells. Importantly, the complex feedback interactions among regulatory components in this model system can be subjected to multiple levels of cellular control, including transcriptional and posttranslational mechanisms, as well as spatial mechanisms that promote molecular clustering through scaffold assembly. Such potentially facile cellular control over component stoichiometry may additionally affect the relative impact of intracellular Ca^{2+} ion gradients on calcineurin function in different cell types.

Evidence indicates that RCANs are required for some, but not all, cellular pathways of calcineurin-dependent signal transduction and may additionally support calcineurin-independent functions. While RCN1 expression in S. cerevisiae is calcineurin dependent, expression of RCN1 in C. neoformans is not, consistent with the notion that the Rcn1 protein may be responsive to signaling regulators other than calcineurin (75). RCAN1 expression in higher eukaryotes is proposed to have calcineurin-independent effects in cell cycle regulation, the activation of superoxide dismutase gene expression and function, and the posttranscriptional induction of GSK3β expression (42–44, 83). Some of these functions might occur in connection with a putative 80-amino-acid RNA-binding domain near the N-terminal region of RCAN1 which may confer transcriptional activity and the predominantly nuclear localization of RCAN1 in mammalian cells (100, 102, 112). Although a role for calcineurin in the induction of RCAN1 gene expression in higher eukaryotes has not been investigated, it is regulated by a Ca^{2+}-dependent mechanism (83). When phosphorylated, RCAN1 is also capable of directly interacting with protein effectors other than calcineurin (1). Considered together, the studies described above suggest that Rcn1 in fungi and its functional homologs in higher eukaryotes mediate stress-activated damage control, repair, and other related activities by both calcineurin-dependent and -independent signaling pathways.

**HSPs AS CALCINEURIN REGULATORS**

As with the functional interactions of calcineurin, increased knowledge about its regulatory relationships is rapidly expanding the current understanding of the breadth and complexity of calcineurin function in eukaryotes. An example in this regard is recent work elucidating a central role for heat shock proteins (HSPs) as an upstream component of calcineurin signal transduction in fungi (29, 71). HSP90 in mammals, or its homologs in lower eukaryotes, is the most abundantly expressed cytosolic protein of eukaryotic cells and functions as a major molecular chaperone regulating structure, stability, function, and transport of many essential signaling molecules (22).

*S. cerevisiae* encodes two HSP90 homologs, Hsp82 and Hsc82, that are 97% identical at the amino acid level and that share 63% amino acid identity with mammalian HSP90 (19, 46). The Hsp90 yeast homologs are independently and differentially expressed under basal growth conditions and in response to specific stress stimuli; both are constitutively expressed, with Hsc82 expression approximately 10 times greater than Hsp82 expression under basal growth conditions and Hsp82 expression differentially induced during cell stress (19). The Hsc82 and Hsp82 proteins are functionally redundant, with at least one functional homolog required for cell viability and reproduction (19). Yeast that lack expression of either Hsp82 or Hsc82 (jointly referred to as Hsp90 for the remainder of this review) exhibit defects in high-temperature growth that derive from gene dosage rather than functional specificity (19). Additional studies examining the functional impact of HSP overexpression on stress signaling found that increased HSP90 dosage resulted in hypersensitivity to sodium (1 M), chloride (1 M), lithium (0.2 M), calcium (0.6 M), heat shock (50°C, 30 min), and the naturally occurring toxic amino acid derivative canavanine (100 µg/ml) (71). Of interest was the notable similarity in phenotype between fungi expressing enhanced HSP dosage and cells genetically inactivated (or drug inhibited) for calcineurin.

Further analyses have demonstrated a direct interaction between Hsp90 and the catalytic subunit of calcineurin, thus implicating Hsp90 in the molecular stabilization of calcineurin (31). Accordingly, it was found that Hsp90 interacted constitutively with calcineurin under nonsignaling conditions and regulated calcineurin turnover (71). This interaction suggested a mechanism not only for the cellular effects of HSP90 dosage
but also for the posttranslational regulation of calcineurin gene expression. The relative significance of the latter was suggested by the inhibition of calcineurin signal transduction with a 30% reduction in wild-type Hsp90 levels and, at still lower HSP90 expression levels, ablation of CNA2 expression with CNB1 expression unaffected (71). HSP90-calcineurin association is essential for calcineurin stability, activation, and effector-pathway engagement. Studies examining the functional effects of this association suggest that HSP90 inhibits calcineurin activity in the absence of stress stimuli (71). Exposure to relevant stress factors, such as excess sodium, promotes the dissociation of Hsp90-calcineurin complexes and the subsequent engagement of calcineurin-dependent signaling pathways (71).

When considered together, these studies provide insight into the mechanism of calcineurin regulation during normal cell growth and suggest that Hsp90-calcineurin interactions function analogously to constitutive Rcn1-calcineurin interactions, in that both complexes are essential to the relative stability and cellular maintenance of nascent calcineurin and Rcn1 proteins. As with Rcn1-calcineurin interactions, the functional nature of Hsp90-calcineurin interactions is multifaceted, affecting not only structural stability but also protein activation and possibly effector function (Fig. 2).

Recent investigations examining Hsp90 function in S. cerevisiae and other fungi have disclosed a central, facilitating role for calcineurin in the development of antifungal resistance (29). This novel function of calcineurin is Hsp90 dependent, antifungal specific, and selectively engaged in vitro according to method of drug exposure. Hsp90-calcineurin signaling is also mechanistically related to the well-described role of the calcineurin/Crz1p pathway in stress signaling, which contributes to inductive intrinsic mechanisms of antifungal drug resistance. The role of Hsp90 in fluconazole resistance was investigated using an experimental approach in S. cerevisiae based on the Cre-LoxP system to allow genetic regulation of HSP90 expression in an hsp82Δ hsc82Δ background. This system allowed for controlled low- or high-level ectopic expression of HSP90 alleles from noninducible promoters and permitted genetic alterations in HSP90 expression through inducible recombination events.

Using this system in combination with Hsp90 inhibitors (geldanamycin or radicicol) and genetic and biochemical modulation of calcineurin activity, reliance on Hsp90-dependent drug resistance was shown to function biochemically through a calcineurin-dependent signaling mechanism (29). Additionally, Hsp90-calcineurin-dependent drug resistance was antifungal and species specific, depending on the antifungal dose and length of exposure. For example, Hsp90-calcineurin was shown to be essential for the development of drug resistance in S. cerevisiae, or in isolates of Candida albicans, when organisms were grown in the presence of high concentrations of fluconazole (128 µg/ml). All of the drug-resistant S. cerevisiae isolates studied were mutated in ERG3, which encodes a 5,6-desaturase responsible for azole-induced toxic accumulation of 3,6-diol. By contrast, Hsp90 inhibitors did not affect C. albicans resistance to the echinocandin or caspofungin or the drug-resistant growth of Aspergillus terreus on fluconazole or voriconazole (29).

In addition, whereas the rapid, acute exposure to high concentrations of fluconazole invoked Hsp90-calcineurin dependent mechanisms of drug resistance, drug-resistant strains of S. cerevisiae cultivated with lower concentrations of fluconazole (16 µg/ml) developed resistance by an Hsp90-independent mechanism. Other studies additionally indicate a transitional role for Hsp90 in molecular mechanisms conferring azole resistance in C. albicans. For example, a clinical isolate of C. albicans demonstrated an Hsp90-calcineurin-dependent fluconazole resistance during the early stages of a 2-year course of drug therapy that gradually evolved toward Hsp90-calcineurin independence (29, 121), possibly indicating the medical relevance of this pathway in fungal disease.

Mutational analysis of major calcineurin effectors against an erg2Δ background in S. cerevisiae indicated that Hsp90-calcineurin drug resistance derives from the concerted action of multiple, distinct effector pathways operating downstream of calcineurin (28). The calcineurin-specific transcription factor Crz1p and the endoplasmic reticulum transmembrane protein high pH I (Hph1) in S. cerevisiae represent different pathways that function as minor and major contributors, respectively, to Hsp90-calcineurin antifungal drug resistance. Hph1 contains a calcineurin docking motif, similar to that of Crz1p, through which its function is regulated by calcineurin phosphatase activity (63). Hph1 activity also involves the functionally redun-
Calcineurin regulates the genetic and biochemical adaptation of fungi to specific environmental signals (reviewed in reference 49). Current studies suggest this is accomplished by diverse mechanisms of calcineurin signal transduction that implicate this enzyme in the modulation of existing networks and the formation and stabilization of genetically altered signaling complexes that support novel, genetically evolved phenotypes. Calcineurin-dependent gene expression directs the synthesis of components of stimulus-induced stress-response pathways that function in cell wall integrity/repair and other processes required for cell survival. This mechanism of calcineurin signaling regulates stress responses in fungi and has species-specific roles in fungal pathogenesis (Table 1).

Investigations with the human fungal pathogens *C. neoformans*, *Aspergillus fumigatus*, and *C. albicans* have illustrated the specialization of stress-activated calcineurin function between fungal pathogens relative to model yeasts such as *S. cerevisiae* and *Schizosaccharomyces pombe* (79). Calcineurin-deficient strains of *C. neoformans* are unable to grow at 37°C but are capable of growth at 25 to 30°C, though not in the presence of alkaline pH or CO₂ concentrations typical of in vivo conditions (34, 47, 98). In this organism, calcineurin function is also required for the yeast-to-hypha transition during diploid differentiation, asexual fruiting, and mating and consequently the production of basidiospores, the proposed transmissible particles responsible for initial host infection (31).

Similarly, *A. fumigatus* mutants lacking functional calcineurin displayed major defects in polarized growth and filamentation in vitro, as well as in conidial morphology and sporulation (36, 110). Functional calcineurin was required for efficient utilization of inorganic phosphate in this pathogen and was implicated in the induction of the phosphate nutrient-sensing pathway through derepression of phosphate transporter genes (36). A potential role for this pathway in the pathogenesis of *A. fumigatus* was indicated by the inability of calcineurin mutants to grow in 10% serum in the absence of supplemented phosphate (36). Functional calcineurin was also required for the invasive growth and virulence of *A. fumigatus* in low-dose mouse models of invasive aspergillosis using inhalation, paranasal, or intravenous delivery systems (110).

Calcineurin-regulated transcriptional activator proteins have yet to be identified in *C. neoformans* and *A. fumigatus* but likely contribute to calcineurin-mediated virulence in both organisms. While functional calcineurin is required for the in vivo growth and pathogenesis of *C. neoformans* and *A. fumigatus*, this enzyme is conditionally required for organ-specific colonization of *C. albicans*. Calcineurin regulates the virulence of *C. albicans* by promoting its survival in the calcium-rich environment of serum and the spread of infection between organ systems (13, 14, 17). The limited role of calcineurin in the pathogenesis of *C. albicans* is further indicated by studies showing that the major virulence properties of this pathogen, including host cell adherence, filamentous growth, and germ tube formation, occur independently of calcineurin signal transduction (17).

The identification and characterization of the conserved calcineurin regulator Rcn1 in *C. neoformans* have provided insight into the complexity of calcineurin regulation during pathogenesis by elucidating calcineurin-dependent pathway-specific functions of Rcn1 that may partially account for organism virulence. For example, Rcn1 expression is required for full virulence in a mouse model of cryptococcosis (58) but does not appear to be involved in the calcineurin-dependent expression of major virulence properties, including growth at 37°C (48). Instead, Rcn1 directs the calcineurin-dependent processes of mating and tolerance to elevated concentrations of free Ca²⁺ (48). Each of these properties may contribute to host infection through the respective generation of potentially infectious basidiospores and pathogen dissemination in calcium ion-rich tissues, a property of the calcineurin stress response that is also implicated as essential in the virulence of *C. albicans* in mouse models of disseminated candidiasis (13, 16, 17).

In the recently identified HSP90-calcineurin pathway, relatively little is known regarding the nature of alterations that occur within biosynthetic and other signaling networks that, together with HSP90 and calcineurin, contribute to drug-resistant phenotypes after rapid selection on azole-rich media. In the case of *S. cerevisiae*, mutations are acquired in at least one component of the ergosterol biosynthetic pathway and serve to remediate the otherwise toxic intracellular effects of azoles in a manner dependent on HSP90-calcineurin function. The molecular details underlying interactions between calcineurin and such dynamically altered signaling networks remain unknown.
but could indicate that calcineurin is capable of differentially engaging the established effector pathways discussed above, as well as supporting random changes in the genetic regulation, structural organization, or physiological function of existing signaling networks to facilitate both the expression and fixation of new traits. The length of stimulus exposure appears to be critical in determining the relative genetic stabilities of Hsp90-calcineurin traits (29). For example, the Hsp90-calcineurin-driven biochemical changes induced following stimulus exposure over limited periods may become genetically stable with prolonged antifungal drug exposure and thus contribute to novel survival traits that become conserved in subsequent generations regardless of growth conditions. As antifungal drug resistance in C. albicans can evolve from a state of Hsp90-calcineurin dependence to one of stable expression and Hsp90-calcineurin independence over a 2-year course of fluconazole therapy (29, 121), these results could indicate a mechanism for the continued evolution of C. albicans virulence in humans under defined clinical conditions and, consequently, a potentially cooperative basis for the development of calcineurin-dependent and -independent virulence properties in this and other fungal pathogens. Further insights into the species-specific regulation of calcineurin will undoubtedly come with discoveries of additional calcineurin regulators and effectors in C. neoformans, A. fumigatus, C. albicans, and other fungal pathogens. A detailed knowledge of calcineurin regulation in fungi and higher eukaryotes, together with a greater understanding of mechanisms underlying the development of fungal pathogenesis, virulence traits, and antifungal resistance, will likely contribute to new, more effective drug therapies for the treatment of fungal disease, as well as autoimmune and neurodegenerative disorders.

ACKNOWLEDGMENTS

We thank Jim Cutler, Ping Wang, and Mike Ferris for comments and discussions.

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