NOTE

Genetic Architecture of Hsp90-Dependent Drug Resistance

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Hsp90 potentiates the evolution of azole resistance in the model yeast Saccharomyces cerevisiae and the opportunistic pathogen Candida albicans via calcineurin. Here, we explored effectors downstream of calcineurin regulating this Hsp90-dependent trait. Using S. cerevisiae erg3 mutants as a model, we determined that both Crz1 and Hph1 modulate azole resistance.

The molecular chaperone Hsp90 is essential, abundant at normal temperatures, and induced by stress in eukaryotes. Under physiological conditions, Hsp90 dynamically interacts with a diverse set of inherently unstable, or metastable, client proteins (20, 21, 28). It is involved in the maturation and intracellular transport of many regulators of growth and development, including transcription factors and kinases. Under stressful conditions, Hsp90 is induced, but the increased cellular demand for its chaperone functions can exceed its induction (24). This likely occurs because associations between Hsp90 and client proteins are less stable during stress and because Hsp90 is diverted to assist stress-damaged proteins.

By chaperoning regulators of cell signaling in an environmentally contingent manner, Hsp90 is poised to influence the evolution of new traits. In fungi separated by ~800 million years of evolution, we found that Hsp90 potentiates the rapid evolution of drug resistance (7). In both the model yeast Saccharomyces cerevisiae and the opportunistic pathogen Candida albicans, Hsp90 enables the rapid evolution of resistance to the azole antifungal drugs. The azoles inhibit Erg11 and thereby block the biosynthesis of ergosterol, the predominant sterol of fungal membranes (17). Inhibition results in the accumulation of toxic intermediates in ergosterol biosynthesis, culminating in severe membrane stress. Resistance to azoles is a complex trait that can arise by multiple mutations whose phenotypic consequences are contingent on cellular stress responses (7) and interactions with genetic variants in particular genomes (1, 2).

We determined that the key mediator of Hsp90-dependent azole resistance is calcineurin (7), an Hsp90 client protein and a conserved calcium-activated protein phosphatase. In fungi, calcineurin regulates cell cycle progression, morphogenesis, and virulence (10). Hsp90 binds the catalytic subunit of calcineurin, keeping it stable and poised for activation (12, 14). Calcineurin activation is required for tolerance of a myriad of environmental stresses, including the membrane stress exerted by azoles (8, 22). By chaperoning calcineurin, Hsp90 regulates membrane stress responses that are crucial for cells to survive in the presence of azoles, thereby enabling the phenotypic consequences of new resistance mutations.

Here, we explored the genetic architecture of Hsp90-dependent drug resistance by dissecting the contribution of downstream effectors of calcineurin, Crz1 and Hph1. S. cerevisiae strains with resistance to the widely deployed azole fluconazole acquired by loss of function of the ergosterol biosynthetic enzyme Erg3 provide the ideal model due to the exquisite dependence of their resistance on both Hsp90 and calcineurin (7). This resistance mechanism blocks the accumulation of toxic sterol intermediates, resulting in altered membrane sterol composition (1). To validate this model, we used a selection regimen favoring Erg3 mutations. Plating ~10^4 cells of a strain with wild-type levels of Hsp90 (H90) on medium with a high concentration of fluconazole (128 μg/ml) yields three classes of colonies (Fig. 1A) (7): (i) large colonies (≥1.6 mm^2) with increased fluconazole resistance (H90-R; Fig. 1B), defined by an increase in the MIC at which growth is inhibited by 50% (MIC50) relative to the drug-free growth control (all tested had mutations in Erg3 [7]); (ii) intermediate colonies with no change in resistance but with increased tolerance (H90-T; Fig. 1B), defined by growth at drug concentrations above the MIC50; and (iii) small abortive colonies (≤0.7 mm^2). A concentration of an Hsp90 inhibitor that did not impair growth on its own (7) completely blocked the emergence of both fluconazole resistance and tolerance (Fig. 1A). As expected if Hsp90 enables the evolution of azole resistance by chaperoning calcineurin, an inhibitor of calcineurin function also blocked the evolution of both resistance and tolerance to fluconazole (Fig. 1A). Furthermore, inhibitors of Hsp90 or calcineurin abolished both resistance and tolerance phenotypes that had been acquired in their absence (Fig. 1C).

MIC testing and selection experiments were performed as previously described (7). Selection plates were photographed under standard conditions, and images were processed using the free software program CellProfiler (www.cellprofiler.org [15]) in order to measure colonies and classify them as resis-
tant, tolerant, or abortive. The following steps were carried out automatically (without user intervention) on all images: (i) the red channel was extracted from each color image; (ii) the plate was located by alignment with a template; (iii) aligned images were cropped to standard dimensions; (iv) gradients in illumination across each plate were corrected, and background was subtracted; (v) the plate rim was removed by cropping; (vi) colonies were identified using an algorithm detecting local maxima in the distance transform of the image; (vii) dividing lines between clumped colonies were identified using a watershed on the distance-transformed image; (viii) the area of the identified colonies was measured; and (ix) colonies were classified and color coded by size. CellProfiler software provides a powerful analytical tool for high-throughput selection experiments.

The cellular circuitry underlying erg3-mediated resistance is of particular interest as azole-resistant clinical isolates of \textit{C. albicans} harboring this resistance mechanism have been recovered from patients treated with azoles (23). Since \textit{erg3} mutants were not recovered in \textit{C. albicans} in response to the acute selection regimen described above (7), we constructed a \textit{C. albicans} homozygous \textit{erg3} deletion mutant by disrupting the remaining \textit{ERG3} allele in a heterozygous deletion mutant, as previously described (7). Strikingly, the exquisite Hsp90- and calcineurin-dependent azole resistance phenotype of \textit{erg3} mutants identified in \textit{S. cerevisiae} was conserved in \textit{C. albicans} (Fig. 2).

![FIG. 1. Hsp90 and calcineurin mediate both fluconazole (FL) resistance and tolerance acquired by acute selection. (A) Pharmacological inhibitors of Hsp90 (radicicol [RAD]) or calcineurin (cyclosporine [CsA]) block the emergence of both FL resistance and tolerance. Approximately 10^4 cells of a strain with wild-type levels of Hsp90 (Hi90) were plated onto synthetic defined (SD) medium with 128 \mu g/ml FL supplemented with 1 \mu M RAD or 20 \mu M CsA, as indicated, and incubated for 7 days at 23°C. Photographs of selection plates were analyzed with CellProfiler software. The large colonies (>=1.6 mm^2, shown in yellow) had resistant phenotypes (Hi90-R, Fig. 1B), the intermediate colonies (turquoise) were tolerant and the smallest colonies (<=0.7 mm^2, purple) were abortive. (C) Inhibitors of Hsp90 (geldanamycin [GdA]) or calcineurin (CsA) abrogate both FL resistance and tolerance. Resistance was measured at 23°C by broth microdilution in SD medium supplemented with 5 \mu M GdA or 20 \mu M CsA, as indicated. Optical densities at 595 nm of MIC test plates were averaged for duplicate measurements and normalized relative to the FL-free controls (see color legend).](image)
Cnb1 (Fig. 3A). Pharmacological impairment of Hsp90 or calcineurin function abolished the residual tolerance of erg3Δ crz1Δ double mutants (Fig. 3A). Related findings for C. albicans cells with wild-type Erg3 function indicate that Crz1 has only a modest effect on azole tolerance (13, 18). These results suggest that additional downstream effectors of calcineurin modulate cellular responses to azoles.

An ideal candidate for a second downstream effector of calcineurin mediating Hsp90-dependent azole resistance is Hph1. The tail-anchored integral membrane proteins Hph1 and Hph2 serve redundant roles in promoting survival during stress, including alkaline pH, high salt, and cell wall stress; they function independently of Crz1 and have a greater impact on growth under alkaline conditions than does Crz1 (11). Calcineurin directly dephosphorylates Hph1, altering its distribution within the endoplasmic reticulum, but does not interact with or dephosphorylate Hph2 (11). Strikingly, erg3Δ hph1Δ double mutants (n = 4), constructed by mating single mutants in the BY4741/2 background followed by sporulation, all showed a complete loss of resistance comparable to that observed with loss of calcineurin function in the erg3Δ background (Fig. 3B). Deletion of HPH2 did not alter the sensitivity of the erg3Δ hph1Δ double mutant (n = 4) or the resistance of the erg3Δ mutant (n = 4); this resistance was abrogated by inhibition of Hsp90 or calcineurin (Fig. 3B). These findings provide the first evidence for a key role for Hph1 in azole resistance in S. cerevisiae. With no apparent Hph1/2 homologs in C. albicans, the key effectors of Hsp90- and calcineurin-dependent azole resistance remain to be identified in this organism.

Drug resistance is often a complex trait affected by natural genetic variation in yeast (19). We examined the potential for genetic variation affecting these signaling pathways among S. cerevisiae strains by creating the double and triple mutants in hybrid backgrounds between BY4741 and W303. The striking result was that meiotic segregants of the same genotype showed two different growth responses to fluconazole. For example, three of the seven erg3Δ meiotic progeny exhibited the expected resistance phenotype, while the remainder exhibited sensitivity (Fig. 4). The erg3Δ crz1Δ progeny segregated between tolerance (n = 5) and sensitivity (n = 6). The erg3Δ hph1Δ progeny consisted of both resistant (n = 2) and sensitive strains (n = 4), as did the erg3Δ hph2Δ progeny. Finally, the erg3Δ hph1Δ hph2Δ progeny consisted of both tolerant (n = 6)
new targets for therapeutics directed against diverse fungal pathogens (3, 7, 16). Inhibitors of Hsp90 or calcineurin function may enhance the efficacy of existing antifungals, rendering recalcitrant pathogens more responsive to treatments. Hsp90 inhibitors are in currently in phase II clinical trials as antican-
cancer agents, and calcineurin inhibitors are widely deployed as immuno-suppressants. Given that Hsp90 and calcineurin are highly conserved regulators of cell signaling in eukaryotes, successful deployment of inhibitors of their function in anti-
fungal therapy may be compromised by toxicity to the host. Identifying fungus-specific components of this cellular circuitry with a global impact on drug resistance and virulence would provide novel therapeutic targets.

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