Rapid Redistribution of Phosphatidylinositol-(4,5)-Bisphosphate and Septins during the *Candida albicans* Response to Caspofungin

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We previously showed that phosphatidylinositol-(4,5)-bisphosphate [PI(4,5)P2] and septin regulation play major roles in maintaining *Candida albicans* cell wall integrity in response to caspofungin and other stressors. Here, we establish a link between PI(4,5)P2 signaling and septin localization and demonstrate that rapid redistribution of PI(4,5)P2 and septins is part of the natural response of *C. albicans* to caspofungin. First, we studied caspofungin-hypersusceptible *C. albicans* *irs4* and *inp51* mutants, which have elevated PI(4,5)P2 levels due to loss of PI(4,5)P2-specific 5′-phosphatase activity. PI(4,5)P2 accumulated in discrete patches, rather than uniformly, along surfaces of mutants in yeast and filamentous morphologies, as visualized with a green fluorescent protein (GFP)-pleckstrin homology domain. The patches also contained chitin (calcofluor white staining) and cell wall protein Rbt5 (Rbt5-GFP). By transmission electron microscopy, patches corresponded to plasma membrane invaginations that incorporated cell wall material. Fluorescently tagged septins Cdc10 and Sep7 colocalized to these sites, consistent with well-described PI(4,5)P2-septin physical interactions. Based on expression patterns of cell wall damage response genes, *irs4* and *inp51* mutants were firmly positioned within a group of caspofungin-hypersusceptible, septin-regulatory protein kinase mutants. *irs4* and *inp51* were linked most closely to the *gin4* mutant by expression profiling, PI(4,5)P2-septin-chitin redistribution and other phenotypes. Finally, sublethal 5-min exposure of wild-type *C. albicans* to caspofungin resulted in redistribution of PI(4,5)P2 and septins in a manner similar to those of *irs4*, *inp51*, and *gin4* mutants. Taken together, our data suggest that the *C. albicans* Irs4-Inp51 5′-phosphatase complex and Gin4 function upstream of PI(4,5)P2 and septins in a pathway that helps govern responses to caspofungin.

*C*andida albicans is the most common fungal pathogen of humans, causing a wide range of superficial, mucosal, and systemic diseases. Candidemia and other types of systemic candidiasis are associated with mortality rates approaching or exceeding 40% (35). The echinocandin antifungals (caspofungin, anidulafungin, and micafungin) have emerged as front-line therapy against systemic candidiasis (40). These agents inhibit β-1,3-D-glucan synthase, an enzyme that synthesizes a major component of the *Candida* cell wall, and thereby kill cells by disrupting cell wall integrity. Independent of its essential role in maintaining cellular viability, the cell wall is central to the pathogenesis of candidiasis. It forms the interface of the pathogen-host interaction, elaborates virulence factors, and makes complex contributions to cellular morphogenesis and resistance to host defenses (2, 3, 11, 16, 21, 22, 33, 41, 44, 50, 57, 58). Not surprisingly, *C. albicans* cell wall regulation is among the most dynamic areas of translational and basic research in medical mycology.

The *C. albicans* cell wall consists of three layers. Echinocandins target the middle layer, which constitutes a backbone comprised largely of cross-linked β-1,3- and β-1,6-glucans. A mannan- and mannoprotein-rich outer layer is the favored target of the innate immune system. Cell wall rigidity is provided by an inner layer of the polymer chitin, which lies in proximity to the plasma membrane. Much of our understanding of *C. albicans* cell wall synthesis and regulation comes from work with the model yeast *Saccharomyces cerevisiae* (8, 24–27). There are many examples in which orthologous *S. cerevisiae* and *C. albicans* genes involved in cell wall regulation have the same biological function (4). However, *de novo* gene discovery in *C. albicans* has increasingly revealed nonconservation of function (7, 19, 29, 32, 39, 51, 61), which has prompted analysis of cell wall-related genes and genetic relation-

ships in *C. albicans* itself (38, 42). In addition to their clinical implications, studies of *C. albicans* responses to echinocandin exposure are important for understanding cell wall regulation in general and defining mechanisms of susceptibility and resistance to cell wall stress.

In this study, we focus on the relationship between phosphatidylinositol-(4,5)-bisphosphate [PI(4,5)P2] and septins in the regulation of *C. albicans* cell wall integrity in response to caspofungin. We began our studies of PI(4,5)P2 regulation after demonstrating that *C. albicans* Irs4 was among a group of proteins that was recognized by sera from patients with candidiasis (2, 3, 10, 48). We selected Irs4 for further study for two reasons: (i) it has an EH domain, a protein binding motif implicated in cell wall regulation (2, 3, 34), and (ii) it is the sole homologue of *S. cerevisiae* ScIrs4 and ScTax4, which jointly interact with the 5′-phosphatase ScInp51 to regulate levels of PI(4,5)P2 (2, 3, 34). We established that *C. albicans* Irs4 and Inp51 physically interact and that disruption of *IRS4* or *INP51* causes elevated intracellular PI(4,5)P2 levels but does not affect levels of phosphatidylinositol 4-phosphate [PI(4)P], PI(3)P, or PI(3,5)P2 (3). In addition, Irs4 and Inp51 are

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TABLE 1 *C. albicans* strains

Strain	Parent	Description	Research purpose	Reference
SC5314		*C. albicans* reference strain	Wild type	15
irs4_2KO	SC5314	*irs4*Δ/*irs4*Δ	*irs4* null mutant	This study
irs4_2KO_Reins	irs4_2KO	Complemented, *irs4*Δ/IRS4	IRS4 reinsertion	This study
inp51_2KO	SC5314	*inp51*Δ/*inp51*Δ	*inp51* null mutant	3
inp51_2KO_Reins	Inp51_2KO	Complemented, *inp51*Δ/INP51	INP51 reinsertion	3
gin4_2KO*	SC5314	*gin4*Δ/*gin4*Δ	*gin4* null mutant	This study
gin4_2KO_Reins*	gin4_2KO	Complemented, *irs4*Δ/IRS4	GIN4 reinsertion	This study
SC_AGPH	SC5314	RP10/rp10::CaPHx2-GFP	PH-GFP expression	This study
irs4_AGPH	irs4_2KO	*irs4*Δ/*irs4*Δ, RP10/rp10::CaPHx2-GFP	PH-GFP expression	This study
inp51_AGPH	inp51_2KO	*inp51*Δ/*inp51*Δ, RP10/rp10::CaPHx2-GFP	PH-GFP expression	This study
gin4_AGC10	gin4_2KO	*gin4*Δ/*gin4*Δ, RP10/rp10::CaPHx2-GFP	PH-GFP expression	This study
SC_AGC10	SC5314	RP10/rp10::CDC10-GFP	CDC10-GFP expression	This study
irs4_AGC10	irs4_2KO	*irs4*Δ/*irs4*Δ, RP10/rp10::CDC10-GFP	CDC10-GFP expression	This study
inp51_AGC10	inp51_2KO	*inp51*Δ/*inp51*Δ, RP10/rp10::CDC10-GFP	CDC10-GFP expression	This study
SC_AGS7	SC5314	RP10/rp10::SEP7-GFP	SEP7-GFP expression	This study
irs4_AGS7	irs4_2KO	*irs4*Δ/*irs4*Δ, RP10/rp10::SEP7-GFP	SEP7-GFP expression	This study
inp51_AGS7	inp51_2KO	*inp51*Δ/*inp51*Δ, RP10/rp10::SEP7-GFP	SEP7-GFP expression	This study
CAI_R5G	CAI4	*ura3*::imm434/*ura3*::imm434 *iro1/iro1*::imm434, Rbt5sig.GFP.GPI	RBT5-GFP expression	31
rs4_AGR5	CAI_R5G	Same as parent, *irs4*Δ/*irs4*Δ	RBT5-GFP expression	This study
inp51_AGR5	CAI_R5G	Same as parent, *inp51*Δ/*inp51*Δ	RBT5-GFP expression	This study
SC_AGPHRC10	SC_AGPH	RP10/rp10::CaPHx2-GFP, CDC10-RFP	PH-GFP and CDC10-RFP expression	This study
irs4_AGPHRC10	irs4_AGPH	*irs4*Δ/*irs4*Δ, RP10/rp10::CaPHx2-GFP, CDC10-RFP	PH-GFP and CDC10-RFP expression	This study
inp51_AGPHRC10	inp51_AGPH	*inp51*Δ/*inp51*Δ, RP10/rp10::CaPHx2-GFP, CDC10-RFP	PH-GFP and CDC10-RFP expression	This study
gin4_AGPHRC10	gin4_AGPH	*gin4*Δ/*gin4*Δ, RP10/rp10::CaPHx2-GFP, CDC10-RFP	PH-GFP and CDC10-RFP expression	This study
SC_AGPHRS7	SC_AGPH	RP10/rp10::CaPHx2-GFP, SEP7	PH-GFP and SEP7-RFP expression	This study
irs4_AGPHRS7	irs4_2KO	*irs4*Δ/*irs4*Δ, RP10/rp10::CaPHx2-GFP, SEP7	PH-GFP and SEP7-RFP expression	This study
inp51_AGPHRS7	inp51_2KO	*inp51*Δ/*inp51*Δ, RP10/rp10::CaPHx2-GFP, SEP7	PH-GFP and SEP7-RFP expression	This study
SC_AGV4	SC5314	RP10/rp10::Vrg4-GFP	VRG4-GFP expression	This study
irs4_AGV4	irs4_2KO	*irs4*Δ/*irs4*Δ, RP10/rp10::Vrg4-GFP	VRG4-GFP expression	This study
inp51_AGV4	inp51_2KO	*inp51*Δ/*inp51*Δ, RP10/rp10::Vrg4-GFP	VRG4-GFP expression	This study
SC_S1SPG	SC5314	RP10/rp10::SAP1_SP-GFP	SAP1-GFP (secretory) expression	This study
irs4_S1SPG	irs4_2KO	*irs4*Δ/*irs4*Δ, RP10/rp10::SAP1_SP-GFP	SAP1-GFP (secretory) expression	This study
inp51_S1SPG	inp51_2KO	*inp51*Δ/*inp51*Δ, RP10/rp10::SAP1_SP-GFP	SAP1-GFP (secretory) expression	This study
SC_AGV4	SC5314	RP10/rp10::Arf1-GFP	ARF1-GFP expression	This study
irs4_AGV4	irs4_2KO	*irs4*Δ/*irs4*Δ, RP10/rp10::Arf1-GFP	ARF1-GFP expression	This study
inp51_AGV4	inp51_2KO	*inp51*Δ/*inp51*Δ, RP10/rp10::Arf1-GFP	ARF1-GFP expression	This study
SC_S1SPG	SC5314	RP10/rp10::Arf2-GFP	ARF2-GFP expression	This study
irs4_S1SPG	irs4_2KO	*irs4*Δ/*irs4*Δ, RP10/rp10::Arf2-GFP	ARF2-GFP expression	This study
inp51_S1SPG	inp51_2KO	*inp51*Δ/*inp51*Δ, RP10/rp10::Arf2-GFP	ARF2-GFP expression	This study
BWP17		*C. albicans* reference strain (auxotropically marked)		59
DAY185	BWP17	*C. albicans* reference strain (auxotrophies corrected)	Wild-type control	59
DAY_AGPH	DAY185	RP10/rp10::CaPHx2-GFP	PH-GFP expression	This study
cdc10$^{-/-}$ (YAW7)	BWP17	*cdc10*Δ/*cdc10*Δ	*cdc10* null mutant	55, 56
cdc11$^{-/-}$ (YAW11)	BWP17	*cdc10*Δ/*cdc10*Δ	*cdc10* null mutant	55, 56
ckb2$^{-/-}$ (JJH351)	BWP17	*ckb2*Δ/*ckb2*Δ	*ckb2* null mutant	6
CKB2$^{-/-/+}$	ckb2Δ/ckb2Δ	Complemented, *ckb2*Δ/CKB2	CKB2 reinsertion	6
ckb2_AGPH	ckb2Δ/ckb2Δ	*ckb2*Δ/*ckb2*Δ, RP10/rp10::CaPHx2-GFP	PH-GFP expression	This study
cla4$^{-/-}$ (JJH262)	BWP17	*cla4*Δ/*cla4*Δ	*cla4* null mutant	6
CLA4$^{-/-/+}$	cla4Δ/cla4Δ	Complemented, *cla4*Δ/CLA4	CLA4 reinsertion	6
cla4_AGPH	cla4Δ/cla4Δ	*cla4*Δ/*cla4*Δ, RP10/rp10::CaPHx2-GFP	PH-GFP expression	This study
gin4$^{-/-}$ (JJH276)	BWP17	*gin4*Δ/*gin4*Δ	*gin4* null mutant	6
GIN4$^{-/-/+}$	gin4Δ/gin4Δ	Complemented, *gin4*Δ/GIN4	GIN4 reinsertion	6
gin4_AGPH	gin4Δ/gin4Δ	*gin4*Δ/*gin4*Δ, RP10/rp10::CaPHx2-GFP	PH-GFP expression	This study
hsl1$^{-/-}$ (JJH377)	BWP17	*hsl1*Δ/*hsl1*Δ	*hsl1* null mutant	6
HSL1$^{-/-/+}$	hsl1Δ/hsl1Δ	Complemented, *hsl1*Δ/HSL1	HSL1 reinsertion	6
hsl1_AGPH	hsl1Δ/hsl1Δ	*hsl1*Δ/*hsl1*Δ, RP10/rp10::CaPHx2-GFP	PH-GFP expression	This study
kin3$^{-/-}$ (JJH381)	BWP17	*kin3*Δ/*kin3*Δ	*kin3* null mutant	6
KIN3$^{-/-/+}$	hsl1Δ/hsl1Δ	Complemented, *kin3*Δ/KIN3	KIN3 reinsertion	6
kin3_AGPH	hsl1Δ/hsl1Δ	*kin3*Δ/*kin3*Δ, RP10/rp10::CaPHx2-GFP	PH-GFP expression	This study
tpk1$^{-/-}$ (JJH384)	BWP17	*tpk1*Δ/*tpk1*Δ	*tpk1* null mutant	6
TPK1$^{-/-/+}$ (JJH233)	tpk1Δ/tpk1Δ	Complemented, *tpk1*Δ/TPK1	TPK1 reinsertion	6
bck1$^{-/-}$ (JJH256)	BWP17	*bck1*Δ/*bck1*Δ	*bck1* null mutant	6
BCK1$^{-/-/+}$ (JJH246)	bck1Δ/bck1Δ	Complemented, *bck*Δ/BCK1	BCK1 reinsertion	6
ire1$^{-/-}$ (SF008P)	BWP17	*ire1*Δ/*ire1*Δ	*ire1* null mutant	6
IRE1$^{-/-/+}$ (SF008C)	ire1Δ/ire11Δ	Complemented, *ire1*Δ/IRE1	IRE1 reinsertion	6

required for the progression but not the initiation of disseminated candidiasis in a murine model (2, 3).

A connection between PI(4,5)P2 and cell wall organization was first made in *S. cerevisiae*, in which *irs4/tax4* and *inp51* mutations have synthetic interactions with known cell wall integrity regulators (34). However, the role of PI(4,5)P2 in cell wall integrity is well buffered in *S. cerevisiae* (34). Indeed, extremely sensitive competitive growth assays failed to reveal hypersensitivity of an
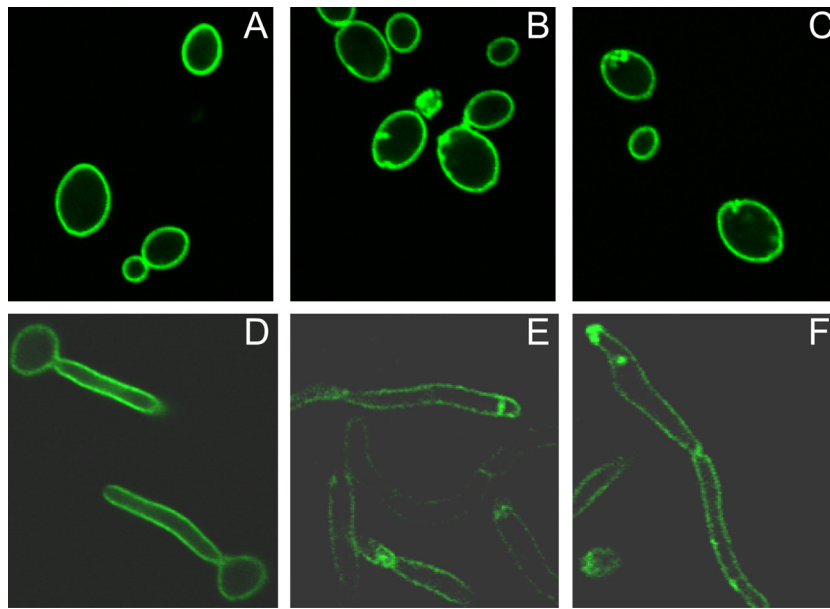
FIG 1 PI(4,5)P2 accumulates in discrete patches in *irs4* and *inp51* mutants. *C. albicans* SC5314 (wild-type) and *irs4* and *inp51* mutants expressing CaPH-GFP were grown in yeast (A to C, respectively; YPD medium at 30°C) or hyphal (D to F, respectively; YPD medium + 5% fetal bovine serum [FBS] at 37°C) morphologies in 35-mm-diameter glass-bottom dishes and imaged using confocal microscopy. As shown, CaPH-GFP was distributed uniformly in SC5314 (A and D) but concentrated in discrete patches in *irs4* (B and E) and *inp51* (C and F) mutants.

inp51 mutant to caspofungin (18). In contrast, PI(4,5)P2 has a highly prominent role in *C. albicans* cell wall organization: *irs4* and *inp51* null mutants are hypersusceptible to caspofungin, do not exhibit paradoxical growth at high caspofungin concentrations, overactivate the protein kinase C–mitogen-activated protein kinase (PKC-MAPK) cell integrity pathway, and exhibit aberrant sites of chitin accumulation (2, 18, 48). Thus, the study of PI(4,5)P2 signaling in *C. albicans* provides a unique opportunity to advance our understanding of both cell wall integrity and echinocandin susceptibility.

Septins are cytoskeletal proteins that serve as scaffolds for cytokinesis and other dynamic events at the plasma membrane (20, 30, 53). They were first connected to cell wall biogenesis in *S. cerevisiae* by the finding that they are required for localization of chitin synthase Chs3 (13, 45). Septin-defective mutants display aberrant sites of chitin deposition (13). We were led to *C. albicans* septins through a screen of protein kinase mutants for cell wall integrity defects (6). Among 24 protein kinase mutants that were hypersensitive to caspofungin, one group had cohesive properties: they displayed increased expression of numerous cell wall integrity genes, and in many cases their *S. cerevisiae* orthologs were implicated in the function of the septin ring. We extended these observations with the finding that *C. albicans* septin mutants are hypersensitive to caspofungin and by showing that a newly characterized protein kinase gene of this group, *KIN3*, is required for septin ring integrity. We observed that the septin ring becomes disorganized and delocalized upon caspofungin treatment. These results suggested that septins may be involved in the natural response to caspofungin and other cell wall inhibitors (6).

S. cerevisiae septins interact specifically with PI(4,5)P2, and the presence of PI(4,5)P2 promotes septin polymerization and organization (5). As such, we hypothesized that the role of PI(4,5)P2 in *C. albicans* cell wall integrity may be mediated by septins or their regulators. Here we present phenotypic comparisons that reveal close similarities between *irs4*, *inp51* and septin regulatory protein kinase mutants. In addition, we observe that PI(4,5)P2 and septin localization is altered in wild-type *C. albicans* upon caspofungin treatment. Our findings argue that redistribution of PI(4,5)P2 and septins is part of the natural cell wall integrity response in *C. albicans*.

MATERIALS AND METHODS

Strains and growth conditions. *C. albicans* strains used or constructed in this study are listed in Table 1. *C. albicans* SC5314 was the wild-type control for *irs4*, *inp51*, and *gin4* null mutants created using the SAT-flipper method (see below). For our previously created septin regulatory protein kinase mutants, the wild-type control strain was DAY185 (6, 59). Strains were routinely grown in yeast extract-peptone-dextrose (YPD) liquid medium (1% yeast extract, 1% Bacto peptone, 2% [α-^{35}C]glucose) or on YPD or Sabouraud dextrose agar (SDA) at 30°C. For embedded growth, ~100 cells of overnight-grown *C. albicans* were mixed into 20 ml of YPD-reverse agar and incubated for 3 days at 30°C or 37°C. Reverse agar (BASF pluronic polyol F-127; kindly provided by Fungal Genetics Stock Center [http://www.fgsc.net]) is a lock polymer of polyoxypropylene and polyoxyethylene that can be used as a replacement for conventional agar in solid medium. It is a liquid in solution at 4°C and solidifies to a viscous solution at room temperature. For invasive growth, cells were grown in liquid medium in 35-mm-diameter glass-bottom dishes (Matek, Ashland, MA) coated with 10 μg/cm^2 of Cell-Tak (BD Biosciences, Bedford, MA) to adsorb a thin layer. *C. albicans* cells immobilized in the layer were covered with ~100 μl of YPD or YPD plus 5% fetal bovine serum and incubated at 30°C or 37°C.

Gene disruption. *C. albicans* *irs4* and *gin4* null mutant strains were created by the standard SAT-flipper method, as used previously to create our *inp51* null mutants (3, 43). In brief, fragments at the 5′ (F1) and 3′ (F2) ends of the targeted genes were amplified by PCR using primers that introduced appropriate restriction sites. These fragments were subcloned into pSFS-2A between the KpnI and XhoI sites and the SacII and SacI sites,
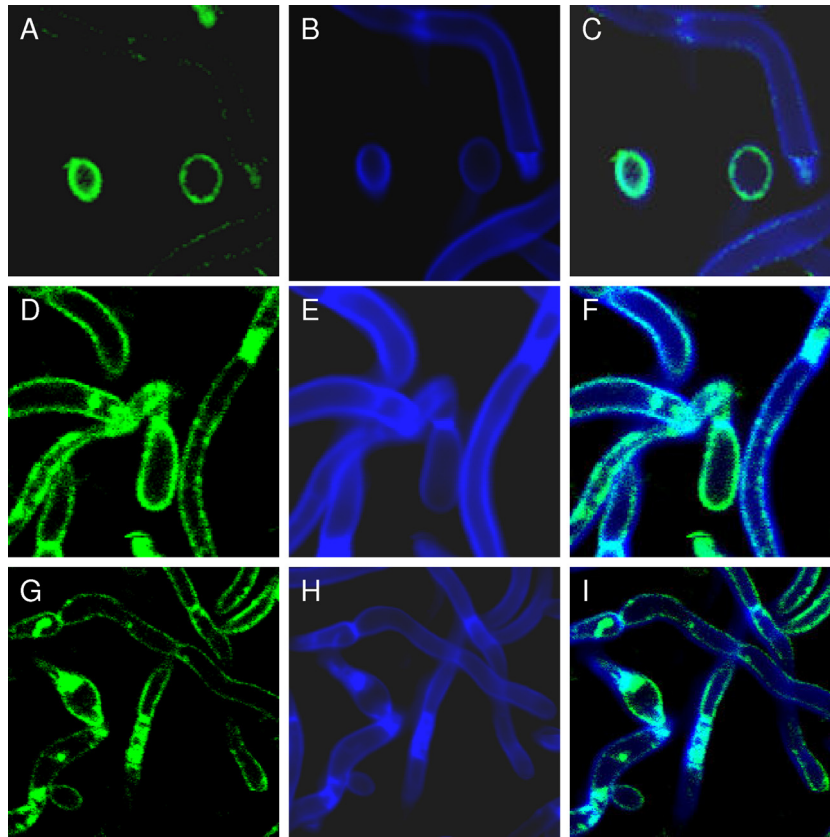
FIG 2 PI(4,5)P2 and chitin colocalize at aberrant patches in *irs4* and *inp51* mutants. PI(4,5)P2 was localized in *C. albicans* SC5314 and *irs4* and *inp51* mutants using a CaPH-GFP reporter (A, D, and G, respectively), as in the experiments shown in Fig. 1. Chitin was localized in the same cells by calcofluor white staining (B, E, and H, respectively). Overlay images revealed that CaPH-GFP and calcofluor white colocalized to aberrant sites in *irs4* (F) and *inp51* (I) mutants (cyan color).

respectively. In parallel, the full gene with 1 kb of downstream noncoding sequence was amplified and used as F1 for gene reinsertion. The resulting plasmids were extracted and linearized with KpnI and SacI and introduced into competent *C. albicans* cells by electroporation. Transformants were grown on selective medium plates containing 200 μg/ml streptothricin (Alexis Biochemicals) for 2 to 3 days and then screened by PCR. Positive transformants were grown overnight in maltose-based medium to induce excision of the plasmid cassette, plated on selective medium plates for 3 to 4 days, and screened for positive excisions to enable the strain for another round of targeted recombination. All positive events of homologous recombination and excision were confirmed by Southern blotting. We verified previously described phenotypes for the *irs4* and *inp51* mutants, including normal growth in liquid media, impaired invasive growth and hyphal formation in contact with solid agar, increased susceptibility to caspofungin, zymolase, and SDS, and distribution of chitin in patches along the cell surfaces of yeast and filamentous morphologies (2, 3). The *irs4* and *inp51* mutants were indistinguishable in each of these assays. The *gin4* mutant was indistinguishable from *gin4* mutants previously created in our lab using *C. albicans* strain BWP17 (6). In all instances, gene reconstitutions corrected the aberrant phenotypes. Similar results were obtained using independently created null mutant and reconstitution strains.

Caspofungin exposure. Sensitivity to caspofungin (Merck Research Laboratories) was measured in 48-well microtiter plates. *C. albicans* strains grown overnight in YPD at 30°C were diluted to an optical density at 600 nm (OD_{600}) of 0.1 in YPD. Caspofungin was added at concentrations ranging from 0.075 to 20 μg/ml and transferred to the microtiter plates (800 μl per well). The plates were incubated at 30°C with shaking at 250 rpm, and the OD_{620} was measured every hour. The MIC of caspofungin was determined using the broth macrodilution method recommended by CLSI (48, 49). To assess the impact of caspofungin exposure on PI(4,5)P2 and septin localization, we used our previously described methods with the following modifications (6). Cells were exposed to the MIC of caspofungin (0.125 μg/ml) in YPD for 5 min, followed by washing twice with phosphate-buffered saline (PBS). This regimen was verified to be nonlethal to *C. albicans* cells by colony count enumerations prior to caspofungin exposure, as well as after exposure and washing. PI(4,5)P2 and septin were visualized as described below.

CaPH-GFP and other fluorescent reporters. Pleckstrin homology (PH) domains bind specifically to PI(4,5)P2 (17, 46). To create a green fluorescent protein (GFP)-tagged *C. albicans* PH domain, the sequence of human PLCδ1 PH was modified to account for noncanonical CTG codon usage (encoding serine instead of leucine). The *C. albicans* PH domain (CaPH; 549 bp) was synthesized using the custom gene synthesis service of Integrated DNA Technologies, Inc. (Coralville, IA), and it was cloned into pZEro-2. For the other fluorescently labeled reporters listed in Table 1, the respective open reading frames were amplified from *C. albicans* SC5314 genomic DNA and fused to yeGFP (*C. albicans*-optimized GFP, as described in reference 12), CaGFP (*C. albicans*-optimized GFP, as described in reference 60), or red fluorescent protein (RFP) (47). In the case of RFP, the sequence of the photostable TagRFP-T version of RFP was optimized for expression using the codon preference of *C. albicans* and was chemically synthesized by Integrated DNA Technologies Inc. The fusion genes were digested with the appropriate restriction enzymes and cloned into pSFS under the control of ADH1p and ADH1t (43). pSFS plasmids carried target fragments for genomic integration by homologous recombination
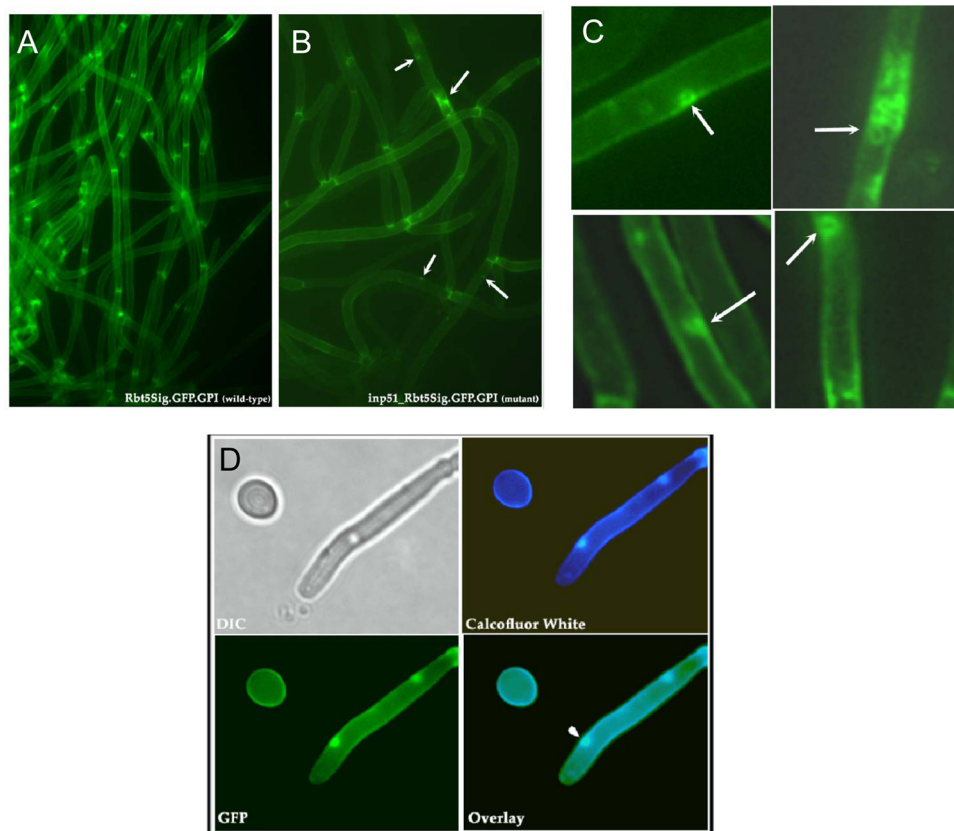
FIG 3 The GPI-anchored cell wall protein Rbt5 colocalizes with chitin at aberrant patches in mutant cells. *C. albicans* CAI4 and *irs4* and *inp51* mutants expressing an Rbt5-GFP fusion were visualized by confocal microscopy as in earlier experiments. *inp51* mutant cells are shown as a representative. Rbt5-GFP was distributed normally in CAI4 cells (A) but was concentrated within aberrant patches (white arrows) in the *inp51* mutant (B and C [higher-resolution images]). Rbt5-GFP colocalized with calcofluor white in the *inp51* mutant (D, cyan color, arrowhead). The *irs4* mutant resembled the *inp51* mutant in all experiments.

into either the *RP10* locus or a noncoding region in chromosome 1 upstream to *RP10* (bp 25000 to 27000). For targeted homologous recombination in *C. albicans*, we used transformation by electroporation (3, 43). *C. albicans* wild-type strain CAI4 expressing Rbt5-GFP (strain CAI_R5G) was generously provided by Brian Wong. As previously described, CAI_R5G expresses a *C. albicans*-optimized GFP fused in frame to the signal peptide and GPI sequence from the N and C termini, respectively, of Rbt5 (31). CAI_R5G was used to generate *irs4* and *inp51* null mutants expressing GFP-Rbt5 by the SAT-flipper method (41).

Chitin staining and endocytosis assays. For chitin staining, cells were incubated for 5 min at room temperature in 0.1 mg/ml calcofluor white and then washed three times in cold PBS. Since calcofluor white may bind other glycan polymers in addition to chitin, we also localized chitin with white germ agglutinin during electron microscopy experiments (described below). For endocytosis assays, cells were resuspended in either a 50-μg/ml solution of FM4-64 (catalog no. T3166; Molecular Probes) or a 6 mg/ml solution of Lucifer yellow (catalog no. 62642; Fluka). Following incubation for 1 h at room temperature or 30°C, cells were washed 3 times in cold PBS with sodium azide and sodium fluoride (10 mM each).

Microscopy. For microscopy of fluorescently labeled cells, respective strains were grown in 35-mm-diameter glass-bottom dishes (Matek, Ashland, MA) for 2 to 3 h, as described above for invasive growth. For chitin colocalization, cells were fixed with 2% formaldehyde and stained with calcofluor white. Microscopy was performed at the University of Pittsburgh Center for Biologic Imaging, using established protocols. For fluorescence microscopy, optical sections were captured using an Olympus Fluoview 1000 confocal laser scanning microscope. Images were captured with an exposure time of 1 s on a Coolsnap HQ^2 (Photometrics) camera

using Axiovision (Zeiss) software. For transmission electron microscopy, cells were collected from within reverse agar (3) and fixed at 4°C in 0.1 M sodium cacodylate buffer (pH 7.2) containing 2% glutaraldehyde and 2% paraformaldehyde (37). The samples were then dehydrated through a graded series of ethanol and embedded in Lowicryl K4M (Electron Microscopy Sciences, Hatfield, PA). Thin sections were imaged with a Zeiss EM902 electron microscope. Wheat germ agglutinin localization of chitin was performed using previously described methods (52).

Quantitative reverse transcription-PCR (6). Overnight cultures of cells were diluted to an OD_{600} of 0.2 in 50 ml fresh YPD medium. Cultures were allowed to grow at 30°C with shaking until the culture density reached an OD_{600} of ~1. Cells were then harvested by vacuum filtration and flash frozen in a dry ice/ethyl alcohol (EtOH) bath. Cells were kept frozen on filters at −80°C until RNA extraction. RNA was extracted using a RiboPure-Yeast kit (Ambion) following the manufacturer's instructions with the following modifications. Cells were resuspended from filters with 1.5 ml ice-cold distilled water (dH_2O) followed by 10 to 15 s of vigorous vortexing. Resuspended cells were transferred to a 1.5-ml tube and spun down following the manufacturer's protocol. Furthermore, during the cell disruption step, cells were beaten with a Next Advance Bullet Blender for 3 min at 4°C to maximize cell lysis. Ten micrograms of the resulting total RNA was treated with the DNA-free kit (Ambion) followed by first-strand cDNA synthesis from half of the DNA-free RNA using the AffinityScript multiple temperature cDNA synthesis kit (Stratagene). Absence of DNA contamination was confirmed using control sets for which reverse transcriptase was omitted from the cDNA reaction. Primer3 software (http://frodo.wi.mit.edu/) was used to design primers for 14 genes (*TDH3*, *DDR48*, *SOD5*, *STP4*, *FDH2*, *AOX2*, *STF2*, *ECM331*, *RTA4*,

SKS1, *RHR2*, *ORF19.4445*, *ALS1*, and *HGT6*) (6). Then, 2× iQ SYBR green Supermix (Bio-Rad), 1 µl of first-strand cDNA reaction mixture, and 0.1 µM primers were mixed in a total volume of 50 µl per reaction, and real-time PCR was performed in triplicate for each sample on an iCycler iQ real-time PCR detection system (Bio-Rad). Product amplification was detected using SYBR green fluorescence, and specificity of the reaction was monitored by melt-curve analysis following the real-time program. *TDH3* was used as a reference gene for normalization of gene expression, which was determined using Bio-Rad iQ5 software ($\Delta\Delta C_T$ method [C_T, threshold cycle]). Cluster analysis was performed with the Multiexperiment Viewer (MeV 4.3) from the J. Craig Venter Institute. Hierarchical clustering was performed with average linkage and a Manhattan distance metric. Each mutant strain was normalized to its respective complement strain to account for strain background differences.

RESULTS

PI(4,5)P2 and chitin are redistributed in *C. albicans irs4* and *inp51* mutants at sites of plasma membrane and cell wall derangements. Building upon our observation that *C. albicans* Irs4 and Inp51 interact to regulate PI(4,5)P2 levels (3), we hypothesized that PI(4,5)P2 distribution would be abnormal in *irs4* and *inp51* mutants. To localize PI(4,5)P2, we constructed a reporter comprised of a *C. albicans*-optimized PI(4,5)P2-binding PH domain tagged with GFP (CaPH-GFP). In wild-type *C. albicans* SC5314, CaPH-GFP was distributed uniformly along the surfaces of yeast and filamentous cells and at septae. In contrast, PI(4,5)P2 was concentrated in discrete patches at the cell surfaces of *irs4* and *inp51* mutants, which appeared to extend intracellularly (Fig. 1). The aberrant distribution of PI(4,5)P2 resembled the chitin accumulations we observed previously (2, 3). Using the CaPH-GFP reporter and calcofluor white, we demonstrated that PI(4,5)P2 and chitin were colocalized in these patches (Fig. 2). Reconstitution of *IRS4* and *INP51* in the respective null mutants restored the uniform distribution of PI(4,5)P2 and chitin.

Since the null mutants are hypersusceptible to caspofungin (2, 3), we hypothesized that the PI(4,5)P2-chitin accumulations were associated with cell wall derangements. To test this hypothesis, we first visualized the GPI-anchored cell wall protein Rbt5 in mutant cells. Indeed, an Rbt5-GFP fusion colocalized with the chitin accumulations (Fig. 3). Next, we used transmission electron microscopy to assess the cell walls of mutant cells in greater detail. The cells demonstrated plasma membrane and cell wall invaginations into the cytoplasm (Fig. 4). The invaginations concentrated immunogold-labeled wheat germ agglutinin, consistent with the accumulation of chitin.

Septins colocalize with PI(4,5)P2 and chitin in *irs4* and *inp51* mutants. Consistent with the observation that PI(4,5)P2 directly interacts with septins (5), we demonstrated that PI(4,5)P2 and chitin colocalized with fluorescently tagged septins Cdc10 and Sep7 in *irs4* and *inp51* mutant cells (Fig. 5 and 6). To test the hypothesis that PI(4,5)P2 and septins are connected functionally, we studied *cdc10* and *cdc11* septin mutants for cell wall-related abnormalities previously demonstrated for *irs4* and *inp51* mutants (2, 3). Similar to *irs4* and *inp51*, *cdc10* and *cdc11* mutants were hypersusceptible to caspofungin and, as previously shown, impaired in the ability to invade solid agar (2, 3, 55). Along these lines, we previously showed that *irs4* and *inp51* mutants were impaired in invasive growth into mouse kidneys during the course of hematogenously disseminated candidiasis, similar to observations with *cdc10* and *cdc11* mutants (55, 56).

In addition to their roles in cell wall regulation, EH domain
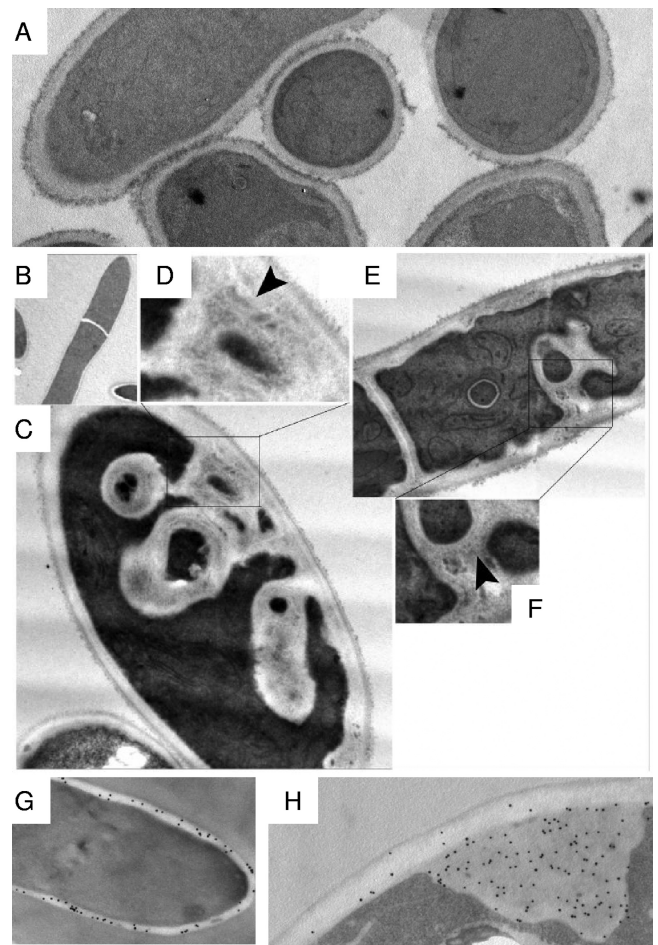
FIG 4 Mutant cells exhibit plasma membrane and cell wall abnormalities that correspond to sites of chitin accumulation. *C. albicans* SC5314 exhibited normal plasma membrane and cell wall (A) and septa (B) by transmission electron microscopy. The *inp51* mutant, on the other hand, had striking invaginations of the plasma membrane and cell wall (invaginations in panels C and E are shown at higher power [arrowheads] in panels D and F, respectively). Immunogold-labeled wheat germ agglutinin, which binds chitin, was distributed normally in SC5314 cells (G, black dots) but accumulated within the plasma membrane and cell wall invaginations of the mutant (H). The *inp51* mutant is shown as a representative; similar results were obtained for the *irs4* mutant.

proteins are known to contribute to the regulation of endocytosis and intracellular membrane trafficking and secretion. For this reason, we tested whether the plasma membrane and cell wall derangements of *irs4* and *inp51* mutant cells were sites of dysregulated endocytosis or secretion. The chitin-containing structures in mutant cells were clearly distinct from endocytic vesicles, as evident by calcofluor white and FM4-64 or Lucifer yellow costaining (see Fig. S1 in the supplemental material). Moreover, we found that mutant cells did not mislocalize GFP-tagged Vrg4 (a cytoplasm-to-Golgi transporter), GFP-Arf1 or GFP-Arf2 (ADP-ribosylation factors, involved in intracellular transport), or GFP-tagged secretory signals from either Rbt5 or the secreted aspartyl protease Sap1 (see Fig. S2 in the supplemental material).

The septin regulatory protein kinase Gin4 acts upstream of Irs4 and Inp51 in regulating cell wall integrity responses. We previously identified several protein kinases with roles in cell wall
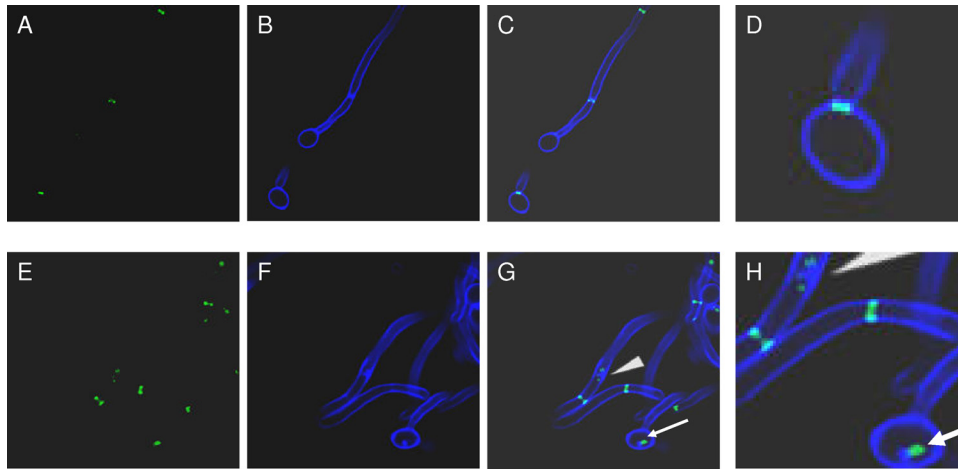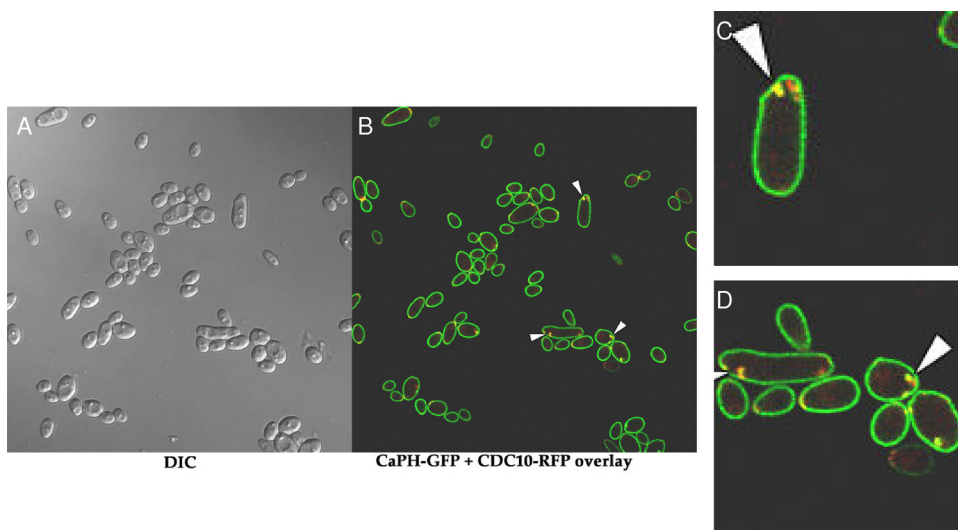
FIG 5 Septins and chitin colocalize in mutant cells. *C. albicans* SC5314 and *irs4* and *inp51* mutants expressing Cdc10-GFP were visualized by confocal microscopy, and chitin was localized by calcofluor white staining. Cdc10-GFP and calcofluor white were distributed normally in SC5314 cells (A and B, respectively), but were concentrated within aberrant patches in the *inp51* mutant (E and F, respectively). As evident in the overlay images, Cdc10-GFP colocalized with calcofluor white in the *inp51* mutant (G and H [higher resolution], cyan color and arrowheads).

integrity that also are involved in septin localization, ring function, or signaling (6). In the absence of stress, mutants of these protein kinases displayed a common cell wall damage response gene expression profile. In this study, we used quantitative RT-PCR to assess the expression of 14 damage response genes by *irs4* and *inp51* mutants, septin regulatory protein kinase mutants (*gin4*, *kin3*, *ckb2*, *cla4*, and *hsl1*), and other cell wall-related protein kinase mutants (*ire1*, *bck1*, and *tpk1*). The *irs4* and *inp51* mutants were firmly positioned with the septin mutants by hierarchical analysis of the expression data (Fig. 7), clustering most tightly with each other and the *gin4* mutant. The *ire1* mutant, in contrast, was a distinct outlier, consistent with its positioning in our earlier analysis (6).

The results suggest that the septin regulatory protein kinases are linked with PI(4,5)P2 and septins within a network that regulates cell wall integrity. If this interpretation is correct, septin regulatory protein kinase and *irs4* and *inp51* mutants should exhibit a number of similar cell wall-related phenotypes. Indeed, phenotypic analysis of the mutant strains showed variation in the degree of similarity (Table 2; see Fig. S3 in the supplemental material). Of particular note, *gin4*, *irs4*, and *inp51* mutant strains exhibited similar phenotypes for each assay, consistent with our hierarchical clustering analysis and suggesting a tight correlation between the functions of these genes. In fact, *gin4* was the only septin regulatory protein kinase mutant to demonstrate aberrant PI(4,5)P2 and chitin patches (Fig. 8). Taken with our previous demonstration that septins were also delocalized in the *gin4* mutant, the data suggest that this protein kinase acts upstream of PI(4,5)P2 and septins in regulating *C. albicans* responses to cell wall stress imposed by caspofungin.

FIG 6 Septins and PI(4,5)P2 colocalize in mutant cells. *C. albicans inp51* colocalized Cdc10-RFP and CaPH-GFP (yellow color and arrowheads in overlay images in B; higher-resolution images in C and D). Images of *irs4* mutants expressing Cdc10-RFP and CaPH-GFP, and both mutants expressing Sep7-RFP and CaPH-GFP, were similar (not shown).
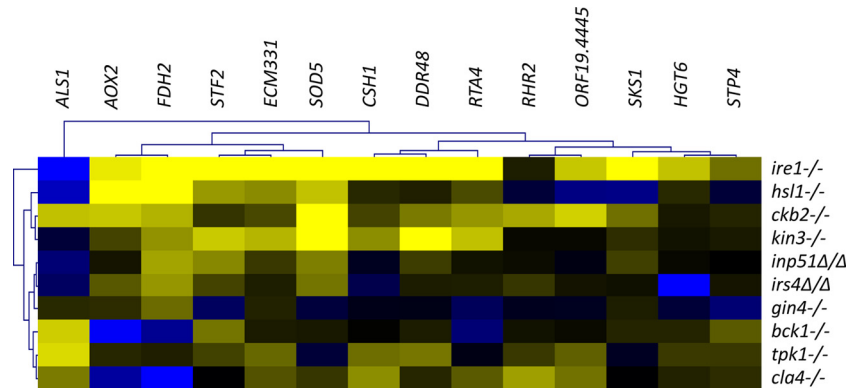
FIG 7 *C. albicans irs4* and *inp51* mutants display cell wall damage response gene expression profiles that are similar to those of septin regulatory protein kinase mutants. The expression of 14 damage response genes was analyzed in mutant strains in the absence of cell wall stress. The expression of *TDH3*, a housekeeping gene involved in glycolysis, was used to normalize expression between strains. Strains were further normalized to their complemented strain to reduce strain background errors. Resultant values were log base 2 transformed. As shown, *irs4* and *inp51* mutants were firmly positioned within the septin regulatory protein kinase functional group by hierarchical analysis and clustered most tightly with *gin4*. The cell wall-related protein kinase mutant *ire1* was an outlier from the other mutants. Blue and yellow on heat map refer to log base 2 transformed values of −2 and 2, respectively.

***C. albicans* rapidly redistributes PI(4,5)P2 and septins in response to caspofungin.** In our earlier study, caspofungin treatment of wild-type *C. albicans* cells resulted in septin ring delocalization. To determine if PI(4,5)P2 is also mislocalized in response to caspofungin, we performed PI(4,5)P2 and septin colocalization experiments. First, we identified a brief caspofungin exposure that was sublethal to a CaPH-GFP, Cdc10-RFP expressing wild-type *C. albicans* strain. Indeed, a 5-min exposure to the MIC (0.125 μg/ml) of caspofungin did not kill the reporter strain, as established by colony count enumerations. At the same time, this treatment regimen resulted in redistribution of PI(4,5)P2 and septins to common sites, which was evident in 50 to 75% of *C. albicans* cells (Fig. 9).

Taken together, therefore, our data demonstrate that a network that includes the protein kinase Gin4 and the Irs4/Inp51 phosphatase complex regulates PI(4,5)P2 and septin localization and is essential for maintaining *C. albicans* cell wall integrity in response to caspofungin. Moreover, *C. albicans* cells redistribute PI(4,5)P2 and septins rapidly as part of the cell wall stress response to caspofungin.

DISCUSSION

In our previous studies, we demonstrated that PI(4,5)P2 regulation and septin ring formation both play major roles in the maintenance of *C. albicans* cell wall integrity (2, 3, 6). Here, we present a series of observations that tie PI(4,5)P2 signaling to septin localization, in keeping with *in vitro* studies showing that PI(4,5)P2

directly interacts with septins and promotes septin filament organization and stability (5). Our most important finding is that PI(4,5)P2 signaling and septin localization are part of the natural response of *C. albicans* to cell wall inhibition by caspofungin. We propose that a PI(4,5)P2-septin regulatory network relays a signal that helps govern responses to cell wall stress and the inhibition of cell wall synthesis (Fig. 10).

We have explored PI(4,5)P2 function through deletion mutations of *IRS4* and *INP51*, genes that encode the PI(4,5)P2-specific 5′-phosphatase and its EH domain-containing regulatory subunit, respectively. As predicted, the mutations resulted in the same biochemical consequence—accumulation of PI(4,5)P2, which we demonstrated by high-performance liquid chromatography (HPLC) analysis (3). In the present study, we extend these observations by showing that the excess PI(4,5)P2 accumulates in discrete plasma membrane patches in budding cells and hyphae of *inp51* and *irs4* mutant cells. These patches do not represent sites of disrupted endocytosis or secretion but, rather, correspond to aberrant deposition of cell wall components, including chitin and the GPI-linked protein Rbt5. We previously observed that *inp51* and *irs4* mutants were hypersensitive to caspofungin and other stressors that cause cell wall disruption, a phenotype that is consistent with indirect effects on cell wall biogenesis. In this context, our current data indicate that PI(4,5)P2 directs deposition of at least some cell wall components. In fact, upregulation of chitin synthesis is a well-recognized adaptive response of *C. albicans* to β-1,3-D-glucan synthase inhibition by caspofungin (54). In our

TABLE 2 Phenotypes of *C. albicans* mutants

Phenotype	Result for *C. albicans* mutant						
	irs4	*inp51*	*gin4*	*kin3*	*ckb2*	*cla4*	*hsl1*
Caspofungin hypersusceptibility	Yes	Yes	Yes	Yes	Yes	Yes	Yes
Impaired invasive growth	Yes	Yes	Yes	No	Yes	Yes	No
Chitin mislocalization	Yes	Yes	Yes	Yes	No	No	No
PI(4,5)P2 mislocalization	Yes	Yes	Yes	No	No	No	No
Septin mislocalization	Yes	Yes	Yes[a]	Yes[a]	No[a]	±[a,b]	No[a]

[a] As demonstrated by Blankenship et al. (6).
[b] Septins mislocalized in hyphal morphology only (23).
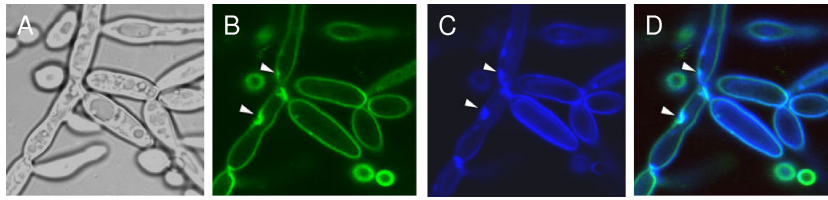
FIG 8 The *gin4* mutant (A) demonstrates aberrant chitin and PI(4,5)P2-containing patches. CaPH-GFP (B) and calcofluor white (C) colocalized in aberrant patches in *C. albicans gin4* mutant cells (D, cyan color and arrowheads in overlay image), similarly to *irs4* and *inp51* mutants. *gin4* mutants were created using the SAT-flipper method and *C. albicans* SC5314.

model for the PI(4,5)P2-septin network, chitin synthesis may be promoted through PI(4,5)P2 activation of the PKC-MAPK cell wall integrity pathway, as demonstrated in our previous study (3), and by septin-directed deposition of chitin, as suggested in the present study.

The connection of PI(4,5)P2 to septins rests on three lines of evidence. First, septins Sep7 and Cdc10 localize to the sites of aberrant PI(4,5)P2 and chitin accumulation in *inp51* and *irs4* mutants. This finding supports the functional role of septin-PI(4,5)P2 binding established *in vitro* (5). Second, *inp51* and *irs4* mutants have similar damage response gene expression changes to protein kinase mutants that govern septin localization or downstream responses (6). Similarity to the *gin4* mutant was particularly noteworthy and also extended to a defect in invasive growth into solid agar. Third, we observed that the *gin4* mutation causes accumulation of PI(4,5)P2 and chitin patches similar to those observed in the *inp51* and *irs4* mutants (2, 3). Gin4 is known to phosphorylate septins directly to govern their localization (1, 28, 36), but it has not previously been connected to PI(4,5)P2. Therefore, our observations suggest that Gin4 may have a second role in regulating PI(4,5)P2 synthesis. Alternatively, there may be a reciprocal septin-PI(4,5)P2 regulatory mechanism, whereby PI(4,5)P2 governs septin distribution, and septins also control PI(4,5)P2 distribution.

Our data supporting the PI(4,5)P2-septin regulatory relationship have two major clinical implications. First, the relationship appears to be an element of the natural response of *C. albicans* to cell wall inhibition by caspofungin. We demonstrated that altered PI(4,5)P2-septin distribution occurs within 5 min of caspofungin exposure and therefore represents a rapid response to β-1,3-D-glucan synthase inhibition. In principle, this altered localization might result from various indirect effects of cell wall perturbation.

However, most caspofungin-hypersensitive mutants that have been tested to date do not affect septin localization (6) or, as we showed here, PI(4,5)P2 localization. The specificity and rapid onset of the PI(4,5)P2-septin response lead us to believe that it mediates a particular rather than generalized signal and that it plays a natural role in cell wall dynamics. The response may serve a protective purpose, such as by preventing accumulation of unbalanced cell wall components on the cell surface, which may lead to cell rupture, or by directing cell wall biogenesis to specific sites of need. Alternatively, PI(4,5)P2-septin redistribution may represent an early step in a mechanism of cell killing. In either case, the phenomenon provides an assay for some of the earliest events in caspofungin-treated *C. albicans* cells, which should prove useful in unraveling complex cell wall integrity responses.

The second clinically relevant contribution of the PI(4,5)P2-septin response is to the progression of invasive candidiasis within organs following hematogenous dissemination of *C. albicans*. We previously showed that *IRS4* and *INP51* were expressed within mouse kidneys after injection of *C. albicans* into the lateral tail vein (2, 3). The respective null mutants colonized kidneys as well as wild-type *C. albicans* but thereafter caused lower mortality and tissue burdens and elicited less inflammation. While the mutants initiated hyphal formation at the early stages of invasive disease, they did not form the dense collections of matted hyphae seen with wild-type strains at later time points within the kidneys. Rather, the mutants were largely found as yeast and truncated filaments. Similarly, *C. albicans* septin mutants have been shown to colonize kidneys but not invade the renal parenchyma (55). Since *gin4* mutants exhibit significant growth defects *in vitro*, we did not test the relative virulence of these strains in the mouse model. Nevertheless, it is logical that mutations that diminish cell wall integrity are associated with decreased ability of *C. albicans* to
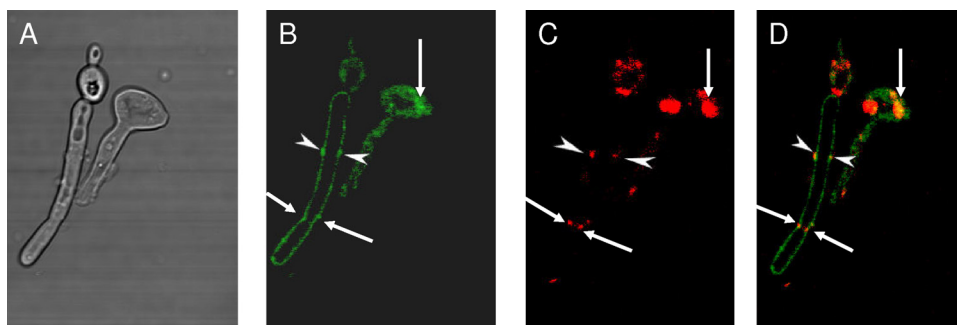
FIG 9 Brief exposure to caspofungin results in redistribution of PI(4,5)P2 and septins to aberrant patches in wild-type *C. albicans* cells. A 5-min exposure to caspofungin at the MIC (0.125 μg/ml) did not kill a wild-type *C. albicans* strain (A) but resulted in redistribution of CaPH-GFP (B) and Cdc10-RFP (C) to aberrant patches (D, overlay image). PI(4,5)P2-septin colocalization was similar to that seen in *irs4*, *inp51*, and *gin4* mutant cells in the absence of cell wall stress.
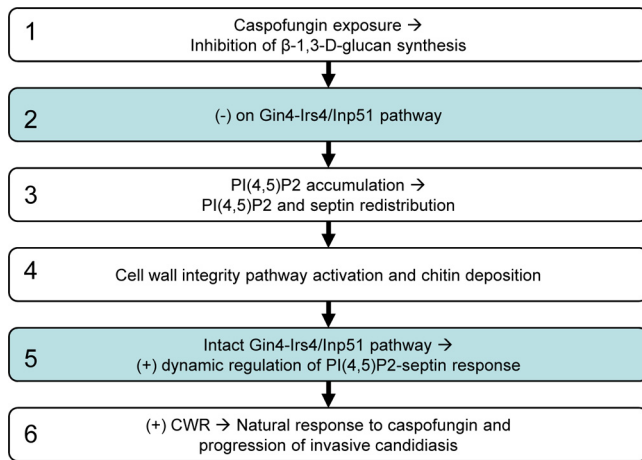
FIG 10 Model for the PI(4,5)P2-septin cell wall regulatory network. Exposure of *C. albicans* to caspofungin inhibits β-1,3-D-glucan synthesis and creates cell wall stress (box 1). Upon sensing this stress, cells rapidly redistribute PI(4,5)P2 and septins (box 3). PI(4,5)P2 accumulation activates the PKC-MAPK cell wall integrity pathway, and PI(4,5)P2 and septins direct the deposit of chitin and other cell wall components at sites of colocalization (box 4). The redistribution of PI(4,5)P2 and septins in response to caspofungin is similar to that observed in *gin4*, *irs4*, and *inp51* mutants, which are hypersusceptible to caspofungin and exhibit highly similar cell wall damage response gene expression profiles. Therefore, the data suggest that (i) Gin4 and the Irs4/Inp51 5′-phosphatase complex function upstream of PI(4,5)P2 and septins in a pathway that relays the cell wall stress signal (box 2), and (ii) an intact Gin4-Irs4/Inp51-PI(4,5)P2-septin pathway is required for the maintenance of cell wall integrity in the face of ongoing caspofungin exposure (box 5). Cell wall regulation (CWR) through the pathway is part of the natural response to caspofungin and, as our earlier mouse data indicated (2), necessary for the progression of invasive candidiasis after the initial stages of tissue colonization (box 6). White and blue boxes represent components of this model that have been established or suggested by our data, respectively.

invade both solid agar and target organs. Taken together, our data indicate that the *C. albicans* PI(4,5)P2-septin network is activated upon both caspofungin exposure and tissue invasion and is necessary for successful long-term adaptation to these cell wall stresses.

Finally, our study offers further evidence that *C. albicans* has more extensive cell wall regulatory circuitry than *S. cerevisiae*, as well as novel cell wall integrity pathways that are relevant to virulence and drug susceptibility. Studies of *S. cerevisiae* have defined biogenesis mechanisms for major classes of cell wall components (24), established the connection between cell polarity and cell wall synthesis (8, 27), developed a detailed mechanistic model of cell wall integrity signaling through the PKC-MAPK cascade (27), and defined genetic and chemical-genetic networks that integrate cell wall integrity into other cell biological processes (14, 24–26). Novel *C. albicans* pathways may include components that do not have clear *S. cerevisiae* orthologues, such as the transcription factor Cas5 (7, 9). Alternatively, *C. albicans* pathways may either be rewired for cell wall regulation or adapted to this purpose as a result of increased "current" through them (6, 14). The role of Gin4, for example, suggests rewiring, in which the protein kinase may be at least partially distinct from its *S. cerevisiae* orthologue in the nature of its target genes and the signals to which it responds. On the other hand, Irs4 and Inp51 may perform comparable basic functions, which are uniquely adapted in *C. albicans* as a result of greater stress upon or flux (i.e., "current") through the pathways

that regulate cell wall integrity. In this regard, our data are the foundation for future mechanistic investigations into cell wall regulatory responses that distinguish *C. albicans* as a versatile opportunistic pathogen and have relevance for both the pathogenesis and treatment of invasive candidiasis.

ACKNOWLEDGMENTS

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