The *Candida albicans* phosphatase Inp51p interacts with the EH domain protein Irs4p, regulates phosphatidylinositol-4,5-bisphosphate levels and influences hyphal formation, the cell integrity pathway and virulence

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We previously identified *Candida albicans* Irs4p as an epidermal growth factor substrate 15 homology (EH) domain-containing protein that is reactive with antibodies in the sera of patients with candidiasis and contributes to cell wall integrity, hyphal formation and virulence. In this study, we use a yeast two-hybrid method and co-immunoprecipitation to show that Irs4p physically interacts with the phosphatase Inp51p. Disruption of the Inp51p Asn-Pro-Phe (NPF) motif eliminates the interaction, suggesting that this motif is targeted by Irs4p. Both *inp51* and *irs4* null mutants exhibit significantly increased levels of phosphatidylinositol-4,5-bisphosphate [PI(4,5)P₂] without changes in levels of other phosphoinositides. Like the *irs4* mutant, the *inp51* mutant demonstrates increased susceptibility to cell wall-active agents, impaired hyphal formation and abnormal chitin distribution along hyphal walls during growth within solid agar. Moreover, the *inp51* and *irs4* mutants overactivate the cell wall integrity pathway as measured by Mkc1p phosphorylation. As anticipated, mortality due to disseminated candidiasis is significantly attenuated among mice infected with the *inp51* mutant, and tissue burdens and inflammation within the kidneys are reduced. Hyphal formation and chitin distribution *in vivo* are also impaired, consistent with observations of embedded growth *in vitro*. All phenotypes exhibited by the *inp51* and *irs4* mutants are rescued by complementation with the respective genes. In conclusion, our findings suggest that Irs4p binds and activates Inp51p to negatively regulate PI(4,5)P₂ levels and the cell integrity pathway, and that PI(4,5)P₂ homeostasis is important for coordinating cell wall integrity, hyphal growth and virulence under conditions of cell wall stress.

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Abbreviations: EH, epidermal-growth factor substrate 15 homology; HA, haemagglutinin; MAP, mitogen-activated protein; PI(4,5)P₂, phosphatidylinositol-4,5-bisphosphate; PI(3)P, phosphatidylinositol-3-phosphate; PI(4)P, phosphatidylinositol-4-phosphate; PI(3,5)P₂, phosphatidylinositol-3,5-bisphosphate; PKC, protein kinase C.

Two supplementary figures, showing expression of the *MEL1* reporter gene and generation of the *inp51* null mutant, are available with the online version of this paper.

INTRODUCTION

The yeast *Candida albicans* is an opportunistic pathogen that causes a wide range of cutaneous, mucosal and systemic diseases in susceptible hosts. In causing diverse diseases, *C. albicans* does not depend upon the expression of a dominant virulence factor (Calderone & Fonzi, 2001). Rather, the pathogenesis of candidiasis requires the coordination of multiple processes in a manner that optimizes proliferation, invasion and tissue damage within a given *in vivo* environment (Mahan *et al.*, 2000; Staib *et al.*, 2000). Several properties of *C. albicans* that contribute to

virulence have been characterized, including niche-specific regulation of central metabolic pathways, adhesion to host cells, secretion of hydrolytic enzymes, iron sequestration, phenotypic switching and morphogenesis (i.e. reversible transitioning between single-cell blastospores and filamentous pseudohyphae or hyphae) (Barelle *et al.*, 2006; Calderone & Fonzi, 2001; Cheng *et al.*, 2007; Fradin *et al.*, 2003; Lorenz *et al.*, 2004; Rubin-Bejerano *et al.*, 2003). Morphogenesis, in particular, has been extensively studied as a determinant of virulence. The disease-causing capacity of mutant *C. albicans* strains that are unable to switch between yeast and hyphal morphologies is generally attenuated (Calderone & Fonzi, 2001; Kumamoto & Vines, 2005). The identification of proteins and protein complexes that might be responsible for coordinating the multiple cellular processes that contribute to efficient morphogenesis has become an active area of research (Brand *et al.*, 2007; Hausauer *et al.*, 2005; Li *et al.*, 2005; Martin & Konopka, 2004; Oberholzer *et al.*, 2006; Walther & Wendland, 2004; Zheng *et al.*, 2003).

In previous reports, we identified *C. albicans* Irs4p as a protein that is reactive with antibodies in the sera of patients with candidiasis (Badrane *et al.*, 2005; Nguyen *et al.*, 2004). We showed that disruption of *IRS4* results in abnormalities of cell wall integrity and chitin distribution, impaired hyphal formation during contact with solid agar and within murine kidneys, and attenuated virulence during disseminated candidiasis. Irs4p consists of 638 amino acids and contains a predicted epidermal growth factor substrate 15 homology (EH) domain. EH domains are highly conserved eukaryotic protein-binding regions that target the amino acid motif Asn-Pro-Phe (NPF) and form the framework of the EH network, an extensive protein interaction network that coordinates pathways regulating cell wall biogenesis and other cellular processes (de Beer *et al.*, 1998; Confalonieri & Di Fiore, 2002; Salcini *et al.*, 1997; Santolini *et al.*, 1999; Tang *et al.*, 2000). *C. albicans* Irs4p is the sole homologue of the duplicated *Saccharomyces cerevisiae* proteins Irs4p and Tax4p, which were recently shown to bind and activate Inp51p, a phosphatidylinositol-(4,5)-bisphosphate [PI(4,5)P₂] 5-phosphatase (Morales-Johansson *et al.*, 2004). Levels of PI(4,5)P₂ are elevated in both *S. cerevisiae* *irs4/tax4* and *inp51* null mutants. These strains, however, do not exhibit readily apparent phenotypes unless they are constructed in the presence of mutations to other phosphatases or disturbances in the cell integrity pathway (Böttcher *et al.*, 2006; Morales-Johansson *et al.*, 2004; Singer-Kruger *et al.*, 1998; Stefan *et al.*, 2002, 2005; Stolz *et al.*, 1998a, b).

Like *S. cerevisiae* INP51, *C. albicans* INP51 encodes a synaptojanin-like protein with a C-terminal 5-phosphatase domain and an N-terminal Sac1-like domain. *C. albicans* Inp51p also possesses an NPF motif, suggesting that it is likely to be targeted by the EH domain of Irs4p. In the present study, we tested the hypothesis that *C. albicans* Inp51p and Irs4p interact and regulate levels of PI(4,5)P₂. In addition, we investigated the effects of INP51 disruption

on cell wall integrity, chitin distribution, hyphal formation and virulence during murine disseminated candidiasis. Finally, we determined whether Inp51p and Irs4p regulate activation of the cell integrity pathway.

METHODS

Strains, media, growth conditions and transformation. *C. albicans* strains used or constructed in this study are described in Table 1 (Badrane *et al.*, 2005; Fonzi & Irwin, 1993; Gillum *et al.*, 1984). The *inp51* null mutant strains were created using the SAT1 flipper method (details below), and *C. albicans* SC5314 served as reference wild-type strain. The *irs4* null mutant had been created previously using the ura-blaster method (Badrane *et al.*, 2005), and *C. albicans* CAI-12 was used as reference wild-type strain. All strains were routinely grown in yeast peptone dextrose (YPD) liquid medium (1 % yeast extract, 1 % bacto-peptone, 2 % α[D]-glucose), or on YPD agar or Sabouraud dextrose agar (SDA) at 30 °C. To induce hyphal formation in liquid media, *C. albicans* strains grown overnight on YPD were subcultured into liquid YPD supplemented with 5 or 10 % fetal calf serum (FCS) or liquid RPMI-1640 (Sigma-Aldrich) at 37 °C. To induce hyphal formation on solid media, overnight-grown *C. albicans* were spotted on Spider medium, Medium 199 (M-199) (Gibco-BRL, adjusted to pH 7.5), modified Lee's, and YPD medium supplemented with 5 % FCS and grown at 37 °C. To evaluate growth under embedded conditions, ~100 *C. albicans* cells subcultured at early exponential phase were mixed into 20 ml molten YPD, YPD + 5 % FCS, YPD-reverse [BASF pluronic polyol F-127, kindly provided by the Fungal genetics Stock Center (FGSC); www.fgsc.net], Spider or M-199 agar, and incubated for 3 days at 30 or 37 °C. Reverse agar is a lock polymer of polyoxypropylene and polyoxyethylene that can be used as a replacement for conventional agar in solid media. For targeted homologous recombination in *C. albicans*, we used either transformation by electroporation (Reuss *et al.*, 2004) or the lithium/caesium acetate protocol provided with the Alkali-Cation Yeast kit (QBiogene).

Yeast two-hybrid system and co-immunoprecipitation. *C. albicans* IRS4 was subcloned into pAlter-1 for use with the Altered Sites II *in vitro* mutagenesis system (Promega). We substituted five CTG codons with TCT for correct translation in *S. cerevisiae*. These CTG codons encode serine residues at positions 101, 125, 361, 414 and 429. *C. albicans* INP51 has only one CTG codon (encoding a serine residue at position 856), which we did not substitute. The Matchmaker two-hybrid system 3 (Clontech) was used to test the interaction between Irs4p and Inp51p in *S. cerevisiae*, as per the manufacturer's instructions. Briefly, *C. albicans* IRS4 and *C. albicans* INP51 were subcloned in pGBKT7 and pGADT7-Rec, respectively, and plasmids were extracted and co-transformed, using the lithium acetate protocol, in strain AH109 (Trp⁻, Leu⁻, Ade⁻, His⁻). Transformants were plated in SDA medium without Trp, Leu, Ade and His. The interaction was confirmed using the Profound c-myc tag IP/Co-IP kit (Pierce) after co-transcription/translation using TnT T7 Quick for PCR DNA (Promega). Primers were designed to introduce optimal signals for *in vitro* transcription/translation and to amplify IRS4 and INP51 tagged with c-Myc and haemagglutinin (HA), respectively. TnT T7 was used as per manufacturer recommendations along with EasyTag L-[³⁵S]methionine (Perkin Elmer) to generate labelled Irs4p-c-Myc and Inp51p-HA. Since expression of the full-length INP51 resulted in low yields, a C-terminal fragment encompassing the last 475 residues of Inp51p was amplified and tagged with HA (Inp51p-C-HA). Samples (10 µl) of each of the labelled products were mixed and incubated at room temperature with gentle shaking for 1 h; then, 10 µl of anti-c-Myc or anti-HA antibody-coupled agarose was added to the mixture and incubated at

Table 1. Strains and plasmids used in this study

Host or parental strain	Name	Description	Source
<i>C. albicans</i> strains			
SC5314	SC5314	Wild type	Gillum <i>et al.</i> (1984)
SC5314	CAI-4	ura3 Δ :: λ imm434/ura3 Δ :: λ imm434	Fonzi & Irwin (1993)
CAI-4	CAI-12	ura3 Δ :: λ imm434/URA3	Fonzi & Irwin (1993)
CAI-4	irs4_2KO	<i>irs4</i> mutant, <i>irs4</i> Δ / <i>irs4</i> Δ	Badrane <i>et al.</i> (2005)
CAI-4	2KO_Re-IRS4	Complemented, <i>irs4</i> Δ /IRS4	Badrane <i>et al.</i> (2005)
SC5314	inp51_2KO	<i>inp51</i> mutant, <i>inp51</i> Δ / <i>inp51</i> Δ	This work
SC5314	2KO_Re-INP51	Complemented, <i>inp51</i> Δ /INP51	This work
<i>S. cerevisiae</i> strains			
PJ69-2A	AH109	MATa, trp1-901, leu2-3, 112, ura3-52, his3-200, gal80 Δ , LYS2::GAL1UAS-GAL1TATA-HIS3, GAL2UAS-GAL2TATA-ADE2, gal4 Δ , URA3::MEL1UAS-MEL1TATA-lacZ, MEL1	BD Matchmaker (Clontech)
Plasmids			
NovaBlue	pGBKT7_IRS4	<i>In vivo/in vitro</i> expression cassette for <i>IRS4</i>	This work
NovaBlue	pGADT7-Rec_INP51	<i>In vivo/in vitro</i> expression cassette for <i>INP51</i>	This work
NovaBlue	pSFS-2A_INP51-F1-F2	<i>INP51</i> disruption cassette	This work
NovaBlue	pSFS-2A_INP51-Full1 kb-F2	<i>INP51</i> complementation cassette	This work
NovaBlue	pSFS-2A_INP51-1.7-F2	<i>INP51</i> 5-phosphatase domain deletion cassette	This work

4 °C for 2–14 h, after which the reaction was passed through the column and washed three times with Tris-buffered saline supplemented with 0.05 % Tween-20 (TBS-T). Retained proteins were eluted with 25 μ l 2 $\times$ non-reducing sample buffer heated at 95–100 °C for 5 min and pulse-centrifuged at 20 000 *g* for 10 s. A 2 μ l volume of 2-mercaptoethanol was added to samples for SDS-PAGE. After the SDS-PAGE gel was run, it was fixed, rinsed and dried on a Whatman paper, then exposed to an X-ray film overnight at room temperature.

Gene disruption. The SAT1 flipper tool was used for *INP51* gene disruption (Reuss *et al.*, 2004). Briefly, fragments at the 5' (F1) and 3' (F2) ends of the targeted gene were amplified by PCR using primers that introduced appropriate restriction sites. These fragments were subcloned into pSFS-2A between *Kpn*I and *Xho*I, and *Sac*II and *Sac*I sites, respectively. In parallel, the full gene with 1 kb of downstream non-coding sequence was amplified and used as F1 for gene reinsertion. The resulting plasmid was extracted and linearized with *Kpn*I and *Sac*I, and introduced into competent *C. albicans* cells by electroporation. Transformants were grown on selective medium plates containing 200 μ g streptothricin ml⁻¹ (Alexis Biochemicals) for 2–3 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 3–4 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.

Myo-[2-³H]inositol labelling, extraction of inositol lipids, and HPLC of deacylated lipids. For phosphoinositide analysis, we followed a procedure reported elsewhere (Dove *et al.*, 1997). For labelling of inositols, *C. albicans* mid-exponential cultures were diluted to 5 $\times$ 10⁴ cells ml⁻¹ in inositol-free SD medium supplemented with 10 μ Ci (370 kBq) myo-[2-³H]inositol ml⁻¹ (American Radiolabelled Chemicals) and grown to 2–3 $\times$ 10⁶ cells ml⁻¹. Cells were harvested after addition of methanol to 50 %. Lipids were extracted by vortexing in the presence of glass beads and 100 %

methanol, followed by twice adding a mixture of chloroform, methanol and HCl-tetrabutylammonium hydrogen sulfate to recover the lower phases, and drying in a speed vacuum. Dried lipids were deacylated by vigorous vortexing with methylamine reagent, incubated at 53 °C for 30 min then dried again. Samples were resuspended in H₂O, extracted twice with butanol/petroleum-ether/ethyl formate, and dried and suspended in 0.1 mM EDTA by vigorous vortexing. Samples were processed by HPLC, either immediately or after a few days storage at -70 °C. The sample analysis was performed using an HPLC system consisting of a quaternary pump (Series 200, Perkin Elmer) and a manual injector with 5 ml stainless steel loop (Rheodyne). Chromatographic separation was achieved on a Partisphere SAX column (5 μ m, 4.6 mm $\times$ 125 mm, Whatman) using a gradient of distilled water (solvent A) and 1.25 M NaH₂PO₄ (solvent B) at a flow rate of 1 ml min⁻¹. The buffer was filtered through a 0.22 μ m filter (Millipore) and de-gassed in a sonicator (Fisher Scientific) for 15 min before use. The dried samples were reconstituted in 500 μ l buffer, vortex-mixed for 1 min, and 1 ml distilled water was added to make up the volume to 1.5 ml. The sample was injected onto the HPLC. The gradient was run and fractions were collected at 0.5 min intervals up to 86 min in scintillation vials (Fisher Scientific). Each fraction was added to 2 ml Packard Ultima Flo AP (Perkin Elmer) liquid scintillation cocktail and mixed gently. The radioactivity in samples was determined on a scintillation counter (Beckman LS 6500, Beckman Coulter). Each strain was tested twice and phosphoinositide levels were normalized to the total signal.

Sensitivity to cell wall agents

Each of the following experiments was performed in triplicate.

SDS and calcofluor white. Cultures from overnight grown cells were subcultured in YPD liquid medium with 1 % glucose until the exponential phase and diluted to OD₅₉₉ 0.1. Samples (4 μ l) of undiluted and serial 10-fold dilutions of each culture were spotted onto YPD plates containing calcofluor white (40 μ g ml⁻¹) or SDS (0.02 %). The plates were incubated at 30 °C for 72 h.

Zymolyase. Exponentially grown *C. albicans* cells at OD₅₉₉ ~0.8 were incubated with 100 µg zymolyase 20T ml⁻¹ (Sigma-Aldrich) in 10 ml Tris-HCl, pH 7.5. An aliquot was removed at timed intervals and the OD₅₉₉ was measured. The OD₅₉₉ was plotted against time of incubation.

Caspofungin. Sensitivity to caspofungin (Merck Research Laboratories) was measured in a 48-well microtitre plate. Organisms grown overnight in YPD at 30 °C were diluted to OD₆₀₀ = 0.1 in YPD. Caspofungin was added at concentrations ranging from 0.075 to 20 µg ml⁻¹ and transferred to the microtitre plate (800 µl per well). The plate was incubated at 30 °C with shaking at 250 r.p.m. and OD₆₂₀ was measured every hour.

Staining of chitin. *C. albicans* cells were grown either as a suspension in YPD liquid culture to exponential phase or embedded in solid medium for 3 days. Cells were washed three times for staining and imaging. For chitin staining, cells were incubated for 5 min at room temperature in 0.1 mg calcofluor white ml⁻¹, and then washed three times in cold PBS (1 ×) (3.2 mM Na₂HPO₄, 0.5 mM KH₂PO₄, 1.3 mM KCl, 135 mM NaCl, pH 7.3). Slides were viewed in a Zeiss AxioKop2 microscope equipped for epifluorescence, using the appropriate filter for each fluorescent compound, and images were digitized with a camera connected to Epilab software, at ×1000 magnification.

Murine disseminated candidiasis and histopathology. Groups of 10–12 seven-week-old male ICR mice (Harlan-Sprague) were inoculated by intravenous injection of the lateral tail vein with 1 × 10⁶ c.f.u. *C. albicans*. For mortality studies, mice were followed until they were moribund, at which point they were sacrificed, or for 42 days. Survival curves were calculated according to the Kaplan–Meier method using the PRISM program (GraphPad Software) and compared using Newman Keuls analysis; a *P* value of <0.05 was considered significant. For tissue burden, mice were infected as above with 5 × 10⁵ c.f.u. Mice (6–8 per group per time point) were sacrificed 24 h and 4 days post-inoculation, and their kidneys were aseptically removed. The kidneys were weighed, homogenized in 2 ml sterile PBS (1 ×) (3.2 mM Na₂HPO₄, 0.5 mM KH₂PO₄, 1.3 mM KCl, 135 mM NaCl, pH 7.3), and serial dilutions were plated on SDA plates containing piperacillin (60 µg ml⁻¹) and amikacin (60 µg ml⁻¹). The plates were incubated at 30 °C for 48 h, after which the number of c.f.u. was determined. Values were expressed as log(c.f.u. per gram kidney). The differences in kidney burden between strains were determined by the Wilcoxon rank sum test; a *P* value <0.05 was determined to be statistically significant.

For staining of *C. albicans* cells recovered from murine kidneys, aliquots from homogenized kidneys were fixed in formalin, washed in cold water and stained with calcofluor white as described above. Samples were visualized under a fluorescence microscope to search for *C. albicans* hyphae. Histopathology was performed by a pathologist blinded to the experimental design. Kidneys were collected 4 days post-inoculation, and fixed with formalin and embedded in paraffin, after which thin sections were prepared and stained with periodic acid–Schiff (Churukian *et al.*, 1986; Churukian & Schenk, 1977). For each strain, kidneys from three mice were chosen for image analysis. TIFF images were captured of all the tissue on each of the slides. The images were analysed on a Windows XP PC using the public domain National Institutes of Health (NIH) image program Image J (<http://rsb.info.nih.gov/nih-image/>) developed at the NIH. For each image, outline splines were traced around the total area(s) of tissues, and another series of outline splines was traced around the area(s) involved in acute inflammation. At least 20 images for each kidney were analysed. The percentage of the total area with the inflammation was calculated and expressed as mean ± SD. Values were compared by *t* test.

Protein extraction and Western blotting. *C. albicans* cells were grown to mid-exponential phase in YPD medium, then harvested and centrifuged at 4 °C, and processed as previously described (Navarro-Garcia *et al.*, 1998). Protein concentration was determined using the Bio-Rad Protein Assay and equal amounts of proteins (usually 150–200 µg per lane) were loaded. After SDS-PAGE migration and transfer to a nitrocellulose membrane, Mkc1p was detected with either an anti-Mkc1p (kindly provided by Jesús Pla, Universidad Complutense de Madrid) or the anti-phospho-p44/42 MAP kinase (Thr202/Tyr204) antibody (Cell Signaling Technology).

RESULTS

C. albicans Irs4p and Inp51p physically interact

Our first objective was to determine whether *C. albicans* Inp51p interacts with Irs4p. As an initial step, we adapted a yeast two-hybrid system (Matchmaker two-hybrid system 3; Clontech), in which the Inp51p (bait) and Irs4p (prey) interaction would activate expression of *ADE2*, *HIS3* and *MEL1* (α -galactosidase) reporters in *S. cerevisiae* strain AH109. Such experiments are complicated by *C. albicans* non-canonical codon usage, in which CTG codons encode serine instead of leucine. To account for this, we substituted five CTG codons in *C. albicans* *IRS4* with TCT to match universal codon usage. *C. albicans* *INP51* and the modified *IRS4* were subcloned into pGBKT7-Rec (binding domain vector, expressing *TRP1* as a selection marker) and pGADT7 (activation domain vector, expressing *LEU2*), respectively. Co-transformation of *S. cerevisiae* AH109 (auxotroph for Trp and Leu) with both plasmids yielded viable transformants on SDA (Trp⁻, Leu⁻, Ade⁻, His⁻) medium, as did the positive control plasmids carrying SV40 large T antigen-HA and murine p53-c-Myc. In this system, the adenine and histidine nutritional selection markers add a level of stringency that minimizes false positives, as *S. cerevisiae* AH109 transformants containing both pGBKT7-Rec and pGADT7 vectors will only grow on SDA (Trp⁻, Leu⁻, Ade⁻, His⁻) medium if the tested proteins interact physically. Moreover, the transformants were positive for α -galactosidase activity, as indicated by the presence of blue colonies in the presence of X- α -gal. As negative control, co-transformation of AH109 with pGADT7 carrying the modified *IRS4* and pGBKT7-Rec carrying *INP51* in the reverse orientation did not yield transformants on SDA (Trp⁻, Leu⁻, Ade⁻, His⁻) medium, since the *ADE2* and *HIS3* reporters were not activated. As anticipated, the negative control was able to grow on SDA (Trp⁻, Leu⁻) medium. Upon the addition of X- α -gal to this medium, the large T antigen/p53 positive control and Inp51/Irs4 co-transformants clearly grew as blue colonies, whereas the negative control co-transformants did not (see Supplementary Fig. S1).

To verify the Inp51p–Irs4p interaction that was suggested by the yeast two-hybrid results, we performed co-immunoprecipitation experiments following co-transcription/translation. We initially amplified complete ORF sequences of *IRS4* and *INP51* from their plasmids so that

the resulting fragments encompassed optimal signal sequences for *in vitro* transcription and translation, as well as c-Myc and HA tags, respectively. Expression of the full-length *INP51*, however, resulted in low yields. For this reason, the C-terminal fragment encompassing the last 475 residues of Inp51p (Inp51p-C) was then amplified, and sufficient yield was obtained after *in vitro* expression (Fig. 1a). The *in vitro* expression products were mixed, and both Irs4p-c-Myc and Inp51p-C-HA were immunoprecipitated using either anti-c-Myc or anti-HA antibodies (Fig. 1b). As positive controls, SV40 large T antigen-HA and murine p53-c-Myc were co-immunoprecipitated using either of the two antibodies, consistent with the anticipated interaction between these proteins. As negative controls, Irs4p-c-Myc was mixed with SV40 large T antigen-HA and Inp51p-C-HA with p53 c-Myc. Upon immunoprecipitating the first mixture with anti-HA, only large T antigen was pulled down (Fig. 1b). Likewise, only p53 was pulled down when the second mixture was immunoprecipitated with anti-c-Myc.

The predicted sequence of *C. albicans* Inp51p reveals a C-terminal NPF motif at residue 965. To determine whether the region of Inp51p that includes the NPF motif is responsible for the interaction with Irs4p, we expressed Inp51p-C- Δ NPF-HA, which has a deletion of the last 22 amino acids including the NPF motif. This truncated version of Inp51p-C no longer showed the interaction with Irs4p-c-Myc (Fig. 1b). Sufficient expression of Inp51p-C- Δ NPF-HA was confirmed by immunoprecipitation with anti-HA antibody (Fig. 1b).

Irs4p and Inp51p negatively regulate levels of PI(4,5)P₂

To determine whether *C. albicans* Inp51p and Irs4p regulate PI(4,5)P₂, we measured intracellular phosphoinositide levels for isogenic null mutant and complemented strains using HPLC (Dove *et al.*, 1997). In our previous study, using the ura-blaster method, we created an *irs4* null mutant strain, which was then complemented with the full

Fig. 1. *C. albicans* Irs4p and Inp51p interact physically. (a) *IRS4* and *INP51-C* (C-terminal fragment) were cloned into vectors conferring T7 modules for *in vitro* expression in addition to c-Myc and HA tags, respectively. Cassettes were PCR-amplified and expressed *in vitro* using the co-transcription/translation TnT system in the presence of [³⁵S]methionine (see Methods), and separated by SDS-PAGE. SV40 large T antigen HA and murine p53 c-Myc genes encode interacting proteins that were used as a positive control in co-immunoprecipitation experiments. (b) Products of *in vitro* expression from candidate genes for interaction were mixed, then immunoprecipitated with anti-c-Myc (lanes 1, 3, 5 and 7) or anti-HA (lanes 2, 4, 6 and 8) antibodies. Irs4p-c-Myc and Inp51p-C-HA were co-immunoprecipitated by either of the two antibodies, consistent with a physical interaction between them (lanes 1 and 2). The physical interaction was no longer apparent when Irs4p was mixed with Inp51p-C- Δ NPF-HA, a truncated version of Inp51p-C-HA in which 22 amino acids (including the NPF motif) at the C terminus were removed (lanes 3 and 4). As negative controls, Inp51p-C-HA was mixed with p53-c-Myc and Irs4p-c-Myc was mixed with large T antigen HA. As anticipated, anti-c-Myc immunoprecipitated only p53-c-Myc and anti-HA immunoprecipitated only large T antigen (lanes 5 and 6). As positive control, large T antigen HA and p53-c-Myc were co-immunoprecipitated using either of the two antibodies (lanes 7 and 8).

IRS4 ORF (Badrane *et al.*, 2005). In this study, we used the SAT1 flipper technique for targeted homologous recombination to successively knock out both alleles of *INP51*, creating the null mutant strain *inp51_2KO*. The full gene with 1 kb downstream non-coding DNA was then reinserted in the null mutant background (creating strain 2KO_Re-*INP51*), and all recombination events were verified using Southern blotting (see Supplementary Fig. S2). For both genes, the null mutant and complemented strains showed growth rates that were indistinguishable from those of wild-type strains when cultured in YPD media.

We were able to detect four different phosphoinositides in all strains: phosphatidylinositol 3-phosphate (PI3P), phosphatidylinositol 4-phosphate (PI4P), phosphatidylinositol 3,5-bisphosphate [PI(3,5)P₂], and PI(4,5)P₂. Under routine

growth conditions, PI4P was the major peak (7.1 % of total signal), followed by PI(4,5)P₂ (4.9 %), PI3P (2.7 %) and PI(3,5)P₂ (<0.1 %). The number and relative distribution of phosphoinositides was similar to that of *S. cerevisiae*, with significantly lower levels of PI(3,5)P₂ than the others (Bonangelino *et al.*, 2002; Morales-Johansson *et al.*, 2004). In both *C. albicans inp51* and *irs4* null mutant strains, we observed a significant increase in PI(4,5)P₂ levels to approximately twice the levels of wild-type strains (Fig. 2a). Levels of PI(4,5)P₂ were re-established to wild-type levels in the *irs4* null mutant complemented with one copy of *IRS4*. The complementation was partial upon reintroduction of one copy of *INP51* to the *inp51* null mutant, indicating a possible dosage effect. The null mutants did not show any significant changes in the three other detected phosphoinositides (Fig. 2b).

Fig. 2. Disruption of *IRS4* or *INP51* increases intracellular levels of PI(4,5)P₂. Cells were grown at 30 °C in the presence of myo-[2-³H]inositol, lipids were extracted, deacylated and separated by HPLC, and relative levels of phosphoinositides were quantified. CAI-12, *irs4* and Re-*IRS4* are wild-type, null mutant (*irs4Δ/irs4Δ*) and complemented strains (*irs4Δ/IRS4*) for *IRS4*. SC5314, *inp51* and Re-*INP51* are wild-type, null mutant (*inp51Δ/ inp51Δ*) and complemented strains (*inp51Δ/INP51*) for *INP51*. (a) The y axis represents PI(4,5)P₂ levels (³H c.p.m.) and the x axis shows the respective strains. Asterisks indicate that PI(4,5)P₂ levels of both *irs4* and *inp51* null mutants were significantly higher than those of corresponding wild-type strains ($P < 0.01$, *t* test). The PI(4,5)P₂ levels of the *IRS4*-complemented strain are similar to those of CAI-12. The *INP51*-complemented strain, on the other hand, showed levels that were significantly lower than those of the null mutant but higher than those of SC5314 ($P < 0.05$). (b) Disruption of *IRS4* or *INP51* does not affect intracellular levels of other phosphoinositides.

Disruption of *INP51* leads to cell wall defects, impaired contact-induced hyphal formation and abnormal chitin distribution

We next assessed the effects of *INP51* disruption on cell wall integrity, hyphal formation and chitin distribution. The *inp51* null mutant strain (*inp51_2KO*) was more susceptible to the cell wall-active agents calcofluor white, SDS, zymolyase and caspofungin than the wild-type (*C. albicans* SC5413) and complemented strains (data not shown). When compared with the wild-type strain during growth on the surface of solid agar or embedded within agar, the null mutant was impaired in hyphal formation. The ability to form hyphae was restored upon complementation with one copy of *INP51* (*2KO_Rei-*INP51**) (Fig. 3). Like the *irs4* null mutant, the *inp51* mutant was indistinguishable from strain SC5314 when grown under hyphal-inducing conditions in liquid media (5 and 10% FCS, data not shown). Finally, calcofluor white staining of *inp51* mutant cells grown under embedded conditions revealed abnormal distribution of chitin along the hyphal walls (Fig. 4). No defects in chitin distribution were detected in yeast, hyphal or pseudohyphal cells among our strains when grown in YPD liquid media at 30 or 37 °C (data not shown). For each of the phenotypes tested, the effects of disrupting *INP51* were identical to those previously observed with *IRS4* disruption (Badrane *et al.*, 2005). Moreover, similar results were obtained using

Fig. 3. Disruption of *INP51* results in repressed hyphal formation during growth in physical contact with a matrix. Cultures of *C. albicans* strains were spotted on Lee's medium and grown at 30 °C for 4–5 days. Three representative sets of figures show that *inp51* null mutant strains (*inp51_2KO*) are impaired in hyphal formation in comparison with the wild-type strain (*C. albicans* SC5413) or complemented strains (*2KO_Rei-*INP51**). Similar results were obtained when the strains were embedded within reverse agar and grown for 2–3 days at 30 °C (data not shown).

independently created *inp51* null mutant and complemented strains.

INP51 contributes to the pathogenesis of disseminated candidiasis

We assessed the contribution of *INP51* to the pathogenesis of disseminated candidiasis by studying multiple end points in intravenously infected mice. First, we infected groups of 8–12 ICR mice via the lateral tail vein with 1×10^6 c.f.u. per mouse and followed survival over 42 days. As expected, SC5314 killed all mice by 7 days (Fig. 5). In contrast, null mutant strains (*inp51_2KO*) killed only one-third of mice by the end of the study ($P < 0.001$). The complemented strain killed all mice within 8 days ($P < 0.001$ versus null mutant).

Next, we quantified viable *C. albicans* within the kidneys of non-moribund mice 24 h and 4 days after intravenous inoculation of 5×10^5 c.f.u. per mouse (groups of 6–8 mice per time point). At both time points, the kidneys of mice infected with wild-type and complemented strains exhibited significantly higher *C. albicans* concentrations than those of mice infected with the null mutant strain (Table 2).

Finally, we performed histopathological evaluations on the kidneys recovered after day 4 from three mice infected with each isolate. The extent of inflammation was significantly greater for the kidneys of mice infected with the wild-type strain compared with the null mutant, as measured on thin section images by a pathologist blinded to the experimental design (Table 2). The complemented strain yielded intermediate results. Extended filamentous morphologies of both wild-type and complemented strains were clearly evident within areas of acute inflammation (Fig. 6). The null mutant, on the other hand, was difficult to locate within areas of inflammation, and filamentous forms were rarely identified. The null mutant also demonstrated abnormal chitin distribution along the hyphal walls upon calcofluor white staining of cells recovered from infected kidneys, consistent with the findings during *in vitro* embedded growth (Fig. 7).

The cell integrity pathway is overactivated in *irs4* and *inp51* mutants

The *C. albicans* cell integrity pathway was recently shown to be necessary for hyphal formation during contact with solid agar but not in liquid media (Kumamoto, 2005). The regulation of PI(4,5)P₂ levels by *S. cerevisiae* Irs4p–Inp51p potentially plays a role in repressing the cell integrity pathway, but only in the presence of mutations to other negative regulators of the pathway (Bickle *et al.*, 1998; Morales-Johansson *et al.*, 2004). To determine if the cell integrity pathway was affected in our *irs4* and *inp51* mutant strains, we assayed the phosphorylation of Mkc1p, a MAP kinase that is phosphorylated upon activation of the pathway. Under conditions in which the pathway is known to be inactive in wild-type cells (growth as yeast in liquid

Fig. 4. Disruption of *INP51* results in an abnormal distribution of chitin in the *C. albicans* cell wall. Calcofluor white staining of wild-type (SC5314) and the complemented (2KO_Rei-INP51) strains during embedded growth reveals a normal distribution of chitin along the cell wall and at septae. In the *inp51* null mutant, on the other hand, there are numerous abnormal accumulations of chitin along the hyphal cell wall (indicated by arrowheads).

YPD medium at 30 °C) (Navarro-Garcia *et al.*, 1998), it was also inactive in the mutant strains (Fig. 8a). As

Fig. 5. Disruption of *INP51* attenuates mortality during murine disseminated candidiasis. Mice infected via the lateral tail vein with the *inp51* null mutant (INP51_2KO) strain survived significantly longer than mice infected with wild-type (SC5413) or complemented (2KO_Rei-INP51) strains ($P < 0.001$).

anticipated, the pathway was activated in wild-type strains during embedded growth and in the presence of 10 mM H₂O₂ (Kumamoto, 2005; Navarro-Garcia *et al.*, 1998). We also demonstrated activation of the pathway in the wild-type strain grown under hyphae-inducing conditions in liquid media (YPD + 10 % FCS, 37 °C). Under each of the activating conditions, excess phosphorylated Mkc1p accumulated in both mutant strains, consistent with pathway overactivation (Fig. 8a, b). This overactivation was greater in the *irs4* mutant than in the *inp51* mutant. It is of note that the expression of Mkc1p did not differ between wild-type and mutant strains for any of the conditions.

DISCUSSION

In the present study, we demonstrated that *C. albicans* Irs4p and Inp51p physically interact and regulate PI(4,5)P₂ homeostasis. Targeted disruption of either *IRS4* or *INP51* resulted in significantly increased levels of PI(4,5)P₂ but no changes in PI3P, PI4P or PI(3,5)P₂ levels, consistent with results reported for the corresponding *S. cerevisiae* null mutants (Morales-Johansson *et al.*, 2004). At the same time, *C. albicans* *irs4* and *inp51* null mutant strains exhibited phenotypes that have not been reported in *S. cerevisiae*, including impaired contact-induced hyphal formation, increased sensitivity to cell wall-active drugs and attenuated virulence. Moreover, the cell integrity

Table 2. Tissue burdens and areas of acute inflammation within murine kidneys

Strain	Tissue burdens [log(c.f.u. per gram kidney)]		Inflammation (percentage area)*
	24 h	4 days	
Wild type	4.97 ± 0.41	5.52 ± 0.41	31.0 ± 8.1
<i>inp51</i> mutant	4.16 ± 0.33†	4.22 ± 0.29‡	2.8 ± 0.7§
Complemented strain	5.08 ± 0.42	5.13 ± 0.23	11.0 ± 7.1

*The percentage of the total area with the inflammation expressed as mean ± SD.

† $P=0.001$ versus both wild-type and complemented strain.

‡ $P<0.0001$ and $=0.001$ versus wild-type and complemented strain, respectively.

§ $P=0.03$ and 0.09 versus wild-type and complemented strain, respectively.

pathway was overactivated in the *C. albicans* *irs4* and *inp51* mutants in response to stress and hyphal-inducing stimuli, whereas the *S. cerevisiae* mutants only demonstrate such overactivation in the presence of mutations to other negative regulators of the pathway (Morales-Johansson *et al.*, 2004). The abnormal phenotypes of the *C. albicans* null mutants were reversed upon reintroduction of the respective genes and the accompanying reductions in PI(4,5)P₂ levels. Taken together, our findings suggest that the interacting *C. albicans* proteins Irs4p and Inp51p are important in coordinating cellular processes that contribute to invasive growth and virulence under conditions of cell wall tension, such as those encountered within solid agar or during tissue invasion.

It is likely that Irs4p and Inp51p mediate their cellular effects, at least in part, through negative regulation of PI(4,5)P₂ levels. PI(4,5)P₂ is one of several phosphoinosi-

tides that play structural roles in membrane biogenesis and have emerged in the past decade as membrane sensors and second messengers (Daum *et al.*, 1998). PI(4,5)P₂ and other phosphoinositides are synthesized in different cell compartments by phosphatidylinositol kinases and phosphatases, and they have the ability to interact with specific domains of target proteins (Sprong *et al.*, 2001). Because phosphoinositides are rapidly modified by headgroup phosphorylation–dephosphorylation, they can transiently and locally activate or deactivate signalling pathways. In so doing, they coordinate diverse processes, including cell wall organization, cell signalling, cytoskeleton regulation, and endocytic and exocytic trafficking (Martin, 1998; Lemmon, 2003). It is of note that efficient hyphal formation depends upon the careful coordination of these cellular processes. As such, dysregulation of PI(4,5)P₂ is consistent with cell wall abnormalities, impaired hyphal formation and attenuated virulence, as seen in our mutants. In interpreting our

Fig. 6. The *inp51* null mutant causes significantly less disease within the kidneys of mice during disseminated candidiasis than the complemented or wild-type strains. Mice infected with the respective strains were sacrificed on the fourth day of disseminated candidiasis and kidneys were harvested. (a) The null mutant was extremely rare to absent within areas of inflammation. Rare filamentous forms of the null mutant were identified. The arrow points to a yeast (appearing red) within an area of inflammation (periodic acid–Schiff; ×400 magnification). (b) The complemented strain was evident as clumps of extended filaments (appearing red) within widespread areas of acute inflammation in both tubules and glomeruli (arrow) (periodic acid–Schiff stain; ×400 magnification). Filamentous forms were abundant (arrow). Similar results were obtained with strain SC5314 (not shown). Bars, 60 μm.

Fig. 7. The abnormal chitin distribution is also expressed *in vivo*. Mice were infected via the lateral tail vein with either wild-type or *inp51* null mutant (INP51_2KO) strains, and sacrificed at day 4 post-infection. They were dissected, kidneys were harvested and homogenized in saline solution, and an aliquot was stored in formalin. Samples were washed in cold water, stained with calcofluor white, washed again and visualized under a fluorescence microscope to search for *C. albicans* hyphae. In addition to the expected staining of the hyphal cell walls and septae, the null mutant hyphae exhibit abnormal staining with calcofluor white similar to findings *in vitro*. Magnifications of a representative calcofluor white-stained hypha of the wild-type and mutant strains are shown to the right, and abnormal chitin depositions in the mutant are indicated by the white arrowheads. Bars, 10 μ m.

results, it is important to recognize that global cellular levels of phosphoinositides, as measured in this study, might be less relevant to the observed phenotypes than local subcellular derangements.

Our data support a model in which Irs4p binds the NPF motif of Inp51p. We used a yeast two-hybrid system that we adapted to account for *C. albicans* non-canonical codon usage to demonstrate a likely interaction between full-length Irs4p and Inp51p. We then confirmed our findings by co-immunoprecipitating Irs4p (Irs4p-c-Myc) and the C terminus of Inp51p (Inp51p-C-HA). Deletion of the NPF motif from Inp51p eliminated the interaction, as Inp51p-C- Δ NPF-HA failed to co-immunoprecipitate with Irs4p-c-Myc. It is of note that Irs4p contains an EH domain between residues 532 and 627, which is a highly conserved eukaryotic signalling module that binds NPF motifs (de Beer *et al.*, 1998; Confalonieri & Di Fiore, 2002; Salcini

et al., 1997; Santolini *et al.*, 1999; Tang *et al.*, 2000). In future studies, we will use site-directed mutagenesis to conclusively demonstrate that the Irs4p–Inp51p interaction is mediated by the EH domain and NPF motif, respectively, and that the interaction is directly responsible for the regulation of PI(4,5)P₂ levels.

The attenuation of virulence seen with the disruption of *INP51* was evident from multiple end points during intravenously disseminated candidiasis in mice, including mortality, tissue burdens and inflammation. Interestingly, the wild-type *C. albicans* strain caused significantly higher burdens of infection and larger areas of inflammation than the null mutant within the kidneys after 4 days of disseminated candidiasis, whereas the complemented strain showed trends toward intermediate results. These observations were consistent with the relative levels of PI(4,5)P₂ for the strains, suggesting a possible dose effect on

Fig. 8. Mkc1p is hyperphosphorylated in *irs4* and *inp51* mutants under conditions of cell integrity pathway activation. Western blots were performed as described in Methods, using anti-phospho-p44/42 MAP kinase to detect the phosphorylated form of Mkc1p (top rows of a and b). Mkc1p was detected using anti-Mkc1p (bottom rows of a and b). Experimental conditions and strains are indicated in the figure. For each of the experimental conditions, expression of Mkc1p did not differ significantly between the strains. (a) Control conditions in which the cell integrity pathway is known to be inactive (yeast growth in YPD liquid medium at 30 °C) or activated (hyphal induction in YPD supplemented with 10% FCS at 37 °C, to which 10 mM H₂O₂ is added for 10 min) were tested. In both wild-type and mutant strains (*irs4* and *inp51*), phosphorylated Mkc1p is undetectable during growth at 30 °C. Upon activation of the cell integrity pathway, Mkc1p in the mutant strains is hyperphosphorylated compared with the wild type. (b) Mkc1p in the wild-type strain is phosphorylated during hyphal growth in liquid medium (YPD supplemented with 10% FCS at 37 °C) and embedded in solid agar. Mkc1p in mutant strains is clearly hyperphosphorylated during hyphal growth. The *irs4* mutant displays higher activation of the cell wall integrity pathway than the *inp51* mutant.

proliferation and invasion within the kidneys. It is worth noting that the impaired hyphal formation and abnormal hyphal wall chitin distribution exhibited by *irs4* and *inp51* mutants within the kidneys after 4 days resembled the phenotypes observed during embedded growth within agar over a similar time period. Indeed, it has been hypothesized that embedded conditions *in vitro* resemble those encountered by *C. albicans* *in vivo*, where the organism grows in contact with a solid matrix under reduced oxygen concentrations (Ernst, 2000).

In addition to confirming that *inp51* null mutants resembled *irs4* mutants in their increased susceptibility to cell wall-active agents, impaired hyphal formation, abnormal chitin distribution and attenuated virulence, we demonstrated that disruption of either gene resulted in overactivation of the cell integrity pathway. The fungal cell integrity pathway is responsible for initiating and controlling responses to cell wall stresses (Delley & Hall, 1999; Jung & Levin, 1999). Our findings indicate that the Irs4p–Inp51p interaction exerts some degree of control over the pathway, but this effect is not dominant since it was only observed under conditions in which the pathway was already activated. It has been proposed that effects of *S.*

cerevisiae Irs4p/Tax4p and Inp51p on the cell integrity pathway are exerted through the binding of PI(4,5)P₂ to the pleckstrin homology domain of the GDP/GTP exchange protein Rom2p (Morales-Johansson *et al.*, 2004). In the cell integrity pathway, Rom2p is an upstream activator of the PKC–MAP kinase cascade (Ozaki *et al.*, 1996), which culminates in Mkc1p. Since the pathway components are conserved in *C. albicans*, a similar hypothesis is plausible.

The *C. albicans* cell integrity pathway has been linked to the regulation of morphogenesis. *C. albicans* Mkc1p has been shown to be required for morphogenesis (Navarro-Garcia *et al.*, 1998), and the pathway is activated in candidal cells grown in contact with agar (Kumamoto, 2005). A connection between the *S. cerevisiae* cell integrity/PKC and RAS/cAMP pathways has been recently demonstrated through the shared protein Rom2p (Kuranda *et al.*, 2006; Park *et al.*, 2005; Verna *et al.*, 1997). In *C. albicans*, the RAS/cAMP pathway is a major regulator of hyphal formation (Ernst, 2000; Zhao *et al.*, 2002). As such, dysregulation of the *C. albicans* cell integrity pathway might be associated with dysregulation of hyphal-inducing pathways. Nevertheless, dysregulation of the cell integrity pathway cannot fully account for the phenotypes we

observed, as the *irs4* and *inp51* mutant strains overactivated Mkc1p in liquid media but did not display impaired hyphal growth or abnormal chitin deposition.

In conclusion, we propose a model in which the interaction between Irs4p and the phosphatase Inp51p negatively regulates PI(4,5)P₂ levels in *C. albicans*. The specific increases in PI(4,5)P₂ levels in *irs4* and *inp51* null mutants are associated with abnormal chitin deposition and impaired hyphal formation during contact-induced growth *in vitro* and within murine kidneys, and attenuated virulence during disseminated candidiasis of mice. Lack of Irs4p and Inp51p also results in overactivation of the cell integrity pathway under normal activating conditions. Future studies of *irs4* and *inp51* null mutant strains that assess processes such as cell wall biogenesis, secretion, endocytosis, exocytosis and, in particular, the temporal-spatial regulation of phosphoinositides under diverse biological conditions will provide unique insights into the pathogenesis of candidiasis.

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