Candida albicans IRS4 contributes to hyphal formation and virulence after the initial stages of disseminated candidiasis

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Candida albicans is a common cause of mucosal and bloodstream infections. As a screening strategy to identify novel candidal virulence factors, sera recovered from HIV-infected patients with active oropharyngeal candidiasis (OPC) were previously used to probe a C. albicans genomic expression library. IRS4 was identified as a gene that encodes an immunogenic protein. In the present study, the presence of IRS4 transcripts was verified within OPC pseudomembranes recovered from patients. Having confirmed that the gene is expressed during human candidiasis, gene disruption strains were created and this implicated IRS4 in diverse processes, including hyphal formation on solid media and under embedded conditions, cell wall integrity and structure, and adherence to human epithelial cells in vitro. IRS4 disruption, however, did not influence hyphal formation or virulence in a murine model of OPC. Rather, the gene was found to be necessary for normal morphogenesis and full virulence during murine intravenously disseminated candidiasis (DC). IRS4’s effects on hyphal formation and virulence during DC were not evident on the first day after intravenous inoculation, even though transcripts were detected within murine kidneys. After 4 days, however, an irs4 null mutant strain was associated with attenuated mortality, diminished tissue burdens, less extensive infections, impaired C. albicans hyphal formation and decreased kidney damage. Taken together, these findings suggest that IRS4 makes distinct temporal-spatial contributions to the pathogenesis of candidiasis, which appear to vary between different tissue sites as well as within a given tissue over time.

INTRODUCTION

Candida albicans causes a wide range of common cutaneous, mucosal and systemic diseases. Oropharyngeal candidiasis (OPC), for example, is the most common opportunistic infection among human immunodeficiency virus (HIV)-infected persons (Greenspan, 1994), and candidaemia is among the most frequent bloodstream infections among hospitalized patients in North America and Europe (Edmond et al., 1999; Richet et al., 2002). In most humans, C. albicans is a harmless commensal organism of mucosal surfaces. To succeed in this capacity, it has adapted to coexist with the host and other microbes within stressful in vivo environments. When the normal equilibrium of these environments is disturbed, such as with suppression of the immune system or alterations in microbial flora due to antibiotics, C. albicans can invade and damage host tissues. The exact mechanisms of pathogenesis under such conditions remain incompletely understood. Although disruptions of genes involved in classic virulence-associated functions such as tissue adhesion, secretion of hydrolytic enzymes and iron sequestration diminish the organism’s disease-causing potential, no specific virulence factor is dominant (Calderone & Fonzi, 2001). Rather, pathogenesis depends upon the coordinated expression of multiple genes in a manner that facilitates proliferation, invasion and tissue damage within the given in vivo milieu (Mahan et al., 2000; Staib et al., 2000a, b; Fradin et al., 2003). A number of C. albicans genes are likely to play roles in the pathogenesis of candidal disease at diverse tissue sites (Staab et al., 1999; Sundstrom et al., 2002; Fu et al., 2002). Since each tissue site is a unique ecological niche that changes over the course of the disease process, however, other genes are likely to

Abbreviations: DC, disseminated candidiasis; FCS, fetal calf serum; FOA, fluoroorotic acid; GMS, gomori methenamine silver; HIV, human immunodeficiency virus; MAP, mitogen-activated protein; OMP, orotidine 5’-monophosphate; OPC, oropharyngeal candidiasis; P(4,5)P3, phosphatidylinositol (4,5) bisphosphate; PMN, polymorphonuclear cell; SAP, secreted aspartyl proteinase; UMP, uridine 5’-monophosphate.
make distinct temporal-spatial contributions to virulence (Ghannoum et al., 1995; Muhlschlegel & Fonzi, 1997; De Bernardis et al., 1998).

We have adapted an antibody-based screening strategy to identify C. albicans genes of diverse functions that are expressed within humans. In earlier studies, we used sera recovered from HIV-infected patients with active OPC to screen a C. albicans genomic DNA expression library, identifying genes that encode immunogenic antigens (Cheng et al., 2003a; Nguyen et al., 2004). We demonstrated that not5, a previously uncharacterized gene, was necessary for normal hyphal formation and complete virulence during disseminated candidiasis (DC) (Cheng et al., 2003a, b). In this study, we characterize a previously unreported in vivo expressed gene identified upon further screening.

C. albicans open reading frame IPF8663 is 1917 bp in length and is the sole homologue of two closely related Saccharomyces cerevisiae genes, IRS4 and TAX4. Since IPF8643 is annotated as encoding a protein similar to scls4p by the Agabian lab. at UCSF (http://agabian.ucsf.edu/canodb/anno.php), we have named the corresponding gene C. albicans IRS4. The putative 639 aa protein encoded by C. albicans IRS4 contains a single identifiable domain (an EH domain) of approximately 71 aa at positions 553–624. In other eukaryotes, EH domain-containing proteins regulate diverse cellular processes, including cell wall organization (Santolini et al., 1999). Recently, S. cerevisiae IRS4 was demonstrated to contribute to the negative regulation of the cell integrity pathway, which controls cell wall biosynthesis and repair, as well as chitin distribution (Morales-Johansson et al., 2004). A C. albicans cell integrity pathway has been inferred to contribute to hyphal formation in vitro and virulence during murine DC (Navarro-Garcia et al., 1995; Diez-Orejas et al., 1997; Navarro-Garcia et al., 1998). For these reasons, we hypothesized that disruption of C. albicans IRS4 would result in dysregulation of hyphal formation and cell wall integrity. If so, we further hypothesized that IRS4 would play a role in candidal virulence.

**METHODS**

**Strains and media.** C. albicans strains used or constructed in this study are described in Table 1. All strains were routinely grown in yeast peptone dextrose (YPD) liquid medium (1% yeast extract, 1% bactopeptone, 2% d-glucose), or on YPD or Sabouraud dextrose agar (SDA) at 30°C. To induce hyphal formation in liquid media, C. albicans strains grown overnight on YPD agar were subcultured into liquid YPD supplemented with 5% fetal calf serum (FCS) or liquid RPMI-1640 at 37°C. To induce hyphal formation on solid media, overnight grown C. albicans were subcultured onto 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 cells of overnight grown C. albicans were mixed into 20 ml molten YPD, YPD + 5% FCS, YPD-reverse [BASF pluronic polyol F-127; kindly provided by FGSC (www.fgsc.net)], Spider or M-199 agar, and incubated for 3 days at 30 or 37°C. Reverse agar is a polymer of polyoxypropylene and polyoxyethylene that can be used as a replacement for conventional agar in solid medium.

**Construction of IRS4 mutants.** IRS4 was disrupted using the Ura+blaster strategy (Fonzi & Irwin, 1993). A proximal fragment from nucleotide positions +820 to +1185 bp relative to ATG of IRS4 was amplified by PCR using the primers N-F1F (5′-AAAACTGCAGGGAATAAGCATTGAGTCACA-3′) and N-F1R (5′-GACGGGTTCGACCGCAAACTTGGAACTGTGTTG-3′); the restriction sites PstI and SphI (underlined) were introduced into N-F1 and N-F1R, respectively. A distal fragment from nucleotide positions +1570 to +1849 bp relative to ATG of IRS4 was amplified by PCR using the primers C-F1F (5′-GGGTAGAAGTACTCGAGAAGTCGACGTTG-3′) and C-F1R (5′-GGCGCTAGCTCTCCAAAACCGACATTATCGAC-3′); the restriction sites BglII and SacI (underlined) were introduced into C-F1F and C-F1R, respectively. Following amplification, the fragments were digested with the appropriate restriction enzymes and ligated sequentially into the plasmid pMB-7, flanking the hisG-URA3-hisG disruption cassette. The resulting plasmid was digested with PstI and SacI and transformed into C. albicans strain CAI4 by electroporation. Ura+ transformants were selected on SDA plates lacking uracil. After confirmation of disruption by Southern analysis, a Ura+ transformant was screened for segregants on 5-fluoroorotic acid (5-FOA) plates. The resulting Ura+ strain was transformed with the same disruption cassette to disrupt the second copy of IRS4. In the final step, a Ura+ strain was obtained following 5-FOA selection and confirmed by Southern analysis. URA3 was then reintroduced to its original locus by transforming a 4 kb BglII–PstI fragment isolated from plasmid pUR3 (kindly provided by William Fonzi, FGSC).

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<th>Table 1. Strains used in this study</th>
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<td><strong>Strain names</strong></td>
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<td>SC5314</td>
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<td>CAF2-1</td>
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<td>CAI-12</td>
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<td>CAI4</td>
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<td>ΔIRSs</td>
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<td>ΔIRSs-U (heterozygous mutant)</td>
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<td>ΔIRSr (reinsertion)</td>
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C. albicans IRS4 encodes a virulence factor

Georgetown University Medical Center, Washington DC, USA) into a Ura− strain by electroporation (Cheng et al., 2003b). The success of transformations was confirmed by Southern analysis.

In order to obtain a reconstituted strain of one IRS4 allele, we first constructed a modified pMB7 (pMB7-1), in which one copy of hisG had been eliminated by treatment with Xbal followed by religation. Then, nucleotide positions from + 820 to + 2817 bp relative to ATG of IRS4 were amplified using the primers N-F1F and Reinsert-R (5′-TTGGACGTCGACCTGCTTCATTCTATGGA-3′), introducing a SalI restriction site (underlined). The amplified product was digested with the appropriate restriction enzymes and cloned into pMB7-1 at the PstI/SalI site. The IRS4-URA3-hisG cassette was released by treatment with SphI/SacI and used to transform the Ura− irs4 null mutant. Again, results were confirmed by Southern analysis.

**Growth rates in vitro.** The in vitro growth rates were determined in YPD and SD media at 30 and 37°C in microtitre plates as described in our previous publications (Cheng et al., 2003a, b). We used a BioMate 3 spectrophotometer (Thermo Spectronic) and disposable polystyrene cuvettes (USA Scientific).

**Sensitivities to cell wall agents**

Each of the following experiments was performed in triplicate.

**SDS and calcofluor white.** Cultures from overnight grown organisms were subcultured in YPD liquid medium with 1% glucose until the exponential phase, and diluted to an OD599 of 0.1. Four microlitres of undiluted and serial 10-fold dilutions of each culture were spotted onto YPD plates containing calcofluor white (40 μg 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 (Sigma) ml−1 in 10 ml Tris/HCl, pH 7.5. An aliquot was removed at timed intervals and the OD599 was measured. We used a BioMate 3 spectrophotometer (Thermo Spectronic) and disposable polystyrene cuvettes (USA Scientific). The OD599 was plotted against time of incubation.

**Caspofungin.** Sensitivity to caspofungin (Merck) was measured in a 48-well microtitre plate. Organisms grown overnight in YPD at 30°C were diluted to OD600 of 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. We used a Beckman Coulter AD340 spectrometer and 48-well tissue culture cluster plates.

**Chitin staining and microscopy.** Cells were grown at 30 or 37°C, either embedded in YPD-reverse agar for 3 days or in liquid YPD to exponential phase. For chitin staining, cells were resuspended in a 0.1% calcium chloride white solution and incubated at room temperature for 2–5 min, then washed three times in cold PBS. Assays were performed on three separate occasions.

**Adherence assay.** We evaluated the adherence ability of C. albicans strains using the colonic adenocarcinoma cell line HT-29, cervical carcinoma cell line HeLa and pharyngeal squamous cell carcinoma cell line FaDu (purchased from ATCC). HT-29, HeLa and FaDu cells were cultivated in ATCC complete growth medium (minimal essential medium; Eagle) with 2 mM L-glutamine and Earle’s balanced salt solution adjusted to contain 1.5 g sodium bicarbonate l−1, 0.1 M non-essential amino acids and 1.0 mM sodium pyruvate supplemented with 10% fetal bovine serum. Cells (5 × 104) were seeded in each well of six-well plastic dishes. The cells were grown to confluency at 37°C and 5% CO2. Monolayers were washed twice with 2 ml Dulbecco’s PBS (DPBS), incubated with 150 C. albicans cells in 1 ml DPBS for 45 min at 37°C and 5% CO2. Monolayers were washed three times with warm DPBS to remove non-adhering cells, then covered with 2 ml YPD agar. Yeast colonies appearing after 48 h of growth at 30°C were counted. Each experiment was performed in triplicate, on three separate occasions. Adherence for each strain was determined as the percentage of fungal cells attached to monolayers. Results for each strain were expressed as the percentage of the adherence noted for control strain CAI-12.

**Murine OPC.** As described by Kamai et al. (2001), 7-week-old male ICR mice (Harlan-Sprague) were immunosuppressed with 4 mg cortisone acetate (Sigma Aldrich) in saline with 0.05% Tween 80 administered subcutaneously on the day before, 1 and 3 days after inoculation. Mice received tetracycline hydrochloride (Fisher) in their drinking water (0.5 mg ml−1) starting the day before inoculation. For inoculation, mice were anaesthetized by intraperitoneal injection with 3 mg pentobarital sodium solution (Abbott Laboratories), and cotton wool balls (3 mm diameter) containing 106 c.f.u. of C. albicans were placed sublingually in the oral cavity for 2 h. To determine tissue fungal burden, the mice were sacrificed at day 7 post-infection and the mandibular soft tissue, including the tongue, was dissected free of the teeth and bone. The excised tissue was homogenized in saline, after which serial dilutions were plated onto SDA plates containing 60 μg amikacin ml−1 for colony counting. For the histopathological study, tissues were fixed with formalin and embedded in paraffin, after which thin sections were prepared and stained with gomori methenamine silver (GMS) stain.

**Murine DC.** Seven-week-old male ICR mice (Harlan-Sprague) were inoculated by intravenous injection of the lateral tail vein with 1 × 106 c.f.u. of C. albicans. Mice were monitored until they were moribund, at which point they were sacrificed, or for 30 days. Survival curves were calculated according to the Kaplan–Meier method using the Prism program (GraphPad Software) and compared using the Newman Keuls analysis; a P-value of <0.05 was considered significant. To determine C. albicans tissue burdens, mice were infected in the preceding manner. Mice in each group were sacrificed 20 h or 4 days after intravenous inoculation and their kidneys were aseptically removed. The kidneys were weighed, homogenized in 2 ml sterile PBS, and serial dilutions were plated onto 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 10 c.f.u. g−1 kidney. The difference in kidney burden between strains was determined by Wilcoxon rank sum test; a P-value <0.05 was determined statistically significant. In a separate experiment, murine kidneys collected 20 h and 4 days after intravenous inoculation were fixed with formalin and embedded in paraffin, after which thin sections were prepared and stained with GMS stain (Churukian & Schenk, 1977; Carson, 1996).

**RT-PCR**

OPC. Pseudomembranes were recovered from the oral cavities of HIV-infected patients, total RNA extracted and treated with RNase-free DNase, and RT-PCR performed as previously described (Cheng et al., 2003a). We used the following PCR protocol: 1 min at 94°C, 1 min at 50°C and 1 min at 72°C, preceded by denaturation for 5 min at 94°C and followed by a final extension cycle for 10 min at 72°C. Reactions of 35 and 40 amplification cycles were performed using primers IRS-1300F (5′-CTACCCAGAATCTTCCCTGAGAT-3′) and IRS-CRev (5′-CAGTGCATATTAAACCAATCTCAGT-3′), which should result in an amplification product of 633 nt. We included the constitutively expressed housekeeping gene EFB1 (elongation factor 1β) as an internal mRNA control using primers EFB1-For (5′-AATTCTTGCCTGAAACATACCATAC-3′) and EFB1-Rev (5′-CTAAAGCATCTTTCAACACAGTT-3′) (Schaller
et al., 1998; Naglik et al., 1999; Ripeau et al., 2002). In the event of genomic DNA contamination, an 891 bp fragment containing an intron would be amplified. PCR products were migrated and visualized in a 1:5% agarose gel.

Murine kidneys. Mice were infected intravenously with strain CAI-12 (10⁶ c.f.u.) as described for the DC model, and sacrificed at 20 h (three mice) or 4 days (two mice) post-infection. Kidneys were harvested aseptically and homogenized, and total RNA extracted using RiboPure-Yeast kit (Ambion), which includes RNase-free DNase. First-strand cDNA synthesis using the primers listed in the preceding paragraph was performed with SuperScript III reverse transcriptase (Invitrogen) following the manual. PCR amplification of the targeted genes was performed for 30 cycles using Taq DNA polymerase (Eppendorf) and the manufacturer’s instructions, using the amplification protocol described above. As in the OPC RT-PCR experiments, EFB1 was included as an internal mRNA control, and PCR products were migrated and visualized in a 1:5% agarose gel.

RESULTS
C. albicans IRS4 is expressed in the oral cavity during OPC

In order to verify that IRS4 is expressed during the course of OPC, we collected pseudomembranes from the oral cavities of three HIV-infected patients. Pure cultures of a single C. albicans strain were isolated from each patient. We extracted total RNA from the pseudomembranes and detected IRS4 transcripts using RT-PCR (Fig. 1).

IRS4 is required for normal hyphal formation on solid media and under microaerophilic conditions of embedded growth

Next, we used the Ura-blaster method to construct C. albicans strains, in which one or both copies of the gene were disrupted (Table 1). The disruption cassette was inserted at nucleotide position +1185 bp relative to ATG, assuring that the sequence encoding the potential EH domain was eliminated. We then reintroduced the URA3 selection marker to its native locus in the Δirs4::hisG/Airs4::hisG Δura3::imm434/Δura3::imm434 background, thereby creating an irs4 null mutant strain. We also created an IRS4 reinsertion strain, in which a full copy of the gene was reintroduced to a disrupted native locus. The null mutant strain exhibited logarithmic-stage growth rates that were indistinguishable from the parent strain CAI-12, heterozygous mutant and reinsertion strains in YPD and SD liquid media at 30 and 37 °C (data not shown). In addition, the null mutant and CAI-12 did not differ in activity of orotidine 5′-monophosphate decarboxylase, the enzyme encoded by URA3 (mean ± SD, n = 6: 0.0026 ± 0.0007 vs 0.0023 ± 0.0011 U mg⁻¹ protein, respectively) (Myers et al., 1995; Lay et al., 1998; Cheng et al., 2003b).

In YPD supplemented with 5% FCS and RPMI-1640 liquid media, we noted no significant differences in the formation of true hyphae by CAI-12 and the heterozygous mutant, null mutant and reinsertion strains after 4 h of incubation at 37 °C. Furthermore, all strains reverted to yeast morphology after 10–12 h of incubation. Clear phenotypic differences, however, were noted when strains were grown in contact with solid media, both on the surfaces of agar and under microaerophilic conditions of embedded growth. During surface growth, CAI-12 exhibited extensive hyphae after 2–4 days on YPD + 5% FCS, Spider, modified Lee’s and M-199 agar at 37 °C. The null mutant strain, on the other hand, either did not form hyphae or exhibited markedly truncated hyphae (Fig. 2). The heterozygous mutant and reinsertion strains exhibited intermediate phenotypes under these conditions. Similar findings for the strains were noted after 3 days of growth under embedded conditions within YPD, YPD + 5% FCS, YPD-reverse, Spider and M-199 agar at 30 and 37 °C (Fig. 2). Of note, differences between strains in hyphal formation on the surface of and within solid agar continued to be maintained until plates were disposed of after 7 days, indicating that the earlier observations reflected differences in morphogenesis rather than growth rates.

Disruption of C. albicans IRS4 alters cell wall integrity and structure, as well as adherence to human epithelial cells

We found the irs4 null mutant to be more sensitive to the detergent SDS (0.02%) and the chitin-binding agent calcofluor white (40 μg ml⁻¹) than CAI-12, heterozygous mutant or reinsertion strains, which did not differ significantly from one another (data not shown). We also demonstrated that the null mutant strain was more susceptible to the β-1,3-glucanase preparation zymolyase 20T (100 μg ml⁻¹) and the antifungal agent caspofungin, which targets β-1,3-glucan synthase (Fig. 3). Finally, calcofluor white staining of cells grown under embedded conditions in YPD-reverse agar for 3 days revealed abnormal distribution of chitin along the walls of hyphae in the null mutant (Fig. 4). Of note, these abnormalities in chitin were not observed during growth at 30 or 37 °C in YPD liquid media (not shown).

Since the cell wall is the point of contact between C. albicans and host tissues, we assessed whether disruption of IRS4 attenuated adherence to human epithelial cell lines in vitro.
Taking strain CAI-12 as a reference in each experiment, the relative adherence of the irs4 null mutant to FaDu (oesophageal), HeLa (cervical) and HT-29 (colonic) cells was significantly diminished compared with the parental strain (respectively, mean ± SD, n = 6: 68·1 ± 12·1, P = 0·02; 48·4 ± 19·5, P = 0·0008; 64·6 ± 15·7 %, P = 0·0004).

**Disruption of C. albicans IRS4 attenuates hyphal formation and virulence during murine DC but not OPC**

To study the contribution of IRS4 to virulence in vivo, we first used a murine model of OPC. We applied cotton balls saturated with 10⁸ c.f.u. to the oropharynges of 7-week-old ICR mice immunocompromised with subcutaneous cortisol acetate (strain CAI-12, 12 mice; irs4 null mutant, 13 mice) (Kamai et al., 2001). After 7 days, the mice were sacrificed, and the oropharynges and oesophagi removed for c.f.u. enumeration. We demonstrated that there were no significant differences in the candidal tissue burdens for mice infected with CAI-12 or the null mutant strain (log₁₀ c.f.u. g⁻¹ tissue mean ± SD, n = 6: 5·61 ± 0·60 vs 5·91 ± 0·64 %, respectively; P = not significant). Furthermore, histopathological studies of resected tongues and oesophagi revealed no differences in candidal morphologies, with clusters of yeast and hyphae present for both strains (Fig. 5). Similarly, we found no differences in the degree of tissue invasion, numbers of infecting organisms or host tissue damage. We obtained similar results for both tissue burdens and histopathology upon repeating the murine experiments.

**Fig. 2.** Disruption of IRS4 results in repressed hyphal formation on the surface of solid media and during embedded growth. Hyphal formation by C. albicans CAI-12 and the irs4 null mutant strain is compared after 3 days of growth at 37 °C on the surface of YPD agar, as well as after 3 days of growth embedded at 30 °C within YPD-reverse agar and Spider agar (a). For surface growth, similar results were obtained at 37 °C on YPD supplemented with 5 % FCS, modified Lee’s and M-199 media. For embedded growth, results were similar at 37 °C within YPD-reverse agar and Spider agar, and at both 30 and 37 °C within YPD+5 % FCS, YPD and modified Lee’s media. Under all conditions tested, the single copy IRS4 reinsertion strain exhibited intermediate phenotypes, as pictured in (b) embedded within YPD-reverse agar at 30 °C and on the surface of YPD agar at 37 °C (labelled as irs4_IRS4).

**Fig. 3.** IRS4 disruption increases sensitivity of C. albicans to zymolyase (a) and caspofungin (b and c). As shown in (a), strain CAI-12 and an IRS4 reinsertion strain were significantly more resistant to killing by zymolyase 20T (100 µg ml⁻¹) after 20, 30 and 40 min than the irs4 null mutant strain. (b and c) Microtitre wells with growth of CAI-12 and the null mutant, respectively. For each strain, the first well at the left is a control (without caspofungin), and the remaining wells from left to right contain caspofungin concentrations increasing from 0·075 to 1·25 µg ml⁻¹. As with the zymolyase experiments, CAI-12 is more resistant to caspofungin than the null mutant.

**Fig. 4.** Disruption of IRS4 results in an abnormal distribution of chitin in the C. albicans cell wall. Calcofluor white staining of strain CAI-12 during embedded growth reveals a normal distribution of chitin along the cell wall and at septae (a). In the irs4 null mutant, on the other hand, there are abnormal accumulations of chitin along the hyphal cell wall (b) and (c).
Next, we used a murine model of DC to assess the effects of IRS4 disruption on mortality, tissue burden and the histopathology of kidneys. In the mortality study, we infected groups of 8–12 ICR mice intravenously via the lateral tail vein with 10^6 c.f.u. per mouse. All mice infected with strain CAI-12, the heterozygous mutant strain, and the reinsertion strain died by day 10 (Fig. 6). In contrast, 50% of mice infected with the null mutant strain were still alive at the termination of the study on day 30. The survival of mice infected with the null mutant strain was significantly prolonged compared with mice infected with any of the other strains (*P* < 0.0001). These studies were repeated on two other occasions, with similar findings.

In a separate experiment, we quantified viable *C. albicans* within murine kidneys following the lateral tail vein inoculations with CAI-12 (12 mice) or the null mutant strain (10 mice). First, we sacrificed five mice from each group 20 h post-infection and demonstrated that there were no significant differences in *C. albicans* concentrations within the kidneys (mean log_{10} c.f.u. g^{-1} kidney ± SD: 5.88 ± 0.77 vs 5.61 ± 0.56, respectively; *P* = not significant). Then, five non-moribund mice from each group were sacrificed on day 4. At this time, mice infected with CAI-12 exhibited significantly higher tissue burdens than mice infected with the null mutant strain (mean log_{10} c.f.u. g^{-1} kidney ± SD: 6.04 ± 0.06 vs 4.98 ± 0.42, respectively; *P* = 0.0002). Of note, two mice infected with CAI-12 that were moribund on day 4 were excluded from the data analysis.

Finally, we recovered kidneys from non-moribund mice infected with either strain after 20 h and 4 days for histopathological evaluation (three mice per group). Our results were consistent with the tissue burden data. At 20 h, all kidneys exhibited small clusters of *C. albicans* growing as yeasts, germ tubes and short hyphae, which were widely separated (Fig. 7). Normal tissue histopathology was maintained. Furthermore, kidneys from mice infected with CAI-12 and the null mutant could not be distinguished based on the number of infected clusters, number of cells within clusters or candidal morphology. By 4 days, however, differences were striking (Fig. 7). Kidneys infected with CAI-12 showed multiple clusters of *C. albicans*, consisting of dense mats of hyphae interspersed with yeasts. These were surrounded by a brisk inflammatory response that disrupted normal tissue architecture. Kidneys infected with the null mutant, on the other hand, showed widely dispersed pockets composed of yeast cells that stained poorly with GMS. Germ tubes and truncated hyphae were extremely rare, isolated and very poorly stained. The inflammatory response and damage to host cells within the kidneys were minimal. Of note, the length of hyphal elements and the number of germ tubes and hyphae for the null mutant strain were markedly less at 4 days than at 20 h.

IRS4 is expressed within murine kidneys at both 20 h and 4 days of DC

We hypothesized that the variable contribution of IRS4 to virulence during DC might be explained by a lack of gene expression at 20 h. In a separate experiment from those described above, we performed RT-PCR using total RNA extracted from the kidneys of mice infected with strain CAI-12 (three mice at 20 h; two mice at 4 days). At both time points, IRS4 transcripts were detected in all mice (Fig. 8).
DISCUSSION

In this study, we demonstrated that a newly identified \textit{C. albicans} gene, IRS4, was expressed under diverse conditions \textit{in vivo} and makes distinct temporal–spatial contributions to the pathogenesis of candidiasis. Despite our detection of IRS4 transcripts in pseudomembranes recovered from the oral cavities of HIV-infected patients, we did not implicate the gene in virulence during murine OPC. Indeed, there were no differences between an \textit{irs4} null mutant and parent strain CAI-12 in candidal cell morphology, tissue burdens or histopathology within the oral and oesophageal mucosa of mice. Strikingly, however, we demonstrated that IRS4 was necessary for normal hyphal formation and complete virulence during intravenous murine DC. Moreover, we showed that IRS4 mediates these effects after the initial establishment of infection in the kidneys. During the first day following intravenous inoculation of \textit{C. albicans}, disruption of IRS4 did not influence tissue burdens, extent of infection or host tissue damage within the kidneys. The cell morphologies of the null mutant strain were indistinguishable from those of CAI-12, growing as mixtures of yeasts, germ tubes and short hyphae. After 4 days, on the other hand, the null mutant was associated with attenuated mortality, diminished tissue burdens, less extensive infections, decreased kidney damage and impaired \textit{C. albicans} hyphal formation. Of note, we detected IRS4 transcripts within infected kidneys at both the early and later time points. Taken together, these data suggest that the contribution of Irs4p to virulence varies between different tissue sites as well as within a given tissue over time.

Our findings support a paradigm in which the pathogenesis of diverse candidal diseases depends upon both generalized virulence factors and those that function in specific environments dictated by tissue site, immune function and other host factors. The former group, for example, would include the transglutaminase substrate encoded by \textit{HWP1} and the adhesin protein encoded by \textit{ALS1}, which contribute to virulence at mucosal and deep organ sites (Staab \textit{et al.}, 1999; Sundstrom \textit{et al.}, 2002; Kamai \textit{et al.}, 2002; Fu \textit{et al.}, 2002). It is notable that IRS4 appears to differ from a number of other genes in the latter group, for which temporal–spatial contributions to virulence are determined.
by expression patterns. For example, \textit{PHR1} and \textit{PHR2} encode cell surface proteases and are expressed at neutral–alkaline and acidic pH, respectively. Consistent with this expression, \textit{phr1} null mutant strains are avirulent during murine DC and \textit{phr2} null mutants are avirulent during vaginal candidiasis in rats (Ghannoum et al., 1995; Muhlschlegel & Fonzi, 1997; De Bernardis et al., 1998). Individual members of the SAP family of secreted aspartyl proteases and genes encoding phospholipases are also regulated at the transcriptional level \textit{in vivo}, with expression patterns varying depending on tissue sites and stages of infection (Staib et al., 2000a; Fradin et al., 2003; Naglik et al., 2003). Presumably, Irs4p is present and performs functions in the oral cavity and in the kidneys in the first day of DC, but these do not contribute to hyphal formation, tissue invasion or damage. Within the unique \textit{in vivo} milieu of the kidneys after 4 days of DC, however, the protein appears to play a role in these processes. In this regard, our data indicate that the host environment is likely to be the principle determinant of whether Irs4p contributes to virulence.

The mechanisms by which \textit{IRS4} contributes to virulence in the appropriate settings are not clear from this study. It is possible that the \textit{irs4} null mutant strain’s attenuated virulence correlates, at least in part, with its impaired hyphal morphogenesis. The precise relationship between yeast–hyphal morphogenesis and virulence remains controversial (Gow et al., 2002; Sudbery et al., 2004). As with other genes that regulate hyphal growth, it is uncertain whether the attenuated virulence of the \textit{irs4} null mutant strain in the kidneys after 4 days stems from defects in morphogenesis per se, repression of hyphal-associated gene expression, or pleiotropic effects of gene disruption. Nevertheless, it is interesting that the impaired hyphal formation by the null mutant within the kidneys resembles the phenotype observed during embedded growth within agar rather than in liquid media. Indeed, it has been hypothesized that embedded conditions \textit{in vitro} resemble those encountered by \textit{C. albicans} \textit{in vivo} (Brown et al., 1999; Ernst, 2000; Doedt et al., 2004), where the organism grows under reduced oxygen tension, in contact with surrounding extracellular matrix. Our data suggest that the phenotypes exhibited by the null mutant during embedded growth might be more relevant to the pathogenesis of invasive candidiasis than those seen in liquid media. We will investigate this possibility in future studies of gene expression patterns under embedded conditions.

The difficulty in establishing a definite link between \textit{IRS4}’s roles in morphogenesis and virulence is highlighted by our demonstration that the gene is also required for normal cell wall structure and integrity. In fact, cell wall remodelling is integral to hyphal formation, so it is not surprising that \textit{IRS4} might contribute to both processes. At the same time, cell wall integrity is likely to play an important role in growth and survival of \textit{C. albicans} \textit{in vivo}, independent of cell morphology. For this reason, it might reasonably be hypothesized that \textit{IRS4}’s effects on the cell wall are as important to its virulence function as are the effects on morphogenesis. For example, alterations in the cell wall might contribute to the diminished adherence of the \textit{irs4} null mutant to oesophageal, vaginal and colonic adenocarcinoma cells \textit{in vitro}. Alternatively, mutant cells might form hyphae whose altered cell walls lack sufficient strength to penetrate or cause damage deep within the tissue. Indeed, this could also explain the differences in virulence during OPC and DC, since OPC lesions are normally superficial, as well as the discrepancy between hyphal formation in liquid media and under embedded conditions. Interestingly, our observations in many ways resemble those of investigators studying \textit{C. albicans} septin mutants, which are only mildly defective for hyphal growth in liquid media but significantly impaired in invasive growth into agar and murine kidneys (Warenda et al., 2003). These investigators have suggested that the ability to undergo invasive growth entails the coordination of hyphal formation and multiple other processes, and is likely to be more important to pathogenesis than hyphal growth alone.

The contribution of \textit{IRS4} to virulence in conducive settings is likely to be multifactorial and indirectly mediated. \textit{S. cerevisiae} \textit{IRS4} has recently been shown to encode a component of a complex that negatively regulates the cell integrity mitogen-activated protein (MAP) kinase pathway (Morales-Johansson et al., 2004). This negative regulation is effected through activation of a phosphatase that down-regulates levels of the second messenger phosphatidylinositol (4,5) bisphosphate \textit{[PI(4,5)P2]} \textit{[PI(4,5)P2]}, in turn, is an important mediator of signal transduction that is involved in diverse cellular processes. As a follow-up to the present study, we will investigate whether \textit{C. albicans} \textit{IRS4} similarly regulates phosphoinositide levels.

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