Reintroduction of the PLB1 gene into Candida albicans restores virulence in vivo

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Phospholipases have been proposed to contribute to the virulence of Candida albicans. Recently, a candidal strain deleted for PLB1, the gene encoding the predominant phospholipase B (Plb1) secreted by C. albicans, was constructed and its virulence in an intravenous murine model of disseminated candidiasis was evaluated. In the present study, the PLB1 gene was reintroduced back into the plb1 null mutant to generate the revertant strain, which showed similar growth and morphology to its isogenic parent strain. Virulence of the revertant strain was found to be comparable to that of the parent strain in an intravenous murine model of disseminated candidiasis. To compare the abilities of the plb1 null mutant, the revertant and the isogenic parent strains to cross the gastrointestinal (GI) tract and cause systemic infection, an oral–intragastric infant mouse model of candidiasis was used. Histological examinations and analysis of c.f.u. of the pathogen in liver homogenates revealed that the parental and revertant strains were able to invade and traverse the GI mucosa to a significantly greater extent than the plb1 null mutant. Immunofluorescence and immunoelectron microscopic studies of infected host tissue using anti-Plb1 antibody showed that Plb1 is secreted during invasion of the gastric mucosa by the parental and revertant strains. In contrast, little or no labelling was observed in the null mutant strain. The results indicate that the Plb1 secreted by C. albicans enhances the ability of this organism to cross the GI tract and disseminate haematogenously. These studies provide unequivocal evidence supporting a role for Plb1 during the course of infection by C. albicans.

Keywords: phospholipase B, virulence factor, candidal transmigration, in vivo localization of Plb1

INTRODUCTION

Infections caused by Candida albicans, the most common fungal pathogen of humans (Odds, 1994), have increased significantly in several patient populations, especially immunocompromised individuals (Odom, 1994; Jarvis, 1995; Viscoli et al., 1999). Candidaemia is the fourth leading cause of bloodstream infections in many intensive care units (Edmond et al., 1999; De Marie, 2000). Administration of antifungal agents, particularly amphotericin B and fluconazole, is the current mode of therapy used to treat candidal infections (Klepser et al., 1998; De Marie, 2000). However, renal toxicity associated with amphotericin B and resistance to azole antifungals (including fluconazole) are inherent drawbacks in current antifungal therapies based on these drugs (Patterson & Andriole, 1989). Even when the ideal antifungal treatment protocol has been established, mortality still persists at an unacceptably high range of 38–50% (Wey et al., 1988, 1989; Pagano et al., 1999). In view of these obvious limitations of treatment with antifungal agents, it is evident that new approaches based on microbial virulence need to be developed for effective and less toxic antifungal drugs.

To develop such new therapies and treatments for candidiasis, it is essential to dissect the infectious process of C. albicans. Several virulence factors have been proposed for C. albicans, which may represent novel molecular targets for antifungal development. These

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Abbreviations: GI, gastrointestinal; PAS, periodic acid–Schiff reagent.
factors include extracellular phospholipases and proteinases (Hube et al., 1998; Ghannoum, 2000; Mukherjee & Ghannoum, 2001). Several bacterial and protozoan phospholipases have also been shown to contribute to the infectious processes of these pathogens (Ravdin et al., 1985; Saffer & Schwartzman, 1991; Silverman et al., 1992; Schmiel & Miller, 1999; Zhou et al., 2001). Like their bacterial and protozoan counterparts, the phospholipases of C. albicans are also considered important virulence determinants (Ibrahim et al., 1995), and could potentially facilitate increased penetration of fungal hyphal elements by directly damaging host cell membranes. To determine if phospholipases have a role in candidal virulence, we have previously cloned and disrupted PLB1, the gene encoding candidal phospholipase B (Plb1), and showed that virulence of the plb1 null mutant was significantly attenuated compared to that of its isogenic parental counterpart when tested in a murine model of haematogenously disseminated candidiasis (Leidich et al., 1998).

C. albicans is a member of the gastrointestinal (GI) microflora in normal individuals. In immunocompromised hosts, migration of this fungus across the GI tract represents one of the mechanisms by which disseminated candidiasis is established (Cole et al., 1989). An oral–intragastric infant mouse model of GI candidiasis has previously been established which is designed to mimic the process by which C. albicans traverses the human GI tract and haematogenously disseminates to other body organs (Cole et al., 1990, 1996). This model allows precise control of challenge dose and maintains the natural host barriers, e.g. gastric and intestinal secretions, peristalsis and mucin (Cole et al., 1996).

In our earlier investigations, we showed that the plb1 null mutant is attenuated in its virulence (Leidich et al., 1998). However, these studies did not involve a revertant strain containing the reintroduced PLB1 gene, due to the unavailability of such a strain at that time. To unequivocally prove the association of plb1 genes with the functional PLB1 gene back into the plb1 null mutant, and determine if the revertant has similar virulence to the parental strain. In this study, we report the successful construction of the PLB1 revertant strain and show that it has similar virulence to the wild-type strain in an intravenous murine model of haematogenously disseminated candidiasis. Additionally, using an oral–intragastric infant mouse model of candidiasis, we provide evidence that Plb1p is secreted during transmigration of the GI tract. Furthermore, deletion of the PLB1 gene results in significant reduction in the ability of the pathogen to traverse the stomach mucosa and disseminate haematogenously to the liver.

METHODS

Strains, plasmids and culture media. The C. albicans strains used in this study are listed in Table 1. Bacterial strain Escherichia coli DH5α was used for propagation of all plasmids. The plasmid pPCR-Script Amp (SK+) (Stratagene) was used for subcloning experiments. Yeast strains were maintained at 4 °C on Sabouraud dextrose agar (SDA; 1 % neopeptone, 2% glucose, 2% agar) (Difco Laboratories) and subcultured onto fresh SD medium every 1–2 weeks. Minimal defined media consisted of yeast nitrogen base (YNB) supplemented with 2% glucose. Media were supplemented with uridine (50 µg ml⁻¹), and solidified with 2% agar as required. Unless noted otherwise, all cultures were incubated at 30 °C.

Ura⁺ auxotrophs were selected on agar plates containing 5-fluoro-orotic acid and uridine (50 µg ml⁻¹), as described previously (Boeke et al., 1984; Leidich et al., 1998). Dilutions of a suspension of PLB1Δ cells were spread on these 5-fluoro-orotic acid plates, and scored after 3–4 d incubation. The loss of the URA3 gene in the resulting strains was confirmed by PCR and Southern blot analyses. A representative strain (PMY53) was randomly selected from among these Ura⁺ derivatives for further experiments.

Reagents. Restriction endonucleases, T4 DNA ligase, DNA polymerase (Klenow fragment), calf intestinal alkaline phosphatase and Taq DNA polymerase were purchased from Boehringer Mannheim. Oligonucleotide DNA primers were synthesized by Bio-Synthesis. Glass beads (25–600 µm), used for total chromosomal DNA extraction, and all other chemicals were obtained from Sigma. Guinea pig anti-Plb1 polyclonal antiserum was produced by Pocono Rabbit Farm & Laboratories.

Standard procedures were followed for the propagation and selection of plasmids, the growth of their bacterial hosts, and for the subcloning of DNA fragments (Sambrook et al., 1989).

Construction of plasmids. To reintroduce the PLB1 gene, the integration plasmid pPMB3 was constructed as follows. Plasmid pMB-7, which contains the C. albicans URA3 gene flanked by 1 kb direct repeats of the Salmo nella typhi murium hisG DNA, was kindly provided by Dr W. Fonzi (Georgetown University, Washington, DC, USA). A 2.9 kb fragment containing the URA3 gene was isolated by digesting pMB-7 with BamHI and cloned into the BamHI site of the plasmid pPCR-Script Amp (Stratagene) to generate plasmid pPMB1. A SacI/Apal genomic fragment containing the intact PLB1 gene was ligated into the EcoRI site of plasmid pcDNA3.1 (Invitrogen) to form plasmid pGN. Digestion of pGN with EcoRI and XhoI led to the release of a 2.2 kb PLB1 fragment, which was subcloned into the EcoRI/SalI sites of plasmid pPCR-Script Amp (Stratagene) to generate plasmid pPMB2. Next, plasmid pPMB2 was digested with PstI/XhoI to release the 2.2 kb PLB1 fragment, which was then cloned into the PstI/XhoI sites of plasmid pPMB1 to generate the integration plasmid pPMB3. The presence of URA3 and PLB1 genes in pPMB3 was confirmed by restriction enzyme analysis using URA3- or PLB1-specific restriction enzymes (data not shown). Integration plasmid pPMB3 was used in transformation of the plb1-disrupted strain of C. albicans.

Reintroduction of the C. albicans PLB1 gene. The integration plasmid (pPMB3, constructed as described above), containing the functional PLB1 and URA3 genes, was used to transform the null mutant strain in order to reintroduce these two genes. Plasmid pPMB3 was digested with BglII, which has a single recognition site in PLB1 but not in the URA3 or vector regions (Fig. 1a). This linearized plasmid was then transformed into the PLB1α⁻ derived Ura⁺ strain (PMY53, generated by selection on 5-fluoro-orotic acid as described above) using a lithium acetate-based transformation protocol (Rose et al., 1990). Ura⁺ prototrophs were selected on minimal medium lacking uridine. Total chromosomal DNA was isolated (Rose et al.,
Table 1. C. albicans strains

<table>
<thead>
<tr>
<th>Strain</th>
<th>Genotype</th>
<th>Source/reference</th>
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<tbody>
<tr>
<td>SC5314</td>
<td>Δura3::imm434/Δura3::imm434</td>
<td>Gillum et al. (1984)</td>
</tr>
<tr>
<td>CAI-4</td>
<td>Δura3::imm434/Δura3::imm434</td>
<td>Fonzi &amp; Irwin (1993)</td>
</tr>
<tr>
<td>PLB1A</td>
<td>Δura3::imm434/Δura3::imm434</td>
<td>Leidich et al. (1998)</td>
</tr>
<tr>
<td>PMY53</td>
<td>Δura3::imm434/Δura3::imm434</td>
<td>This study</td>
</tr>
<tr>
<td>PMY106</td>
<td>Δura3::imm434/Δura3::imm434</td>
<td>This study</td>
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1990) from cultures produced by the growth of individual colonies (transformants). Insertion of the functional PLB1 gene into the null mutant as a result of spontaneous recombination was confirmed by Southern blot analyses.

**PCR amplification of the PLB1-specific region.** Total chromosomal DNA isolated from the respective strain was subjected to PCR with oligonucleotide primers identical to the 751 bp region of the PLB1 gene. Oligonucleotide primers (5'-Synthesis) used in the PCR reactions were: forward, 5'-ATGATTTTGCATCATTTG-3' and reverse, 5'-AGTATCTG-GAGCTCTACC-3'. PCR amplification reactions consisted of 100 µl volumes containing 10 mM Tris-HCl (pH 8.4), 50 mM KCl, 1.5 mM MgCl₂, dNTPs (1 mM each), template DNA (10–100 ng) and 2 U Taq DNA polymerase. The PCR conditions were as follows: initial denaturation (95 °C, 2 min) followed by 33 cycles of denaturation (94 °C, 1 min), annealing (50 °C, 1 min) and extension (72 °C, 3 min), ending with a 10 min extension step at 72 °C. Reaction mixtures were analysed for the presence of a 751 bp DNA fragment, representing the 5' half of the PLB1 gene, on 1% agarose gels stained with ethidium bromide.

**Southern blot analyses.** Total chromosomal DNA was isolated from the respective C. albicans strains as described previously (Rose et al., 1990). The isolated DNA was digested with (i) KpnI and SacI or (ii) KpnI only (6 µg DNA µl⁻¹), electrophoresed through 1.2% agarose and transferred to nylon membranes (Boehringer Mannheim) following standard protocols (Sambrook et al., 1989). The transferred DNA was cross-linked to the membrane using a UV Stratalinker 228 (Stratagene) delivering 120000 µJ. The probes used were (a) a 3 kb HindIII fragment containing a portion of the 5'-terminus of the intact PLB1 gene (b) a 736 bp BamHI fragment corresponding to the portion of PLB1 deleted during the disruption process (BamHI internal) (Fig. 1a) or (c) a 1.3 kb Xbal–PstI fragment of the pPMB1 plasmid which contains the S. typhimurium hisG region. Probes were labelled with digoxigenin (DIG)–dUTP using the DIG High Prime DNA Labelling and Detection Kit (Boehringer Mannheim) according to the manufacturer's instructions. Cross-linked membranes were prehybridized for 1 h at 65 °C, then hybridized overnight with DIG-labelled probe (30 ng ml⁻¹) at 65 °C. After stringency washes, hybridizing DNA fragments were detected using the kit mentioned above, according to the manufacturer's instructions.

Western blot analysis and assays for Plb1 enzyme activities performed with culture supernatants from the respective strains, as described previously (Leidich et al., 1998).

**Haematogenous model of disseminated candidiasis.** The protocol described earlier by our group was followed (Ghannoum et al., 1995). Briefly, strains of C. albicans were grown in SD medium at 30 °C until mid-exponential phase. Cells were harvested, washed, counted and resuspended at a density of 1.25 × 10⁷ cells ml⁻¹ in PBS. Female BALB/c mice (7–8 weeks old) were injected intravenously via the lateral vein with 200 µl (5 × 10⁵ blastospores) of this fungal suspension. Cages were checked twice daily for dead or moribund mice. Mice were categorized as moribund if they displayed the following symptoms: lethargy, wasting and vertigo. Such mice were killed by CO₂ asphyxiation. To determine tissue fungal burden, mice were injected with the respective C. albicans strain as described above. Mice in each group were killed 48 h post-infection and their kidneys were removed. The organs were weighed, homogenized separately in 5 ml sterile PBS, and serial dilutions were plated on SDA plates supplemented with chloramphenicol (50 µg ml⁻¹; Sigma). The plates were incubated at 30 °C for 24–48 h, after which the number of c.f.u. was determined.

**GI infection of mice (oral–intragastric model).** Outbred mice [crl:CFW (SW) BR] obtained from Charles River Farms (Wilmington, MA, USA) were used to establish a breeding colony and the offspring of these animals were used in all experiments. Infant mice, 6 d old (3–4 g), were separated from their mother 4 h prior to challenge. Candidal cells grown on SDA slants at 30 °C were harvested, washed with saline, and the challenge dose was standardized by haemocytometer counts. The inoculum size was confirmed by dilution plating. Infant mice were inoculated with 2 × 10⁸ blastospores in a total volume of 0.05 ml by the oral–intragastric route using a 24-gauge animal feeding needle (Popper & Sons) as described previously (Cole et al., 1990). Following inoculation, the pups were kept at 35 °C for 1 h before being returned to their mother. We determined that C. albicans delivered by this route was not recovered from the lungs or blood of the pleural cavity when these tissues were cultured within 10 min post-challenge, thus making it unlikely that spread to body organs resulted from faulty inoculation technique or aspiration.

**Faecal pellet assay and separation of test groups.** Mice infected by oral–intragastric inoculation with C. albicans were selected for further study on the basis of the presence of yeast in their faecal pellets at 9 d post-challenge (Cole et al., 1989). Animals were marked for identification using picric acid at the time of the assay. Fresh faecal pellets were immediately homogenized in 1/0 ml chilled, sterile saline and 0.1 ml of the homogenate was plated on SDA (Difco) supplemented with 50 µg chloramphenicol ml⁻¹. The plates were incubated at 37 °C for 48 h. Mice identified as culture positive for C. albicans were immunocompromised as described below and used to compare the systemic spread of the parental (SC5314), plb1 null mutant (PLB1A) and revertant (PMY106) strains from the site of colonization in the GI tract.
Immunosuppression. An immunocompromising treatment was initiated on day 11 after oral–intragastric challenge as previously reported (Cole et al., 1989). Mice selected on the basis of positive faecal pellets received cyclophosphamide (Adria Laboratories) by the intraperitoneal route at doses of 0.2 mg (g body wt)$^{-1}$ and 0.1 mg (g body wt)$^{-1}$, on days 11 and 14 post-challenge, respectively. In addition, 1.25 mg cortisone acetate (Merck, Sharp & Dohme) was administered by the intraperitoneal route on days 11 and 14 post-challenge. This combined drug treatment was chosen on the basis of its severe immunocompromising effects, which lead to systemic spread of C. albicans from sites of colonization in the GI tract (Guentzel & Herrera, 1982; Cole et al., 1991a). Mice were killed at day 20 post-challenge by CO$_2$ asphyxiation. Stomachs and livers were aseptically removed and prepared for microscopic analyses or quantification of fungal c.f.u. as described previously (Pope et al., 1979). For enumeration of candidal c.f.u., the stomach and liver of each infected animal were removed under aseptic conditions, visually inspected for gross candidal foci, and homogenized separately in 5–0 ml sterile saline (Travenol Laboratories) using a tissue grinder equipped with a Teflon pestle (Cole et al., 1989). The c.f.u. of C.


** albicans** in each organ were determined by plating serial dilutions of the respective homogenate on SDA supplemented with chloramphenicol (50 μg ml⁻¹) as above. Plates were incubated at 37 °C for 48 h and then colonies were counted.

**Light microscopy.** The stomachs of five animals from each group (parental, plb1 null mutant and revertant strain infected) were used for light microscopic histological and immunofluorescence labelling. In addition, stomachs of five more animals from each group were used for electron microscopic and immunogold labelling studies. Following removal, stomachs were immediately placed in chilled saline and dissected open to expose the cardia-atrial fold (Cole et al., 1990). Tissues were fixed in 4% paraformaldehyde (v/v) in PBS; 0.1 M, pH 7.4) for 12 h at 4 °C. Tissues were then washed with buffer, dehydrated in ethanol, and embedded in paraffin wax. Sections were stained with periodic acid–Schiff reagent (PAS) for demonstration of fungal elements (Luna, 1968).

**Transmission electron microscopy.** C. albicans-infected stomachs were chemically fixed for 12 h at 4 °C in glutaraldehyde (3%, v/v) and paraformaldehyde (2%, v/v), each prepared separately in cacodylate buffer (0.1 M, pH 7.4) and mixed just before use. The tissues were rinsed five times in buffer, post-fixed in 2% osmium tetroxide (2 h) prepared in the same buffer, dehydrated and embedded in Spurr’s low viscosity resin as described previously (Seshan & Cole, 1994). Thick sections (approx. 1 μm) were stained with azure 11-methylene blue for light microscopy as reported by Cole et al. (1989). Thin sections were mounted on copper grids, stained with uranyl acetate and lead citrate and examined with a Phillips CM-10 transmission electron microscope. Osmium tetroxide post-fixation was omitted for tissues intended for immunofluorescence and immunogold labelling described below.

**Production of antibodies.** Guinea pig anti-Plb1 polyclonal antibodies were produced commercially (Pocono Rabbit Farm & Laboratory). A standard protocol was followed for the antibody production. Briefly, on day 0 a preimmune (base-line) bleed was performed. The preimmune serum was stored at −80 °C until assayed. On the same day, 50 μg purified Plb1 was injected intramuscularly in Freund’s complete adjuvant. On day 14, 10–20 μg Plb1 was injected intramuscularly in Freund’s incomplete adjuvant. A test bleed was performed on day 42 post-immunization and anti-Plb1 antibody levels were titrated. Guinea pigs were boosted every 4 weeks with 10 μg Plb1 antigen until peak antibody titres were achieved. Antiserum samples were stored frozen (−80 °C) until assay.

**Immunofluorescence microscopy.** Immunofluorescence detection of Plb1 was performed on chemically fixed, resin-embedded stomach tissue harvested from mice infected with the parent, *plb1* null mutant or revertant strains. Ten thick (10 μm) sections of stomach tissues of each strain cut with an ultramicrotome (MT-5000; Dupont) were mounted on a specially prepared gelatin-coated glass slide for immunolabelling. Sections were blocked with 1% bovine serum albumin in PBS (0.15 M, pH 7.4) for 20 min, reacted with primary antibody (guinea pig anti-Plb1) diluted 1:500 in PBS for 1 h and incubated with goat anti-guinea pig IgG-FITC secondary antibody (Sigma; diluted 1:30 in PBS) for 1 h. The sections were washed and examined with a Zeiss microscope equipped with an FITC filter set. Control sections were reacted either with FITC alone, or with normal guinea pig preimmune serum followed by secondary antibody–FITC conjugate. These experiments were repeated five times.

**Cryofixation and freeze-substitution.** To better preserve the association between the soluble Plb1p antigen and the fungal cells, the infected tissue was subjected to cryofixation and freeze-substitution (Cole et al., 1991b). In brief, blocks of dissected stomach tissue (approx. 3 mm³) from the region of the cardia-atrial fold were dropped onto a polished copper block prechilled with liquid nitrogen. The frozen tissue was then immediately transferred to a vial containing the substitution fluid (anhydrous acetone plus 0.05% uranyl acetate), which was maintained at −80 °C. The tissue was then freeze-substituted at −80 °C for 48 h, −40 °C for 24 h, −20 °C for 24 h, 4 °C for 4 h, and then gradually brought to room temperature. After two rinses with absolute acetone, the tissues were embedded in Spurr’s resin (Spurr, 1969).

**Immunogold labelling for localization of Plb1.** Stomach tissue was prepared for immunoelectron microscopy as previously described (Kruse & Cole, 1992). Briefly, three to four thin sections of cryofixed, infected stomach tissue were mounted on a Formvar-coated nickel grid. A total of 15 grids containing about 45–60 thin sections from each group were used for immunological labelling. The grids were floated on 10% ovalbumin in 20 mM Tris/HCl (pH 7.4) for 10 min. The grids were then transferred to droplets of the guinea pig anti-Plb1 primary antibody (diluted 1:500) prepared in 1% ovalbumin in Tris/HCl buffer. Control sections were incubated in preimmune guinea pig serum diluted as above. Sections were incubated for 48 h at 4 °C. The grids were rinsed with buffer and incubated on droplets of goat anti-guinea pig secondary antibody conjugated with colloidal gold (20 nm diameter). The secondary antibody was prepared in Tris/HCl buffer (pH 8.2) and diluted 1:20 in 1% ovalbumin. The grids were finally washed in buffer followed by distilled water, and then stained with uranyl acetate and lead citrate. The specimens were examined with a transmission electron microscope as described above.

**Statistical analysis.** The numbers of c.f.u. per organ were expressed on a log scale. Because these values did not fall into normal distribution, the Mann–Whitney U test was used to compare medians. Statistical comparisons were performed using the StatView version 4.5 software package for Windows 95, or SPSS version 9.0 software package for Windows.

**RESULTS**

The strategy followed for the reintroduction of the *PLB1* gene into the mutant PLB1A² strain is shown in Fig. 1(a). Spontaneous recombination between the chromosomal DNA and the BglIII-linearized integration plasmid (pPMB3) is expected to result in the formation of the revertant strain PMY106, which contains both the *PLB1* and *URA3* genes.

**Reintroduction of the functional *PLB1* gene**

Strain PMY53 (the Ura⁻ derivative of the *plb1* null mutant) was selected as the recipient for transformation with the BglIII-digested integration plasmid pPMB3 containing a functional *PLB1* gene. Growth and morphology of the resulting transformants were compared and found to be similar (data not shown): one of these transformants, PMY106, was selected as the representative revertant strain and further analysed. PCR analysis was performed to determine the correct insertion of the functional *PLB1* gene into PMY53. Total chromosomal DNA was isolated from PMY106 and subjected to PCR using a *PLB1*-specific 751 bp region as template. Fig. 1(b) shows that the revertant (PMY106) and...
monitored for 21 d. checked twice daily for dead or moribund mice. Survival was

To confirm the reintroduction of the functional PLB1 gene into the mutant strain, total chromosomal DNA isolated from the transformant PMY106 was analysed by Southern blotting using a C. albicans PLB1-specific, as well as a bacterial hisG-specific probe. Fig. 1(c) shows that the 3·6 kb band corresponding to the functional PLB1 gene present in the parent SC5314 and CAI-4 strains is also detected in PMY106, thus proving the introduction of functional PLB1 in the transformant. To make sure that PMY106 was not a contaminant strain, total chromosomal DNA from the revertant strain was digested with KpnI, and hybridized with a hisG-specific probe. An 8 kb band representing the URA3–hisG–Δplb1::hisG fragment and a 4·7 kb band representing the Aplb1::hisG fragment were detected (Fig. 1d). The parent strains (SC5314 and CAI-4) did not show any detectable fragment, which was expected since the probe used (hisG) represents a bacterial sequence. These results clearly show that the PLB1 gene was successfully reintroduced into the mutant strain.

Western blot analysis of the culture supernatant from the revertant, mutant and parental strains was performed to detect the Plb1 protein secreted by these strains. Plb1p was detected in both the parental and revertant strains, but not in the mutant strain (data not shown). Additionally, the relative levels of Plb1 enzyme activity in culture supernatant from the parental, revertant and mutant strains were 100%, 98% and 1%, respectively, as determined by the colorimetric free fatty acid assay procedure. This indicated that the enzyme activity in the revertant strain was similar to that of the parental strain.

Reintroduction of PLB1 restores candidal virulence (haematogenously disseminated model)

A murine model of haematogenously disseminated candidiasis was employed to evaluate the effect of PLB1 reintroduction on virulence. All mice injected with either the wild-type strain SC5314 or the revertant PMY106 strain succumbed to candidal infection within 10 d (Fig. 2). In contrast, 80% of the mice injected with PLB1A2 were alive at day 10. The mean survival time (± sd) for mice infected with SC5314 was 5·30 ± 0·790 d, while that for mice infected with the revertant strain was 6·30 ± 0·59 d. In contrast, the mean survival time for mice infected with the plb1-disrupted mutant was 15·80 ± 1·27 d. Statistical comparisons of the survival curves by Logrank (Mantel–Cox) analysis revealed that survival of mice infected with strain SC5314 did not differ significantly from that of mice infected with PMY106 (P = 0·48). In contrast, mice infected with the PLB1A2 mutant strain survived significantly longer than mice infected with either SC5314 or PMY106 (P <

Fig. 2. Survival of mice following experimentally induced candidiasis (haematogenously disseminated model). The virulence of the revertant (×, PMY106) strain was compared with the parental (○, SC5314) and plb1 null mutant (□, PLB1A2) strains in an intravenous murine model of haematogenously disseminated candidiasis. BALB/c mice (10 per group) were infected via the lateral tail vein with 5 × 10⁵ blastospores of the respective C. albicans strain. Cages were checked twice daily for dead or moribund mice. Survival was monitored for 21 d.

Fig. 3. Box plot representation of the number of c.f.u. detected in the stomach and liver of immunocompromised mice (oral–intragastric model). Mice were challenged by the oral–intragastric route with the parental, revertant or Plb1-deficient strains of C. albicans. The boxes indicate the 25th and 75th percentiles, and the bars show the 5th and 95th percentiles. The line within the box indicates the median.
PLB1 reintroduction restores candidal virulence

Fig. 4. Histological comparison of the invasiveness of the parental and PLB1-deficient strains of *C. albicans*. (a) PAS-stained section of the stomach of a non-infected, 4-week-old mouse in the region of the cardial-atrium fold (ge, glandular epithelium; ke, keratinized epithelium; L, lumen). (b) Section of the cardial-atrium fold of a mouse infected with the parental strain showing the extensive invasion of hyphal and yeast elements within the mucosal (M) and submucosal (SM) layers. (c) Blood vessel in the submucosal region of the stomach shown in (b) which reveals a hyphal element (arrow) that has crossed the endothelial layer. (d) Same region of the murine stomach as (b) but infected with the PLB1-deficient *C. albicans* strain showing superficial and less abundant hyphal elements. Bars, 200 µm (a) and 100 µm (b, c, d).

0·0001 for both comparisons). Candidal c.f.u. were recovered from the kidneys of mice challenged with either the parental (SC5314) or revertant (PMY106) strain. No significant difference was found (*P* = 0·54) between c.f.u. count for mice challenged with the revertant strain [1·3 ± 0·12 × 10⁶ c.f.u. (g tissue)⁻¹] and those challenged with the parental strain [1·2 ± 0·28 × 10⁶ c.f.u. (g tissue)⁻¹]. Strain PMY106 recovered from the kidneys of infected mice was incubated in serum for 2 h, and no difference was found in its ability to form germ tubes, as compared to SC5314 (data not shown). Revertant and wild-type strains
Fig. 5. For legend see facing page.
passed through mice were further analysed by Southern hybridization, which confirmed that passage through animals had no effect on the genetic composition of these strains (data not shown). These results show that the reintroduction of the PLB1 gene restores the virulence properties of C. albicans in the haematogenously disseminated model of candidiasis.

**Fungal colonization of stomach and liver (oral–intragastic model)**

The numbers of C. albicans c.f.u. recovered from the stomach and liver of mice challenged with the parental, plb1 null mutant or revertant strains were compared. c.f.u. counts of the three strains in the stomachs of infected mice were not significantly different, as revealed by the box plots in Fig. 3. However, the c.f.u. in the liver were significantly greater in mice challenged with the parental (P = 0.0001) or revertant (P = 0.006) strains compared to mice challenged with the plb1 null mutant. In addition, the number of mice that developed systemic candidiasis infection differed markedly following challenge with the three strains. Ninety per cent and 70 % of mice challenged with the parental or revertant strains exhibited liver colonization, respectively. In contrast, only 45 % of mice infected with the plb1 null mutant exhibited liver involvement, and low numbers of c.f.u. were recovered from organ homogenates from these mice.

**Candidal invasion of gastric mucosa**

The histopathological appearance and localization of C. albicans in the immunocompromised mice challenged with the parental, revertant and mutant strains showed marked differences. The focus of these studies was the cardial-atrium fold of the stomach (Fig. 4a), where C. albicans is known to concentrate in mice after oral–intragastic challenge (Cole et al., 1990). In PAS-stained sections of the stomach of mice infected with the parental strain, both hyphal and yeast elements occurred in large numbers in the gastric mucosa and submucosa (Fig. 4b). Hyphal elements, which had apparently penetrated the endothelial layer of blood vessels, were frequently found (Fig. 4c). Similar observations were recorded for mice challenged with the revertant strain (data not shown). In contrast, hyphae of the plb1 null mutant appeared to be restricted to the lumen and exposed epithelial surface of the gastric mucosa (Fig. 4d), and were very rarely observed deeper in the submucosa.

Thin sections of the stomach of mice in the region of the cardial-atrium fold which were infected with the parental strain of C. albicans consistently revealed invasive hyphae and yeast cells associated with necrotic host tissue (Fig. 5a). In sharp contrast, most hyphae of the plb1 null mutant were associated with the keratinized layer in this same region of the murine stomach, and apparently were unable to penetrate into the gastric submucosa tissue layers (Fig. 5b). Thin sections of the stomach of mice infected with the revertant strain (Fig. 5c) showed essentially the same morphological features as the gastric tissue of mice infected with the parental strain.

**Phospholipase B expression in vivo**

Expression of Plb1p during the course of infection in the stomach was investigated using indirect immunofluorescence as well as immunoelectron microscopy. Guinea pig antiserum raised against the purified Plb1 protein reacted with hyphal and yeast cell walls of the parental strain in vivo, as revealed by immunofluorescence (Fig. 6a) as well as by immunoelectron microscopy (Fig. 6b). The plb1 null mutant, on the other hand, showed little to no label (Fig. 6c, d). The intensity of label in the walls of hyphae and yeast cells of the revertant strain was comparable to that of the parental strain (Fig. 6e, f). Control sections incubated with preimmune serum followed by secondary antibody did not produce any detectable fluorescence (Fig. 6g) or immunogold label (Fig. 6h).

**DISCUSSION**

The results of this study provide further evidence in support of Plb1 as a virulence determinant for C. albicans. We constructed a PLB1 revertant strain by reintroducing the PLB1 gene into the null mutant (PLB1A2), and showed that the revertant strain had similar growth, morphology and Plb1p secretion properties to the parental SC5314 strain. Successful construction of the revertant strain provided us with a set of isogenic strains differing only at the PLB1 locus. Using this set of isogenic strains in a haematogenously disseminated murine model of candidiasis, we showed that the revertant strain had similar virulence to the parental strain, but significantly greater virulence than the null mutant. Additionally, employing an oral–intragastic infant mouse model of disseminated candidiasis, we showed that the revertant strain is similar to SC5314 in its efficiency of invading the GI tract and colonizing the host liver. In contrast, the plb1 null mutant was much less efficient at invading and colonizing host tissues, compared to the SC5314 or revertant strains. These findings from two different animal models of disseminated candidiasis are in agreement with our previous studies in which disruption of the PLB1 gene led to the attenuation of candidal virulence in a murine model of candidiasis (Leidich et
Fig. 6. For legend see facing page.
The finding that the revertant strain can restore virulence in *C. albicans* to near wild-type levels has also been observed in other studies. Thus, the pathogenicity of *C. albicans* was restored to a near wild-type degree upon reintroduction of the VPS34 (vacuolar protein sorting) gene into the corresponding null mutant (Bruckmann et al., 2000). Additionally, a *Klaac* null mutant of the fungus *Kluyveromyces lactis* was functionally complemented by the introduction of a single copy of the *Saccharomyces cerevisiae* AAC1 (ADP/ATP carrier) gene (Viola et al., 1999). In another study, the cgf1/CGT1 (*C. albicans* mRNA 5'-guanylyltransferase) heterozygote was found to be equally virulent for mice and guinea pigs when tested in an intravenous infection model of disseminated candidiasis (De Backer et al., 2000). Thus, at least some genes in *C. albicans* do not appear to be dose-dependent in expressing their phenotypic characteristics.

The mechanism/s by which Plb1p contributes to virulence is not well understood, but probably involves direct damage or degradation of host cell membranes. Such host cell injury would be expected to facilitate candidal penetration. In this context, Klotz et al. (1983) used an *in vitro* model to depict the earliest events of metastatic *C. albicans* infection and showed that the pathogen first adheres to, and then penetrates the endothelium. During transmigration, endothelial cell continuity was found to be disrupted by the yeasts. As destruction of the endothelium progressed, the fungus penetrated deeper into the substance of the vascular tissue. These authors attributed the dissolution of a portion of the endothelial cells to phospholipase activity. In another study, Pugh & Cawson (1977) reported that phospholipase activity is generally localized at the tips of developing hyphae, i.e. in the direction of candidal penetration. Consistent with these findings, our immunolabelling studies showed that Plb1 localized mostly to the hyphal tips. Although the cell wall of *plb1* null mutant cells reacted slightly with the immunolabel, the intensity of the signal was much lower than that of the parental and revertant strains. The low level of reactivity may be due to non-specific binding or the presence of another minor phospholipase with homology to Plb1p. In this regard, we reported the cloning of caPLB2, the second PLB gene in *C. albicans* (Sugiyama et al., 1999).

Our results suggest that Plb1 may directly degrade the phospholipid constituents of host cell membranes. Such injury to the protective cell membrane may provide fungal hyphae with rapid access to the cytoplasm. Similar evidence for enzymic activity by *C. albicans* in the penetration of glossal epithelium in both rats (Howlett & Squier, 1980) and humans (Montes & Wilborn, 1968) has been reported, and studies have been performed that document this process in murine cervical tissue and skin (Scherwitz, 1982). We previously reported a significant difference in the ability of parental and Plb1-deficient strains to penetrate endothelial and epithelial cells *in vitro* (Leidich et al., 1998). The wild-type parental strain penetrated at twice the rate of the Plb1-deficient strain. However, that study did not include the revertant strain, which was necessary in order to unequivocally ascertain the role of Plb1 in candidal virulence, according to Molecular Koch’s Postulates (Falkow, 1988). The successful construction of the revertant and analysis of its virulence in two different animal models enabled us to satisfy Molecular Koch’s Postulates and conclusively prove that Plb1p is an important virulence factor in disseminated candidiasis. In the present study, both the parental and the revertant strains penetrated deep into the gastric mucosal and submucosal tissues. In contrast, the Plb1-deficient mutant was not as invasive and was generally sequestered to the stomach lumen. The invasiveness of the parental and revertant strains increased their access to the gastric vasculature, thus allowing the organisms to haematogenously disseminate in the bloodstream more efficiently than the Plb1-deficient strain. Consistent with this interpretation, hyphal elements were observed in blood vessel lumens following challenge with the parental or revertant strains, as compared to the mutant. These differences in penetration and dissemination were reflected by the number of candidal c.f.u. recovered from the liver of mice infected with the parental, revertant or Plb1-deficient strains. c.f.u. counts in livers isolated from mice infected with the revertant strain were similar to those obtained from mice infected with the parental strain. In contrast, c.f.u. counts obtained in livers isolated from mice infected with the revertant or parental strain were significantly higher than those found in liver harvested from mice infected with the Plb1-deficient mutant. Similar observations were reported earlier using genetically unrelated strains, which differed in their ability to secrete phospholipase (Barrett-Bee et al., 1985; Ibrahim et al., 1995). The results of our study, using a set of isogenic strains, suggest a possible role for Plb1 in the transmigration of *C. albicans* across the GI tract, and its ability to cause systemic candidiasis.

Our results show that deleting the PLB1 gene attenuates candidal virulence and reduces the extent of candidal infection in two clinically relevant murine models (intravenous and oral–intra gastric models), although this targeted disruption does not render the strains completely avirulent. The fact that this attenuation in virulence is the result of deletion of the PLB1 gene is borne out by our results, which show that when the functional PLB1 gene is reintroduced into the null
mutant, the strain (revertant) regains virulence properties. The PLB1\# strain retains the ability to produce low-grade infections, which is expected because C. albicans virulence is believed to be multifactorial. Other putative virulence factors for C. albicans have been the subject of several gene disruption studies. Targeted deletion of genes encoding such factors does not always result in complete avirulence, suggesting that more than one factor may be required for infection. For example, disruption of INT1 supports this concept since int1 null mutants have a dual phenotype (loss of adherence and germination), both of which have been implicated in candidal virulence (Gale et al., 1998). The int1 null mutants are essentially avirulent (90% reduction in mouse mortality). Further investigations regarding the mechanism/s of PLB1 action and how this secreted enzyme relates to other virulence factors will provide important clues about the pathobiology of C. albicans. Uncovering the key components and steps involved in the infectious process of C. albicans should provide an impetus for the development of new antifungal agents and improved therapeutic modalities for efficient treatment of disseminated candidiasis.

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