Differential expression of *Candida albicans* phospholipase B (*PLB1*) under various environmental and physiological conditions

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Candida is the fourth most common organism responsible for bloodstream infections in many intensive care units, with *Candida albicans* being the most predominant species isolated in such cases. It has previously been shown that candidal phospholipase B, encoded by the *PLB1* gene, is an important virulence factor for *C. albicans* pathogenesis. In this study, the effects of environmental factors (carbohydrate source and pH) and physiological conditions (serum, phospholipids and temperature) on the expression of *PLB1* by *C. albicans* cells grown in rich [Sabouraud dextrose broth (SB) or yeast extract/peptone/dextrose] or chemically defined [Lee’s, RPMI-1640 or yeast nitrogen base (YNB)] media were investigated. Northern blot analyses revealed that *PLB1* mRNA was expressed in *C. albicans* cells grown in rich media at 30 °C but not at 37 °C. However, the protein Plb1p was detected in fungal cells growing at 37 °C in SB, as determined by Western blot analysis, indicating that although the mRNA for this gene was not detected, the actual gene product was present at this temperature. Expression of *PLB1* was detected in cells grown in YNB/glucose at 30 °C but not at 37 °C. However, growth of *C. albicans* in YNB/glucose supplemented with serum and phospholipids resulted in expression of *PLB1* at 37 °C also. Additionally, acidic pH induced higher levels of *PLB1* mRNA expression compared to neutral pH, while the morphological form of *C. albicans* did not have any influence on the expression of this gene. The studies described here show that the expression of *PLB1* is regulated by nutritional supplementation, environmental factors and the growth phase of the *C. albicans* cells, as well as by physiological conditions. The differential expression of *PLB1* in response to environmental factors may be correlated to host-specific components available to *C. albicans* during infection.

**INTRODUCTION**

The incidence of *Candida* bloodstream infections has increased significantly in the last two decades (Wey *et al.*, 1988; Beck-Sagué & Jarvis, 1993; Stamos & Rowley, 1995; Colombo *et al.*, 1999). A prospective, active population-based survey for candidemia in two cities in the USA showed that the rate of occurrence of this disease is higher than that of various serious invasive bacterial or fungal infections, such as invasive meningococcal, group B streptococcal or cryptococcal diseases (Kao *et al.*, 1999). Mortality rates among candidemic immunocompromised patients have been estimated to be between 38 and 50 %, even with therapy (Wey *et al.*, 1988; Espinel-Ingroff, 1997). One study showed that candidal infections are associated with the highest crude morality compared to other nosocomial bloodstream pathogens (Edmond *et al.*, 1999).

Several virulence factors potentially representing molecular targets for new anti-*Candida* drugs have been proposed for *Candida albicans* infections, which include extracellular phospholipases, secretory aspartic proteinases (Saps), adhesion proteins and proteins involved in germ-tube formation (Cutler, 1991; Fallon *et al.*, 1997; De Bernardis *et al.*, 1999; Ghannoum, 2000; Calderone *et al.*, 2000; Mukherjee & Ghannoum, 2001; Asleson *et al.*, 2001; Bahn & Sundstrom, 2001). Secretion and activity of candidal Saps have been shown to be influenced by environmental factors including pH, growth media, morphology, nutrients and substrate concentration (Tsuboi *et al.*, 1989; Hube *et al.*, 1994; Wu & Samaranayake, 1999; De Bernardis *et al.*, 1999; Naglik *et al.*, 1999).

We have previously shown that *C. albicans* strains exhibiting increased phospholipase B activity are associated with

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**Abbreviations:** SB, Sabouraud dextrose broth; YPD, yeast extract/peptone/dextrose; YNB, yeast nitrogen base.
higher virulence in murine models of disseminated candidiasis (Ibrahim et al., 1995). We have also cloned and disrupted PLB1, the gene encoding C. albicans phospholipase B, and shown that the Δplb1 null-mutant has significantly attenuated virulence compared to the isogenic parental strain when tested in a murine model of haematogenously disseminated candidiasis (Leidich et al., 1998). The reintroduction of a functional PLB1 gene into the Δplb1 null-mutant restored virulence to levels similar to those observed for the parental strain, in both haematogenously disseminated and oral–intragastric infant mouse models of candidiasis (Mukherjee et al., 2001). These studies showed that candidal Plb1p is an important candidal virulence factor.

To develop a detailed picture of the multifactorial phenomenon of Candida pathogenesis, in this study we analysed the factors affecting the expression of the C. albicans PLB1 gene and the production of the corresponding protein. Our results showed that PLB1 mRNA was detected in C. albicans grown in rich media only at 30 °C, while the corresponding protein was present at 30 and 37 °C. Growth in chemically defined media failed to induce the expression of PLB1 mRNA at either temperature. However, different environmental conditions induced this gene in C. albicans grown in yeast nitrogen base (YNB). Supplementation of YNB with glucose, serum, and a mixture of phospholipids was essential to induce PLB1 expression in C. albicans at 37 °C. Additionally, acidic pH induced higher levels of PLB1 mRNA expression compared to neutral pH, while the morphological form of C. albicans did not have any influence on the expression of this gene. These results indicate that PLB1 is differentially expressed in C. albicans under varying environmental and physiologically relevant conditions.

METHODS

Strains and culture medium. C. albicans 16240 was used in this study; it was a clinical isolate with high virulence and high phospholipase activity. This strain was maintained on yeast extract/peptone/ dextrose (YPD) agar (Difco Laboratories) and subcultured onto fresh medium when needed.

Reagents. Restriction endonucleases and Taq DNA polymerase (Klenow fragment) were purchased from Roche Molecular Biochemicals. Oligonucleotide DNA primers were custom-ordered from BioSynthesis (Lewisville, TX, USA). Glass beads (25–600 μm diameter), used for total chromosomal DNA extraction, and all other chemicals were purchased from Sigma.

Growth conditions. C. albicans cells were grown in rich or defined media under different nutrient and environmental conditions. The rich media used for cell growth were Sabouraud dextrose broth (SB; 1 % yeast extract, 1 % polypeptone, 4 % glucose) and YPD (1 % yeast extract, 2 % peptone, 2 % dextrose). The chemically defined media used were Lee’s medium (Lee et al., 1975), RPMI-1640 (Cellgro MediaTech) and YNB (Difco Laboratories). Media were supplemented as required with glucose, mannose, galactose, serum, phosphatidylethanolamine (PE), phosphatidylcholine (PC), phosphatidylinositol (PI) or a phospholipid mixture (PE:PC:PI, 1:2:1). C. albicans cells (6×10⁶ cells ml⁻¹) were grown with shaking (200 r.p.m.) up to mid-exponential phase (7 h), at which time the cells were harvested for RNA extraction. C. albicans cell morphology under each growth condition was monitored microscopically at the end of the incubation period.

PCR amplification of the PLB1-specific region. Total chromosomal DNA isolated from C. albicans cells was subjected to PCR amplification with oligonucleotide primers targeting a 751 bp region representing the 5’ half of the PLB1 gene, as described previously (Mukherjee et al., 2001). The oligonucleotide primers were 5’-ATGATTTTGCATATTGG-3’ (forward) and 5’-AGTATCCTGGACCGTCACC-3’ (reverse). PCR amplification reaction mixtures consisted of 100 μl volumes containing 10 mM Tris/HCl (pH 8.4), 50 mM KCl, 1·5 mM MgCl₂, dNTPs (1·0 mM each dNTP), oligonucleotide primers (1 μM each), template DNA (10–100 ng) and 2 U Taq DNA polymerase. The PCR conditions used were an 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), with a final extension (72 °C, 10 min). Reaction mixtures were analysed for the presence of the 751 bp DNA fragment on 1-2 % agarose gels stained with ethidium bromide.

Northern blot analyses. Total RNA was extracted from C. albicans cells as described by Collart & Oliviero (1993). Briefly, 2 mg harvested cells were resuspended in 1·0 ml buffer and lysed by vortexing with 0·5 mm diameter glass beads; the lysate was recovered by centrifugation. Total RNA was isolated from the lysate using the RNeasy Kit (Qiagen) according to manufacturer’s instructions. Ten micrograms of the isolated total RNA were electrophoresed through 1 % agarose/formaldehyde gels with circulating buffer and then transferred to nylon membranes (Boehringer Manheim). The transferred RNA was cross-linked to the membrane using a UV Stratalinker 228 (Stratagene), pre-hybridized for 1 h and then hybridized overnight with the PLB1-specific PCR-amplified 751 bp probe at 42 °C. After stringency washes with 0·2×SSC, the hybridized RNA signals were detected by autoradiography.

Western blot analyses. Immunodetection of the Plb1p protein secreted by C. albicans cells into the media was performed by Western blot analyses as described previously (Leidich et al., 1998; Mukherjee et al., 2001). Briefly, extracellular proteins secreted by the C. albicans cells were concentrated using a Millipore Ultrafilter centrifugation column (molecular mass cut-off, 10000 Da). Ten micrograms of total protein were separated on a 4–12 % polyacrylamide gel by SDS-PAGE and transferred to a nitrocellulose membrane using a SemiDry TransBlot apparatus (Bio-Rad) according to manufacturer’s instructions. The transferred blots were incubated with 5 % non-fat dry milk, and probed with an anti-Plb1p antibody. Plb1p on the nitrocellulose membrane was detected using goat-anti-rabbit IgG as the secondary antibody (1:1000 dilution) and a chemiluminescence assay (ECL Amersham) according to the manufacturer’s instructions.

RESULTS

Expression of PLB1 mRNA in C. albicans is induced in a temperature-specific manner in rich media but not in chemically defined media

To determine the effect of different media on PLB1 expression in C. albicans, fungal cells were grown in rich (SB and YPD) or chemically defined (Lee’s, RPMI-1640 and YNB) media under different temperature conditions (25, 30 or 37 °C). Total RNA was isolated from these fungal cells and subjected to Northern blot analyses using a PLB1-specific probe. PLB1 mRNA was detected in cells grown in rich
Regulation of C. albicans PLB1 expression

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**Fig. 1.** Effects of rich and defined media on the expression of PLB1 in C. albicans grown under different environmental conditions. Total RNA was extracted from C. albicans cells grown in SB, YPD, Lee’s, RPMI-1640 or YNB media at different pH and temperature conditions. Extracted RNA was analysed by Northern blot using a PLB1-specific PCR-generated probe as described in Methods. Actin expression was used as a control for total RNA loading. Expression of PLB1 was normalized against the levels of actin mRNA.

media (SB or YPD) at 30 °C, with 1.5-fold more detected in YPD as compared to cells grown in SB (Fig. 1). In contrast, growth in chemically defined media (Lee’s, RPMI-1640 or YNB) did not induce expression of PLB1, irrespective of the growth temperature (Fig. 1). The morphology of the fungal organism did not influence PLB1 expression, since the yeast and hyphal forms of C. albicans exhibited similar patterns of expression for this gene (data not shown). These results indicate that growth medium and temperature influence the expression of PLB1.

**PLB1 expression in C. albicans cells grown in rich media is influenced by extracellular carbohydrates and pH**

To determine the factors needed to induce in vitro expression of PLB1 in rich medium at 30 and 37 °C, fungal cells were grown in SB at these temperatures under conditions of different carbohydrate source and pH (Fig. 2). Northern blot analyses of total RNA isolated from these fungal cells revealed that supplementation of SB with 2 % (SB/2G) or 4 % (SB/4G) glucose led to expression of PLB1 (Fig. 2), while supplementation with 0-5 % glucose failed to induce expression of this gene (Fig. 2, lanes 1–3). Additionally, PLB1 expression in SB/4G was higher than in SB/2G, indicating that expression of this gene is dependent on the carbohydrate concentration (Fig. 2, lanes 2 and 3). To test whether the carbohydrate source influences PLB1 expression, we investigated PLB1 expression in C. albicans grown in SB supplemented with 4 % (w/v) glucose, mannose or galactose. Exogenously added mannose induced PLB1 mRNA expression in C. albicans, albeit to a lower level than glucose (Fig. 2, lanes 3 and 11). In contrast, galactose supplementation did not induce PLB1 expression (data not shown). Induction of PLB1 was detected in exponential phase C. albicans cells but not in stationary phase C. albicans cells (data not shown). Because extracellular pH is a known virulence factor of C. albicans (Ghannoum *et al*., 1995; De Bernardis *et al*., 1998; Ramon *et al*., 1999), we studied the effects of acidic and neutral pH conditions on PLB1 expression at 30 °C. Induction of PLB1 mRNA was dependent on the pH of the growth media, with highest PLB1 expression noted at pH 5.9 (Fig. 2). The following expression pattern was observed: pH 5.9 > pH 4.5 > pH 7.2 (Fig. 2, lanes 3, 4 and 5, respectively). These studies indicated that the expression of PLB1 in C. albicans grown in rich medium is dependent on both the pH and the concentration of glucose. Additionally, pH 5.9 was found to be optimal for PLB1 expression.

**Effects of physiological factors on PLB1 expression at 37 °C in rich medium**

Since supplementation of SB with a high glucose concentration or changes in the pH failed to induce PLB1 expression in vitro at 37 °C, we investigated whether the addition of host factors such as serum or phospholipids to the growth medium induces expression of PLB1 at this physiologically relevant temperature. Supplementation of rich medium (SB/4G) with 2 % serum induced PLB1 expression at 30 °C but not at 37 °C (Fig. 2). Similar to serum, the addition of 20 µg phospholipid mixture ml⁻¹ led to the expression of PLB1 at 30 °C (Fig. 2) but not at 37 °C (data not shown). Supplementing the rich medium with serum and the phospholipid mixture simultaneously did not induce PLB1 expression at 37 °C (Fig. 2).

**Plb1p is secreted at 37 °C in rich media**

Expression of mRNA is not always correlated at the protein level in yeasts (Gygi *et al*., 1999). Since our results indicated that PLB1 mRNA was not detected at 37 °C in C. albicans cells grown in rich medium under different conditions, we decided to investigate whether this observation was supported at the protein level also. Western blot analyses of proteins secreted by C. albicans under different conditions revealed that Plb1 was produced at 37 °C in C. albicans grown in rich media (SB and YPD) supplemented with 0.5, 2 or 4 % glucose (Fig. 3). Thus, although PLB1 mRNA was not detected at 37 °C, the corresponding protein was present...
at this temperature, suggesting the possibility that the mRNA may be unstable or expressed at an earlier stage of growth.

**PLB1 mRNA is detected in C. albicans cells grown at 37 °C in physiologically relevant conditions**

Rich media such as YPD and SB have complex compositions, which make it difficult to determine the role of individual components in specific processes. Therefore, we selected YNB (a chemically defined medium) to study the effect of serum and phospholipids (two physiologically relevant host factors) on PLB1 expression in *C. albicans* grown *in vitro* at different temperatures. Growth of *C. albicans* cells at 30 °C in YNB supplemented with 0-5 % glucose (YNB/0-5G) did not result in the induction of PLB1 (Fig. 4). However, supplementation of YNB/0-5G with 2 % serum or 20 µg phospholipid mixture ml⁻¹ induced PLB1 expression at 30 °C (Fig. 4). The level of PLB1 mRNA expression induced by serum was less than that induced by the phospholipid mixture (Fig. 4). Addition of individual phospholipids (phosphatidylcholine, phosphatidylethanolamine or phosphatidylinositol) did not induce PLB1 expression at either temperature (data not shown). In contrast, growth of *C. albicans* cells at 37 °C in YNB/0-5G supplemented with 2 % serum and 20 µg phospholipid mixture ml⁻¹ induced PLB1 expression (Fig. 4). These studies indicated that PLB1 is expressed at 30 °C in cells grown in YNB supplemented with serum or phospholipids, while both serum and the phospholipid mixture are essential to induce expression of this gene at 37 °C.

**DISCUSSION**

*Candida* virulence is known to be influenced by environmental factors including pH, temperature and nutrient supplementation (Hazan & Hazen, 1987; Tsuboi *et al*., 1989; Ramon *et al*., 1999; Brown *et al*., 1999; Ernst, 2000; Lane *et al*., 2001; Staib *et al*., 2001). In the current study, we investigated the role of environmental and physiological parameters in regulating the expression of candidal phospholipase B using Northern and Western blot analyses. Growth of *C. albicans* in SB induced PLB1 mRNA expression at 30 °C but not at 37 °C. However, Western blot analysis revealed that the Plb1p protein was present at 37 °C. The observed discrepancy between mRNA and protein analysis
could be because Northern blotting revealed the level of PLB1 mRNA in C. albicans cells at the time point (7 h, mid exponential phase) when RNA was extracted and, hence, was dependent on the growth stage of the culture. In contrast, Western blotting determined the total amount of Plb1p synthesized up to the same time point when the protein was extracted. Thus, it is likely that PLB1 mRNA is unstable under rich conditions at 37˚C making it undetectable, while Plb1p is stable at this temperature and detectable by Western blot analysis. Similar observations have been reported previously for mRNA and protein expression of secretory aspartic proteinases (SAPS) under different environmental conditions (White & Agabian, 1995). Additionally, it is possible that PLB1 mRNA is not expressed at 7 h of growth – the stage when total RNA was isolated from C. albicans in our studies. In this regard, it has been shown previously that C. albicans grown for 3 h in YPD at 37˚C expresses reduced levels of PLB1 mRNA as compared to cells grown at 25˚C (Hoover et al., 1998). Therefore, the expression of PLB1 is likely to be regulated by both the growth phase of C. albicans and the type of growth medium.

Expression of yeast genes at the mRNA level is not always correlated with the amount of the translated functional protein. A study to determine the relationship between mRNA and protein levels for selected genes expressed in the yeast Saccharomyces cerevisiae reported that expression levels of proteins coded by mRNA with comparable abundance varied by up to 30-fold, while mRNA levels coding for proteins with comparable expression levels varied by as much as 20-fold (Gygi et al., 1999). Thus, the inability to detect PLB1 mRNA at 37˚C does not necessarily indicate the absence of the gene product. Indeed, Western blot analysis confirmed this contention since the Plb1p protein was present at 37˚C in rich medium – conditions in which the corresponding mRNA was not detected. Previously, using Western blot analyses, we reported the extracellular secretion of Plb1p by C. albicans cells grown at 30 ˚C (Leidich et al., 1998; Mukherjee et al., 2001). Therefore, PLB1 is expressed at 30 and 37˚C, although its mRNA may not be clearly detectable at the latter temperature. Recently, it was reported that phospholipase activities in another opportunistic fungal pathogen, Cryptococcus neoformans, vary with strain type and growth temperature (Wright et al., 2002). Thus, the temperature-responsive expression of virulence factors may be a common mode of regulation in yeasts.

The role of cellular morphology in the regulation of virulence varies with each determinant. A recent study has shown that phase-specific expression of the transcription

**Fig. 3.** Western blot analysis of extracellular proteins secreted by C. albicans grown in rich media at 37˚C. Lanes 1–6 show SB or YPD media supplemented with glucose at the indicated concentrations; lane 7, Plb1p standard. Extracellular proteins secreted by the C. albicans cells were concentrated using a Millipore Ultrafree centrifugation column (molecular mass cut-off, 10 000 Da). Ten micrograms of total protein were loaded onto each lane, separated by SDS-PAGE and then probed with an anti-Plb1p antibody. Detection of the immune complex was performed using goat-anti-rabbit IgG as the secondary antibody (1:1000 dilution) and a chemiluminescence assay (ECL Amersham) according to the manufacturer's instructions. The results clearly show that Plb1p was detected at all the media and glucose supplementation conditions tested.

**Fig. 4.** Expression of PLB1 in C. albicans grown in YNB medium containing a low concentration of glucose and supplemented with serum or phospholipid mixture. Total RNA was extracted from C. albicans cells grown in YNB medium supplemented as indicated at different pH and temperature conditions. Extracted RNA was analysed by Northern blot using a PLB1-specific PCR-generated probe as described in Methods. Actin expression was used as a control for total RNA loading. Expression of PLB1 was normalized against the levels of actin mRNA.
regulator Tup1p plays an important role in the ‘white-opaque’ phenotypic switching that affects colony morphology, cellular phenotype and expression of a number of phase-specific genes and virulence traits (Zhao et al., 1997; De Bernardis et al., 1995; Muhlschlegel & Fonzi, 1997). White & Agabian (1995) have shown that specific Sap isoenzymes are produced by different cell types and that the level of SAP mRNA detected during hypha formation is low and transient, suggesting that both the yeast–hyphal transition and phenotypic switching influence Sap production. In another study, Hoover et al. (1998) reported that the level of PLB1 mRNA is higher in blastospores and pseudohyphae compared to germ-tube-forming cells of C. albicans grown to early-exponential phase (3 h) in YPD, suggesting that the expression of this gene is morphology-dependent. Our studies revealed that this morphology-dependent expression of PLB1 was not observed after the mid-exponential growth stage (7 h) of C. albicans, since varying morphologies of the fungal cells grown to 7 h did not affect PLB1 expression. The growth-phase-dependent regulation of PLB1 expression by morphogenic transition is notable since Plb1p is believed to aid in host tissue invasion by damaging host cellular membranes (Ghannoum, 2000; Mukherjee et al., 2001), and early expression of this virulence gene under physiological conditions is likely to be an advantage for C. albicans during infection.

In this study, we have shown that PLB1 expression is dependent on the pH of the growth medium. The role of pH in regulating Candida virulence factors such as germination and morphology-switching has been well documented (Brown et al., 1999; Ernst, 2000). The effect of pH on the regulation of C. albicans virulence genes has been shown for PHR1, expressed at pH 5.5 or higher, and PHR2, which is expressed at pH values less than 5.5 (Ghannoum et al., 1995; Muhlischlegel & Fonzi, 1997; De Bernardis et al., 1998). De Bernardis et al. (1998) showed that a Δphr1 null-mutant was avirulent in a mouse model of systemic candidiasis but uncompromised in its ability to cause vaginal infection in rats, while a Δphr2 null-mutant was avirulent in a vaginal-infection model but virulent in a systemic-infection model. Since the systemic pH is near neutrality and the vaginal pH is around 4.5, the studies by De Bernardis et al. (1998) suggested that the pH of the infection site regulates the expression of genes essential to survival within that niche.

Fungal cells grown in chemically defined medium expressed PLB1 mRNA at 30 °C but not at 37 °C. However, we have shown previously that Plb1p is present in the sera of mice infected with C. albicans (Mukherjee et al., 2001) and in sera from patients with systemic candidiasis (M. Hossain, unpublished data). Moreover, Ripeau et al. (2002) reported that PLB1 was expressed constitutively in a murine model of oropharyngeal candidiasis. Therefore, we reasoned that the presence of in vivo physiological factors such as serum and host phospholipids may be essential to induce PLB1 expression in C. albicans at 37 °C (Nelson, 1967; Engen & Clark, 1990; Kurumi et al., 1991). Indeed, supplementing YNB (+0.5% glucose) with serum and a mixture of phospholipids commonly found in mammalian cells (Nelson, 1967; Engen & Clark, 1990) induced PLB1 expression at 37 °C. Interestingly, supplementing the medium with either serum or the phospholipid mixture separately failed to induce PLB1 expression at the physiologically relevant temperature of 37 °C. Thus, in vitro expression of PLB1 mRNA at 37 °C can only be achieved under physiological conditions. This situation differs from that of SAP regulation, where it was reported that pH and hypha production alone are sufficient for SAP expression and that components of serum are not necessary (White & Agabian, 1995).

In conclusion, our results suggest that the expression of PLB1 by C. albicans is a complex and closely coordinated phenomenon involving multiple environmental and physiological parameters. Furthermore, expression of this gene at the physiological temperature (37 °C) necessitates the presence of additional host-associated factors including serum and phospholipids. Understanding the mechanism by which environmental stimuli exert their effect on the regulation of virulence factors is likely to aid in the development of novel antifungal agents with increased efficacy in the management of candidiasis.

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