Isolation and characterisation of the phospholipase B gene of Cryptococcus neoformans var. gattii

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Abstract

Cryptococcus neoformans var. gattii (serotypes B and C) is a human pathogen, ecologically, biochemically, clinically and genetically different from C. neoformans var. grubii (serotype A) and C. neoformans var. neoformans (serotype D). The phospholipase B (PLB1) gene from serotypes B and C was isolated and characterised. It resembled the serotype A and D genes, with an overall sequence homology of more than 85%. The respective open reading frames were 2236 bp (serotype B) and 2239 bp (serotype C) in length. Each contained six introns and encoded a 68-kDa protein destined for secretion. PLB1 was located on the second smallest chromosome in both serotypes. Gene expression, measured as mRNA, was not regulated by temperature, pH or exogenous nutrients.

Keywords: Phospholipase B gene; Serotype B; Serotype C; mRNA expression; Cryptococcus neoformans var. gattii

1. Introduction

Cryptococcus neoformans is a basidiomycetous yeast that causes potentially fatal neurological disease in humans and other animals. Three biochemically and genetically distinct varieties have been recognised [1-3]: C. neoformans var. grubii (serotype A), C. neoformans var. neoformans (serotype D) and C. neoformans var. gattii (serotypes B and C). C. neoformans var. grubii and C. neoformans var. gattii correspond to the teleomorph Filobasidiella neoformans var. neoformans and C. neoformans var. gattii corresponds to the teleomorph F. neoformans var. bacillispora. It has been suggested recently that C. neoformans var. gattii is sufficiently distinct from the other varieties to be classified as a new species, Cryptococcus bacillisporus, based on new sub-typing and sequencing data [4,5].

C. neoformans var. gattii differs from the other varieties in several respects [6]. It has a limited geographical distribution, which is generally congruent with its known ecological niche, and causes disease predominantly in immunocompetent hosts. In this group, cryptococcomas (mass lesions) in the brain and/or lung are more common. The reason for these clinical differences remains unexplained, but in some animal studies C. neoformans var. gattii was less virulent or invasive than the other varieties [7]. Despite its recognition as a primary human pathogen, the study of C. neoformans var. gattii, particularly at a molecular level, has attracted relatively little attention.

Fungal phospholipases, like proteases, are potential determinants of tissue invasion [8,9]. A protein with phospholipase B (PLB), lysophospholipase (LPL) and lyso-phospholipase transacylase (LPTA) activities (collectively referred to as ‘PLB’ in this paper) has recently been purified from C. neoformans var. grubii [10]. A single-copy gene (PLBI) encoding for all three activities was subsequently sequenced from C. neoformans var. grubii strain H99 and proven to be a virulence determinant by targeted gene disruption and reconstitution experiments in mice and rabbits [11]. Phospholipases may enhance cryptococcal virulence by hydrolysing phospholipids in the membrane lipid bilayer of epithelial and endothelial cells. Phospholipids in mammalian cell membranes and in lung surfactant are preferred substrates for secreted PLB from...
C. neoformans and hence are a potential site of membrane attack [12,13].

Recent work in our department has identified PLB, LPL and LPTA activities in culture supernatants of C. neoformans var. gattii. The specific activity of this enzyme was less than that of the enzyme secreted by C. neoformans var. grubii [14], suggesting that gene expression, protein secretion or enzyme activity of the gene product differs between the two varieties. The expression of other cryptococcal virulence factors, such as capsule and melanin production, are regulated by environmental cues [15]. However, Cox et al. observed no change in the level of PLBI expression between cells of the serotype A strain H99 when cultured at 30°C or 37°C [11]. The regulation of PLBI expression by other factors such as growth conditions and pH has not been studied in either C. neoformans var. grubii or C. neoformans var. gattii.

This report describes the isolation, sequencing, characterisation and determination of the chromosomal location of PLBI in C. neoformans var. gattii, serotypes B and C. The effects of selected environmental conditions on PLBI expression in C. neoformans var. gattii are also described.

2. Materials and methods

2.1. Strains and media

The Australian environmental strain WM 276 (TCS-SC1, serotype B, MATa) and a South African clinical isolate M27055 (serotype C), a gift from Valerie Davis, South African Institute for Medical Research, Johannesburg, South Africa, were used in the isolation, localisation and expression studies of the C. neoformans var. gattii PLBI genes. The clinical strains H99 (serotype A), a gift from Prof. John Perfect, Duke University Medical Center, Durham, NC, USA and WM 629 (serotype D), a gift from Drs Brian Dwyer and Bryan Speed, Victorian Infectious Diseases Reference Laboratory, Melbourne, Australia, were used for cluster analysis and comparison in the karyotyping study. Individual isolates of C. neoformans were streaked onto Sabouraud’s agar (SAB) (10 g l⁻¹ peptone, 40 g l⁻¹ glucose, 15 g l⁻¹ agar, pH 5.5) and cultured at 30°C for 2 days. Single colonies were harvested, plated and subcultured overnight prior to each experiment. For experiments requiring cells in broth, fresh colonies were cultured overnight in yeast peptone dextrose (YPD) broth (20 g l⁻¹ n-glucose, 10 g l⁻¹ yeast extract, 20 g l⁻¹ peptone) at pH 7.0. For the gene expression experiments, cells were also cultured in yeast nitrogen broth (YNB) (1.6 g l⁻¹ Yeast Nitrogen Base without amino acids (Sigma, St. Louis, MO, USA) and 0.5% glucose) with the pH adjusted to 4.5. The growth of C. neoformans in the YNB broth with pH 4.5 has been shown to reduce the size of the capsule [16]. The effect of this growth environment on PLBI expression was, therefore, also investigated.

2.2. Construction and screening of a genomic library for C. neoformans var. gattii serotype B

Genomic DNA was extracted from strain WM 276 by mechanical disruption as previously described [17]. The DNA was purified via a cesium chloride gradient and precipitated overnight at −20°C in 96% ethanol and 0.03 vol. of 3-M sodium acetate (pH 5.2). The DNA pellet was then washed in 70% ethanol and reconstituted in sterile distilled water. The purity of the preparation was checked by spectrophotometry and electrophoresis on a 0.8% agarose gel.

The DNA was partially digested with Sau3AI and the first two bases of the overhang were filled in as recommended by Stratagene (La Jolla, CA, USA). DNA fragments ranging from 9 to 23 kb in size were separated on a 0.5% agarose gel and recovered using a Geneclean spin column (Bio101, Carlsbad, CA, USA). Size-fractionated DNA was then cloned, using the λ Fix® II/Xhol partial fill-in vector kit from Stratagene. The resulting phage library was amplified, divided into non-overlapping sections and set up in a microarray format in 10 microtitre plates. The primers PLBC1f1 and PLBC1r1 (see Table 1) were designed from a partial sequence of the C. neoformans var. grubii PLBI gene, provided by Prof. John Perfect and Dr Gary Cox, and were used to amplify a fragment of PLBI from strain WM 276. The resultant PCR amplicon showed high sequence homology to the H99 PLBI fragment. Primers CngPLBI1 and CngPLBI1R were designed from the partial serotype B (WM 276) sequence to amplify a 260-bp fragment of the gene. These primers were then used to screen the genomic library. Wells from which a PCR product of the expected size was obtained were screened by conventional plaque hybridisation, using the var. gattii-derived PLBI gene fragment as a radiolabelled probe.

2.3. Isolation and sequencing of the serotype B PLBI gene

The clone containing the PLBI gene insert was mapped by PCR using the high fidelity DNA polymerase, AmpliTaq GOLD (Applied Biosystems, Branchburg, NJ, USA) and the high fidelity/long range polymerase, PfuTurbo® (Stratagene). To sequence the 5’-end of the gene, the positive clone was used as template in a PCR reaction (using AmpliTaq GOLD) with an internal primer, PLBC1f1R, and a vector primer, T7 (see Table 1). For the 3’-end of the gene, the same strategy, using an internal (CngPLBI1) and a vector primer (T3) (see Table 1), was employed but with PfuTurbo® (Stratagene). Each PCR reaction contained 30 ng of DNA, extracted from the positive λ clone, as template along with 10 μl of 10× Pfu turbo buffer (200 mM Tris–HCl (pH 8.8), 20 mM MgSO₄, 100 mM KCl, 100 mM (NH₄)₂SO₄, 1% Triton X-100 and 1 mg ml⁻¹ nuclease-free bovine serum albumin), 12.5 μl of dNTPs (2 nmol μl⁻¹ of each nucleotide), 25 μl of CngPLBI1 (10 ng μl⁻¹), 25 μl of T3 (10 ng μl⁻¹) and 1.5 μl of PfuTurbo®

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Table 1

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<tr>
<th>Primer</th>
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<th>Length (bp)</th>
<th>Origin</th>
<th>Used for</th>
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</thead>
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<tr>
<td>PLBCm1</td>
<td>5’ TAT CCT CCC TGG CAA GTC CGC 3’</td>
<td>24</td>
<td>H99 (PLB1)</td>
<td>– partial amplification of PLB1 from serotype B</td>
</tr>
<tr>
<td>PLBCm1R</td>
<td>5’ TGG TAA CCA TGG ACG AAG CGC 3’</td>
<td>24</td>
<td>H99 (PLB1)</td>
<td>– partial amplification of PLB1 from serotype B</td>
</tr>
<tr>
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<td>5’ TAG ACT GGT TTA GCT TCT GGC GAA CG 3’</td>
<td>26</td>
<td>WM 276 (PLB1)</td>
<td>– sequencing the 5’-end of the genomic clone of PLB1 from WM 276 (serotype B)</td>
</tr>
<tr>
<td>CngPLB1R</td>
<td>5’ TTG CCC ATC TCC CAC CGC TCA GAC C 3’</td>
<td>25</td>
<td>WM 276 (PLB1)</td>
<td>– sequencing the 3’-end of the genomic clone of PLB1 from WM 276 (serotype B)</td>
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<tr>
<td>CngPLB6</td>
<td>5’ TTG ACG CGA TGG CGT TTG ACG C 3’</td>
<td>25</td>
<td>WM 276 (PLB1)</td>
<td>– isolation of the cDNA clone of PLB1 from WM 276 (serotype B)</td>
</tr>
<tr>
<td>PLBPE1</td>
<td>5’ TTA ACC AAT TTG GCT ATC AC 3’</td>
<td>26</td>
<td>WM 276 (PLB1)</td>
<td>– isolation of the cDNA clone of PLB1 from WM 276 (serotype B)</td>
</tr>
<tr>
<td>PLBDISR2</td>
<td>5’ TTC ATG TCC AAT GGG GGT CAG TTG C 3’</td>
<td>25</td>
<td>WM 276 (PLB1)</td>
<td>– generation of the PLB1 probe B</td>
</tr>
<tr>
<td>PLBDISR2R</td>
<td>5’ ATT GGT ACG GAT CAA CCA GGG AGG 3’</td>
<td>24</td>
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<tr>
<td>PLB3’R</td>
<td>5’ GCA TGC TCC ACA GTG CCT GAA TCA A 3’</td>
<td>25</td>
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<td>– generation of the PLB1 probe B</td>
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<td>T7</td>
<td>5’ GAA ATA CGA TCC ACT ATA GGG C 3’</td>
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<td>λ FIX II vector</td>
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DNA polymerase (2.5 U µl⁻¹). Cycling conditions for the amplification of the 3’-end of the gene included an initial denaturation step at 97°C for 1 min followed by 25 cycles of 95°C for 1 min, 60°C for 10 min and 72°C for 10 min and a final extension step at 72°C for 10 min. Similar parameters were used for the amplification of the 5’-end of the gene except that the annealing and extension cycling steps lasted only 2 min rather than 10. A 2.9-kb fragment was obtained from the 5’-end and a 9-kb fragment from the 3’-end. The two DNA fragments overlapped and were bi-directionally sequenced via primer walking using an Applied Biosystems Model 373 automated sequencer at the Westmead Hospital DNA sequencing facility.

2.4. Isolation and sequencing of the serotype C PLB1 gene

The PLB1 gene from the serotype C strain M27055 was amplified by PCR, using the primers PLBPE1 and PLB3’R (see Table 1) and Herculase®-enhanced DNA polymerase (Stratagene). The amplified product was sequenced as described above.

2.5. Isolation of the cDNA transcript of the PLB1 gene for the serotype B strain WM 276

A cDNA library was constructed into a λ Uni-ZAP® XR bacteriophage vector (Stratagene). Total RNA was extracted using the DRP³ tri-reagent (Progen Industries Ltd., QLD, Australia) from cells grown in YPD broth at 30°C. Following reverse transcription and cloning, DNA was extracted from the λ cDNA library using an in vivo excision protocol (Stratagene), and subjected to PCR with the primers CngPLB1 and PLBDISR2R (see Table 1). A PCR product approximately 1.5 kb in length was obtained and sequenced. The ends of the transcript, absent from the cDNA library, were isolated using 3’ and 5’-RACE and sequenced using the SMART® RACE cDNA Amplification kit from Clontech Laboratories, Inc. (Palo Alto, CA, USA). Primers were carefully chosen so that all amplicons generated included a segment of the gene where an intron had been expected in the genomic sequence. The absence of the intron following sequencing confirmed that the product was from cDNA rather than genomic DNA. The intron/exon boundaries were defined by comparing cDNA and genomic DNA sequences. All fragments were sequenced bi-directionally to avoid the introduction of PCR or sequencing artefacts in the final sequence. The peptide sequence of the gene was predicted using Edit-Seq® 4.00 and subsequently analysed using Protein® 4.00 (DNASTar Inc., Madison, WI, USA), including a hydropathy analysis according to the method of Kyte and Doolittle [18].
2.6. Sequence alignment and clustering of fungal PLB sequences

The PLB1 sequences from the serotype B strain, WM 276 (accession No. AJ238508) and serotype C strain, M27055 (accession No. AJ302038) were aligned to the serotype A PLB1 sequence from strain H99 (accession No. AF223383) and the serotype D sequence from strain B-3501 (accession No. AF238241). The following fungal PLB gene sequences were included in subsequent alignments: Candida albicans caPLB1 (U59710) and caPLB2 (AB010809), Penicillium chrysogenum PLB (X60348) (syn. Penicillium notatum), Schizosaccharomyces pombe PLB1 (Z99258, SPAC4.9A6.03c and SPAC4.9A6.04c), Torulaspora delbrueckii PLB (D32134) (syn. Saccharomyces rosei), Saccharomyces cerevisiae PLB1 (L23089) and PLB2 (AF129165), S. cerevisiae SPO1 (L39372), Neurospora crassa LPL (AF045575) and LPL2 (AF045574) and Kluyveromyces lactis PLB1 (AB014495). In the case of S. pombe, the sequence was derived from a GenBank entry based on cosmid sequencing. The sequence contained two segments with significant homology to other PLB genes and both were included in the analysis. The SPO1 gene from S. cerevisiae was included because of a reported homology with the S. cerevisiae PLB gene [19,20]. It had been included in a study of fungal phylogenetics using PLB genes which comprised relatively few species and no cryptococcal phospholipases [21]. All sequences were edited to exclude introns and any flanking, non-coding sequences, based on data from GenBank. The edited sequences were aligned using the Jotun Hein function of the MegAlign® program within the DNASTar software package (DNASTar Inc., Madison, WI, USA). Alignment of the SPO1 gene to the other fungal phospholipases revealed that it was different from the other genes and was therefore used as an outgroup in the present study. Phylogenetic analysis was performed using parsimony and the heuristic search option of the PAUP*4.0b8a program (Swoford 2020, Florida State University, Tallahassee, FL, USA). The branching was statistically evaluated using bootstrapping with 100 replicates and a 50%-majority rule consensus tree was generated.

2.7. Karyotyping and chromosomal location of the PLB1 gene

Chromosomes were isolated using a method by Halliday [22] modified from established protocols. The chromosomal location of the PLB1 gene was investigated in one strain of each serotype: A (H99), B (WM 276), C (M27055) and D (WM 629). Cells were grown to confluence on SAB plates for 48 h at 30°C. Then 500 µl of packed cells were suspended in 1 ml of SCE buffer (100 mM sodium citrate (pH 5.8), 1 M sorbitol and 10 mM EDTA (pH 8.0)), vortexed and centrifuged at 12 000 rpm for 5 min. The supernatant was discarded and the cells were again
washed in 1 ml of SCE buffer. Spheroplasts were generated by incubation at 37°C for at least 2 h in 1 ml of SCE buffer containing 20 mg of lysing enzyme (from *Trichoderma harzianum*, Sigma), centrifuged as above and washed twice in 500 μl of SCE buffer. The pellet was then resuspended in 500 μl of 1% low-melting point agarose made in 0.5×TBE buffer and cooled to 37°C. The mixture of spheroplasts and molten agarose was quickly poured into pre-chilled plug moulds and held at 4°C until solidified. The plugs were then placed in 5 ml of overlay solution (0.45 M EDTA (pH 9.0), 1% N-lauryl-sarcosine (pH 9.0), 2 mg ml⁻¹ proteinase K, 10 mM Tris–HCl (pH 8.0)), incubated at 50°C for 24 h, transferred to EPS solution (0.5 M EDTA (pH 9.0), 1 mg ml⁻¹ proteinase K and 1% N-lauryl-sarcosine (pH 9.0)) and held at 4°C until required.

For pulsed-field gel electrophoresis, the plugs were washed in 1 ml of electrophoresis running buffer (0.5×TBE buffer) and inserted in the 1.5% pulsed-field grade agarose gel (14×12.5 cm). The gel was run in 3 l of 0.5×TBE buffer in a CHEF-DR® III variable-angle pulsed-field electrophoresis system (Bio-Rad Laboratories, Hercules, CA, USA) at 3.3 V cm⁻¹ and 4°C. A switch time ramping from 100 to 300 s and a switch angle of 120° for 34 h was used, followed by 40 h with a switch time ramping from 400 to 600 s, with a switch angle of 115°. The gel was stained with ethidium bromide in 1×TBE buffer. The number of chromosomes in each karyotype was determined by the number of bands. Bands that appeared more intense than others in the same lane were considered to be doublets and were counted as two chromosomes. To identify the chromosomal location of *PLB1*, the karyotypes were transferred to a charged nylon membrane and probed overnight at 65°C. A DNA fragment, 589 bp in length, was amplified from WM 276 using the primers CngPLB6 and PLBDist2R, and labelled using the Ran-
dom Primers DNA Labelling System (Gibco BRL, Rockville, MD, USA), with [\(\alpha\)-\(^{32}\)P]dCTP. The labelled fragment was used as a probe (probe A). The results obtained were confirmed in the serotype A and B strains, with a different probe (probe B), about 1.5 kb in length, which had been amplified using the primers PLBDisr2 and PLBDisr2R.

2.8. Northern analysis

All experiments were performed in duplicate. Cells were grown in 10 ml of YPD broth at pH 7.0 or in YNB broth at pH 4.5 overnight at either 30°C or 37°C. 500 \(\mu\)l of this cell suspension were added to 50 ml of fresh broth and cultured at 30°C or 37°C until the OD\(_{600}\) reached 1.5. Total RNA was extracted from the cells using the DRP3 reagent (Progen). 20 \(\mu\)g of RNA per sample were loaded on gels containing 1.1% formaldehyde (Technical bulletin #137, Hoefer Scientific Instruments, San Francisco, CA, USA). The RNA was blotted onto a charged nylon membrane and probed with a 672-bp PLB1 gene fragment from strain WM276 and labelled using the Random Primers DNA Labelling System (Gibco BRL, Rockville, MD, USA), with [\(\alpha\)-\(^{32}\)P]dCTP (probe C). The hybridisation solution used was ULTRAhyb® (Ambion, Austin, TX, USA). Following the detection of the hybridisation bands, membranes were stripped of the PLB1 probe and re-probed with a labelled actin fragment generated from strain WM276. The intensity of the bands was quantitatively by phosphor-imaging and expressed as a percentage of the intensity of the respective bands obtained with the actin probe. The experiment was repeated with the total RNA that had been extracted previously. Thus the probe was amplified and labelled on two independent occasions, to probe two independent blots.

3. Results

3.1. Isolation and characterisation of the C. neoformans var. gattii PLB1 genes

The PLB1 gene of C. neoformans, serotype B (strain...
was 2236 bp in length (submitted to EMBL under accession No. AJ238508, see Fig. 1) with four potential TATA boxes and two potential CAAT boxes upstream from the start codon. The cDNA sequence was 1905 bp in length (submitted to EMBL under accession No. AJ302039) and revealed the presence of six introns within the gene. The CCAAT motif, demonstrated to be an important DNA-binding region in the transcriptional regulation of \( CNLAC1 \) [23], was identified —58 bp upstream from the start codon of \( PLB1 \). The upstream consensus transcriptional control sequence, AATATATAAA of the \( C. albicans \) \( PLB1 \) gene [24], was not identified —58 bp upstream from the start codon of \( PLB1 \). The upstream consensus transcriptional control sequence, AATATATAAA of the \( C. albicans \) \( PLB1 \) gene [24], was not identified —58 bp upstream from the start codon of \( PLB1 \). At the 5’-end of the gene, the polyadenylation signals AATAA and AATAAAA were observed at 192 and 263 bp downstream from the stop codon [21,24] (Fig. 1). The peptide sequence was predicted to be 634 amino acid residues in length, with an estimated molecular mass of 68 280 Da, an overall negative charge at pH 7 and an isoelectric point of 4.12. A hydrophathy plot revealed a stretch of 21 hydrophobic residues at both the amino and carboxyl ends of the peptide sequence (Fig. 2). Seventeen possible N-glycosylation sites (Asn-X-Ser/Thr) and four potential O-glycosylation sites (Ser-Thr) were identified. Typical lipase (GLSGGS) and phospholipase (AGGGXRAML) catalytic motifs were also present (Fig. 1).

The genomic sequence of the \( PLB1 \) open reading frame from \( C. neoformans \), serotype C, was 2239 bp in length and also contained six introns (submitted to EMBL under accession No. AJ302038). Two potential CAAT boxes were identified but no TATA boxes were observed since only 65 bp of sequence were upstream obtained from the start codon (data not shown). Two polyadenylation signals (ATAA) were present at 6 bp and 19 bp downstream from the stop codon. The sequence contained 17 N-linked glycosylation sites but only 3 O-glycosylation sites. Both the lipase and phospholipase catalytic motifs were present. The predicted peptide sequence was 634 amino acids long with an estimated molecular mass of 68 186 Da, an overall negative charge at pH 7 and an isoelectric point of 4.14. A string of negatively charged amino acid residues was observed at both the amino-terminal (21 residues) and carboxyl end (22 residues) of the peptide sequence (Fig. 2). The predicted peptide sequences of \( C. neoformans \) var. \( gattii \) had a maximum homology of 91.7% with the PLB peptide of \( C. neoformans \) var. \( grubii \).

3.2. Sequence alignment and cluster analysis of fungal PLB genes

Alignment of the predicted amino acid sequences from fungal \( PLB \) genes revealed substantial heterogeneity between them. The obvious homologies were restricted to the lipase and phospholipase motifs. The tree generated using the edited nucleotide sequence alignment is shown in Fig. 3 and though not a conventional phylogenetic tree, it indicates the relatedness of the sequences. One tree consisting of 6716 steps, with a consistency index (CI) of 0.5864, a homoplasy index (HI) of 0.4136 and a retention index (RI) of 0.5988 was obtained.

3.3. Karyotyping and chromosomal location of PLB1 gene in \( C. neoformans \)

The karyotypes of the four cryptococcal serotypes are shown in Fig. 4I,II. An estimated 11 chromosomes were present in serotype A, 12 in serotype B, 12 in serotype C and 12 in serotype D. Serotypes B and C contained three chromosomal bands smaller in size than the smallest chromosome in serotype A. The size range of chromosomes

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**Fig. 5. Northern hybridisation patterns of the PLB and actin probes to total RNA extracted from serotype B and C cells grown under different conditions.**

I: Total RNA run on a formaldehyde gel with an RNA ladder. II: Hybridisation of the \( PLB1 \) probe (probe C) to the blot. III: Hybridisation of an actin probe to the same blot.
was similar in the serotype A and D strains used, but their profiles were distinctly different from each other and from the other karyotypes (Fig. 4). The PLB1 gene was located on the smallest chromosome (approximately 700 kb in size) in serotype A, and on the second smallest chromosome in serotypes B (620 kb), C (620 kb) and D (760 kb, see Fig. 4 III,IV). When the experiment was repeated on the serotype A and B strains using a different PLB1 probe, similar results were obtained (Fig. 4IV).

3.4. PLB1 gene expression in C. neoformans var. gattii

Fig. 5II,III shows Northern blots probed with PLB and ACT gene fragments. In the YPD (nutrient-rich) medium, PLB1 gene expression decreased by 1–4% in cells grown at 37°C compared with growth at 30°C. In contrast, when grown in YNB (nutrient-poor) medium at pH 4.5, PLB1 expression increased by 3–4% between cells grown at 30°C and 37°C. PLB1 gene expression at 37°C also increased by 4–5% when the cells were grown in YNB at pH 4.5 as compared with growth in YPD at pH 7.0. At 30°C, gene expression in serotype C was unaffected by the growth medium while in serotype B, expression increased by 5% during growth in YPD. The highest rate of PLB1 expression was 14% that of actin expressed under the same conditions.

4. Discussion

A single gene exhibiting between 87.3 and 88.4% sequence homology with PLB1 from C. neoformans serotype A and D, respectively, and containing typical lipase and phospholipase motifs, was identified in representative strains of C. neoformans var. gattii serotypes B and C. The use of the C. neoformans var. gattii sequences to query GenBank/EMBL non-vertebrate databases recovered many fungal phospholipase genes with significant homologies, confirming their identity as PLB1 genes. In addition, a secreted protein with PLB activity has recently been purified from the serotype B strain of C. neoformans var. gattii used in the present study. N-Terminal amino acid sequencing revealed a high degree of homology with the predicted peptide sequence derived from PLB1. Additional stretches of amino acids homologous with those from other PLB peptides were also identified [14].

In common with other genes of C. neoformans, a relatively high number of introns was present in the PLB1 gene from all serotypes. The six introns present within the serotype B genomic sequence corresponded well with the introns reported in the serotype A sequence by Cox et al. [11]. As with the serotype B sequence, the serotype C sequence contained six introns that matched the serotype B introns in location and size. The N-terminal hydrophobic residues present in both the serotype B and C predicted PLB sequences have also been reported in the gene from C. albicans (caPLB1 and caPLB2), S. cerevisiae (PLB1 and PLB2), T. delbrueckii (syn. S. roset) and C. neoformans var. grubii [9,11,21,25–27]. This stretch of negatively charged residues is thought to act as a signal sequence to indicate that the protein is destined for secretion. The hydrophobic region specifies the transfer to the endoplasmic reticulum (ER) and Golgi and eventually the secretion into the extracellular space. The hydrophobic carboxyl end of the protein, on the other hand, is thought to act as a signal sequence for the attachment of a glycosylphosphatidylinositol (GPI) anchor. The negatively charged amino acids are thought to be cleaved off in the ER by the action of enzymes. The PLB peptides of S. cerevisiae [26,27], P. chrysogenum (syn. P. notatum) [28] and T. delbrueckii (syn. S. roset) [25] also contain hydrophobic residues at the carboxyl end, which may be used for the attachment of GPI anchors. The Kyte and Doolittle hydropathy plot function of the Protein program (DNAStar, Inc., Madison, WI, USA) was used to find a similar GPI anchor site in both the C. neoformans var. grubii and C. neoformans var. neoformans sequences. These observations are consistent with biochemical studies in which cryptococcal PLB was characterised as a heavily glycosylated protein which is mostly cell-associated but is secreted into the surrounding medium over time [12,13]. By contrast, the GPI anchor signal sequence is absent in either of the two C. albicans PLB sequences [9,21], suggesting that the candidal PLB is not membrane-bound.

As with the other reported fungal phospholipases, the C. neoformans var. gattii predicted peptide sequences were found to contain several potential N-linked and O-glycosylation sites. The exact function of oligosaccharide chains on proteins is not known, although glycosylation is thought to enhance stability and aid in the transport of the protein to the cell surface. In C. neoformans, they are essential for enzyme activity since deglycosylation of the pure PLB protein completely destroys the enzyme activity in a serotype A strain [12].

In every case where two copies of PLB occur within a particular species, the two copies cluster closely together, as can be seen for S. cerevisiae, C. albicans, S. pombe and N. crassa (Fig. 3). The C. neoformans sequences behaved like outgroup sequences in the analysis due to the fact that they are the only representatives of basidiomycetes in the group while all the other sequences originate from ascomycetous species. It is clear from the cluster analysis that the four C. neoformans sequences are more closely related to each other (90%) than to other fungal PLB sequences, and that they represent the same gene since they are more closely related than two PLB genes from the same organism, i.e. S. cerevisiae and C. albicans. The percentage homology between PLB1 and PLB2, from S. cerevisiae and C. albicans, was about 65% [9,21,26,27], suggesting that if two copies exist in C. neoformans, the second copy could be identified by sequence comparison. However, a second phospholipase gene has not been found to
date in C. neoformans. Cox et al. failed to identify a PLB2 gene in C. neoformans var. grubii by Southern hybridisation following the disruption of PLB1 [1], although the deletion mutant showed some minimal residual activity, suggesting the possibility of a second PLB gene (Wright and Sorrell, pers. commun.). BLASTN searches of the recently completed C. neoformans genome project database at the Stanford Genome Technology Centre, using the C. neoformans var. gattii PLB1 gene sequences as query, identified contigs relating to the PLB1 gene only and not to any other phospholipase genes.

The karyotype pattern generated for H99 in the experiments described in this paper corresponded to that previously obtained by Perfect et al. [29] using the same isolate, and was used as a quality control since its karyotype and band sizes have been published. Each of the strains used had a distinct karyotype, with the number and size of the chromosomes obtained for each serotype being within the range defined by others for C. neoformans [30,31]. As reported by several researchers [30–33], the karyotypes of C. neoformans var. gattii isolates were different from those of the C. neoformans var. grubii and C. neoformans var. neoformans isolates, in that they had chromosomes of smaller size. Although karyotypes are known to be unstable [34], the preparation and use of the H99 and WM 276 chromosomes in two independent experiments served to confirm the karyotype patterns obtained as well as the identity of the chromosomes harbouring PLB1. The hybridisation of two different PLB1 probes (probes A and B) to the chromosomes generated from the serotype B strain, showed that the phospholipase B gene is located on the smallest chromosome in C. neoformans var. grubii but on the second smallest chromosome for C. neoformans var. gattii and C. neoformans var. neoformans. This result is inconsistent with that of Cox et al. [11], who have found that the PLB1 gene is located on the third largest chromosome of the H99 strain. This discrepancy between the hybridisation patterns cannot be explained. The same H99 strain was used in the experiments described here, and the same karyotype was obtained. Both serotype B probes used in this study hybridised to exactly the same chromosome for each of the four strains, confirming the chromosomal location. The only obvious difference between the two reports was the probes used. Cox et al. have used a probe generated from the serotype A strain H99, whereas the experiments described here used two independent probes generated from the serotype B strain WM 276. Given the high homology (> 85%) between all four PLB genes of C. neoformans, this should not account for the discrepancy. In each case, only one band was obtained suggesting that hybridisation occurred between the probe and only one chromosome. This result is congruent with that of Cox et al. and suggests that the PLB1 gene either exists as a single-copy gene, or if two alleles of this gene are present they are located on the same chromosome.

The expression of the PLB1 gene in C. neoformans var. gattii isolates was low compared to the expression of the actin gene, as measured by phosphor imaging. The highest level of PLB1 expression was approximately 14% of actin expression. At the mRNA level, temperature and pH had little effect on the expression of PLB1 in serotype B and C strains of C. neoformans. Our results are in accordance with those obtained for C. neoformans var. grubii [11], where no change in gene expression was reported between cells grown at 30°C and 37°C. It is possible that temperature and pH may regulate expression in vivo but this possibility was not translated to an in vitro setting. We had hypothesised that growth in a nutrient-poor medium (YNB) at pH 4.5 might have repressed PLB1 expression, as is the case for capsule production [16]. However, our results showed a minor increase in expression at 37°C between cells grown in YPD and those grown in YNB. The fact that the PLB1 gene is expressed constitutively in each serotype suggests that this gene plays an important role in vegetative growth, perhaps by maintaining cell structure and viability. On the other hand, plb1 deletion mutants in C. neoformans var. grubii were viable [11]. Redundancy among fungal phospholipases may be possible, as has been found with other fungal genes.

In conclusion, the phospholipase B gene of C. neoformans var. gattii serotypes B and C has extensive homology to known fungal phospholipase B genes. The cryptococcal genes contain six introns, a potential secretion signal and a potential segment for GPI anchoring, both of which are characteristics of proteins intended for secretion. The observation of several potential glycosylation sites is consistent with biochemical evidence, suggesting that the secreted protein is heavily glycosylated. The PLB1 gene is located on the smallest chromosome for serotype A and the second smallest chromosome in serotypes B, C and D. It is either a single-copy gene, or if two copies exist they are linked on the same chromosome. Finally, PLB1 expression at the mRNA level in serotypes B and C does not appear to be regulated by temperature, pH or nutrient availability in the medium.

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