Phospholipase B (Plb1) is secreted by pathogenic fungi and is a proven virulence determinant in Cryptococcus neoformans. Cell-associated Plb1 is presumptively involved in fungal membrane biogenesis and remodelling. We have also identified it in cryptococcal cell walls. Motif scanning programs predict that Plb1 is attached to cryptococcal membranes via a glycosylphosphatidylinositol (GPI) anchor, which could regulate Plb1 export and secretion. A functional GPI anchor was identified in cell-associated Plb1 by (G)PI-specific phospholipase C (PLC)-induced release of Plb1 from strain H99 membrane rafts and inhibition of GPI anchor synthesis by YW3548, which prevented Plb1 secretion and transport to membranes and cell walls. Plb1 containing β-1,6-linked glucan was released from H99 (wild-type strain) cell walls by β-1,3 glucanase, consistent with covalent attachment of Plb1 via β-1,6-linked glucans to β-1,3-linked glucan in the central scaffold of the wall. Naturally secreted Plb1 also contained β-1,6-linked glucan, confirming that it originated from the cell wall. Plb1 maintains cell wall integrity because a H99 deletion mutant, ∆Plb1, exhibited a morphological defect and was more susceptible than H99 to cell wall disruption by SDS and Congo red. Growth of ∆Plb1 was unaffected by caffeine, excluding an effect of Plb1 on cell wall biogenesis-related signaling pathways. Environmental (heat) stress caused Plb1 accumulation in cell walls, with loss from membranes and reduced secretion, further supporting the importance of Plb1 in cell wall integrity. This is the first demonstration that Plb1 contributes to fungal survival by maintaining cell wall integrity and that the cell wall is a source of secreted enzyme.

Cryptococcus neoformans is an environmental basidiomyceteous yeast, which, following inhalation by humans, can disseminate from the lung and cause a potentially fatal meningitis, especially in patients with AIDS. The secreted enzyme, phospholipase B (Plb1), is an established cryptococcal virulence determinant (1). It facilitates invasion of host lung tissue (2), most likely by hydrolyzing phospholipids in mammalian cell membranes and pulmonary surfactant (3–6) and is essential for hematogenous dissemination of infection (2). In the cryptococcal cell, Plb1 is localized in specialized membrane domains known as rafts (7). These domains contain phospholipid substrates, consistent with the putative role of Plb1 in cryptococcal membrane homeostasis and remodelling. It has been assumed that cell membranes are the source of secreted enzyme, based on studies of the Plb1 homologue in Saccharomyces cerevisiae (8), and our observation that incubation of cryptococci with the raft-disrupting agent, methyl β-cyclodextrin, releases Plb1 into the culture supernatant (7). We discovered recently that cryptococcal Plb1 is concentrated in the cell wall, but its function there is not known (9).

The Plb1 gene sequence contains a putative glycosylphosphatidylinositol (GPI) anchor motif at the C terminus (1) which, as demonstrated by heterologous expression studies in S. cerevisiae, is necessary for localization of Plb1 in the cell membrane and cell wall (9). It is therefore probable that in C. neoformans Plb1 is tethered to the cell surface by a GPI anchor, which influences its export and secretion (9). However, such an attachment has never been demonstrated directly, nor have mechanisms of Plb1 release from GPI anchors been investigated. Evidence from studies in the model yeast, S. cerevisiae and the pathogenic yeast, Candida albicans, demonstrate that GPI anchors direct the transport of the attached proteins to the plasma membrane (10, 11) where they are rendered hydrophobic by attachment to the lipid membrane anchor (12). Some of these proteins are released as hydrophilic mannoproteins, for example, following cleavage by GPI-specific phospholipase C ((G)PI-PLC) (13, 14), and routed to the cell wall (15) where they form much of its outer layer. In S. cerevisiae, C. albicans, and Aspergillus species these proteins attach specifically to β-1,6-linked glucans in the central layer of the cell wall. The β-1,6-linked glucans are attached to β-1,3-linked glucans which are in turn, attached to an inner layer comprised of chitin (16–20).
GPI-anchored proteins maintain cell wall integrity by either remodelling the cell wall during growth and cell division or by providing a protective “coat,” which confers osmotic stability (21). There is evidence that cryptococcal Plb1 is covalently bound to β-glucans in the cell wall because it is released from cell walls of both C. neoformans and S. cerevisiae expressing heterologous cryptococcal Plb1, by treatment with fungal β-1,3 glucanase (9). This raises the possibility that cryptococcal Plb1 serves dual functions in the cryptococcal cell, namely, that it is involved in membrane homeostasis and remodelling and in the maintenance of cell wall integrity. Both functions would contribute to fungal survival in the environment and the mammalian host. In addition, secreted Plb1, which facilitates tissue invasion, may be directly derived from the cell wall, in which case, the cell membrane is not, as is currently believed, the only source of extracellular Plb1.

**EXPERIMENTAL PROCEDURES**

**Reagents**—Bicinchoninic acid (BCA) protein assay reagents were obtained from Pierce Chemical Co., 1-α-phosphatidylcholine, dipalmityl (C16:0) (DPPC), 1-α-lyso phosphatidylcholine, palmityl (C16:0) (LysoPC), and β-1,3 glucanase (Trichoderma hazarum) from Sigma Aldrich. 1,2-di[1-14C]palmitoyl phosphatidylcholine, 1-[14C]palmitoyl lyso-PC, and protein G-Sepharose and ECL reagents, from GE Healthcare (Rydalmere, NSW, Australia). (G)PI-PLC (Bacillus cereus and B. thuringiensis) were obtained from Molecular Probes (Eugene OR) and Prozyme (San Leandro, CA), respectively. Zirconia/silica beads (0.5 mm) were purchased from Biospec Products, Inc. (Daintree Scientific, TAS, Australia) and Immobilon™, PSQ 0.2 μm Membrane from Millipore Corp. NuPAGE™ 4–12% Bis-Tris Gel and loading reagents were obtained from Invitrogen (Mulgrave, Vic, Australia), Coomassie Brilliant Blue R-250, from Bio-Rad and YW3548 was a gift from Taroh Kinoshita (Institute for Microbial Diseases, Osaka University, Japan).

**Antibodies**—Antibodies to Plb1 were prepared as described (7, 9). Antibodies to β-1,6-linked glucan were supplied by Frans Klijs (Swammerdam Institute for Life Sciences, University of Amsterdam). Donkey anti-goat, rabbit anti-mouse, and goat anti-rabbit IgG-HRP conjugates were purchased from Santa Cruz Biotechnology and GE Healthcare, respectively.

**Strains**—A highly virulent, high Plb1-producing strain (H99) of C. neoformans var. grubii serotype A (wild type), its PLB1 deletion mutant (ΔPLB1) and the reconstituted strain, ΔPLB1<sup>rec</sup>, were supplied by Dr. Gary Cox, Duke University.

**Subcellular Fractionation**—Cryptococcal total and raft membranes were prepared as described (7). Briefly, cryptococcal cells, either grown on SAB agar for 72 h or in YNB/2% glucose for 24 h, were homogenized in MES-buffered saline (MBS; 25 mM MES, 150 mM NaCl, 2 mM EDTA, pH 6.5) containing protease inhibitors and 0.1% Triton X-100. The cell lysate was centrifuged at 3500 × g for 10 min at 4 °C, and the supernatant collected. The pellet was further disrupted by probe sonication and the centrifuged pellet was retained for preparation of cell walls. The supernatant was centrifuged at 135,000 × g for 1 h at 4 °C to separate the pellet (total membranes) and supernatant (cytosol). Membranes were incubated with Triton X-100 (1%) prior to fractionation into raft and non-raft fractions on a sucrose gradient. Gradient fractions containing each membrane type were pooled and pelleted by ultracentrifugation at 135,000 × g. Cell wall material (3500 × g pellet) was washed with 1 M NaCl (washes kept), resuspended in 100–300 μl of β-1,3 glucanase made up as a 20 mg/ml solution in water or MBS/protease inhibitors and incubated with agitation for 1–2 h at 37 °C. Supernatants, containing cell wall proteins, were collected following centrifugation (14,000 × g for 15 min at 4 °C) and either analyzed by Western blot for Plb1 and laccase or immune-captured with antibodies to β-1,6-linked glucan (see below).

**(G)PI-PLC-mediated Release of Plb1 from Membranes**—Total and raft membranes were washed with 1 mM NaCl to remove cytosolic material and collected by ultracentrifugation (135,000 × g for 1 h at 4 °C). Pellets were resuspended in 100–200 μl of (G)PI-PLC incubation buffer (50 mM Tris-HCl, pH 7.5, MBS pH 6.5 or 25 mM HEPES pH 7, 0.5 mM EDTA, protease inhibitors) with/without (G)PI-PLC (0.5–1U) from either B. cereus or B. thuringiensis. In some instances Tris–HCl was replaced with HEPES or MBS with no difference in release of GPI-anchored Plb1. Incubation was carried out at 37 °C for 1 h with agitation. Released proteins were separated from the membrane-associated proteins by ultracentrifugation.

**Investigation of the Association of Plb1 with Membranes**—Membranes were resuspended in (G)PI-PLC incubation buffer with/without (G)PI-PLC, incubated for 1 h and ultracentrifuged. Membrane pellets and supernatants were subjected to phase-separation with TX-114 from which contaminating detergents had been removed (22). Briefly, a solution of chilled MBS/protease inhibitors and 1% (v/v) purified TX-114 was used to solubilize membranes or proteins released from membranes, by incubation at 4 °C for 1–2 h. Insoluble debris was cleared by uncentrifugation. The suspension was incubated at 37 °C for 1 h to achieve phase separation and centrifuged for 3 min at room temperature. The aqueous phase was collected, and the organic phase was back-washed three times with MBS/protease inhibitors. The proteins in both phases were precipitated with trichloroacetic acid (10% v/v) in preparation for SDS-PAGE and Plb1 Western blotting.

**YW3548 Inhibition Studies**—YW3548 is a terpenoid lactone, which inhibits synthesis of GPI anchors and prevents transport of yeast GPI-anchored proteins to the cell surface (10). Cells from an overnight culture of C. neoformans in 2× YNB/2% glucose, were washed three times with 50 ml of saline, resuspended in 50 ml of 1× YNB/2% glucose and incubated for 8 h. YW3548 (or methanol solvent) was then added to 0.01, 0.05, and 0.1 μM, and incubation was resumed for 12 h. Preliminary experiments had established that YW3548 concentrations of up to 0.1 μM did not affect growth or viability of C. neoformans. Cells were washed, subcellular fractions prepared, and proteins released from membranes/cell walls by incubation with (G)PI-PLC/β-1,3 glucanase, respectively. Fractions were analyzed for the presence of Plb1 or laccase 1 protein by Western blotting.

**Western Blotting**—Trichloroacetic acid-precipitated proteins were resuspended in SDS sample buffer and loaded on to
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a 4–12% NUPAGE Bis-Tris gradient gel (Invitrogen). Following SDS-PAGE (100 mA, 1 h), proteins were visualized with Coomassie Brilliant Blue R-250 or electrotransferred to polyvinylidene difluoride membrane. Plb1 and laccase were detected by incubation with anti-Plb1 peptide antibody (1 μg/ml) or anti-laccase 1 antibody (2 μg/ml), respectively, for 1 h, then with secondary antibody diluted 1:2,000 (donkey anti-goat IgG-HRP or rabbit anti-mouse IgG-HRP) for a further hour. Bands were visualized using ECL on Hyper film.

Immune-Capture—Proteins in cryptococcal secretions or released from cell wall fractions by high salt-washing (1 M NaCl) or treatment with β-1,3-glucanase were diluted with 10× TNET buffer to give a final concentration of 1× TNET buffer (100 mM Tris-HCl, pH 8.0, 100 mM NaCl, 5 mM EDTA, and 1% Triton X-100). Anti-β-1,6-glucan antibody (2 μg) and protein G-Sepharose beads (100 μl of a 50% slurry in 1× TNET buffer) were added, and incubation was carried out overnight with rotation at 4 °C. Beads were collected by centrifugation at 200 g, and supernatants aspirated. Beads were washed with 5× 1 ml of 1× TNET (with 0.5% Triton X-100) and a final time with 1 ml of 10 mM Tris-HCl, pH 7.5. Proteins were eluted by boiling in SDS sample buffer and subjected to SDS-PAGE/Western blotting with anti-Plb1 or anti-laccase antibodies.

Activity Assays—Phospholipase B1 activities (LPL, LPTA, and Plb) in cryptococcal secretions were assessed radiometrically in a final volume of 125 μl at 37 °C as described (5). Superoxide dismutase (SOD) activity was assayed as described (7, 23). Briefly, SOD activity was assayed using a kit (Dojindo, Japan) according to the manufacturer’s instructions. SOD activity was calculated as the rate of inhibition of reduction of the tetrazolium salt WST-1 where one unit was defined as a 50% decrease from the control value (no SOD) over 30 min at 37 °C. The results are presented as total SOD activity.

Determination of Cell Wall Integrity—The integrity of cryptococcal cell walls was assessed after 48 h of growth on SAB agar plates containing 0.05% SDS, 0.5% Congo Red or 0.5 mg per ml laccase (24). Capsules were visualized by Transmission electron microscopy as described (25).

RESULTS

Plb1 Is Released from Membranes by Exogenous (G)PI-PLC and Partitions into TX-114—We have shown previously that Plb1 is present in cryptococcal membranes (7). DNA sequencing, hydrophathy plotting, and GPI anchor motif scanning using the DGPI/GPI-SOM prediction programs, predict that cryptococcal Plb1 is attached to the cell membrane by a GPI anchor. We used two classical approaches to confirm this, namely, release from the membrane following incubation with GPI-specific phospholipase C ((G)PI-PLC) and partitioning into the detergent, TX-114. (G)PI-PLC cleaves between the α-1–6-myoinositol-PO₄ and the diacylglycerol (DAG) group within the anchor sequence and converts the GPI-anchored protein, which is hydrophobic due to the DAG lipid anchor, to a soluble (hydrophilic) form. Each form partitions differently with the biphasic detergent, TX-114.

The membrane-associated virulence determinant, superoxide dismutase (SOD), which lacks the predicted GPI anchor consensus motif as per the DGPI/GPI-SOM prediction programs, was used as a control.

Following incubation with (G)PI-PLC, all membrane-associated Plb1 was released into the supernatant (Fig. 1A, lanes 4 and 6); ~10% was released by washing with 1 M NaCl (lane 1) and ~10–15%, by incubation in the absence of (G)PI-PLC (lanes 2 and 3). Plb1 release was not affected by the addition of protease inhibitors (results not shown), confirming that neither endogenous membrane proteases nor proteases that may be potential contaminants in the (G)PI-PLC preparation were responsible for the effect.

As expected for a GPI-anchored protein, the TX-114 partitioning experiments confirmed that Plb1 is integrally associated with the membrane. Fig. 1B (compare lanes 5 and 6) indicates that membrane-associated Plb1 partitioned exclusively into the hydrophobic (detergent) phase. In experiments where Plb1 had been released from membranes by preincubation with (G)PI-PLC prior to incubation with TX-114, no membrane Plb1 protein was detected in either the aqueous or the detergent phase (Fig. 1B, lanes 1 and 2). The Plb1 protein was now present in the supernatant and associated exclusively with the hydrophilic (aqueous) phase, indicating that the hydrophobic lipid component of the protein had been removed (Fig. 1B, compare lanes 3 and 4).

Only a small amount of SOD activity (~10%) was released from membrane preparations by (G)PI-PLC and controls (see Fig. 2) confirming that the membrane association of SOD is not GPI-anchor-mediated and supporting the specificity of the results for Plb1.

Plb1 Is Attached to Raft Membranes via a GPI Anchor—We next investigated whether Plb1 is tethered to raft membranes, in which it is known to be concentrated (7), by the GPI anchor. As shown in Fig. 3, a large proportion of membrane-associated Plb1 was derived from lipid rafts (compare lanes 3 and 7). Following (G)PI-PLC treatment, all of the raft-associated Plb1 was released into the supernatant (compare lanes 1–4). A small
Inhibiting GPI Anchor Biosynthesis Prevents Plb1 Transport and Secretion—YW3548 inhibits α,1,2-mannosyltransferase, which adds the third mannose to the core GPI structure. Consequently, the supply of anchors to proteins displaying a GPI anchor consensus motif is reduced and transport of GPI-anchored proteins from ER to Golgi is prevented (10). Incubation of cryptococci with non-toxic concentrations of YW3548 inhibited transport of Plb1 to the cell membrane, cell wall, and secretions (Fig. 4A). Fractions of cytosol were run following β-1,3 glucanase treatment, revealed a similar contribution of each to the total pool of cell wall-associated Plb1 (Figs. 5A and 7B). No Plb1 was detected in the corresponding fractions prepared from ΔPLB1 (not shown).

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amount of Plb1 protein that associated with non-raft membrane fractions was resistant to cleavage by (G)PI-PLC (compare lanes 6 and 8); this may be explained by partial inhibition of (G)PI-PLC by the higher concentrations of Triton X-100 in mixed membrane micelles.

Plb1 Is Covalently Linked to β-Glucans in the Cryptococcal Cell Wall—If, as in other yeast, GPI-anchored proteins are covalently attached to β-1,6-linked glucans in the outer cell wall and β-1,6-linked glucans are themselves attached to β-1,3-linked glucans, we reasoned that incubating cell walls with β-1,3 glucanase would release cryptococcal Plb1 containing β-1,6-linked glucan. Fig. 5A demonstrates that after removal of non-covalently bound Plb1 from cell wall preparations of wild-type H99 with 1M NaCl, Plb1 was released by β-1,3 glucanase. In C, culture supernatants (secretions) from cells grown at 25 °C were dialyzed and lyophilized and the Plb1 present was detected by Plb1 Western blotting before and after immune-capture with antibodies to β-1,6-glucan. No bands were detected in fractions prepared from ΔPLB1 before immune-capture (not shown).
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FIGURE 6. Plb1 has a role in cell wall integrity. Comparison of colony morphology (A) and cell morphology (B) of wild-type (H99), ΔPLB1, and ΔPLB1rec strains cultured for 3 days on SAB agar at 30 °C. In B, cells were stained with India ink to emphasize the capsule. C, growth of each strain in the presence of SDS and Congo Red. Cells were serially diluted 10-fold, spotted onto agar plates (10^5–10^1 cells per spot) and cultured for 3 days at 30 °C.

β-glucanase. Cytosolic Plb1 was devoid of β-1,6-linked glucan, indicating that it did not originate from cell walls. No Plb1 was detected in any fractions prepared from ΔPLB1.

Control experiments to detect laccase 1 released from cell walls by both high salt washing and treatment with β-1,3 glucanase, did not yield a protein containing β-1,6-linked glucan as expected, because laccase 1 has no GPI anchor (results not shown).

Plb1 Is Released from Cryptococcal Cell Walls by Endogenous β-Glucanase(s)—We next investigated Plb1 in the secretions of H99 for the presence of β-1,6-linked glucan. We chose a secretion temperature of 25 °C as Plb1 secretion is greatest at this temperature (see Fig. 7B). Fig. 5C shows that Plb1 secreted at 25 °C is recognized by antibody to β-1,6-linked glucan indicating that cell wall-associated Plb1 is a source of endogenously secreted Plb1. No Plb1 was secreted by ΔPLB1.

Plb1 and Cryptococcal Cell Wall Integrity—GPI-anchored proteins play a role in maintaining cell wall integrity in S. cerevisiae. Indicators of compromised cell wall integrity include altered cell and colony morphology, clumping (27, 28) and reduced growth in the presence of SDS, Congo Red, or caffeine (24, 29, 30). The microscopic morphology and appearance of colonies of ΔPLB1, cultured on SAB agar, revealed an abnormal wrinkled (rather than smooth) appearance and flocculation (clumping), unlike the wild-type H99 and the reconstituted strain (ΔPLB1rec) (Fig. 6, A and B). In addition, ΔPLB1 was more sensitive to SDS and Congo Red (Fig. 6C) but not to 0.5 mg/ml caffeine (not shown).

As the capsule of C. neoformans is anchored to cell wall components (31) and was reduced in density in a PKC-defective strain of C. neoformans with a cell wall integrity defect (32), the capsule densities of cells from H99 wild type, ΔPLB1, and ΔPLB1rec were compared using transmission electron microscopy (see supplemental information). Cells from ΔPLB1 colonies had capsules of a lower density than cells from H99 wild type and ΔPLB1rec. However, the diameter of the capsule and cell wall, relative to the diameter of the cell, was similar in all three strains (p = 0.7242 and p = 0.0932, respectively, by non-parametric Mann-Whitney two-tailed analysis).

Effect of Temperature Stress on Plb1 Distribution—Cell wall-associated Plb1 was increased at least 3-fold under conditions of heat stress (30–37 °C), with a concomitant decrease in secreted protein (Fig. 7). Plb1 in cell membranes also decreased with increasing temperature, with a concomitant increase in cytoplasmic Plb1 (Fig. 7). Total Plb1 protein produced at each temperature (25, 30, and 37 °C), remained constant (~65,500, 53,500, and 80,000 density units, respectively).

We also measured the effect of temperature on secreted Plb1 activity, by radiometric assay (Fig. 8). Despite the reduced amount of Plb1 protein secreted at 30–37 °C, the LPL specific activity (and total activity (not shown)), increased 3-fold at 30 °C compared with 25 °C and decreased slightly when the temperature was increased from 30 to 37 °C, suggesting that Plb1 is more active at higher temperatures. Similar results were obtained for LPTA (not shown). PLB activity was below the level of detection.

FIGURE 7. Plb1 is concentrated in the cell wall during heat stress. Cells were grown at 25, 30, and 37 °C in YNB for 24 h. Culture supernatants (secretions) were collected, and cells were fractionated into cell walls, membranes, and cytosol. Cell wall fractions were washed with 1 M NaCl to release noncovalently associated Plb1 and treated with β-1,3 glucanase to release covalently associated Plb1. Plb1 was released from membranes with (G)PI-PLC. Each fraction was subjected to Plb1 Western blotting (A). The Plb1 in each fraction was quantified by densitometry (B). For each temperature, the total Plb1 was ~65,500, 53,500, and 80,000 density units, respectively. Results are representative of one experiment with identical results obtained in a second experiment, and are normalized to the total value obtained at 37 °C.

FIGURE 8. Specific activity of secreted Plb1 is increased during heat stress. Cells were grown at 25, 30, and 37 °C, and the culture supernatants (secretions) were dialyzed, lyophilized, and analyzed for Plb1 activity. Only LPL activity is shown as the same trend was observed for LPTA activity. Activity is given as μmol LysoPC substrate degraded per min per mg protein. Results represent the mean and standard error of the mean (n = 3). * indicates that the increase in LPL activity from 25 to 30 and 37 °C is statistically significant (p < 0.05) using an unpaired, parametric t test.
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DISCUSSION

The pathogenic potential of fungi such as *C. neoformans*, *C. albicans*, and probably *Aspergillus* spp., depends on host factors and a fungal virulence composite that includes secreted Plb1. Using *C. neoformans* as a model pathogen, this study provides the first demonstration that Plb1 contributes to fungal survival by maintaining integrity of the cell wall and that the cell wall is a source of secreted enzyme. The localization of Plb1 in both the membrane and cell wall was shown to depend on the presence of a GPI anchor and was influenced by temperature stress.

We first confirmed predictions from motif scanning programs that cryptococcal Plb1 is attached to raft membranes via such an anchor. Specifically, Plb1 was released from membranes by an exogenous (G)PI-PLC. Membrane-associated Plb1 partitioned into the hydrophobic phase of TX-114 and was converted from a hydrophobic to a soluble form following enzymatic cleavage with (G)PI-PLC. The virulence determinant, SOD, was used as a specificity control. Like Plb1, it is found in membrane rafts (7), but does not contain a secretory leader peptide or a GPI anchor attachment motif. As predicted, SOD was not released by (G)PI-PLC. Inhibition of GPI anchor formation by the terpenoid lactone, YW3548, inhibited transport of Plb1 to, and its release from, the cell surface, indicating that GPI anchor formation is an essential prerequisite for cellular export and release of Plb1. Cellular export of lactase, another virulence determinant that localizes in the cell wall, but which lacks the motifs that predict acquisition of a GPI anchor, was not affected by incubation with YW3548.

Approximately half of the Plb1 recovered from the cell wall fraction of *C. neoformans* was released by high-salt washing and the remainder, by enzymatic digestion with β-1,3 glucanase. Only the latter fraction contained β-1,6-linked glucan, a sugar linkage that is unique to the fungal cell wall, indicating that there is a covalent association between Plb1 and cell wall β-1,6-linked glucan via either a mannose sugar within the Plb1 GPI anchor or an amino acid in the Plb1 C terminus (14, 33). The source of the non-covalently bound Plb1, which lacked the β-1,6-linked glucan, is presumably the cell membrane. Endogenous (G)PI-PLC activity has been associated with the release of GPI-anchored proteins from *S. cerevisiae* protoplasts (14).

The presence of β-1,6-linked glucan on naturally secreted Plb1 implicates endogenous β-glucanases in the release of Plb1 from the cell wall. Furthermore, β-1,3 glucanase activity has been detected in cryptococcal secretions (34), and four putative endo-β-1,3 glucanases are present in the cryptococcal genome data base (35). The product of one gene (CNBC2400), is present in cryptococcal cell walls (36), and the remaining three are predicted to be GPI-anchored (35). Interestingly, no β-1,6 glucanase genes or activity have been found, suggesting that Plb1 is linked via β-1,6-linked glucan to β-1,3-linked glucan in the central scaffold of the cell wall and that endogenous β-1,3 glucanases are responsible for its secretion.

Deletion of cryptococcal *PLB1* compromised cell wall integrity of *C. neoformans*. This was demonstrated by increased sensitivity of Δ*PLB1* to SDS, a detergent and protein destabilizer (24) and to Congo Red, which binds to β-glucans and interferes with cell wall construction (29). Growth of Δ*PLB1* was unaffected by caffeine. In *S. cerevisiae*, caffeine inhibits rapamycin-sensitive Tor1 kinase, activating the Pkc1p-Mpk1p cell wall integrity cascade and producing a fortified cell wall with increased resistance to zymolyases (30). The resistance of Δ*PLB1* to caffeine suggests that neither PLB1 nor its breakdown products affect the cell wall integrity pathway downstream of Tor1 kinase. Taken together, these findings suggest that the role of Plb1 in cell wall integrity is structural and independent of its enzyme activities and that it relates to the control of cell wall porosity, as reported previously for cell wall-associated mannoproteins (21). A second novel function of cell wall-associated Plb1 may be to release protein cargo transported across cell walls inside membrane-bound vesicles. Rodriguez et al. (37) described transport of capsular polysaccharide components in bilayered vesicles enriched in glucosylceramide and sterols (and presumably, phospholipids).

The amount of Plb1 in the cell wall increased, and that in cell membranes and secretions decreased, as the growth temperature rose from 25 to 30–37 °C. Because increasing temperature would reduce cell wall stability (38), the temperature-dependent association between Plb1 and the cell wall supports its role in cell wall integrity. This suggests that Plb1 has a role in defense of *C. neoformans* against temperature stress in the natural environment and the human host. We postulate that release of Plb1 from cell membranes at higher temperatures is stimulated by increased activity of GPI anchor-cleaving enzymes which increases transport of Plb1 to the cell wall, where β-glucanase expression and/or activity, and hence Plb1 release to the exterior, are suppressed. It is noteworthy that although less Plb1 protein was secreted at 30–37 °C, its specific activity was greater than that secreted during culture at 25 °C. This may provide a compensatory mechanism, which allows Plb1 to provide a dual function at host physiological temperature, namely to maintain cell wall integrity and hence survival in the host, while at the same time facilitating invasion of host tissue.

In conclusion, we have identified a novel pool of Plb1 in the cell wall which can serve as a source of secreted enzyme thereby promoting invasion of host tissue. The biological functions of Plb1 in the cryptococcal cell now include not only a role in membrane homeostasis and turnover but also maintenance of cell wall integrity and hence survival, especially during heat stress. These findings open up a new area of investigation into mechanisms of fungal pathogenesis.

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