

Potential Role of Phospholipases in Virulence and Fungal Pathogenesis

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INTRODUCTION

To aid in the invasion of host tissues, microbial cells possess constitutive and inducible hydrolytic enzymes that destroy or derange constituents of host cell membranes, leading to membrane dysfunction and/or physical disruption (167). Since

membranes are made up of lipids and proteins, these biochemicals constitute the target of enzyme attack. Pathogenic fungi (e.g., *Candida albicans*) secrete enzymes which are considered to be integral to their pathogenesis; these are categorized into two main types, proteinases (72, 73), which hydrolyze peptide bonds, and phospholipases (75), which hydrolyze phospholipids. In this review, we focus on fungal phospholipases. It should be emphasized that this field is evolving rapidly and that most of the data reviewed in this article were published recently. New insights into the biology and contribution of these enzymes to fungal virulence will be forthcoming in the near future.

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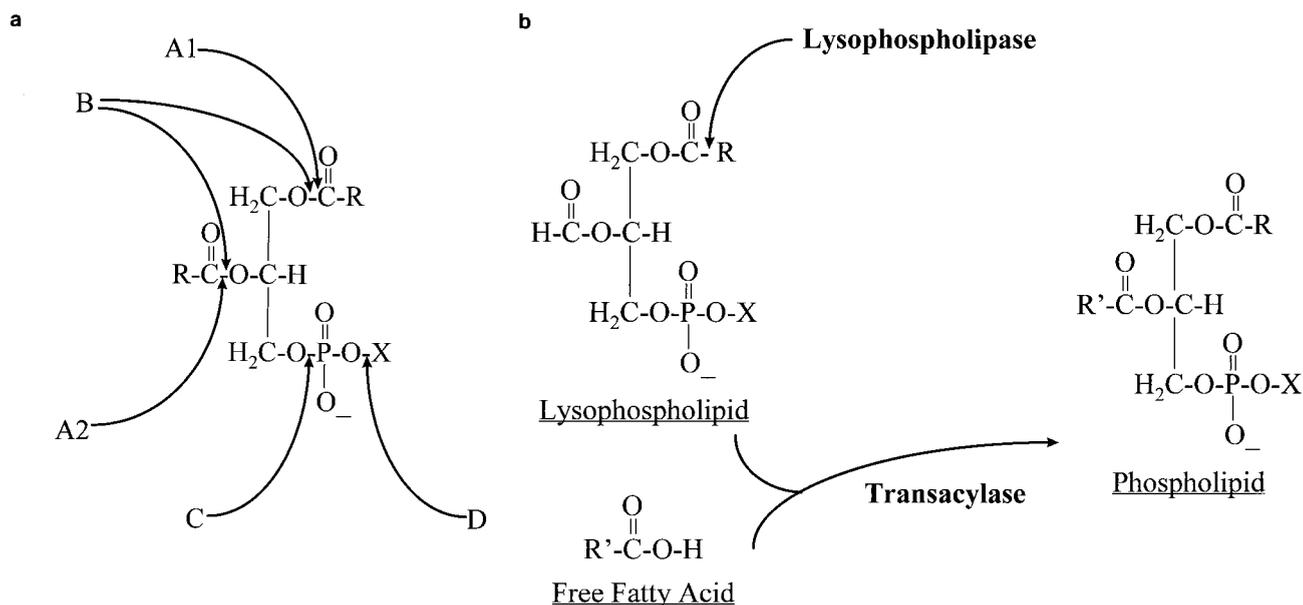


FIG. 1. Sites of action of various phospholipases. (a) A1 and A2, PLA₁ and PLA₂, respectively; B, PLB; C, PLC; D, PLD. (b) Lyso-PL and Lyso-PL transacylase.

DEFINITION OF PHOSPHOLIPASES AND RATIONALE IN CONSIDERING THEM FOR A ROLE IN VIRULENCE

The term “phospholipases” refers to a heterogeneous group of enzymes that share the ability to hydrolyze one or more ester linkage in glycerophospholipids. Although all phospholipases target phospholipids as substrates, each enzyme has the ability to cleave a specific ester bond (Fig. 1a). Thus, qualifying letters, such as A, B, C, and D, are used to differentiate among phospholipases and to indicate the specific bond targeted in the phospholipid molecule (4). For example, phospholipase A₁ (PLA₁) hydrolyzes the fatty acyl ester bond at the *sn*-1 position of the glycerol moiety, while phospholipase A₂ (PLA₂) removes the fatty acid at the *sn*-2 position of this molecule. The action of PLA₁ (EC 3.1.1.32) and PLA₂ (EC 3.1.1.4) results in the accumulation of free fatty acids and 2-acyl lysophospholipid or 1-acyl lysophospholipid, respectively. The fatty acid still linked to the lysophospholipid is, in turn, cleaved by other enzymes termed lysophospholipases (Lyso-PL) (EC 3.1.1.5) (Fig. 1b). Phospholipase C (PLC) (EC 3.1.4.3) hydrolyzes the phosphodiester bond in the phospholipid backbone to yield 1,2-diacylglycerol and, depending on the specific phospholipid species involved, phosphatidylcholine, phosphatidylethanolamine, etc. The second phosphodiester bond is cleaved by phospholipase D (PLD) (EC 3.1.4.4) to yield phosphatidic acid and choline or ethanolamine, again depending on the phospholipid class involved (4).

Although phospholipase B (PLB) (synonyms: lysophospholipase, lysophospholipase-transacylase) refers to an enzyme that can remove both *sn*-1 and *sn*-2 fatty acids, its nomenclature is confusing (4). This confusion arises because PLB has both hydrolase (fatty acid release) and lysophospholipase-transacylase (LPTA) activities. The hydrolase activity allows the enzyme to cleave fatty acids from both phospholipids (PLB activity) and lysophospholipids (lysophospholipase [Lyso-PL] activity), while the transacylase activity allows the enzyme to produce phospholipid by transferring a free fatty acid to a lysophospholipid (Fig. 1b). The finding that PLB has both hydrolase and acyltransferase activities has been reported for enzymes purified from *C. albicans*, *Penicillium notatum*, and

Saccharomyces cerevisiae (100, 122, 164, 224, 225). This complex pattern of activity led to difficulties in the nomenclature of the enzyme, with some authors naming it PLB (as in the case of *S. cerevisiae* and *P. notatum*) and others naming it LPTA (10, 122, 193). Recent cloning and disruption of genes encoding PLB provided further evidence that this enzyme has both PLB and Lyso-PL activities. Deletion of *caPLB1*, the gene encoding PLB from *C. albicans*, showed that both PLB and Lyso-PL activities were reduced in the PLB1-deficient mutant relative to the wild type, confirming that *caPLB1* encodes an enzyme responsible for the PLB and Lyso-PL activities (75, 101). Similar findings were reported for PLB deletion in *S. cerevisiae* (100).

Invasion of host cells by microbes entails penetration and damage of the outer cell envelope. This transmigration process is mediated, most probably, by either physical or enzymatic means or a combination of the two. Phospholipids and proteins represent the major chemical constituents of the host cell envelope. Therefore, enzymes capable of hydrolyzing these chemical classes, such as phospholipases and proteinases, are likely to be involved in the membrane disruption processes that occur during host cell invasion. By cleaving phospholipids, phospholipases destabilize the membrane and cell lysis results (166). Evidence implicating phospholipases in host cell penetration, injury, and lysis by microorganisms has been reported for *Rickettsia rickettsii* (207), *Toxoplasma gondii* (160, 161), *Entamoeba histolytica* (154), and *C. albicans* (101). Consequently, phospholipases have been included among the virulence factors that damage host cells (166).

PHOSPHOLIPASES IN ORGANISMS OTHER THAN FUNGI

Besides fungi, which are the subject of this review, extracellular phospholipases have been implicated as pathogenicity factors for bacteria (Table 1). Since bacterial phospholipases have been the subject of at least two recent reviews (183, 194), to avoid repetition, only a brief description of these enzymes and their role in virulence is covered in this article. Furthermore, including some information about phospholipases from

TABLE 1. Phospholipases from microorganisms other than fungi

Microbial class	Enzyme ^a	Gene cloned	Substrate specificity ^a	Reference(s)
Bacteria				
<i>Clostridium perfringens</i>	Alpha-toxin	Yes	PC, SM, PS, LPC	103, 136, 162, 195, 197
<i>Clostridium novyi</i>	Gamma-toxin	No	PC, SM, LPC, PE, PI, PG	191
	PI-PLC	No		
<i>Clostridium bifermentans</i>	PLC	Yes	Unknown	197
<i>Listeria monocytogenes</i>	PLC-A	Yes	PI	102, 119, 203
	PLC-B	Yes	PC, PE, PS, SM	
<i>Pseudomonas aeruginosa</i>	PLC-H	Yes	PC, LPC, SM	13, 29, 137
	PLC-N			
<i>Pseudomonas cepacia</i>	PLC	Yes		202
<i>Staphylococcus aureus</i>	Beta-toxin	Yes	SM, LPC	38, 76, 148, 157, 216, 217
	PI-PLC	No	PI, LPI	
<i>Bacillus cereus</i>	PC-PLC	Yes	PC, PE	77, 85, 96, 104
	SMase	Yes	SM	
	PI-PLC	Yes	PI, LPI	
<i>Bacillus thuringiensis</i>	PI-PLC	Yes	PI, LPI	68, 78, 192
<i>Mycobacterium tuberculosis</i>	MPC-A ^c		SM, PC	83
	MPC-B ^c		SM, PC	
	PLD		PC	
	PLA			177
<i>Rickettsia prowazekii</i>	PLA			
<i>Corynebacterium pseudotuberculosis</i>	PLD	Yes		118
<i>Arcanobacterium haemolyticum</i>	PLD	Yes		118
<i>Legionella pneumophila</i>	PLC	No	PC	8
<i>Ureaplasma urealyticum</i>	PLC	No		15, 36
Protozoa				
<i>Toxoplasma gondii</i>	PLA	No		160, 161
<i>Entamoeba histolytica</i>	PLA	No		106, 153

^a Abbreviations: PI, phosphatidylinositol; LPI, lysophosphatidylinositol; PG, phosphatidylglycerol; SM, sphingomyelin; LPC, lysophosphatidylcholine; LPG, lysophosphatidylglycerol; PLC-N, nonhemolytic PLC; PLC-H, hemolytic PLC.

organisms other than fungi will show a global perspective of microbial phospholipases.

Bacterial Phospholipases

***Clostridium perfringens*.** PLC (synonyms: alpha-toxin, lecithinase), which is one of the most active bacterial phospholipases, is 1 of at least 12 different soluble antigens referred to as toxins, produced by *Clostridium perfringens*, that may be involved in pathogenesis. The findings that clostridial PLC was a potent toxin (109, 110, 123) with hemolytic (162, 195), lethal (196), dermonecrotic (115), vascular permeabilization (186), and platelet-aggregating (134, 185) properties attracted a large number of investigators to study this secreted protein, making it the most extensively studied bacterial toxin. Thus, it is not surprising that extensive reviews have been written about this enzyme (67, 79, 194), its application to the study of cell membranes (3, 5, 48, 126, 159, 229), and its role in virulence (183).

Over the past 60 years, extensive data have accumulated concerning both the physiology of toxin production by *C. perfringens* (179) and some of the physical and biological properties of the enzyme (115). However, many of the earlier data have been difficult to interpret because of the lack of purity of the toxin preparations used (127). In these earlier studies, many authors used preparations of the enzyme from commercial or other sources with insufficient or no regard for the fact that these preparations, unless produced under rigorous purification standards, may contain as many as 11 contaminating substances (115). Furthermore, purification of this enzyme is difficult. Fortunately, recent molecular biology techniques allowed the cloning and sequencing of the gene encoding clostridial PLC (195, 227) and subsequent synthesis of purified

recombinant enzyme (227). Availability of the gene and the purified protein made studies aimed at defining the role of PLC in the virulence of *C. perfringens* feasible.

To study the pathogenesis of *C. perfringens*-mediated gas gangrene, Awad et al. (6) used reverse genetics to construct a suicide plasmid in which the *plc* gene, encoding PLC, was inactive. Using this plasmid, they isolated mutants that had lost their ability to produce detectable PLC. Comparing the mutants and their parent in a mouse virulence model showed that the mutants were markedly less pathogenic than the parent strain (6). Unlike animals infected with the phospholipase-deficient mutants, which showed minimal swelling, muscle destruction, inflammation, or necrosis of the infected tissues and remained active with an otherwise healthy appearance, animals challenged with the parent strain had extensive swelling of the infected foot, leg, and hip, as well as a severely necrotic infected foot, demonstrable signs of hematuria, and extensive muscle destruction. This study provided definitive genetic evidence of the essential role of PLC in gas gangrene or clostridial myonecrosis. For a more in-depth review of clostridial PLC with regard to its substrate specificity, molecular architecture, interaction with phospholipids and membranes, and role in disease, the reader is referred to excellent reviews by Titball (194) and Songer (183).

***Listeria monocytogenes*.** Similar to *C. perfringens*, *L. monocytogenes* secretes a number of extracellular enzymes including listeriolysin (encoded by *hly*) and two phospholipases: i) PI-PLC or PLC-A, encoded by *plcA*, which is an inositol-specific PLC (21), and (ii) PC-PLC or PLC-B, encoded by *plcB*, which is a broadly active PLC with the ability to hydrolyze most cellular phospholipids (203). Although each phospholipase may contribute to the virulence of *L. monocytogenes* by itself

(21, 119, 203), elegant work by Portnoy and colleagues showed that the two phospholipases play overlapping roles in the pathogenesis of *L. monocytogenes* (178). In their study, mutants harboring a deletion in each PLC, as well as a double mutant lacking both enzymes, were characterized with regard to virulence. Abolishing PI-PLC and PC-PLC resulted in strains that were 2- and 20-fold less virulent than the parent strain, respectively. In addition, these strains were defective in escape from the vacuole and in cell-to-cell spread, depending on which enzyme was deleted. Interestingly, the mutant lacking both PLCs was 500-fold less virulent in mice and was severely diminished in its ability to escape from the vacuole and to spread from cell to cell (178). These findings are consistent with the two enzymes having overlapping functions throughout the course of infection. Importantly, deleting a single gene resulted in only a modest reduction in virulence, while simultaneous deletion of the two genes in the same strain led to a highly significant decrease in the virulence, suggesting that the two enzymes have a synergistic effect on the ability of the organism to invade host tissues. For more details on entry of *L. monocytogenes* into phagocytic cells and the role of secreted enzymes, including phospholipases, in the ability of the organism to escape from the vacuole and transigrate cell-to-cell, the reader is referred to references 144, 183, and 194.

***Pseudomonas aeruginosa*.** Two distinct PLCs are produced by *Pseudomonas aeruginosa*: PLC-N (nonhemolytic) and PLC-H (hemolytic) (183). The genes (*plcN* and *plcS*) encoding these enzymes have been cloned (137). Although the expression and secretion of both enzymes are phosphate regulated, each enzyme has a distinct substrate specificity, with PLC-H hydrolyzing sphingomyelin in addition to phosphatidylcholine (PC), while PLC-N is active in phosphatidylserine and PC degradation (137). This difference in substrate specificity has a bearing on the efficiency with which these enzymes degrade eukaryotic membranes. Ostroff et al. (138) proposed that the two enzymes could work sequentially and synergistically to lyse host cells. PLC-H initiates membrane lysis by hydrolyzing PC and sphingomyelin (the major constituents of the membrane outer leaflet), and this is followed by the action of PLC-N which cleaves phosphatidylserine (the major constituent of the membrane inner leaflet). Furthermore, substrate specificity studies have shown that PLC-H preferentially cleaves phospholipids containing quaternary ammonium groups, such as phosphatidylcholine, which are found primarily in eukaryotic membranes and lung surfactants, but that it has minimal activity toward phospholipids such as phosphatidylethanolamine, which are found in the prokaryotic membrane (14). This selective ability may explain why the invading organism can lyse the host cells without damaging its own membrane.

Correlation between PLC-H and *P. aeruginosa* pathogenicity can be derived from studies showing that the enzyme is secreted in vivo and in experiments comparing the wild type with mutants in which this gene is deleted in animal models. Three lines of evidence suggest that PLC-H is secreted in vivo: (i) clinical isolates from the lungs produce PLC (13), (ii) PLC is produced when the bacterium is grown in bronchial washings (105), and (iii) high titers of antibody against PLC-H are detected in patients with chronic *P. aeruginosa* infection (62).

Mutants disrupted in the *plcS* gene were generated and used to determine the role of PLC-H in pseudomonal virulence (138). In this study, the virulence of wild-type strains and *plcS*-disrupted mutants was compared in a mouse burn model of infection. A reduction was observed in the ability of the disruptant grown under phosphate-limiting conditions to kill mice (200-fold increase in the 50% lethal dose compared with the wild-type strain). Since virulence in these studies was phos-

phate dependent, mutants disrupted in *plcR* (a phosphate-regulatory gene) were generated to investigate its effect alone and combined with *plcS*. These studies showed that when the strains were grown under phosphate-limiting conditions, the virulence of the $\Delta plcS \Delta plcR$ strain was 200- to 10,000-fold lower than that of the parent strain (138). Interestingly, mutants with only *plcR* deleted were also attenuated in virulence even though they produced greater amounts of PLC and hemolysin (138). In spite of this apparent enigma, which suggests a role for *plcR* in pseudomonal pathogenicity, the significantly greater reduction in the pathogenicity of the double disruptant suggests that PLC-H is associated with virulence (194).

***Bacillus cereus*.** Several PLCs, including phosphatidylinositol-specific and PC-preferring enzymes, as well as sphingomyelinase, are produced by *Bacillus cereus* (80, 95, 198, 199). The genes encoding the three different proteins have been cloned and sequenced (84, 85, 96). PC-preferring and sphingomyelinase-encoding genes form a gene cluster (tandemly located) (59), which is not positioned in close proximity to the gene encoding phosphatidylinositol-specific PLC (59, 96). The PC-preferring enzyme has structural homology to the *C. perfringens* alpha-toxin (103), while the phosphatidylinositol-specific enzyme has stretches of sequence homology to other eukaryotic phospholipases (97).

The contribution of phospholipases to the virulence of *B. cereus* has not been investigated in animals. Although PLC of this bacterium is considered to be nontoxic (126), data suggestive of its involvement in host cell lysis have been reported. Gilmore et al. (59) suggested that by creating a duplex hemolysin named cereolysin AB, the PC-preferring phospholipase and the sphingomyelinase act in concert to cause hemolysis. Others (212) showed that *B. cereus* strains producing PLC cause degranulation of human neutrophils with a dose-dependent release of lysosomal enzymes, which may mediate tissue damage. Finally, it has been suggested that *B. cereus* protects itself against phagocytosis by releasing phospholipases (152).

***Rickettsia*.** Two species of the genus *Rickettsia* (*Rickettsia rickettsii* and *Rickettsia prowazekii*) were shown to possess PLA that was suggested to mediate host cell lysis (63, 177, 220, 222). Previously, Winkler and Miller (221) reported that rickettsiae enter cells through a mechanism involving a PLA. Their hypothesis was based on the demonstration that host cells exposed to large numbers of *R. prowazekii* release considerable amounts of lysophospholipids and free fatty acids into the culture medium. Involvement of phospholipases in the penetration and damage of host cells by *R. rickettsii* was first suggested by Walker et al. (207). More recently, Silverman et al. (177) confirmed these findings and provided suggestive evidence that phospholipase activity associated with internalization of this intracellular parasite lies directly with the infecting organism rather than with the host cell. However, as stated by the authors, definitive evidence that the phospholipase originates from these organisms could be provided only by isolation and cloning of specific rickettsial gene(s) involved in the internalization process (177).

***Corynebacterium pseudotuberculosis*.** *Corynebacterium pseudotuberculosis*, the agent of caseous lymphadenitis in small ruminants and ulcerative lymphangitis in horses, produces extracellular PLD, which possibly plays a role in the pathogenicity of this and similar bacteria (*Corynebacterium ulcerans* and *Arcanobacterium haemolyticum*) (1, 34, 118, 184). The genes encoding the PLD in *C. pseudotuberculosis* and *A. haemolyticum* have been cloned and sequenced (118). Targeted mutagenesis of PLD in *C. pseudotuberculosis* reduced the ability of this bacterium to establish a primary infection or cause chronic abscess formation in regional lymph nodes (117). These results

indicate that PLD is a virulence determinant of *C. pseudotuberculosis*, increasing the persistence and spread of the bacteria within the host (117).

Protozoan Phospholipases

Phospholipase A facilitates host cell penetration by the two protozoan species *Toxoplasma gondii* and *Entamoeba histolytica*.

Indirect evidence implicating a calcium dependent-PLA₂ in host cell invasion by *T. gondii* was obtained by Saffer et al. (160), who showed that incorporation of exogenous PLA₂ from snake venom increases host cell penetration by *T. gondii*. Furthermore, penetration of host fibroblasts by the parasite was inhibited following preincubation with PLA₂ inhibitors, such as *p*-bromophenacyl bromide and nordihydroguaiaretic acid, or antisera to this enzyme (160). In another study, the same group extended these findings and demonstrated that treating fibroblasts with fractions of disrupted *T. gondii* led to accumulation of degradation products of the phospholipids (fatty acids and lysophospholipids). The data suggest that PLA may be associated with *T. gondii* cells (161). Moreover, fractions of *T. gondii* that had PLA enzymatic activity also increased host cell penetration (161). Although the findings of Saffer and Schwartzman (161) implicate phospholipases in the process of penetration of host cells by *T. gondii*, these workers used a crude enzyme preparation, which makes interpretation of the results quite difficult. To unequivocally establish a correlation between PLA and *T. gondii* virulence and to elucidate how the enzyme may influence the penetration process of host cells by this parasite, gene cloning and enzyme purification are required.

E. histolytica, similar to *T. gondii*, possesses PLA (106, 153). Ravdin and coworkers (106) reported that this amoeba has two PLA enzymes: a calcium-dependent protein which is associated with the plasma membrane and is most active at an alkaline pH, and a calcium-independent enzyme that is localized predominantly to soluble subcellular fractions of *E. histolytica* and is optimally active at an acidic pH. Amebic cytolytic activity, and thereby virulence, is associated with the calcium-dependent PLA. However, as in the case with *T. gondii*, definitive demonstration of the role of amebic PLA enzymes in the cytolytic event awaits their purification, gene cloning, and disruption.

PHOSPHOLIPASES IN PATHOGENIC FUNGI

Phospholipases of *Candida albicans*

The overall incidence of *Candida* bloodstream infections has increased significantly in the last two decades (9, 140, 143), ranging from a 75% increase in small hospitals to an over 400% increase in some large tertiary-care centers (140). This increase led to a tremendous interest in the study of candidal pathogenesis and strategies for control and prevention of this clinically important fungus. Candidal virulence factors have also attracted interest as a possible means for developing novel therapeutic interventions against candidiasis (33, 52, 139). Such virulence factors include adherence (20, 55, 90), germination (180), extracellular proteinases (71, 72) and phospholipases (75), and phenotypic switching (181, 182).

Early work on candidal phospholipases. (i) Candidal phospholipase. The secretion of extracellular phospholipases by *C. albicans* was first reported in the 1960s by Costa et al. (A. Costa, A. Misefari, and A. Amaro, Abstr. Atti XIV Congr. Naz. Microbiol. Messina, abstr. P35 and P36 1967) and Werner

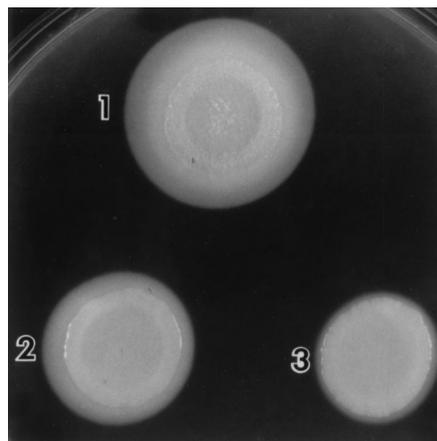


FIG. 2. Production of phospholipase by three different clinical *C. albicans* isolates. Note the difference in the precipitation zones around the three colonies.

(213) by growing the yeast on solid media containing egg yolk or lecithin and analyzing the lipid breakdown products. Later, phospholipase activity was found in many pathogenic *C. albicans* strains by using media containing blood serum and sheep erythrocytes (30). The observation that *C. albicans* secretes phospholipase prompted Pugh and Cawson (150) to develop a lecithin-based cytochemical method to detect this enzyme. In a subsequent study, these authors used this method (149) in conjunction with a chicken chorioallantoic membrane model to evaluate ultrastructural details of candidal invasion and to determine the site of phospholipase production. Invasion was initiated by placing stationary-phase blastospores of *C. albicans* on the membrane, which stimulated cellular changes in the blastospores. Many of the blastospores developed hyphae with phospholipase activity concentrated at the growing tip. The activity was highest where the hyphae were in direct contact with the membrane (149). In general, only hyphae invaded the membrane successfully. Based on these results, the investigators proposed that extracellular phospholipases were important in the invasion of tissue by *C. albicans*.

Subsequent studies by other groups were directed at developing simple methods to detect candidal phospholipases. Odds and Abbott (132) described a biochemical assay to measure intracellular PLA and Lyso-PL activity in *C. albicans*. By using this assay, it was found that PC was hydrolyzed to give Lyso-PC. A small amount of this degradation product was always detected (132). One disadvantage of this assay is that it is time-consuming and therefore unsuitable for testing a large number of isolates. Price et al. (147) described a plate method for the detection of phospholipase activity in *C. albicans* which circumvents this disadvantage. Since egg yolk contains large amounts of phospholipids, predominantly PC and phosphatidylethanolamine, it was incorporated into a Sabouraud dextrose agar-based medium. When grown on this medium, phospholipase-positive candidal isolates produce a distinct, well-defined, dense white zone of precipitation around the colony (Fig. 2). This white zone is probably due to the formation of calcium complex with the fatty acids released by the action of phospholipase on the phospholipids present in the egg yolk (110). In this assay, phospholipase activity (expressed as a P_z value) is defined as the ratio of colony diameter to the diameter of the dense white zone of precipitation around phospholipase positive colonies. This easy plate method became the traditional screening method for phospholipase activity for *Candida* species (66, 99, 168, 215) and other fungi such as

Cryptococcus neoformans (23). However, because egg yolk contains substrates for both phospholipases (phospholipids) and lipases (triglycerides), the egg yolk-based assay is not specific, and therefore its use should be limited to initial screens only (49). Furthermore, the assay is not suitable for the screening of fungal isolates that produce low levels of phospholipase. Confirmation of phospholipase activity necessitates the use of a specific radiometric (10) or colorimetric (122, 176) assay and the use of concentrated culture filtrate, particularly in poorly phospholipase-producing strains. Awareness of this caveat is especially relevant for researchers attempting to determine the phospholipase activity of genetically manipulated mutants, e.g., phospholipase-deficient clones generated by site-specific mutagenesis (49, 101).

(ii) Production of phospholipase by various candidal species. Early studies in which the egg yolk-based assay was used to evaluate the ability of different *Candida* species to produce phospholipase showed that only *C. albicans* and not other species of *Candida* produce extracellular phospholipase. Samaranyake et al. (168) screened 41 *Candida* isolates for phospholipase activity by using a plate assay method and found that 79% of the *C. albicans* strains tested produced extracellular phospholipases whereas no strains of *C. tropicalis*, *C. glabrata*, and *C. parapsilosis* produced the enzymes. The quantity of phospholipase produced by *C. albicans* varied with the specific isolate and correlated with the site of infection. Blood isolates generally produce much higher levels than do isolates from wounds or urine (147). In contrast, Clancy et al. (C. J. Clancy, M. A. Ghannoun, and M. H. Nguyen, Programs Abstr. 36th Annu. Meet. Infect. Dis. Soc. Am., abstr. 317, 1998) recently showed that non-*albicans* *Candida* species produce extracellular phospholipases as determined by both egg yolk-based and colorimetric assays. In this study, 41% of *C. glabrata*, 50% of *C. parapsilosis*, 70% of *C. tropicalis*, 80% of *C. lusitanae*, and 100% of *C. krusei* strains produced detectable phospholipase activity. However, it is important to emphasize that relative to *C. albicans*, the non-*albicans* species secreted significantly smaller amounts of phospholipase (for example, *C. krusei* has approximately 10 times less phospholipase activity [P. Mukherjee and M. Ghannoun, unpublished data]). Although Clancy et al. demonstrated that non-*albicans* species can secrete phospholipase similar to *C. albicans*, it is important to point out that the number of candidal isolates tested was limited ($n = 51$). Therefore, the percentages reported in this study may have been significantly different if a larger number of isolates had been examined. Furthermore, the discrepancy observed by different workers in the phospholipase activity for the non-*albicans* species may be attributed to strain-to-strain variation or differences in the preparation of the egg yolk agar plates used to detect phospholipase secretion in these species.

Lane and Garcia (99), using an egg yolk-based assay, tested the ability of *C. albicans* switching variants (wild type, star, stipple, and ring) to produce phospholipase (181). Star and ring variants produced a similar amount of phospholipase to the wild type. In contrast, the stipple variant produced between 27 and 34% more phospholipase than did the other three strains tested. Although candidal switching is considered to be a virulence factor (181), the relevance of the findings of Lane and Garcia to candidal pathogenicity remains to be shown through *in vivo* experimentation.

(iii) Types of candidal phospholipases. The literature contains contradicting reports on the number and specific types of phospholipase enzymes secreted by *C. albicans*. Costa et al. (Abstr. Atti XIV Cong. Naz. Microbiol. Messina, abstr. P35 and P36, 1967) reported the secretion of both PLA and PLC by this clinically important yeast. Their results were based on the

isolation of palmitic acid and phosphatidylcholine from the proximity of candidal colonies cultured on Sabouraud agar supplemented with serum and sheep erythrocytes. Since this medium is not chemically defined, the sources of the hydrolysis products are uncertain. Banno et al. (10) performed a crude fractionation of the proteins in culture filtrates of *C. albicans* by using DEAE-Sephadex and then assayed the fractions for phospholipase activity. Their data suggested that *C. albicans* secreted three types of phospholipases: Lyso-PL, LPTA, and PLB. Takahashi et al. (193) purified two distinct forms of LPTA from culture filtrates of *C. albicans*. These candidal enzymes differed from mammalian enzymes in amino acid composition; however, the substrate specificities were not determined (193).

To determine the type and substrate specificity of phospholipases secreted by *C. albicans*, a high-phospholipase-producing strain was grown to late log phase and the supernatant was concentrated and assayed for phospholipase activity by using two complementary assays: a specific-substrate radial diffusion assay capable of differentiating between PLA, PLB, and PLC activities (64) and a colorimetric acyl coenzyme A-oxidase system (101). Only PLB activity was observed in the diffusion-based assay (128). In the colorimetric assay, the concentrated supernatant was incubated with a substrate that is specific for PLB (PC) or Lyso-PL (Lyso-PC) activities. Both PLB and Lyso-PL activities were detected in the supernatant, suggesting that there are two enzymes with individual activity, or one enzyme with two activities (see "Definition of phospholipases and rationale in considering them for a role in virulence" above). To resolve this dilemma, protein purification and gene cloning were undertaken.

In collaboration with Yoshinori Nozawa (Gifu University, Gifu, Japan), we purified to homogeneity the protein responsible for candidal extracellular phospholipase activities. This enzyme is a glycoprotein with a molecular mass of 84 kDa on sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The specific activities of the enzyme were 117 $\mu\text{mol}/\text{min}/\text{mg}$ of protein for fatty acid release (hydrolase), and 459 $\mu\text{mol}/\text{min}/\text{mg}$ of protein for PC formation (LPTA activity). The apparent K_m of the hydrolase activity of the enzyme for 1-palmitoyl-*sn*-glycero-3-phosphocholine was 60.6 μM . PLB activity was optimal at pH 6.0. The activity of the purified enzyme was not dependent on divalent cations (Ca^{2+} and Mg^{2+}) and was not inhibited by the addition of EDTA or EGTA (122).

To characterize the functional activity of the purified enzyme, Mirbod et al. (122) used both the radiometric and colorimetric methods described above. In these assays the enzyme was incubated with a substrate that is specific for PLB activity or Lyso-PL activities. In addition, to determine the LPTA activity, Lyso-PC was incubated with the purified enzyme and the rate of production of PC was monitored. These studies revealed that the purified *C. albicans* enzyme has both hydrolase (PLB and Lyso-PL) and LPTA activities. The finding that one enzyme has two hydrolase activities is not unique to *C. albicans*. A similar observation has been reported for PLB secreted by *Penicillium notatum*. Saito et al. (164) reported that *P. notatum* PLB is a glycoprotein with intrinsic Lyso-PL and PLB activities. Similarly, Lee et al. (100) showed that the *Saccharomyces cerevisiae* *PLB1* gene encodes a protein required for Lyso-PL and PLB activities. These data clearly demonstrate that the purified enzyme is a phospholipase with dual activities. Similar to the candidal enzyme, PLB from *S. cerevisiae* is reported to have an acyltransferase activity (224, 225). This complex pattern of activity led to difficulties in the nomenclature of the fungal enzyme (see above). Further evi-

dence that candidal PLB has both PLB and Lyso-PL activities was obtained by assaying phospholipase activities in supernatants from the parent and *PLB1*-deleted mutants (see below for construction and characterization of mutants). Unlike the parent, deletion of *caPLB1* led to loss of both PLB and Lyso-PL activities.

The phospholipase activities described so far are due to secreted candidal enzymes. Recently, McLain and Dolan (116) reported on a membrane-associated enzyme in *C. albicans*. This enzyme has a PLD activity and is capable of performing a transphosphatidylation reaction in the presence of primary alcohols (see below for cloning of candidal *PLD*).

Evidence correlating phospholipases and virulence. Although the contribution of phospholipases to the pathogenesis of bacteria and protozoa has been known for some time, investigations into whether these enzymes are associated with fungal virulence have only recently been undertaken. Barrett-Bee et al. (11) was the first to evaluate the role of extracellular candidal phospholipases in virulence by using a murine model of candidiasis. When phospholipase activity was measured in six yeasts (four strains of *C. albicans* and a single strain each of *C. parapsilosis* and *S. cerevisiae*), a correlation was found between phospholipase activity and two potential parameters of pathogenicity. The *C. albicans* isolates which adhered most strongly to buccal epithelial cells and were most pathogenic in mice had the highest phospholipase activities. Less pathogenic isolates of *C. albicans*, *C. parapsilosis*, and *S. cerevisiae* were less adherent to epithelial cells and less lethal to mice and had lower phospholipase activities (11). Although these findings suggest a correlation between phospholipase activity and candidal virulence, the strains used were not genetically related, and therefore the differences observed in the virulence of the tested strains could be attributed to factors other than phospholipase secretion.

Two strategies were followed by Ibrahim et al. (75) to determine the role of phospholipase in candidal virulence: (i) the ability of blood isolates of *C. albicans* from patients and oral isolates from healthy volunteers to produce phospholipase were compared, and (ii) the pathogenicity of clinical isolates with different levels of phospholipase secretion was compared in a murine model of disseminated candidiasis (75). In the first comparative study, 22 isolates of *C. albicans* were tested (11 were blood isolates obtained from patients with disseminated candidiasis, and 11 were commensal isolates recovered from the oral cavities of healthy volunteers). Marked differences among strains from the two sources were observed. Significantly higher levels of phospholipase production were found in the blood isolates than in the commensals ($P = 0.0081$). In addition, the blood isolates had significantly higher rates of germination ($P = 0.03$), and their germ tubes were longer than the germ tubes formed by the commensal strains ($P = 0.016$). These findings suggest that blood isolates, unlike commensals, may have enhanced the expression of several virulence factors, including both germination and phospholipase production, to enable them to invade host tissues (75).

In the same study, Ibrahim et al. (75) prospectively examined nine blood isolates for expression of virulence factors, including phospholipase and proteinase production, adherence, germination, growth rate, and ability to damage endothelial cells. Additionally, the mortality of mice infected with each of these isolates was determined and the predictive value of each virulence factor for mortality was determined by Cox proportional-hazards analysis. Of the virulence factors studied, only extracellular phospholipase activity was predictive of mortality (75).

To obtain further evidence of the contribution of phospho-

lipases to candidal pathogenicity, the virulence of two *C. albicans* isolates, CA30 and CA87, was compared in an infant-mouse model (28). Strain CA87 failed to cross the bowel wall of the infant mouse after oral-intragastric challenge and did not cause disseminated infection, while strain CA30 was able to cross the bowel wall and to disseminate hematogenously. A variety of putative virulence factors of these two strains were measured in vitro to determine the relationship of these factors to virulence (75). The only apparent difference in expression of virulence factors between the two strains was in the superior ability of CA30 to produce phospholipase (75). Since this strain was distinguished from CA87 by its ability to invade the bowel wall and undergo subsequent hematogenous dissemination and tissue invasion, these data further suggest that phospholipase is involved in the invasion process of *C. albicans*.

Although these studies provide evidence that implicates phospholipases in the pathogenesis of *C. albicans*, they do not prove this association. As in the study of Barrett-Bee et al. (11), the candidal isolates used were not genetically related. The use of such strains does not rule out the possibility that differences observed in the virulence of these strains could be attributed to factors other than phospholipases. Thus, the correlation found between phospholipase and candidal virulence should be confirmed by the use of an isogenic strain pair that differs only in phospholipase production. Molecular cloning of the candidal gene(s) encoding the extracellular phospholipases is the essential first step in the molecular genetic dissection of the role of these enzymes in pathogenesis.

Cloning of the gene(s) encoding candidal phospholipases. Efforts to clone genes encoding candidal phospholipases have resulted in the cloning of two genes encoding PLB (*caPLB1* and *caPLB2*) and one gene (*caPLD*) coding for PLD.

(i) Candidal phospholipases B. (a) *caPLB1*. The *C. albicans* *PLB1* gene was cloned by using a PCR-based approach relying on degenerate oligonucleotide primers designed on the basis of the amino acid sequences of two peptide fragments obtained from a purified candidal enzyme displaying phospholipase activity (122). Sequence analysis of a 6.7-kb *EcoRI*-*ClaI* genomic clone revealed a single open reading frame of 1,818 bp that predicts a preprotein of 605 residues. The size of the candidal PLB1 protein is similar to those reported for other fungal PLBs, which ranged between 612 and 664 amino acids (Table 2). The genomic DNA sequence encodes 17 amino acid residues that are absent from the NH₂ terminus of the mature protein. This stretch of residues represents a possible signal sequence (111, 169). The predicted protein contains seven Asn-X-Ser/Thr motifs (residues 199, 261, 399, 451, 465, 492, and 573) that could potentially be N glycosylated. One possible tyrosine phosphorylation site, Lys-Ser-Asn-Ile-Asp-Val-Ser-Ala-Tyr (residues 369 to 377), was also identified (101). Hydrophobicity analysis of the predicted protein sequence (98) revealed the presence of a single stretch of hydrophobic amino acids present at the amino terminus (residues 1 to 18). This segment of amino acids most probably functions as a single peptide which targets the protein to the endoplasmic reticulum for subsequent processing and, ultimately, secretion. Comparison of the putative candidal phospholipase with other proteins in the redundant database (BLASTP program) revealed significant homology to known fungal PLBs from *S. cerevisiae* (45%), *P. notatum* (42%), *Torulaspora delbrueckii* (48%), and *Schizosaccharomyces pombe* (38%) (Fig. 3). This gene, designated *caPLB1*, was mapped to chromosome 6 (101). Consistent with our findings, Hoover et al. (69a) used degenerate oligonucleotide primers derived from conserved regions of *PLB1* genes from *S. cerevisiae* and other fungi to clone the

TABLE 2. Fungal phospholipases

Fungus	Enzyme	Gene cloned (name)	Substrate specificity ^a	Protein size (amino acid residues)	Molecular mass (kDa)	Potential GPI anchor sequence for attachment	Reference(s)
Pathogenic							
<i>Candida albicans</i>	PLB1	Yes (<i>caPLB1</i>)	PC, 1-Palmitoyl-Lyso-PC, 1-arachidonyl-Lyso-PC, 1-oleoyl-Lyso-PC, fl-Palmitoyl-Lyso-PE, 1-Palmitoyl-Lyso-PI	605	84	No	101, 122, 193
	PLB2	Yes (<i>caPLB2</i>)		608	67 ^b	No	188
	PLD PLB	Yes (<i>caPLD</i>) No		1,710	196.4 ^b		87 Clancy et al., Programs Abstr. 36th Annu. Meet. Infect. Dis. Soc. Am., abstr. 316 and 317 23, 24; Gottfredsson et al., Abstr. 97th Gen. Meet. Am. Soc. Microbiol. 1997
<i>Cryptococcus neoformans</i>	PLB	Yes (<i>PLB</i>)	PC	617			16; Koul et al., Abstr. 98th Gen. Meet. Am. Soc. Microbiol. 1998
<i>Aspergillus fumigatus</i>	PLB	No	PC				16
<i>Aspergillus flavus</i>	PLC	No					16; Koul et al., Abstr. 98th Gen. Meet. Am. Soc. Microbiol. 1998
	PLD	No					
	PLB	No	PC, Lyso-PC				
Nonpathogenic	PLA	No	Unknown				
	PLC	No	Unknown				
<i>Saccharomyces cerevisiae</i>	PLB ^e	Yes (<i>PLB1</i>) ^e		664		Yes	100, 223–225
	PLB1 ^d			200–280			
	PLB2 ^d			220			
<i>Penicillium notatum</i>	PLD	Yes (<i>SPO14</i>)	PC and Lyso-PC	1,683	195	Yes	87, 206
	PLB	Yes (<i>PLB</i>)		603	95	Yes	113, 163
		Yes (<i>PLB</i>)				Yes	
<i>Tortulaspora delbrueckii</i>		Yes ^f	Unknown	649	72	Yes	211
<i>Neurospora crassa</i>		Yes ^f					
<i>Kluyveromyces lactus</i>		Yes ^f					

^a For abbreviations, see Table 1, footnote a.^b Predicted from the amino acid sequence and may vary due to posttranslational modifications.^c Secreted enzyme.^d Membrane-associated enzyme.^e Coding for a core protein moiety of the three isoforms of PLB present in *S. cerevisiae*.^f Gene sequence available in GenBank but not published.

corresponding candidal homolog. Their results confirm our findings and the identity of *caPLB1*.

A deeper analysis of the PLB1 protein sequence revealed a feature that distinguishes the candidal enzyme from the other known fungal PLBs. Unlike the PLBs of the nonpathogenic fungi, *S. cerevisiae*, *S. pombe*, *T. delbrueckii*, and *P. notatum*, candidal PLB1 is characterized by the absence of a hydrophobic COOH terminus (Table 2). Such a hydrophobic COOH-terminal region may ultimately be replaced by a glycosylphatidylinositol (GPI) anchor. Indeed, potential GPI anchor sites, immediate to the hydrophobic COOH-terminus, were identified in fungal PLBs from *S. cerevisiae*, *S. pombe*, *T. delbrueckii*, and *P. notatum* (22, 53). Proteins modified with a GPI anchor may be transiently tethered to the plasma membrane or ultimately cross-linked to the insoluble glucan component of the cell wall (43, 107, 108). Release of proteins associated with the plasma membrane would require the action of a GPI-specific phospholipase. In this view, a GPI anchor may serve to regulate the release of the enzyme to the surroundings. Unlike PLBs from nonpathogenic fungi, the candidal PLB, escaping GPI anchoring, would probably be directly secreted. Such a characteristic may enhance the virulence of *C. albicans*. Further characterization of PLBs from each fungal species will be necessary to clarify whether any are GPI anchored, and what effect this modification may have on the function and subcellular localization of PLBs, as well as on the pathogenicity of these fungi.

(b) *caPLB2*. Attempts to clone *caPLB2* were prompted by evidence suggesting that *C. albicans* may possess more than one gene encoding PLB. This evidence is summarized as follows: (i) two proteins which share phospholipase activity were purified by Takahashi et al. (193); (ii) similarly, three enzymes with PLB activity have been purified and characterized from *S. cerevisiae* (224, 225); (iii) the completion of the sequencing of the *S. cerevisiae* genome revealed that this organism has at least three genes encoding PLB that are highly homologous (GenBank accession no. L23089, S53035, and S66693); (iv) the *S. pombe* genome has two sequences which potentially encode PLB (D89183 and D89204); and (v) deletion of *caPLB1* did not lead to 100% loss of phospholipase activity, suggesting that the residual activity (about 1% and 10% PLB and Lyso-PL activities, respectively) may be due to a second gene.

To clone *caPLB2*, Sugiyama et al. (188) used a PCR-based approach similar to the one used to clone *caPLB1*. A number of similarities are observed between *caPLB1* and *caPLB2* in size, availability of N-glycosylation sites, the presence of a single stretch of hydrophobic amino acids at the amino terminus, and the absence of GPI attachment site (Table 2). The nucleotide sequence of *caPLB2* contained a single open reading frame encoding a putative 608-amino-acid protein with an estimated molecular mass of about 67 kDa. The predicted amino acid sequence contains six potential N-glycosylation sites [Asn-X-(Ser/Thr) motifs] at residues 259, 365, 450, 464, 491, and 572. The deduced amino acid sequence of *caPLB2* was homologous to that of *caPLB1* (65% identity). *caPLB2* was also similar to PLBs from *S. cerevisiae*, *T. delbrueckii*, and *P. notatum* (42, 46, and 42% identity, respectively) (Fig. 3). Hydrophathy analysis of the predicted protein (98) revealed the presence of a cluster of hydrophobic amino acids at the N terminus. Similar to *S. cerevisiae* and *T. delbrueckii* PLBs (100, 211), *caPLB2* possesses a potential signal sequence at the N-terminal region of *caPLB2*, where two polar amino acids (Gln-Ser) are followed by a cluster of six hydrophobic amino acids (Ile-Leu-Leu-Phe-Val-Val). Such sequence may guide proteins to the secretory pathway. Like *caPLB1*, *caPLB2* lacks the GPI attachment site (a cluster of hydrophobic amino acids at the

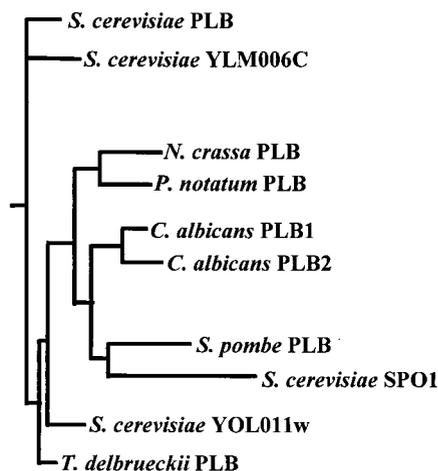


FIG. 4. Phylogenetic tree analysis of fungal PLBs. *S. cerevisiae* PLB (GenBank accession no. L 23089), *S. cerevisiae* SPO1 (P 53541), *S. cerevisiae* YLM006c (S53035), *S. cerevisiae* YOL011w (S66693), *T. delbrueckii* PLB (D 32134), *P. notatum* PLB (P 39457), *S. pombe* PLB (Z 99258), *N. crassa* PLB (AF045575), and *C. albicans* *caPLB1* and *caPLB2*.

carboxy terminal) found in the PLBs from the nonpathogenic fungi such as *S. cerevisiae*, *P. notatum*, and *T. delbrueckii* PLBs (100, 113, 211) (see above). Therefore, *caPLB2*, as with *caPLB1*, is probably not GPI anchored. Since fungal PLBs and mammalian PLA₂ have lysophospholipase activities, it is likely that these enzymes also share common conserved amino acid regions. In this regard, three amino acid residues essential for the catalytic function have been identified in PLA₂ (²⁰⁰Arg, ²²⁸Ser, and ⁵⁴⁹Asp) (142). Among them, the serine residue participates in the catalytic function. Interestingly, three regions surrounding these amino acids are also conserved in PLBs from fungi. The deduced amino acid sequence of *caPLB2* contains the motifs SGGGX⁹⁷RA(M/L), GL¹³³SG(G/S), and ³⁸¹D(S/G)G(E/L)XXXN, which may have a catalytic function (188).

It is clear that fungal PLBs have certain features that are common and others in which they differ. To determine the phylogenetic relationship among various fungal PLBs, Sugiyama et al. (188) constructed a phylogenetic tree of PLBs by using the neighbor-joining method (165). Sequence data from *S. cerevisiae* PLB (GenBank accession no. L23089), *S. cerevisiae* SP01 (P53541), *S. cerevisiae* YLM006c (S53035), *S. cerevisiae* YOLO11w (S66693), *T. delbrueckii* PLB (D32134), *P. notatum* PLB (P39457), *S. pombe* (Z99258), *Neurospora crassa* (AF045575), and *C. albicans* *caPLB1* and *caPLB2* were used in the tree construction. Figure 4 shows that PLBs and potential PLB analogues are contained in a large cluster of PLB family members. *caPLB1* and *caPLB2* are closely related to each other, and *caPLB* genes are more closely related to PLB genes from *S. pombe* and *P. notatum* than to PLBs genes from *S. cerevisiae* and *T. delbrueckii*.

(ii) **Candidal phospholipase D.** PLD catalyzes the hydrolysis of PC to produce phosphatidic acid and choline (Fig. 1a above). Both mammalian and fungal (*S. cerevisiae*) genes encoding PLD have been cloned and characterized (130, 206). Mammalian PLD has emerged as one of the key enzymes in intracellular signaling (44), while PLD from *S. cerevisiae* (encoded by *SPO14*) is essential for meiosis (41, 158, 206). The absence of meiosis in *C. albicans*, which, unlike *S. cerevisiae*, exists as a diploid, indicates that PLD in this clinically important yeast may play other roles than meiosis. McLain and Dolan (116) have recently shown that PLD may be an impor-

tant regulator of the dimorphic transition. Since this yeast-hypha transformation is thought to be an important virulence determinant in *C. albicans* (33, 54, 133), cloning and disruption of candidal *PLD* may contribute to our understanding of the biological role of this phospholipase.

For cloning candidal *PLD*, Kanoh et al. (87) designed two oligonucleotide primers based on the conserved amino acid sequences in human *PLD1a* and *SPO14*. These primers were used in a PCR-based approach to clone the full-length gene. The cloned *PLD* sequence had a potential open reading frame encoding a protein of 1,710 amino acids with a calculated molecular mass of 196.4 kDa. The putative ATG codon was surrounded by the consensus Kozak sequence for translation initiation (94), and the in-frame stop codon and the putative TATA box sequence (TATATAA) were found 6 and 172 bases upstream from the start codon, respectively. The deduced amino acid sequence contained the four conserved regions (I, II, III, and IV) defined by the primary structures of plant, yeast, and human *PLDs* (125). Furthermore, the HKD motif (HxKxxxD) in regions I and IV and a serine residue in the GSRS motif in region IV, which are critical for *PLD* biochemical activity (189), were also completely conserved.

The number of amino acids and the calculated molecular mass of *C. albicans* *PLD* closely resembled those of *SPO14* protein (1,683 amino acids; 195 kDa). Comparison of the primary structure with those of other *PLDs* showed that it had the highest homology to the *SPO14* protein. The overall homology between the amino acid sequences of *C. albicans* *PLD* and the *SPO14* protein was 42%. At the four conserved regions, the homology between *C. albicans* *PLD* and the *SPO14* protein ranged from 65 to 71%, while that between *C. albicans* *PLD* and rat *PLDs* was 42 to 55%. In addition to the four conserved regions found in both fungal and mammalian *PLDs*, candidal *PLD* has seven regions (A to G) of 10 to 91 amino acids that are highly homologous to *SPO14* protein. Of the seven regions, only two, F and G, are conserved in mammalian *PLDs*. Therefore, Kanoh et al. (87) speculated that these five regions (A to E) may compose some functional domains specific for fungi.

A phylogenetic tree for *PLDs* of various species showed three major clusters (87). The first cluster is composed of mammalian *PLD1* and plant (151, 209), nematode, and *Streptomyces* *PLDs*. The second cluster was composed of mammalian *PLD2*, and the third cluster was composed of fungal *PLDs*, including *C. albicans* *PLD* and the *SPO14* protein. Because fungal *PLDs* are in a separate grouping from mammalian and other *PLDs*, it is tempting to speculate that this enzyme may serve a fungus-specific function(s). Unfortunately, elucidation of the biological function of candidal *PLD* awaits the disruption of the gene encoding it.

Disruption of phospholipase B₁. Gene disruption (deletion) is frequently used to determine the functionality of a specific gene. This is particularly relevant in assessing the contribution of a given gene to microbial virulence (45). Several workers have disrupted a number of genes to evaluate their contribution to the virulence of *C. albicans* (37, 61, 74, 170, 228). Most recently, *C. albicans* strains with deletions of the *INT1* gene, which encodes a cell surface protein with similarity to mammalian integrins, were constructed (50). Adhesion, hyphal formation, and virulence were subsequently found to be correlated with the expression of this gene.

The successful cloning of *caPLB1* allowed us to construct *PLB*-deficient mutants by targeted gene disruption by using the *ura*-blaster technique (47) (see reference 101 for details of the method used to disrupt *caPLB1*). Initial disruption of *caPLB1* was complicated by the finding that three rounds of transformation were required to delete the gene, suggesting that strain

CAI-4 (a candidal strain derived from *C. albicans* SC5314 by deletion of the *ura3* gene) was triploid for this locus. This finding seemed plausible since disruption of the *C. albicans* chitin synthase (*CHS2*) and catalase (*CAT1*) genes also revealed that they are triploid (61, 226). However, chromosome-blotting experiments suggested that the triallelic state of the *caPLB1* genetic locus probably originated from a gene duplication/translocation event rather than an inherent triploid state in the parental strain.

Given the above finding, we proceeded to disrupt the *caPLB1* gene a second time, starting with the original CAI-4 strain. Chromosomal analysis was performed at every stage of this second disruption as a means of screening newly constructed strains for genetic translocation or incorrect targeting of transforming DNA. Southern hybridization, chromosomal analysis, and measurement of functional activity confirmed the successful disruption of *caPLB1* and demonstrated that *Candida* is diploid for this locus (101). Although we cannot discount the fact that some candidal genes may be aneuploid (61, 226), our study with *caPLB1* highlights the importance of fully characterizing the nature of a suspected triploidy even if mutant strains are constructed by the generally reliable technique of targeted gene disruption. Similar experience has been encountered by a number of *Candida* researchers (personal communications); therefore, intensive molecular genetic analysis of the constructed mutants should be undertaken so as to disclose potential mutations that otherwise go undetected, especially if disruption of the gene in question produces no obvious phenotypic defect.

Phenotypic characterization of phospholipase B₁-deficient mutants. Phenotypic characterization of the parent and deletion mutants is necessary prior to evaluating the virulence of the isogenic strain pair. Such characterization may provide some measure of assurance that deleting the *caPLB1* gene results in only loss of *PLB* production and not other unrelated phenotypic properties. Comparison of the growth rates and germination capabilities of *PLB*-deficient mutant with that of the parental strain revealed that disruption of *caPLB1* did not affect growth and germination of *C. albicans* (101), indicating that *caPLB1* is not essential for these processes.

Disruption of *caPLB1* reduced the ability of *C. albicans* to secrete the enzyme. When examined by Western blot analysis, *PLB* was found only in culture filtrates produced by parental and *plb1-Δ¹* strains but not in the supernatant produced by strain *plb1-Δ²* (null mutant). Furthermore, assay of supernatants collected from the parent and the *PLB*-deficient mutants for *PLB* and Lyso-*PL* activities by using specific substrates revealed that the two activities were reduced in the *PLB*-deficient mutant by approximately 99 and 80% respectively, relative to the wild type. The residual phospholipase activities secreted by the *caPLB1*-deficient strain may be the result of an additional candidal phospholipase-encoding gene. In this regard, a second gene, *caPLB2*, which has significant homology to *caPLB1*, was recently cloned by our group (see "Candidal phospholipases B" above). Experiments to delete this second gene are under way. Characterization of mutants with *caPLB1* and *caPLB2* deleted may clarify the source of the residual phospholipase activity observed in *caPLB1*-deficient mutant.

Testing of phospholipase B₁-deficient mutants in murine models of candidiasis. Two different models of candidiasis, representing different clinical settings, were used to determine the role of *caPLB1* in the virulence of *C. albicans*: (i) a hematogenous-dissemination murine model (58) and (ii) an oral-intragastric infant mouse model (28).

(i) **Hematogenous-dissemination model.** Leidich et al. (101) challenged BALB/c mice with 5×10^5 yeast cells of either the

parent or the PLB-deficient strains intravenously through the lateral tail vein. Both survival and tissue fungal burden were used to assess the pathogenicity of the infecting strains. Their data showed that all mice infected with parental strain SC5314 succumbed to candidal infection within 9 days. In contrast, 50 and 60% of mice challenged with either the PLB-deficient strain *plb1-Δ¹* or *plb1-Δ²*, respectively, were alive at day 15. The mean survival time \pm standard deviation for mice infected with parent was 4.4 ± 2.1 days, compared to 12.7 ± 2.7 and 13.3 ± 2.6 days for mice infected with *plb1-Δ¹* or *plb1-Δ²*, respectively. Statistical analyses revealed that mice infected with either strain *plb1-Δ¹* or *plb1-Δ²* survived significantly longer than did mice infected with strain SC5314 ($P < 0.0001$ for both comparisons).

Gross inspection of the kidneys showed that numerous visible candidal foci covered the renal cortex of mice infected with the parental strain. In contrast, no candidal foci were visible on renal surfaces of mice infected with either of the PLB-deficient strains. Tissue fungal burden experiments were carried out to assess the severity of infection caused by the wild-type and mutant strains (101). Consistent with the results of the survival experiment, candidal strains *plb1-Δ¹* and *plb1-Δ²* were cleared significantly faster from the kidneys and brain than was parental strain SC5314. For example, the mean fungal burden in the kidneys \pm standard deviation was 6.13 ± 0.05 and 4.33 ± 0.35 CFU/g of tissue for the parent and *plb1-Δ²* mutant, respectively ($P < 0.004$). The relative rates of clearance were as follows: *plb1-Δ²* > *plb1-Δ¹* > SC5314.

Taken together, these findings demonstrate that *caPLB1* is associated with candidal virulence. Survival and tissue fungal burden data showed that mortality of and tissue invasion in mice infected with strain *plb1-Δ¹*, which harbors only a single deleted *caPLB1* allele, was also significantly reduced compared to those for the wild type, suggesting that there may be a dose-dependent relationship between PLB and virulence. Thus, a threshold level of PLB may be required to effectively enhance candidal virulence. In this regard, analysis of culture filtrates for PLB activity revealed that the levels of free fatty acid activity, relative to the parent, released from dipalmitoyl-PC following incubation with culture filtrates obtained from CAI-4, *plb1-Δ¹*, and *plb1-Δ²* were 100, 54, and 1%, respectively. Moreover, the fact that deletion of *caPLB1* did not render *C. albicans* strains completely avirulent underscores the notion that candidal pathogenicity is multifactorial and is regulated by more than one determinant (33).

(ii) Oral-intragastric infant-mouse model. Seshan et al. (174) used an oral-intragastric infant-mouse model to examine the effect of *caPLB1* on the ability of *C. albicans* to traverse the gastrointestinal barrier and colonize systemic target organs, such as the kidneys and liver. This model simulates candidal migration across the gastrointestinal tract, one of the major routes for contracting disseminated candidiasis (131). In these experiments, inbred infant mice [cri:CFW (SW) BR] were inoculated intragastrically with 2×10^8 blastospores of either the wild-type or *caPLB1*-deficient strain. Transmigration of candidal cells across the gastrointestinal tract was monitored microscopically as well as by determining tissue fungal burden of target organs.

The histopathologic appearance of the gastric mucosa of mice infected with the parental strain differed markedly from that of mice infected with the PLB-deficient mutant. Light and transmission electron microscopic examination revealed yeast and hyphal elements in the gastric mucosa 14 days postchallenge. Fungal elements were observed after challenge with both the parental and PLB-deficient strains. However, mucosal invasion in mice challenged with the PLB-deficient strain was

confined to the stomach lumen and inner layers of the gastric mucosa, and a minimal neutrophil-dependent inflammatory response was elicited. Furthermore, several areas of the stomach revealed no demonstrable infection. In contrast, the parental strain invaded the submucosal tissue of the stomach, elicited a neutrophil-dependent inflammatory response, and produced systemic candidiasis to a far greater extent. Additionally, the parental strain was able to transverse the vasculature, since numerous hyphal elements were observed within blood vessel lumens (see below) (174).

The number of mice that developed systemic candidal infections differed markedly following challenge with either the parental or PLB-deficient strain. Of the mice challenged with the parental strain, 90 and 70%, respectively, exhibited liver and kidney colonization. In contrast, only 45 and 27% of mice infected with the PLB-deficient mutant exhibited liver and kidney involvement, respectively (174). This difference was reflected in the number of candidal cells (CFU) recovered from these organs. The relative candidal cells recovered from the kidneys and liver of mice challenged with the parental or *caPLB1*-deficient strain were 2.05 ± 0.4 and 0.77 ± 0.4 CFU/g for the kidney and 2.87 ± 0.4 and 1.33 ± 0.4 CFU/g for the liver, respectively. These differences were statistically significant (P values for kidneys and liver were 0.042 and 0.014, respectively).

The findings of Seshan et al. (174) that the parental strain was more efficient at crossing the gastrointestinal tract and invading internal organs than was its PLB-deficient counterpart suggest a role for PLB in candidal transmigration across the gastrointestinal tract and subsequent dissemination to target organs. Furthermore, their study presents further evidence in support of PLB as a virulence determinant for *C. albicans*. Finally, the results of the studies of Leidich et al. (101) and Seshan et al. (174) indicate that candidal PLB may be critical for dissemination of *C. albicans* by the gastrointestinal and hematogenous routes.

Expression of phospholipase B₁ during host tissue invasion. One of the criteria to prove that a particular gene or its product plays an important role in the disease process is to show that it is expressed by the microorganism during the infectious process (45, 167). Both the hematogenous-dissemination (101) and oral-intragastric mouse (174) models for candidiasis were used to determine whether PLB is secreted in vivo.

In the hematogenous-dissemination model, Leidich et al. (101) challenged mice with either the parental or the *caPLB1*-deleted mutant. Kidneys were harvested, and sections were processed for immunoelectron microscopy by being incubated with either PLB antiserum or goat serum, which served as a negative control. The data revealed that while PLB was secreted from the parental strain during the infectious process, as evidenced by the formation of immunogold complexes following incubation of the tissue sections with PLB antiserum, it was not secreted from the PLB-deficient mutant. In other experiments, *C. albicans* cells were recovered from the kidneys of mice infected with either the parental or the PLB-deficient strain and prepared for immunogold electron microscopy. Sections examination showed that immunogold labeling was observed only with cells recovered from mice infected with the wild type strain and not with cells recovered from animals infected with the PLB-deficient mutant (J. Vitullo, S. D. Leidich, C. J. Jessup, and M. A. Ghannoum, Program Abstr. 98th Gen. Meet. Am. Soc. Med., abstr. F-39, 1998). These data demonstrate that *C. albicans* secretes PLB during the invasion of target organs. Moreover, deletion of PLB abrogate the fungal cell ability to secrete this lytic enzyme (see "Phenotypic and

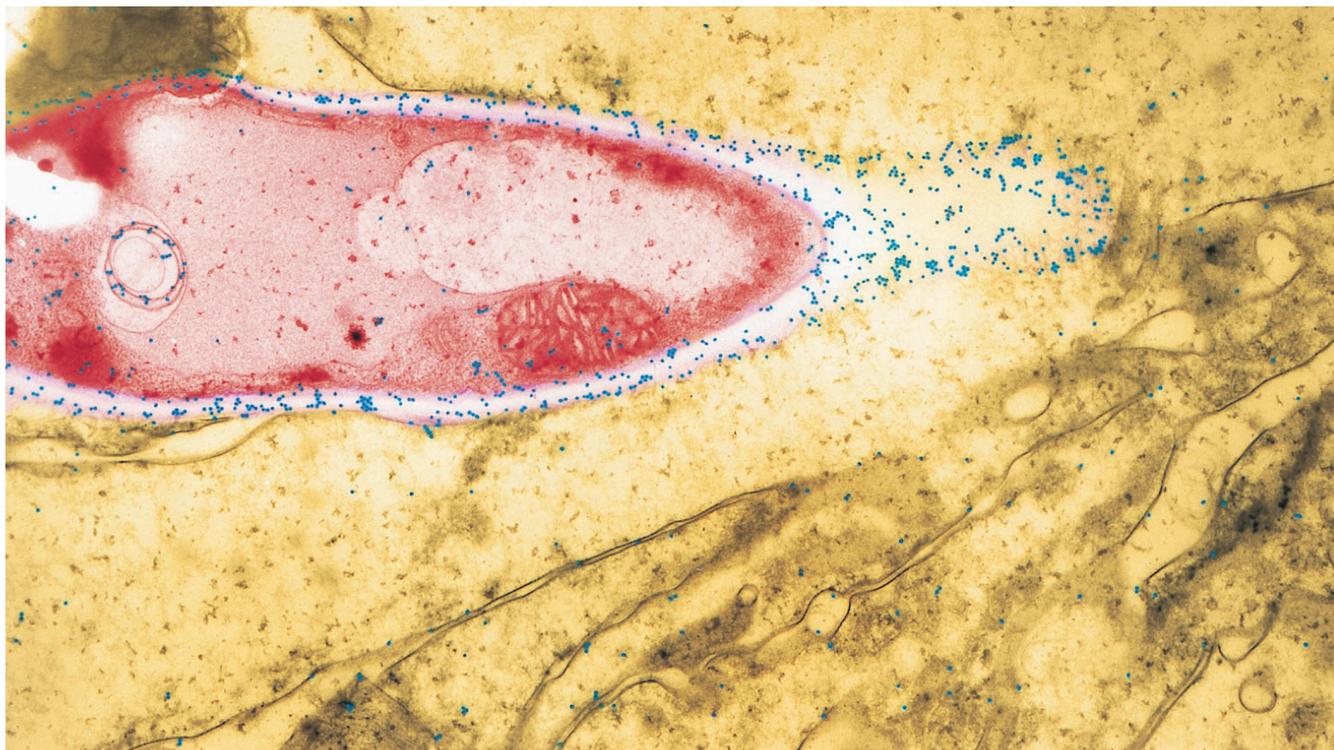


FIG. 5. Expression of PLB during candidal invasion of gastrointestinal tract of an infant mouse. Inbred infant mice [cr1: CFW (CSW) BR] were inoculated intragastrically with 2×10^8 cells of the phospholipase-producing parental strain (SC5314). The stomach was harvested, and sections were prepared for immunogold microscopy. The candidal cell appears in red, and the stomach tissue appears in greenish-yellow. Immunogold complexes that formed following incubation of the sections with PLB antiserum are shown in blue. Magnification, $\times 10,000$.

genotypic characterization of phospholipase B₁-deficient mutants" above).

Using indirect-immunofluorescence microscopy, Seshan et al. (174) investigated the expression of PLB during the course of candidal transmigration across the gastrointestinal tract of infant mice. Following challenge with either the parent or the PLB-deficient strain, mice were sacrificed and their gastric mucosal tissues were removed and prepared for indirect-immunofluorescence microscopy. The data showed that the vast majority of candidal cells secrete PLB. Both hyphal and yeast forms of *C. albicans* secreted the enzyme. Although the periphery of most cells exhibited low-level fluorescence suggestive of PLB secretion, intense fluorescence was observed at the growing tips of mature and developing hyphae, suggesting that PLB secretion is concentrated at the invading hyphal tip. This phenomenon was strikingly clear when immunogold transmission electron microscopy was used to localize PLB secretion in the stomach of infected mice (Fig. 5).

The observation that PLB is expressed by *C. albicans* in murine models prompted us to investigate whether *C. albicans* also secretes this enzyme during human infection. Sera from patients with proven invasive candidal infection (as determined by a positive blood culture) were obtained from Leo Mendoza (Michigan State University, Lansing, Mich.). Using Western blot analysis, we showed that sera from human patients with systemic candidiasis contain antibodies that reacted with purified PLB (data not shown), suggesting that PLB is secreted during an infectious episode. Taken together, the above findings, using two fundamentally different candidiasis murine models and sera from patients with systemic candidiasis, suggest that PLB is secreted during the course of *C. albicans* infection and thereby may be involved in virulence.

Phospholipases of *Candida glabrata*

C. glabrata is recognized increasingly as an important nosocomial pathogen (2, 89, 92, 146, 156, 201, 210, 214, 218, 219); D. W. Warnock, J. Burke, N. J. Cope, E. M. Johnson, N. A. Von Fraunhofer, and E. W. Williams, Letter, *Lancet* **ii**:310, 1988). It ranks third among *Candida* species in the number of reported cases of candidemia, and in one study, *C. glabrata* surpassed *C. tropicalis* to become the most common non-*albicans* species isolated (51). *C. glabrata* is important clinically, not only because of the increase in its frequency but also because it is associated with high complication rates (51) and high mortality rates (7, 12). In one study, the mortality rate of 83% exceeded the observed rates from any other *Candida* species (92). Therefore, identification of factors that contribute to the virulence of this organism may provide new attractive therapeutic targets. Recently, Clancy et al. (Programs Abstr. 36th Annu. Meet. Infect. Dis. Soc. Am., abstr. 317, 1998) reported on the detection of extracellular phospholipase activity in *C. glabrata* and provided data implicating it as a virulence factor in patients with candidemia caused by this yeast. In their study, phospholipase activity (as determined by opacity around colonies growing on egg yolk-containing media) was investigated in 51 non-*albicans* *Candida* species (*C. glabrata*, 22; *C. parapsilosis*, 12; *C. tropicalis*, 10; *C. lusitaniae*, 5; *C. krusei*, 2) recovered from patients in a multicenter study of candidemia. Phospholipase activity was detected in 53% of isolates. An assay involving agar containing specific phospholipid substrates was used to identify the type of phospholipase activity secreted by *C. glabrata* (64). As with *C. albicans*, PLB and Lyso-PL accounted for 100% of phospholipase activity. No PLA or PLC activities were detected in supernatants collected from various

C. glabrata isolates tested. To determine whether a correlation exists between phospholipase activity and clinical virulence, the authors classified the *C. glabrata* isolates into two groups: persistent isolates were strains that remained in the blood despite therapy with antifungal agents effective in vitro and despite removal of all intravenous catheters, and nonpersistent isolates were strains that were cleared. Among non-*albicans* *Candida* species tested, the association between phospholipase activity and persistent candidemia was strongest for *C. glabrata* ($P = 0.004$). Moreover, the median PLB and Lyso-PL activities were higher for persistent isolates (9.5 and 24 pmol/mg, respectively) than for nonpersistent isolates (0 and 5.8 pmol/mg) ($P < 0.0001$ and $P = 0.02$, respectively).

To establish the role of PLB in the pathogenesis of *C. glabrata*, an isogenic strain pair of this yeast which differed only in PLB activity was created (C. J. Clancy, A. Leuin, M. A. Ghannoum, and M. H. Nguyen, Programs Abstr. 36th Annu. Meet. Infect. Dis. Soc. Am., abstr. 316, 1998). Conserved sequences of *scPLB1*, the gene responsible for PLB activity in *S. cerevisiae*, were used to design guessmer primers for PCR with DNA from a clinical *C. glabrata* isolate. A 1,030-bp PCR product that encoded part of a putative protein with 78% homology to *scPLB1* protein was generated. To create an isogenic mutant with disruption of the PLB gene, Clancy et al. first created a *ura3⁻* auxotroph of the parent *C. glabrata* strain. *S. cerevisiae* *URA3*, which is >80% identical to *C. glabrata* *URA3*, was amplified by PCR, *Bam*HI restriction sites were added to either end, and the resulting product was inserted in the unique *Bam*HI site of the 1,030-bp PCR fragment. The disrupted product was liberated from the plasmid by *Eco*RI digestion and was then used to transform *C. glabrata* *ura3⁻* auxotrophs. The resulting mutants were selected by growth on *Ura⁻* media. Targeted disruption was confirmed by Southern blot analysis. Colorimetric assay of phospholipase activity revealed >90% elimination of both PLB and Lyso-PL activities in the mutant isolate (*URA3⁺ plb⁻*) compared to the parent *C. glabrata* (*URA3⁺ plb⁺*), confirming that the cloned gene was responsible for the vast majority of PLB and Lyso-PL activities. Comparative in vivo pathogenicity studies are currently being performed to determine the role of the cloned gene in *C. glabrata* virulence.

Phospholipases of *Cryptococcus neoformans*

Cryptococcus neoformans is the cause of the most common life-threatening fungal infection in patients with AIDS. Depending on the study, estimates of the frequency of cryptococcosis among AIDS patients range from 5% to slightly greater than 10% (25, 42, 172). Although the occurrence has decreased in the last couple of years in the developed world due to the introduction of triple HIV therapy (B. Dupont, Abstr. 4th Congr. Eur. Confed. Med. Mycol., abstr. S1, 1998), this incidence is still high, particularly in developing countries such as Uganda. Given the high rate of relapse after initial antifungal therapy, the current management of *C. neoformans* infections includes lifelong suppressive therapy with antifungals.

Correlation of phospholipase and virulence. The ability of *C. neoformans* to secrete phospholipase was first reported by Vidotto et al. (204). Their data showed that 22 of 23 cryptococcal isolates tested produced phospholipase. As with *Candida*, a wide variation was observed in the ability of various strains to secrete phospholipase (P_z values ranged between 0.271 and 0.49). Although these authors found a correlation between phospholipase production and the size of the capsule in the strains isolated from AIDS patients, the number of

isolates analyzed for these factors was small. Therefore, more isolates should be examined to confirm this notion. In another study, Sorrell and coworkers (23) examined 50 *C. neoformans* isolates for extracellular phospholipase activity by using an egg yolk-based agar assay. Forty-nine of these isolates produced a pericolonial precipitate indicative of phospholipase activity. No difference in phospholipase production was observed between environmental and clinical isolates of *C. neoformans* var. *gattii*. Quantitation of cryptococci in the lungs and brains of BALB/c mice inoculated intravenously with four strains expressing high, intermediate, or low phospholipase activity revealed a correlation between phospholipase activity and virulence (23). Based on this finding, the authors proposed that phospholipases secreted by *C. neoformans* may be associated with cryptococcal virulence.

In a second study, Chen et al. (24), using ^1H and ^{31}P nuclear magnetic resonance spectroscopy combined with thin-layer chromatography analyses, extended their work to define the nature of the phospholipase activity produced by *C. neoformans*. Nuclear magnetic resonance spectroscopy revealed that the sole phospholipid degradation product of the reaction between the substrate PC and cryptococcal culture supernatants was glycerophosphocholine, indicating the presence of PLB. No products indicative of PLA, PLC, PLD, or other lipase activity were detected (24). Thin-layer chromatography analysis confirmed that PLB and Lyso-PL activities were detected in *C. neoformans* supernatants. Additionally, LPTA activity was identified, by the formation of radioactive PC from Lyso-PC, in the supernatants. The Lyso-PL activity was 10- to 20-fold greater than the PLB activity in these supernatants, with mean specific activities \pm standard deviation of 34.9 ± 7.9 and 3.18 ± 0.2 μmol of substrate hydrolyzed/min/mg of protein, respectively. Enzyme activities were stable at acidic pHs (pH 3.8), with pH optima of 3.5 to 4.5. Activities were unchanged in the presence of exogenous serine protease inhibitors, divalent cations, and EDTA. Thus, *C. neoformans* secretes phospholipases with similar activity (PLB, Lyso-PL, and LPTA) to those observed in *C. albicans*.

Cloning of the gene encoding phospholipase B. A PCR-based approach similar to the one used to clone other fungal phospholipases was used to clone cryptococcal PLB (M. Gottfredsson, G. M. Cox, M. Ghannoum, and J. R. Perfect, Abstr. 98th Gen. Meet. Am. Soc. Microbiol. 1998, abstr. F-71, 1998). Multiple degenerate PCR primers were designed to conserved regions in known fungal PLB genes. From one of the primer combinations, a 1.2-kb amplicon was obtained and cloned into plasmid SK. This fragment was sequenced to confirm its identity and used to screen an EMBL3 *C. neoformans* genomic library to isolate the entire gene. The data showed that the cryptococcal PLB gene exists in a single copy of approximately 2.4 kb and the putative protein is made up of 617 amino acids. Comparison of the cloned *C. neoformans* gene to other fungal PLB genes revealed 37, 36, and 36% homology to *S. cerevisiae*, *P. notatum*, and *C. albicans* PLB, respectively, at the amino acid level.

The importance of PLB production for virulence properties in *C. neoformans* is still unknown and awaits the construction of an isogenic strain pair with specific deletion of PLB. Construction of this pair is currently being accomplished.

Phospholipases of *Aspergillus* Species and Their Relation to Virulence

Aspergillus fumigatus is the most pathogenic member of its genus, being responsible for 90% of *Aspergillus* infections. In spite of tremendous efforts, no specific virulence factors have

been identified in this genus. Only two studies attempted to determine whether *Aspergillus* strains secrete phospholipase. Birch et al. (16) investigated the ability of *A. fumigatus* to produce extracellular phospholipases. The use of fast atom bombardment (which compares lipid-containing media, before and at intervals during *A. fumigatus* growth, for the presence of anions corresponding to major classes of phospholipids and fatty acids) enabled the detection of several anions corresponding to phospholipid breakdown products. Based on specific degradation products Birch et al. (16) suggested that *A. fumigatus* secretes multiple extracellular phospholipases, including PLA, PLB, PLC, and PLD. Further characterization of PLC revealed that this activity was initially observed after 30 h of growth and accumulated in broth cultures up to 50 h. Maximal PLC activity coincided with fungal cultures entering the stationary phase, with greater activity occurring at 37°C than at lower temperatures.

Koul et al. studied eight strains of *A. fumigatus* and *A. flavus* (*A. fumigatus*, $n = 5$; *A. flavus*, $n = 3$) for their ability to produce phospholipase (A. Koul, C. J. Jessup, D. J. Deluca, C. J. Elnicky, M. Nunez, R. G. Washburn, and M. A. Ghannoum, Abstr. 987. Gen. Meet. Am. Soc. Microbiol. 1998, abstr. F-78, 1998). Following growth for 3 days at 30 or 37°C in Sabouraud dextrose broth supplemented with 4% glucose, supernatants were concentrated and assayed for phospholipase activity by using a specific substrate radial-diffusion assay capable of differentiating between PLA, PLB, and PLC. Phospholipase activity was detected in all strains regardless of temperature. For both species, PLB was the predominant enzyme. Two of three *A. flavus* strains also secreted PLA and PLC. For *A. fumigatus* grown at 30 and 37°C, quantitative PLB activity was 76 to 98 U/100 ml (mean \pm standard deviation = 84 ± 9) and 67 to 90 U/100 ml (mean 79 ± 11), respectively. Similarly, for *A. flavus*, activities were 50 to 81 U/100 ml (mean 62 ± 17) and 33 to 68 U/100 ml (mean, 45 ± 20), respectively. Unlike Birch et al. (16), Koul et al. did not detect enzymatic activity indicative of PLD. Furthermore, the latter group detected only PLB activity in *A. fumigatus*. This discrepancy could be due to strain-to-strain variation. Alternatively, the discrepancy in the detected activities could be because Birch et al. deduced the type of phospholipase secreted by *A. fumigatus* based on phospholipid degradation products and not assays incorporating specific substrates as utilized by Koul et al.

Although the role of phospholipase in *Aspergillus* virulence must await cloning and disruption of the gene(s) encoding these enzymes, Koul et al. (Abstr. 98th Gen. Meet. Am. Soc. Microbiol. 1998) showed that reagent-grade PLB produced extensive cytolysis of cultured pneumocytes, offering the possibility that this enzyme enhances the virulence of *Aspergillus* by causing damage to host cells.

MECHANISMS BY WHICH PHOSPHOLIPASE AUGMENTS CANDIDAL VIRULENCE

The mechanisms through which the secreted PLB contributes to candidal virulence are currently unknown. Intuitively, it is reasonable to assume that this enzyme is likely to be involved in the early steps of host invasion (adherence, penetration, and damage).

Influence of Phospholipase B₁ on Candidal Adherence to Host Cells

A number of investigators provided evidence implicating phospholipases in microbial adherence to host cells. Silverman et al. (177) suggested that phospholipases mediate adhesion to

and lysis of host cell membranes during infections by *Rickettsia*. Prakobphol et al. (145) presented evidence suggesting that enzymes which use lysophospholipids as substrates, e.g., PLB and Lyso-PL, mediate adhesive interactions between host cells and *C. tropicalis*. Additionally, Barrett-Bee et al. (11) found a correlation between the ability of yeasts, including *C. albicans*, to adhere to buccal epithelial cells and phospholipase activity. In contrast, comparison of two *C. albicans* isolates that differed in their ability to produce phospholipase showed that the low phospholipase producer adhered more avidly to the gastrointestinal tract of infant mice than did the high phospholipase producer (28). These contradictory findings could be because the above studies used genetically unrelated strains. Alternatively, phospholipases may facilitate adherence in some organisms and not in others or in certain specific settings. To determine whether *caPLB1* influences candidal adherence, the abilities of PLB-deficient mutant and parental strain to adhere to epithelial and endothelial cells were compared by using a standard in vitro assay (57). Our data showed that the percent adherence for the PLB-deficient mutant and parent strain to epithelial cells (HT-29) were $36\% \pm 3.9\%$ and $35\% \pm 2.2\%$, respectively. Similarly, no significant difference was noted in the ability of the isogenic strain pairs to adhere to human umbilical vein endothelial cells (HUVEC) (101). These data suggest that PLB does not appear to directly affect the ability of *C. albicans* to adhere to host cells. Susan Fisher (University of California, San Francisco), in collaboration with our group, is currently comparing the adherence capabilities of the parent and mutant strains by using more sensitive adherence assays developed by her group. These investigations should determine if subtle differences exist in the adherence of these strains.

Involvement of Phospholipase B₁ in Host Cell Injury and Penetration

Since phospholipase targets membrane phospholipids and digests these components, leading to cell lysis (166), direct host cell damage and lysis has been proposed as a major mechanism contributing to microbial virulence. Such host cell injury would be expected to facilitate the penetration of the infecting agent. In this context, Klotz et al. (91), using an in vitro model depicting the earliest events of metastatic *Candida* infection, showed that *Candida* organisms first adhere to and then penetrate the endothelium. During transmigration, endothelial-cell continuity was 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 (91). Similar suggestive evidence for enzymatic activity by *C. albicans* in the penetration of the glossal epithelium in both rats (70) and humans (124) has been described, and studies that document this mechanism in murine cervical tissue and skin have been reported (171).

More recently, evidence implicating phospholipase in host cell penetration and injury has been derived from in vitro and in vivo studies. The in vitro studies compared the ability of the parent and PLB-deficient strains to penetrate epithelial and endothelial cell monolayers (101). Also, the abilities of supernatants from cultures of these strains to cause damage to epithelial cells were compared (P. K. Mukherjee, S. D. Leidich, J. Vitulla and M. A. Ghannoum, Program Abstr. 38th Intersci. Conf. Antimicrob. Agents Chemother., abstr. J-81, 1998). Leidich et al. (101), using scanning electron microscopy,

compared the abilities of the parent and *caPLB1*-deficient mutant to penetrate both HUVEC and HT-29 epithelial cells. Their data showed that both the parent and *caPLB1*-deficient mutant formed germ tubes which penetrated HUVEC and HT-29 cells. However, the capacity of the *caPLB1*-deficient strain to penetrate HUVEC and HT-29 host cells was significantly lower than that of the parent. The percentage of penetrating parental hyphae was $66.7\% \pm 1.7\%$ for the endothelial cell line and $57.8\% \pm 2.5\%$ for the epithelial cell line. In contrast, the percentages of penetrating PLB-deficient hyphae were $37.3\% \pm 1.3\%$ and $29.0\% \pm 2.9\%$ for the HUVEC and HT-29 cell lines, respectively ($P < 0.0002$ for HUVEC; $P < 0.0003$ for HT-29) (101).

The ability of supernatant from cultures of the parental or PLB-deficient strains to cause damage to epithelial cells was compared by using a thiazolium blue-based assay (Mukherjee et al., 38th ICAAC). Supernatant from the parental culture was about twofold more efficient in causing cell damage than that from the *caPLB1*-deficient culture (supernatant from the parental strain caused $62.33\% \pm 1.15\%$ damage, while supernatant from the *caPLB1*-deficient strain caused $34.67\% \pm 0.58\%$ damage [$P < 0.0001$]). In the same experiments, damage caused by supernatants obtained from *C. albicans* and the nonpathogenic yeast *S. cerevisiae* was compared. In contrast to the significant damage caused by *C. albicans*, only minor injury was caused by *S. cerevisiae* ($3.95\% \pm 0.48\%$). The above data suggest that PLB may enhance candidal virulence by directly damaging host cell membranes.

Seshan et al. (174) provided in vivo data demonstrating that candidal PLB facilitates candidal penetration of host tissues. Using an oral-intragastric infant-mouse model of candidiasis, these authors showed that the phospholipase-producing parental strain penetrate deep into the gastric mucosal and submucosal tissues following intragastric challenge. In contrast, the *caPLB1*-deficient mutant was not as invasive and was generally sequestered to the stomach lumen. The invasiveness of the parental strain is likely to have increased its access to the gastric vasculature, allowing the organism to hematogenously disseminate more efficiently than the PLB-deficient mutant. Consistent with this notion, more hyphal elements were observed in blood vessel lumens following challenge with the parental strain. These differences in penetration and dissemination were reflected by the number of candidal cells recovered from the livers and kidneys of mice infected with either the parental or *caPLB1*-deficient strains. Organs (kidneys and liver) from mice infected with the parental strain had significantly higher candidal CFU than did organs from mice infected with the PLB-deficient mutant (174) (see above).

Therefore, the decrease in the capability of PLB1-deficient strains to cross the gastrointestinal tract and cause systemic infection, combined with their decreased capability to penetrate host cell monolayers, and the ability of the enzymatic preparation to lyse the host cell indicate that PLB may play a role in candidal virulence by causing direct damage to host cell membranes. Such injury would allow fungal hyphal elements to more effectively traverse the vascular endothelium, ultimately increasing the rapidity of dissemination to and invasion of target organs.

Do Fungal Extracellular Phospholipases Have Other Functions That Facilitate Virulence in Addition to Cell Lysis and Damage?

Evidence accumulated so far suggests that fungal phospholipases enhance virulence by damaging host cell membranes. However, based on findings derived from studies of phospho-

lipases from bacteria, it is likely that these hydrolytic enzymes may have other functions that facilitate virulence, in addition to causing direct tissue damage (183, 194). Clues to the functions and mechanisms of phospholipases during fungal infection will be provided by the availability of purified fungal phospholipases and isogenic strains from different pathogenic fungi. While *C. albicans* strains differing in phospholipase production became available only recently and were tested in vivo for their virulence (101, 174), sets from other pathogenic fungi such as *C. neoformans* are still being constructed, and in some cases (e.g., *A. fumigatus*) the genes encoding phospholipases await cloning. Thus, mechanistic studies represent important areas of future inquiry.

A number of potential investigational areas relevant to the phospholipase mechanism(s) could be proposed, including (but not limited to) the contributions by phospholipases to signal transduction, stimulation of host cells to release cytokines, and the host inflammatory response.

Many lipids and lipid-derived products generated by phospholipases acting on phospholipids present in host cell membranes are implicated as mediators and second messengers in signal transduction (35, 173). Given the substrate specificity of the enzyme and the lipid degradation products, candidal PLB could also be involved in an as yet unidentified signal transduction pathway potentiated at the lysophospholipid levels (187). In this regard, by-products of PLB degradation, such as lysophospholipids, are reported to induce the activation of protein kinase C (135). Activation of protein kinase C could play a role in the deregulation of cell signaling through local effects or as a consequence of global impacts on host cell physiology (178, 183).

Stimulation of host cells to produce cytokines in response to soluble factors of microbial origin is well documented. For example, PLC from *Clostridium perfringens* induces the expression of interleukin-8 (IL-8) synthesis by endothelial cells (18, 19). *Entamoeba histolytica*, which utilizes phospholipase to injure host cells, also stimulates epithelial cells to secrete IL-6 and IL-8 (40). Stimulation of cytokine production in response to lytic enzymes of microbial origin has also been demonstrated in vivo (114). Since phospholipase production is closely associated with candidal host cell injury (see above and reference (101)), it is conceivable that this enzyme may directly or indirectly stimulate host cells to produce specific cytokines. A possible mechanism responsible for the expression of genes encoding IL-6 and IL-8 by epithelial cells following microbial injury was proposed by Eckemann et al. (40). These authors showed that *E. histolytica* injures epithelial cells and causes them to release IL-1 α into the medium. This cytokine stimulates adjacent epithelial cells to produce IL-8 and IL-6. Similarly, Kaplanski et al. (88) reported that injury of endothelial cells following exposure to *Rickettsia conorii* led to the synthesis of IL-6 and IL-8 via a cell-associated IL-1 α -dependent pathway. Thus, it is feasible that fungal phospholipases may mediate cytokine production via a similar mechanism.

Microbial phospholipases have been demonstrated to be potent inflammatory agents, inducing the accumulation of inflammatory cells and plasma proteins and the release of various inflammatory mediators in vivo (120). Moreover, microbial phospholipases are able to mobilize arachidonic acid and subsequent prostaglandin synthesis (208). The availability of purified fungal phospholipases and isogenic strain pairs that differ in phospholipase production only will make it feasible to probe the above exciting areas of investigation.

Role of Fungal Phospholipases in the Antifungal Activity of Amphotericin B Lipid Complex

New lipid formulations of amphotericin B with improved safety and efficacy profiles were approved in the United States in the mid-1990s (175). It has been hypothesized that the enhanced therapeutic index of amphotericin B lipid complex (ABELCET) relative to conventional amphotericin B is due, in part, to selective release of active amphotericin B at sites of fungal infection (81). Moreover, this release may occur through the action of phospholipases that are released by the fungus itself or by activated host cells (81, 200). To test this hypothesis, Minassian et al. generated phospholipase-deficient *C. albicans* mutants by nitrous acid treatment and evaluated them for extracellular phospholipase activity and killing kinetics in the presence of both amphotericin B and amphotericin B lipid complex (ABLC) (B. Minassian, R. K. Flamm, R. R. Whitney, L. Kurselman, L. Bender, R. Summerill, and D. P. Bonner, Program Abstr. 30th Intersci. Conf. Antimicrob. Agents Chemother, abstr. 847, 1990). Compared to the parent the mutants were phospholipase-deficient with increased ABLC MICs and slightly elevated amphotericin B values (121). When tested in vivo, ABLC and conventional amphotericin B were equally efficacious (80 to 90% animals survived following treatment with either agent). Based on these findings, the authors concluded that in vitro release of amphotericin B from ABLC in the case of *C. albicans* may be due to the breakdown of phospholipids by fungal phospholipases. Furthermore, reduced phospholipase may account for the elevated MICs of ABLC compared to amphotericin B. Moreover, the in vitro activity of amphotericin B but not of ABLC correlates with in vivo efficacy of ABLC against certain phospholipase-deficient *Candida*. Finally, it was suggested that standard in vitro susceptibility tests with ABLC itself (rather than conventional amphotericin B) may not accurately predict in vivo activity. More recently, Swenson et al. performed an in-depth analysis of the role of candidal and host phospholipases in the antifungal activity of ABLC (190). To evaluate the contribution of fungal phospholipases, these authors used the same candidal mutants created by Minassian et al. (30th ICAAC) and reached similar conclusions. One drawback to these elegant studies is that they were performed with genetically unrelated strains that were created by chemical mutagenesis and were performed prior to the availability of isogenic candidal strains that differ only in phospholipase activity. It is recognized that chemical mutagenesis may have resulted in other changes besides abrogation of phospholipase and thereby may have complicated the data interpretation.

Recently, our group cloned and disrupted the gene *caPLB1*, which encodes PLB, the dominant phospholipase in *C. albicans*, and constructed an isogenic strain pair which differ only in phospholipase secretion (101). Next, we used the isogenic strain pair to determine whether a difference exists in the susceptibility of ABLC and conventional amphotericin B in vitro. Our data showed that both conventional amphotericin B and ABLC had similar MICs (0.125-0.25 $\mu\text{g}/\text{ml}$) for the phospholipase-producing wild-type strain (SC5314) and the phospholipase-deficient mutant (*plb-1 Δ* ²). This finding was consistent even when different media (RPMI 1640, Sabouraud's dextrose broth, and yeast nitrogen base) were used to assay the in vitro activities of these antifungals (C. Jessup and M. A. Gharroum, unpublished data). Similarly, no difference was observed in the in vitro susceptibility of parent and phospholipase-deficient *C. neoformans* strains (constructed by Gottfredsson, et al., Abstr. 98th Gen. Meet. Am. Soc. Microbiol. 1998) against amphotericin B and ABLC (Jessup and Ghan-

nom, unpublished). Therefore, data derived from our group and that of Perfect clearly demonstrate that deletion of fungal phospholipases does not influence antifungal susceptibility of ABLC. Furthermore, our data support the conclusion of Minassian et al. (30th ICAAC), who suggested that in vivo efficacy of ABLC may be more closely correlated with host phospholipase activity and not with yeast or fungal phospholipase activity. It is conceivable that the effects seen in the studies of Minassian et al. (30th ICAAC) and Swenson et al. (190) could be due to a second phospholipase beside PLB. However, this is unlikely since PLB is the dominant phospholipase and is responsible for about 90% of the extracellular activity of the candidal phospholipase.

FUNGAL PHOSPHOLIPASES AS A THERAPEUTIC AND DIAGNOSTIC TARGET

The validation of PLB as a virulence factor in *C. albicans* by using animal models of hematogenously disseminated (101) and gastrointestinal (174) candidiasis and the detection of this hydrolytic enzyme in other pathogenic fungi including *C. neoformans*, *A. fumigatus*, and *A. flavus* makes it a potential therapeutic target. The finding that phospholipases are secreted by different clinically important fungal genera and evidence that these fungi share the same class of phospholipase, namely, PLB, suggest that these hydrolytic enzymes may represent a common theme utilized by pathogenic fungi as a universal virulence factor (56). Moreover, demonstration that phospholipase secretion is not limited to one fungal genus increases the potential of fungal phospholipases as a therapeutic target.

A number of drug development approaches could be pursued which may lead to the discovery of novel agents targeting fungal phospholipases. These approaches include the development of vaccines, identification of agents that inhibit the production or release of fungal phospholipases (medicinal chemistry synthetic programs, screening of chemical or combinatorial libraries), and rational inhibitor design (based on the tertiary structure of the protein).

The use of microbial phospholipases as vaccines in protection against diseases has been investigated. Clostridial alpha-toxin (PLC) was reported by Kameyama et al. (86) to induce protection against *C. perfringens*-mediated gas gangrene. Williamson and Titball (214a) were able to protect sheep against experimentally induced *Clostridium novyi* and *C. perfringens* gas gangrene by using a toxoid mixture from *C. perfringens*-*C. septicum*-*C. novyi* as a vaccine. Instead of using PLD to develop a vaccine against *C. pseudotuberculosis*, Hodgson et al. (69) constructed a PLD-negative strain (called Toxminus), which was incapable of inducing caseous lymphadenitis, and used it to vaccinate sheep. Sheep vaccination and challenge trials with this mutant showed that it elicited strong humoral and cell-mediated immune response and protected the animals from wild-type challenge (69).

Preliminary attempts to identify inhibitors to candidal phospholipases have been previously undertaken by Hänel et al. (65), who screened for synthetic phospholipase inhibitory substances by using in vitro and in vivo models. Their efforts resulted in the identification of lead structures capable of inhibiting phospholipase activity in vitro (65). Additionally, prolongation of animal survival was observed when these compounds were used in combination with fluconazole (65). The above studies provide some support to the concept of targeting fungal phospholipases for drug discovery and warrant a systematic approach for drug discovery based on these lytic enzymes.

Another potential use of fungal extracellular phospho-

lipases, particularly candidal PLB, is as a diagnostic tool. Since it has been demonstrated that candidal PLB is released during the progression of candidal infection in murine models of candidiasis (101, 174) and that sera from patients with systemic candidiasis contain antibodies which react with purified candidal PLB (see above), it is conceivable that this particular antigen could be applied in an antibody detection assay. PLB possesses a number of advantages which make it attractive for development as a diagnostic tool: (i) it is a naturally secreted protein and not a cell wall or cytoplasmic component as is the case with all fungal antigens, with the exception of aspartyl proteinases (46), under development (for reviews, see references 112 and 155); (ii) it is a purified well-defined antigen rather than a crude extract containing cell wall polysaccharide (112); and (iii) blood isolates generally secrete much higher levels of enzyme than do isolates from the oral cavity of healthy individuals (75) or isolates from wounds or urine (147).

Secretion of PLB by a number of pathogenic fungi, including *Candida*, *Cryptococcus neoformans*, and *Aspergillus*, may pose a challenge for the development of a highly specific and sensitive diagnostic test. However, this challenge could be met as more information on genes encoding PLB from specific pathogens become available and these proteins are purified. As stated by Martinez et al. (112), "with their amino acid sequences readily available and the application of new specific epitope-mapping technique, it is now easier to identify specific epitopes within these antigens and to adapt this knowledge to development of improved serologic tests for the diagnosis of candidiasis".

CONCLUSION AND OUTLOOK

The aim of this review was to evaluate our current knowledge of fungal phospholipases, with special emphasis on their possible role in virulence. It is clear that we are at an early stage in our investigations and that exciting areas for future studies lie ahead. Among the pathogenic fungi, cloning, disruption, and virulence evaluation of genes encoding extracellular phospholipases have been completed only for *C. albicans*. It is also clear that efforts to clone the genes encoding these enzymes in other pathogenic species are being pursued in earnest. Investigations aimed at determining the role of phospholipases in the virulence of different pathogenic fungi will reveal whether these enzymes are universal virulence factor in pathogenic fungi. Elucidation of the mechanism(s) by which phospholipases contribute to virulence is another area that warrants attention. In addition to gene cloning, enzyme purification should be pursued. Purification of fungal phospholipases will affect our mechanistic studies and, in the long term, may help in rational drug design of novel antifungal targets. A word of caution: purification of these enzymes is not a trivial matter. They are produced in small quantities, and therefore large volumes of medium are required to produce reagent amounts of protein. Moreover, the high lytic potency of these enzymes may interfere with recombinant approaches to purify the gene products. Finally, purification procedures could be complicated further by organism-related factors. For example, in *C. neoformans*, the presence of a large capsule may make it difficult to purify the enzyme. There is a lack of knowledge of phospholipase gene regulation in vivo, as well as whether this class of enzymes acts in concert with other secreted proteins, such as aspartic proteinases, to augment fungal virulence. Research into these areas will further our understanding of the pathobiology of fungal infection. Once the importance of phospholipases in fungal virulence is proven, the next challenge will be to use these enzymes in drug discovery efforts to identify and design inhibitors and/or develop a vaccine. The utility of

phospholipases as diagnostic markers of fungal infections is yet another area of study that may prove to be fruitful.

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