2001

Phospholipase D1 as a Regulator of Morphogenesis in Candida albicans

Carol Anne Baker
Medical University of South Carolina

Phospholipase D1 as a Regulator of Morphogenesis in *Candida albicans*

Carol Anne Baker

A thesis submitted to the faculty of the Medical University of South Carolina in partial fulfillment of the requirement for the degree of Masters of Science in Biomedical Sciences in the College of Graduate Studies.

Department of Microbiology and Immunology

2001

Approved by:

Dr. Joseph Dolan
Chairman of Advisory Committee

Dr. Gillian Galbraith

Dr. Jean-Michel Goust

Dr. John Hildebrandt

Dr. Kathryn Meier

ACKNOWLEDGEMENTS

I would like to thank the members of my committee, Drs. Joseph Dolan, Gillian Galbraith, Jean-Michel Goust, John Hildebrandt, and Kathryn Meier for all of their guidance during the course of my thesis work.

I would especially like to thank my mentor, Dr. Joseph Dolan, and Dr. Gillian Galbraith. Dr. Dolan taught me more about benchwork than I ever thought possible. He was able to keep me motivated and pushed me to continually strive to complete this body of work. He gave me confidence in myself by having confidence in me and my abilities. A special thanks goes to Dr. Galbraith for helping me find my direction and always taking the time to listen.

Thank you to my fellow lab members, past and present: Amanda Ayers (my Charleston friend), Michelle Hairfield, Rhoda Ascanio, and Nealoo McLain for their friendship and support. These guys really made work enjoyable.

I would also like to thank Phil Werner and Caroline Westwater for their laboratory wisdom and willingness to help.

Finally, I must thank my family for their overwhelming support and perseverance. To my husband, I would like to say thank you for all of the encouragement and for always telling me what I needed to hear whether I wanted to hear it or not. Lastly, to my mom, thank you for always being there. I love you both very much.

TABLE OF CONTENTS

FIGURES

TABLES

GLOSSARY

Filamentous growth- refers to the presence of hyphae in a culture or sample

Germ tube- newly evaginating hypha

Hyphae- microscopic tube that contains multiple fungal cell units divided by septa; arises from a yeast cell

Pseudohyphae- end-to-end aggregation of yeast cells which are very elongated; resemble true hyphae but have constrictions at septal junctions

Septum- cross wall between the parent and daughter cell units

Yeast cell- round unicellular yeast form; referred to as a blastospore in past literature but found not to be a spore

Chlamydospore- large, thick-walled cell that is subtended from hyphae or pseudohyphae by a cell called a "suspensor cell"

ABBREVIATIONS

ARF- ADP-ribosylation factor

AVP- Arg-vasopressin

BPA- BODIPY phosphatidic acid

BPC- BODIPY phosphatidylcholine

BDG- BODIPY diacylglycerol

CLA4- gene which encodes a serine/threonine kinase activity

DAG- diacylglycerol

DGK- diacylglycerol kinase

DG kinase- diacylglycerol kinase

ET-1- Endothelin-1

LPA- lysophosphatidic acid

PA- phosphatidic acid

PAP- phosphatidic acid phosphohydrolase

Pbu- phosphatidylbutanol

PC- phosphatidylcholine

PDGF- platelet-derived growth factor

PIP_2- phosphatidylinositol 4, 5-bisphosphate

PI4P-5K- phosphoinositol 4-phosphate 5-kinase

PKC- protein kinase C

PLA_2- phospholipase A2

PLC- phospholipase C

PLD- phospholipase D

PLD1- phospholipase D isoform in *Candida albicans*

YEP- yeast extract, peptone medium

YPD- yeast extract, peptone, dextrose medium

ABSTRACT

Phospholipase D (PLD) enzymes play significant roles in phospholipid metabolism, secretion, and lipid signal transduction. The yeast *Candida albicans* possesses a PLD activity that is designated PLD1. This enzyme has been implicated in the regulation of dimorphic transition. *In vivo* assays using sphinganine, propranolol, and staurosporine were used to test the importance of phosphatidic acid (PA) and diacylglycerol (DAG), two downstream products of PLD1 activity. A concentration of 5 μM sphinganine was sufficient to decrease the appearance of germ tubes with no effect on culture doubling times. However, at higher concentrations sphinganine was inhibitory to cell growth. Propranolol concentrations up to 1 mM were able to inhibit germ tube formation without increasing doubling times. Increasing concentrations of propranolol (e.g. 2 mM) did inhibit yeast cell growth. Varying concentrations of staurosporine had no effect on either germ tube formation or cell growth. *In vitro* assays demonstrated a decrease in the conversion of PA to DAG with increasing amounts of propranolol. Since DAG kinase assays showed that DAG levels were not affected, *in vivo*, by the presence of propranolol, phosphatidic acid phosphohydrolase (PAP) activity was measured in the presence of propranolol. No significant change in PAPase function was observed. Wildtype *C. albicans*, SC5314, showed numerous, invasive hyphae when plated on Spider medium, in contrast to a confirmed pld1Δ mutant which showed no visible hyphae. DAG kinase assays comparing DAG levels between the wild type and mutant strain showed significantly higher levels in the null mutant. These results suggest that C. albicans may be compensating for a loss of PLD-derived DAG, possibly through up-regulation of

phospholipase C (PLC). The enzymes monoacyl-glycerol-acyl-transferase (MAGAT) and inositol-phosphoryl ceramide synthase (IPC synthase) may participate in replacement of the DAG pool. The data also implicate PA, not DAG, as the enzyme needed for morphogenesis.

CHAPTER 1

INTRODUCTION

PHOSPHOLIPASE D

Phospholipase D (PLD) is a signal transducing enzyme that has been shown to be involved in the regulation of cellular functions such as differentiation, proliferation, and senescence (1). PLD has been shown to hydrolyze phosphatidylcholine to produce phosphatidic acid (PA) and choline (2, 3) PLD can also be involved in the transfer of a phosphatidate group to a primary alcohol to produce a phosphatidylalcohol (4). Isoforms of PLD have been found in mammals, plants, bacteria (2), and fungi (5, 6).

PA has been implicated as an effector of such processes as secretion (7), DNA synthesis, and cell proliferation (8, 9). Phosphatidic acid can be converted to lysophosphatidic acid (LPA) by the enzyme PLA_2 (3). It can also be broken down by PA phosphohydrolase to produce diacylglycerol (DAG) (10). DAG is known to be a second messenger that can activate some members of the protein kinase C family (11).

It has been shown that inhibitors of PKC partly or totally inhibit activation of PLD in many cell types (12). However, there are also examples in which inhibitors of PKC do not inhibit PLD (13). These results indicate that both PKC-dependent and independent mechanisms are involved in the regulation of PLD. Many substances that stimulate PLD also activate the breakdown of PIP2 by phosphoinositide phospholipase C (PI-PLC). This breakdown results in the production of inositol trisphosphate and diacylglycerol. Diacylglycerol is responsible for activating PKC isozymes. These enzymes, which are Ca^{2+} sensitive, in turn activate PLD (14). Researchers have supported this process of regulation with genetic mutations or disruptions involving PI-PLC and overexpression of PI-PLC. For example, cells expressing mutated PDGF receptors showed a decrease in PI-PLC response to PDGF, leading to a loss of PLD response to PDGF (15). Cells from

mice in which the PI-PLCγ gene had been disrupted exhibited a similar result of decreasing PLD response to PDGF (18, 22). Cells overexpressing PI-PLCγ were shown to have increased PLD response to the growth factor (16). PLD response to PKC isoforms can either both dependent or independent of ATP. Studies using brain PLD have shown activation by PKCα that does not require ATP and is not inhibited by staurosporine (17). This suggests a phosphorylation-independent mechanism for this particular PLD stimulation (14). On the other hand, activation of PLD by PMA in permeabilized human embryonic kidney cells, is inhibited by staurosporine, requires MgATP, and is blocked by certain PKC inhibitors (18).

ADP-ribosylation factors (ARFs) have been shown to regulate certain isoforms of PLD. It has been shown that regulation of PLD in membranes and permeabilized HL60 cells by GTPγS require a cytosolic factor (19). This factor was later identified as ARF1 and ARF3 (20, 21). The presence of an ARF-regulated PLD in the cytosol of HL60 cells was also demonstrated (22). At present, ARF-regulated PLD activity has been found in nuclei (23, 24), Golgi, plasma membranes, (24-26) and cytosol (22, 24, 27).

PLD regulation by small GTP binding proteins of the Rho family has also been demonstrated. This regulation was first shown in studies with neutrophils (28). This group demonstrated that PLD activation by GTPγS required both plasma membrane and cytosol fractions. The activation also required protein factors from both fractions (28). Activity that was specific for GTPγS showed properties of a small GTP binding protein. When a GDP dissociation inhibitor specific for the Rho family was added, PLD activation by GTPγS was inhibited (28, 29). These results show that the Rho family of small GTP binding proteins is involved in PLD activation.

PLD activation has also been shown to be regulated by protein tyrosine kinases. Tyrosine phosphorylation plays an important role in physiological functions such as cell metabolism, differentiation, and growth regulation (30). Receptor tyrosine kinases on the plasma membrane mediate signal transduction of certain growth factors such as PDGF (31). Endothelin-1 (ET-1) and PDGF were both shown to stimulate PLD in smooth muscle cells (32, 33). However activation of PLD by ET-1 was shown to be sensitive to inhibitors of PKC and tyrosine kinases (33), while studies with PDGF suggested a receptor-independent pathway for activation of PLD (32). PLD activation by non-receptor tyrosine kinases was also found using BALB/C 3T3 murine cells transformed with v-src (34). This stimulation of PLD by oncogenes involves an increase in PKC and tyrosine kinase activity (35, 36). An increase in DAG levels has also been observed in v-src transformed cells (37). However, in time courses for these experiments, the increase in DAG levels did not correspond to the amount of inositol phosphate accumulated (38). This discrepancy suggested the possibility of DAG formation via breakdown of another phospholipid, such as phosphatidylcholine. The increase in DAG was shown to be due to PLD activation, which led to the hydrolysis of PA by PA phosphohydrolase to produce DAG (34, 39). In addition, this PLD activation by v-src was found to be PKC-independent, since it was unaffected by the PKC inhibitor staurosporine (34).

PLD ACTIVITY IN YEAST

The first PLD activity in fungi was found in *Saccharomyces cerevisiae* and designed SPO14 (yPLD1). The *SPO14* gene of *S. cerevisiae* encodes a PC-PLD that is capable of performing the transphosphotidylation reaction and has been shown to be regulated by carbon source (5). PC- specific PLDs can also promote the transfer of the

phosphatidyl group of PC to a primary alcohol to produce phosphatidylalcohol, which has been very useful in measurement of PLD activity in mammalian systems (4). However, the transphosphatidylation reaction of yPLD1 is not as efficient as that of the mammalian enzymes (40). This may be due to the fact that phosphatidylethanol is also a substrate of yPLD1 and may be hydrolyzed upon production, making accurate measurements difficult (3). PLD1-deficient mutants cannot successfully complete meiosis and sporulation (41), and there is an absence of PC-PLD activity in these cells (11, 43). A 400 amino acid region near the middle of the *SPO14* gene shows sequence similarity to a castor bean PLD (40). PLD activity has been found in membranes from bacteria (40) and insect cells (42) expressing the *SPO14* gene.

PC-PLD activity has also been found in *C. albicans*, where it is referred to as PLD1. This is a membrane-associated PLD activity that can perform transphosphatidylation with alcohols, is increased during dimorphic transition, and is regulated by carbon sources (43).

CANDIDA ALBICANS

Candida albicans is a pathogenic yeast that has severe consequences in immunocompromised hosts, including patients with AIDS, those receiving immunosuppressive therapy as cancer treatment or after transplant operations, and those undergoing antibiotic therapy (44). It is the most common opportunistic fungal infection in high-risk hosts. Although candidiasis can be treated if diagnosed early, it can be fatal if not recognized (44).

Growth of Candida in the Human Body

Candida species that cause infection can be isolated throughout the animal kingdom. The major source of these yeast species in human disease is endogenous (45). The predominant areas of *Candida* colonization in humans are the mouth, gastrointestinal tract, vagina, and skin. Within the mouth, the tongue is the site of the largest yeast population, followed by the palate and the cheeks (46, 47). However, in those individuals with denture prostheses, the yeast population is greatest on the area of denture fitting (46). Isolation of *Candida* species in fecal samples show very little difference between healthy and unhealthy individuals, and the concentrations found are typically very low (45). Yeast concentrations in the gut are relatively high, similar to those of the oral cavity. The literature varies widely as to precisely what parts of the gut have the highest concentrations of *Candida*, but isolates can be found in all parts of the gastrointestinal tract (45). *C. albicans* carriage frequency from the human vagina of "normal" individuals has been estimated at 22.3%. However, the carriage frequency from patients with vaginitis was estimated at 31.8% (45). These carriage data are approximately a decade old, but are still useful to illustrate the areas that may be most susceptible to excess colonization and candidiasis.

Predisposition to Candidiasis

The phrase "compromised host" is typically used to describe someone that is susceptible to infection by a *Candida* species. This does not mean, however, that the host must have a severe predisposing factor such as immune deficiency. Natural, dietary, mechanical, and iatrogenic medical factors all contribute to an individual's vulnerability

to candidiasis (Figure 1.1) (45). Examples within these classifications include age, microbial infections, vitamin deficiencies, dentures, and antibiotics.

CLASSIFICATION OF DISPOSING FACTOR	EXPLANATION
Natural Factors	1. Infectious, congenital, idiopathic, or other diseases 2. Digressions from normal physiological status
Dietary Factors	1. Excess or deficiency of food that may alter normal microbial flora
Mechanical Factors	1. Trauma 2. Occlusion or maceration of tissues
Iatrogenic Medical	1. Drug treatments that alter normal body flora or host defenses 2. Surgical procedures or prostheses

Table 1.1: Classification of Factors that Predispose Humans to Candidiasis (Adapted from reference 45).

C. albicans Morphology

C. albicans can exist in two major morphologies. One is the budding yeast cell, and the other is the filamentous or hyphal form. This hyphal form can appear either as true hyphae with no segmentation from the parent cell, or as pseudohyphae that have constrictions at the septal junctions (Figure 1.1) (45). *Candida* cells can be induced to undergo morphological changes by various environmental factors including temperature, pH, and nutritional status. Both the yeast and hyphal forms have been associated with infection. However, hyphal cells are the predominant form in most infections. Hyphae have enhanced ability to invade host tissue and disseminate an infection, and hyphal filaments can more effectively destroy phagocytic cells from the inside.

There seems to be some mechanical advantage to the ability to switch morphologies. This advantage may be related to the success of *Candida* as a fungal pathogen. For this reason, much work has been directed toward the regulation of morphogenesis, and some of the proteins and enzymes involved have been indentified (Figure 1.2). These components have been identified as homologues of the MAP kinase pathway, largely by their ability to complement mating defects in *S. cerevisiae* mutants (48-52). Defective hyphal growth on solid medium results from a single disruption in any of these genes. However, these mutants still exhibit characteristic induction of hyphae by human serum (48-50, 52). A *cph1/efg1* double mutant, unlike the single mutants, was unable to form hyphae even in the presence of human serum. This double mutant was also shown to be avirulent in a mouse candidiasis model (53). This same sort of altered virulence can be seen with mutations in the *CLA4* gene, which encodes a Ste20-related kinase. The *cla4*Δ mutants are incapable of forming hyphae and exhibit no virulence in mouse models (54).

Negative regulators of *Candida* morphogenesis have also been identified. Mutations involving either MAP kinase phosphatase or the transcriptional corepressor Tup1 enhance the formation of hyphae (54-56). Identification of these positive and negative regulators of morphogenesis, and the connection being made between a decreased hyphal formation and avirulence, continues to strengthen the argument that the morphogenic switch is needed for infection.

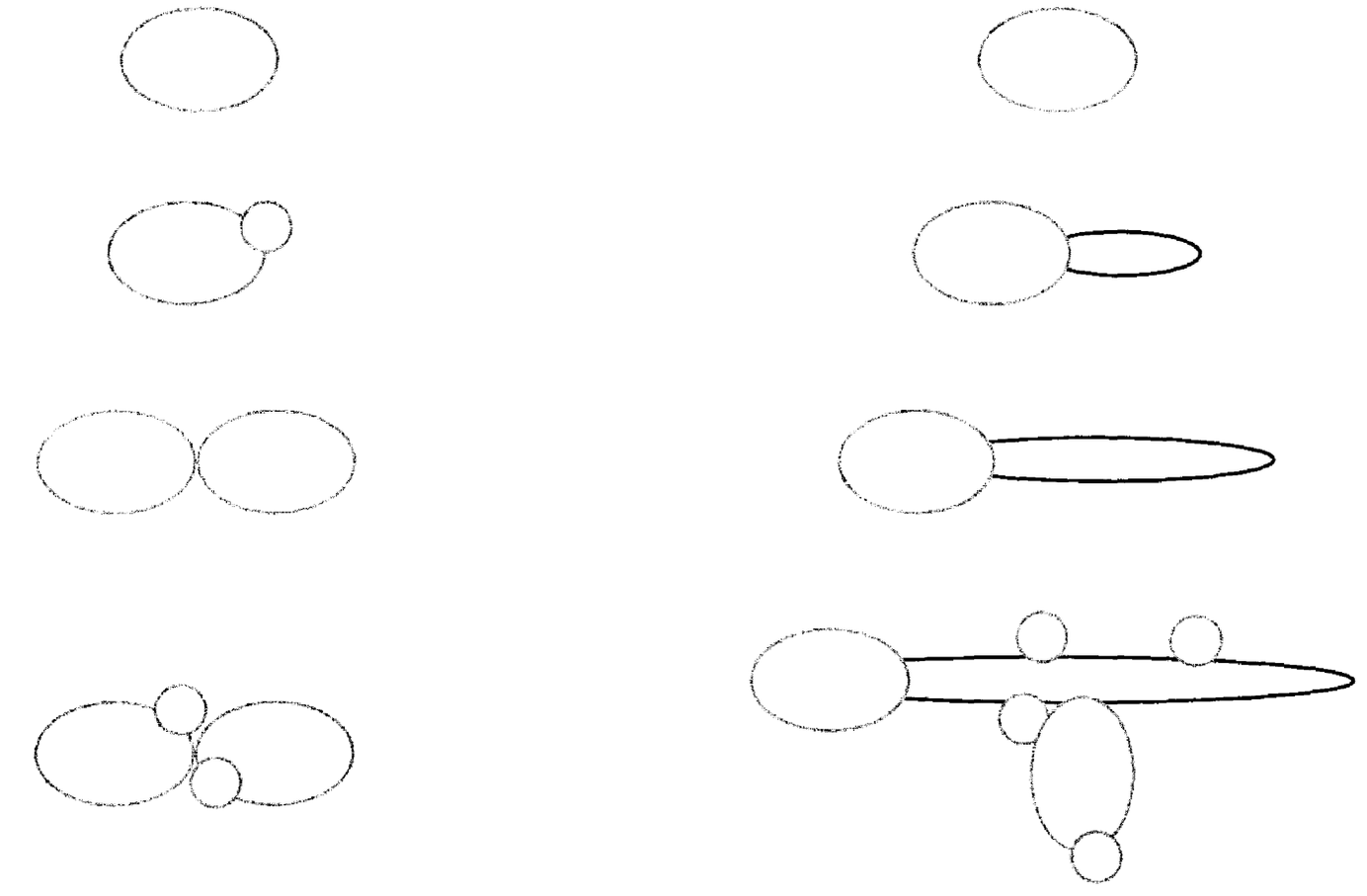

Figure 1.1: Morphogenesis of *Candida albicans*. **A)** The process of budding in *C. albicans*. The mother yeast cell produces a new cell growth at a site near one of the cell poles. The new cell growth or bud will beseparated from the mother cell by a septum, after mitosis. At this point both cells are independent and can undergo the budding process once again. **B)** Hyphal formation in *C. albicans*. The outgrowth of new cellular material appears. This first appearance is referred to as the germ tube. As the germ tube lengthens, septa are laid down behind the extending tip, and buds will form laterally just behind the septa (Adapted from reference 45).

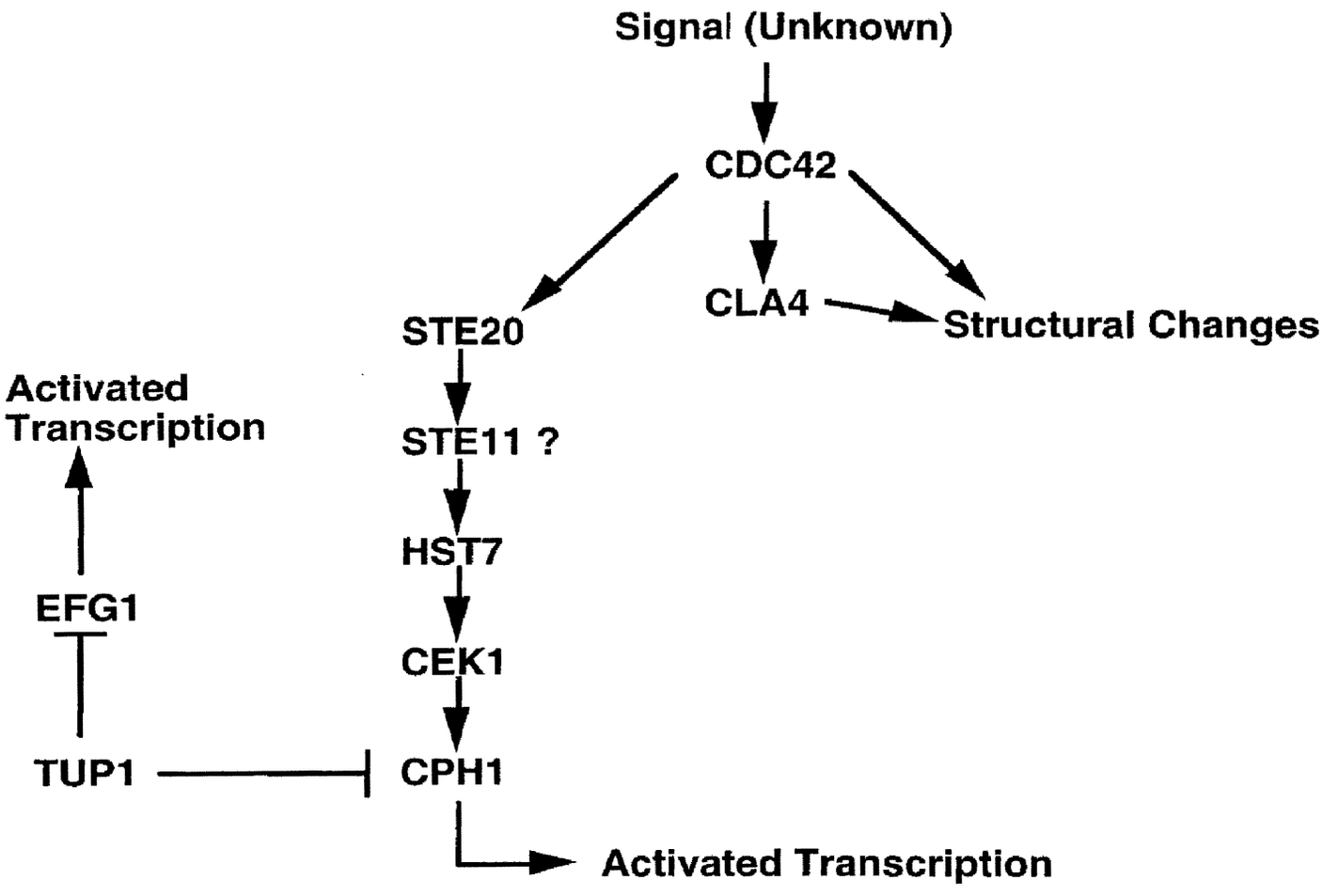

Figure1.2: Morphogenesis Pathway in *C. albicans*.

PROPRANOLOL AS AN INHIBITOR OF PA PHOSPHOHYDROLASE

A significant tool in this study was propranolol, which is an inhibitor of certain forms of PA phosphohydrolase (PAP), the enzyme responsible for the conversion of phosphatidic acid to diacylglycerol. At moderate concentrations (250μM) of propranolol, this inhibition of PAP occurs without affecting PLC and PLD activities in human neutrophils (57)). In mammalian systems, propranolol is used as a β-adrenergic receptor blocking agent, to reduce the action of adrenaline in the body (58). The use of propranolol in PLD studies has been well established in literature concerning mammalian systems. For example, Naro et al. exposed the rat skeletal muscle cell line L6 to Arg-vasopressin (AVP). AVP is a neurohypophyseal hormone that induces myogenic differentiation, which leads to the expression of myogenic regulatory factors. In studying this mechanism of myogenesis, propranolol was used to establish the relevance of 1,2-sn-diacylglycerol produced by PLD activity, independent of PLC activity, in L6 cells (59). Recently, propranolol was used to demonstrate the involvement of a PLD-mediated signaling mechanism in the angiotensin (Ang) II-induced growth of vascular smooth muscle cells (VSMC). Ang II is a peptide that acts as both a vasoconstrictor and a mitogen in VSMC. It mediates its effects through the activation of various signal transduction cascades, such as the activation of PLC (60) and protein tyrosine kinases (61). VSMC cultured from rat aorta were incubated with propranolol (0- 10,000 nM concentrations) both 5 minutes prior to and during stimulation with Ang II. A dose-dependent decrease in DNA synthesis was shown with 10 μM being sufficient to block the VSMC growth (62).

CHAPTER 2

ESTABLISHMENT OF A ROLE FOR PLD1 IN DIMORPHISM IN *C. ALBICANS*

The switch from yeast-form cells to hyphal cells in *C. albicans* has been associated with the establishment of infection (63). PLD1 has been shown to play a role in this transition (43). However, little is known about the specific roles of this enzyme in the *Candida* morphogenesis pathway. Therefore, PLD1 as well as two of its downstream products, DAG and PA, were investigated. We tested the ability of *Candida* to switch morphology in the presence of several pharmacological agents. These agents were chosen based on their effects on the activity of enzymes that might play a role in regulating this switch. Propranolol was used because of its ability to block the conversion of phosphatidate to DAG by inhibiting phosphatidate phosphohydrolase. Sphinganine was used because of its action as a phospholipase D inhibitor (6). We also assayed cells for PLD1 activity when presented with these same agents to measure the effect on downstream products of PLD1. A final pharmacological agent, staurosporine, was used to determine whether PLD1-derived DAG was acting through PKC.

MATERIALS AND METHODS

Assay of Dimorphism Induction

A 2 ml YPD culture was inoculated with *C. albicans* strain 28367 (wild type) and grown 16-18 hours at 30°C. The culture was then adjusted to $1x10^6$ cells/ml in YPD broth. This suspension was divided into culture tubes. The appropriate experimental agents were added (Table 2.1). Each assay set contained a cell-only control. A parallel assay set with the same parameters contained fetal bovine serum (FBS) at a final concentration of 20% (v/v). All tubes were incubated at 39°C in a shaking water bath for 2 hours. At the end of 2 hours, all samples were removed and 0.10 volume of

formaldehyde was added to each tube. Cells were counted, and the percentage of hyphal cells was determined.

Effect of Pharmacological Agents on Growth

Growth curves were performed using each agent shown in Table 2. A 2 ml YPD culture was inoculated with *C. albicans* strain 28367 (wild type) and grown for 16-18 hours at 30°C. Fifty microliters of this overnight culture were used to inoculate a 10ml flask of YPD to an OD of approximately 0.150 to 0.200. This step was repeated until enough flasks had been set up to accommodate a zero control and each concentration of the experimental agent being examined. Absorbance measurements were taken at 600nm using a spectrophotometer (Milton Roy, Spectronic 601). Readings were recorded every hour until the control cell culture had reached log phase growth. Absorbance was then determined and doubling times calculated.

AGENT	FINAL CONCENTRATION
Propranolol	0.5, 1.0, 1.5, 2.0 mM
Sphinganine	5.0, 10.0, 25.0 μM
Staurosporine	1.0, 2.0, 5.0, 10.0 nM

Table 2.1: Pharmacological Agents and Concentrations.

Biochemical Assay of PLD1 Activity

A 10 ml YPD culture was inoculated with *C. albicans* strain 28367 (wildtype) and incubated 16-18 hours at 30°C. Cells were harvested, washed in sterile distilled water, and resuspended in one volume of lysis buffer (20mM Hepes, pH7.5, 150mM NaCl, 2mM EDTA, 1mM DTT, 100mM β-glycerophosphate, 5μg/ml leupeptin, 1μg/ml pepstatin, 0.1mM PMSF). One volume of glass beads was added, and the cell suspension was mixed 10 x 30 seconds using a vortex mixer. The suspension was centrifuged at 12,000xg for 15 seconds to pellet beads and cell debris. The supernatant (whole cell extract) was collected, and protein concentration determined using the Bradford assay. Five micrograms of protein were used in each reaction. A fluorescent analog of glycerophosphocholine, BODIPY-PC (Molecular Probes), was used as the substrate. Final reactions contained 5 ug protein, lysis, resuspension (0.375mM NP-40, 350mM NaCl, 50mM Hepes, pH7.5), and 5x reaction buffer (35mM HEPES, pH7.5, 1mM EDTA, 25mM EGTA, 3mM DTT, 5% butanol) in a final volume of 25 μl. These assays examined the effects of the previously mentioned inhibitory agents (Table 2.1). Experimental agents were added immediately before addition of cell extracts. All samples were assayed in duplicate. Reactions were incubated for 30 minutes at 30°C. After 30 minutes, samples were placed on ice to stop the reaction. Five microliters of each reaction were spotted on silica gel 60 plates and developed with methyl acetate/1-propanol/chloroform/methanol/0.25% KCl/ acetic acid (100:100:100:40:36:2). A Fluorimager (Molecular Dynamics) was used for quantitation. Control and experimental reactions were spotted on the same plate, and experimental products were quantitated relative to the fluorescence of the control, which contained only lysis buffer plus reaction

mixture with no extract. Each experiment was repeated at least three times with independent extracts.

Biochemical Assay For Phosphatidic Acid Phosphohydrolase Activity

This assay was used to detect and quantitate PAPase activity in the presence of propranolol. The protocol used was the same as for the PLD1 activity mentioned previously in this chapter; however, the BODIPY-PC substrate was replaced with β-BODIPY PA (Molecular Probes). Within each experiment, increasing BPA concentrations were used with a fixed propranolol concentration. The data collected was analyzed using a Lineweaver-Burk plot to find Michaelis constant (K_M) and the rate of the catalytic process (k_{cat}).

Quantitation of DAG

Preparation of cells:

A 100 ml YPD culture was inoculated with *C. albicans* strain 28367 (wildtype) and incubated 16-18 hours at 30°C. Propranolol was then added to the culture to a final concentration of 2 mM. Ten milliliter samples were taken at 0, 15, 30, 60, and 120 minutes. Cells were harvested by centrifugation at 600 x g for 5 minutes immediately after each sample was taken and stored at -70°C until the time course was complete.

Extraction of lipids by Bligh and Dyer method:

Once the time course was completed, a lipid extraction was performed on samples obtained at each time point. Methanol was added in a 50:1 ratio (mg pellet:ml methanol), and glass beads were added to each sample in a 100:7 ratio (mg pellet:g glass beads). Cells were broken by milling with a vortex mixer for two 30-second pulses with 1 minute at 4°C between pulses. Chloroform was added to a final ratio of 2:1

chloroform:methanol and stirred for 2 hours at room temperature. The extracts were filtered through Whatman #1 filter paper to remove glass beads and cell debris. Each extract was transferred to a glass screw cap tube and washed with 0.2 volume 0.9% NaCl to remove water-soluble contaminants. The aqueous phase was removed, and the organic phase was stored at 4°C under nitrogen vapor.

Phosphate Assay:

Lipid samples were normalized with respect to total phospholipid concentration. Phospholipids were quantified by measuring the phosphate content of the samples. Phosphate standards were prepared using a 1mM NaH_2PO_4 stock. Lipid samples were prepared using 200 ul of each sample; 100 ul of ashing buffer [10 g Mg $(NO_3)_2$/ 100 ml EtOH] were added to all standards and lipids. The EtOH and chloroform were evaporated from the samples by incubation at 80°C for 20 minutes. Samples were then heated with a Bunsen burner until brown vapors ceased to be released from the reaction. HCl (0.5 N, 0.3 ml) was added to each tube and samples were boiled for 15 minutes. Ammonium molybdate (0.6 ml of 0.42%, w/v in 1 N H_2SO_4) and ascorbic acid (0.1 ml of 10%, w/v) were then added. Samples were mixed vigorously and incubated at 45°C for 30 minutes. Absorbance readings were then taken at 820 nm.

DGK Assay:

Lipid samples were dried in glass screw cap tubes in two different amounts, 100 µl (A) and 250 µl (B), for each time point. Samples were dried by placing the tubes in a 30°C heat block in a vacuum desiccator. Lipids were resuspended in 20 µl 7.5% β-octylglucoside (β-OG) with mixing. Seventy microliters of reaction buffer, which consisted of 50 µl 2xBuffer, 3 mM DTT, 0.44 µl DGK-containing membrane

prpeparation (3.8 μg/μl), and 19.36 μl dilution buffer, were added to each tube and mixed. The 2xBuffer contained 400 mM imidazole pH 6.6, 240 mM LiCl, 25 mM $MgCl_2$, 200 mM EGTA pH 6.6, and 37.5 ml sterile distilled H_2O. Dilution buffer consisted of 40 mM imidazole pH 6.6, 50 μM EDTA, and 39 ml sterile distilled H_2O. The reaction was initiated by adding 10 μl of 2 mM [γ-^{32}P] ATP (4 μCi/ sample) to each tube and incubating at room temperature for 30 minutes. Reactions were terminated by the addition of 3 ml of chloroform: methanol (1: 2) and mixing. Samples were centrifuged at 3,000 rpm for 5 minutes to separate phases. The aqueous phase was removed, and the organic phase was collected and dried in a 30°C heat block under vacuum. Samples were resuspended in 50 μl chloroform; half of each sample was spotted on a TLC plate (Whatman Silica 60A). Spots were air-dried, and the plate was developed in chloroform:acetone:methanol:acetic acid:water (10:4:3:2:1). The plate was air- dried and exposed to film overnight. DAG spots for each lipid sample were scraped from the plate and counted using a 1211 Rackbeta scintillation counter (LBK-Wallac). The DAG amounts (cpm) were then normalized to total phospholipids, which had been determined using the phosphate assay. The results of this normalization were then used the determine percent DAG present at each time point relative to time 0, which was set at 100%.

RESULTS

Assay of Dimorphism Induction

Sphinganine:

Sphingolipids, particularly sphinganine, have been shown to inhibit mammalian PLDs. Consequently, the effect of sphinganine on dimorphic transition was measured. In the morphology assays, sphinganine completely eliminated germ tube formation at 25 μM (Figure 2.1). A concentration of 5 uM was sufficient to show a significant reduction of 15% in the efficiency of morphogenesis. A disruption in cell proliferation would be one way that germ tube formation could be reduced, since a germ tube arises through extension of the mother cell rather than by remodeling of the yeast cell. In determining whether or not this reduction in germ tubes was caused by inhibition of cell proliferation, we found that 5 μM sphinganine had no effect on doubling times (Table 2.2). However, higher concentrations of sphinganine inhibited on growth. This result supports the hypothesis that PLD1 is involved in morphogenesis.

Propranolol:

Propranolol has been used to distinguish the effect of PA from the effect of DAG derived from PA. Propranolol has been demonstrated to inhibit the conversion of PA to DAG, presumably by inhibition of PAP. Increasing propranolol concentrations resulted in increased inhibition of germ tube formation. Two millimolar propranolol completely blocked the formation of hyphae (Figure 2.2). Growth curves in the presence of propranolol suggested that the inhibition of germ tube formation was not due to a block in cell proliferation, since concentrations that were sufficient to reduce morphogenesis

(e.g., 0.5 mM) had no effect on cell doubling times (Table 2.2). However, higher concentrations of propranolol (e.g., 2 mM) did inhibit yeast growth.

Temperature shift, alone, induces one pathway which leads to morphogenesis. However, a temperature shift plus bovine calf serum induces multiple parallel pathways leading to morphogenesis. When serum was introduced, in order to test the significance of propranolol's inhibitory effect, there was a small increase in the number of germ tubes at the 0mM concentration. However, serum appeared to have no effect on germ tube formation in the presence of propranolol.

Staurosporine:

Staurosporine is known to be an inhibitor of protein kinase C and other protein kinases in mammalian systems. For this reason, it was used to determine if protein phosphorylation was required for hyphae formation. Staurosporine had no effect on dimorphic transition, with the negative control showing the same germ tube percentages as the culture with 25 nM staurosporine (Figure 2.3). Staurosporine was also shown to have no effect on doubling times, suggesting that it was not affecting cell proliferation.

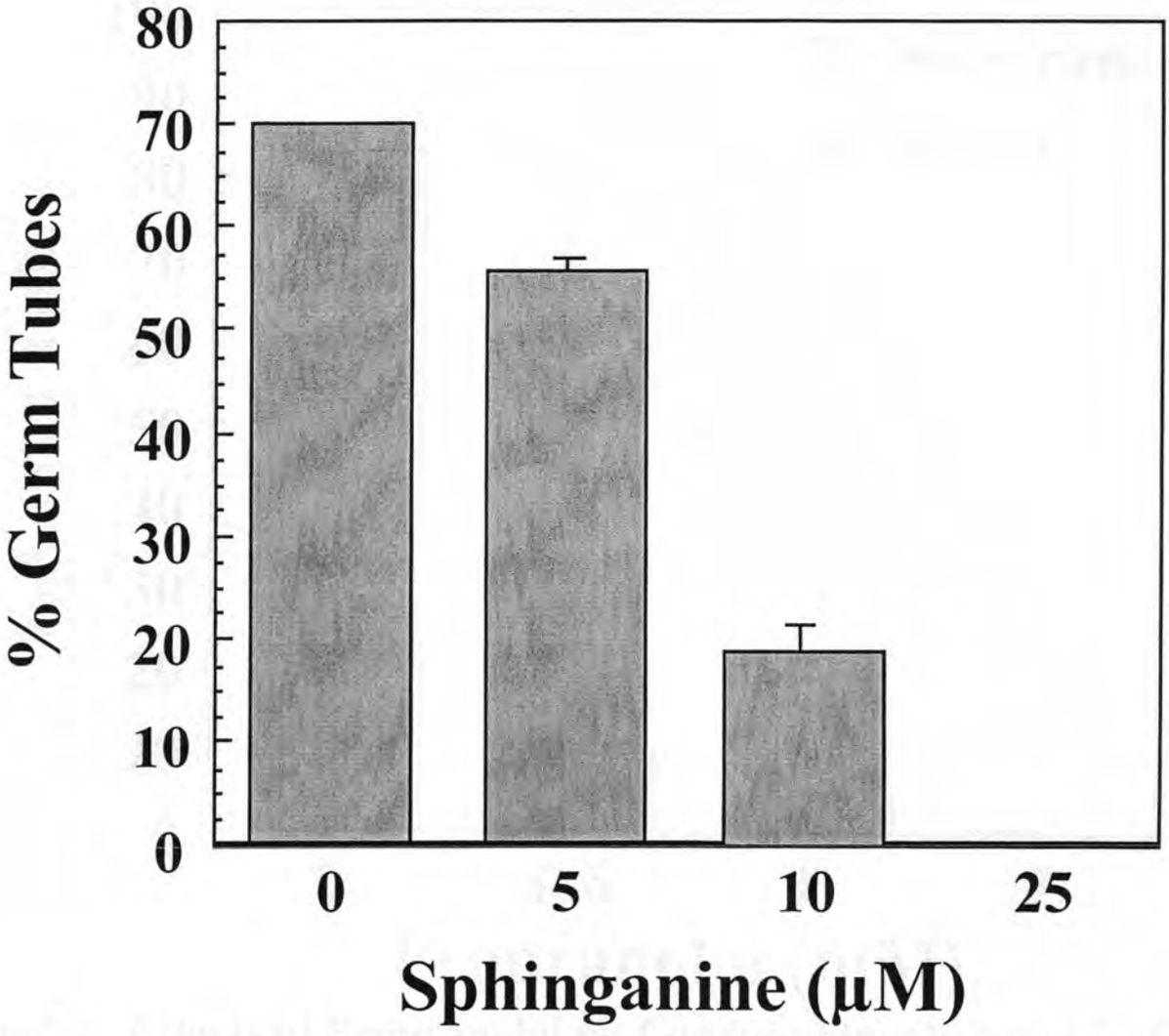

Figure 2.1: Effects of Sphinganine on *Candida* Morphogenesis. Cells were induced to undergo morphogenesis in the presence of the indicated concentrations of sphinganine. The percentage of cells with germ tubes was calculated. Each point represents the mean +/-standard error for 3 independent experiments with duplicate samples within each experiment.

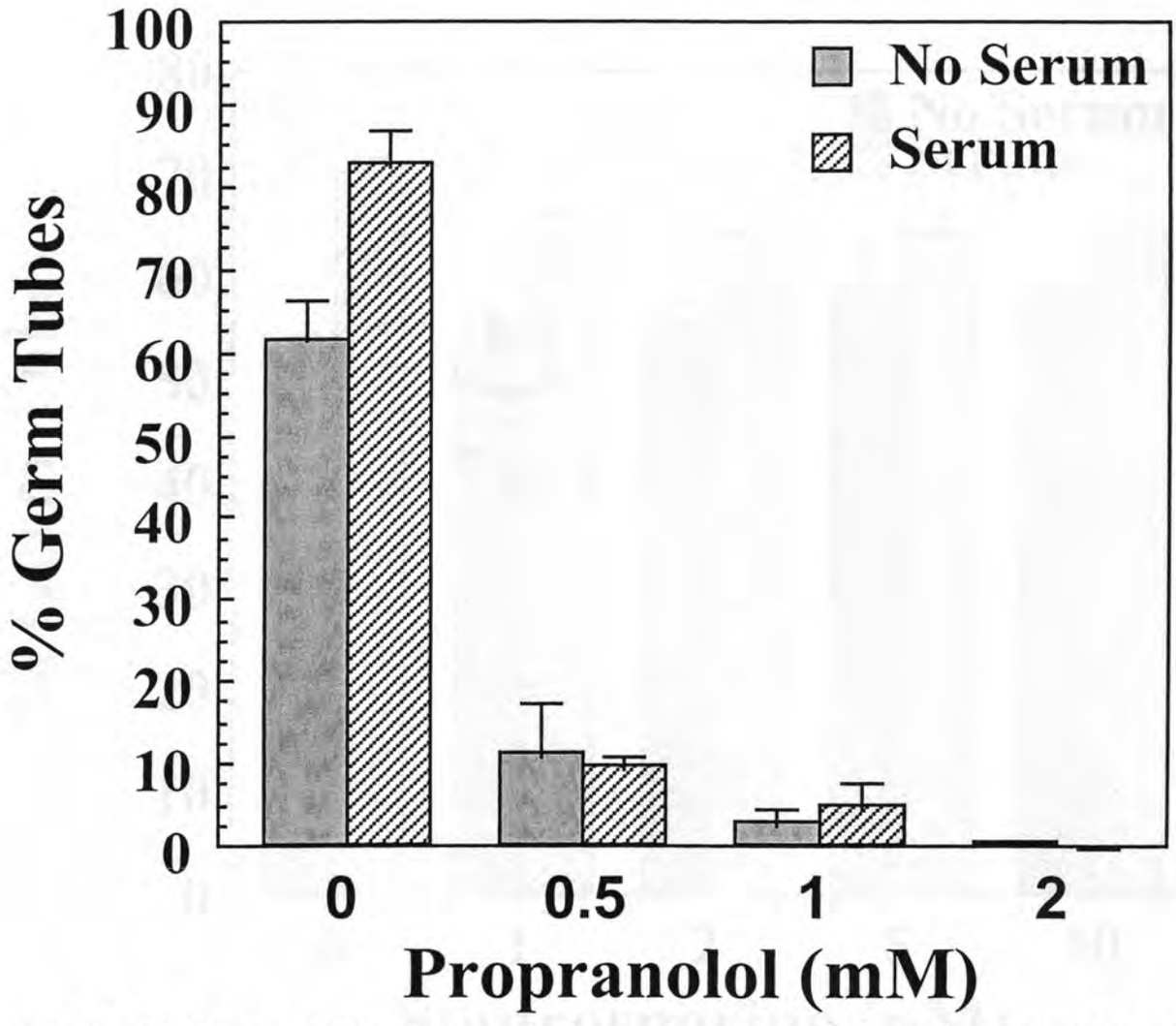

Figure 2.2: Effects of Propranolol on *Candida* Morphogenesis. Cells were induced to undergo morphogenesis in the presence of the indicated concentrations of propranolol. The percentage of cells with germ tubes was calculated. . Each point represents the mean +/-standard error for 4 independent experiments with duplicate samples within each experiment.

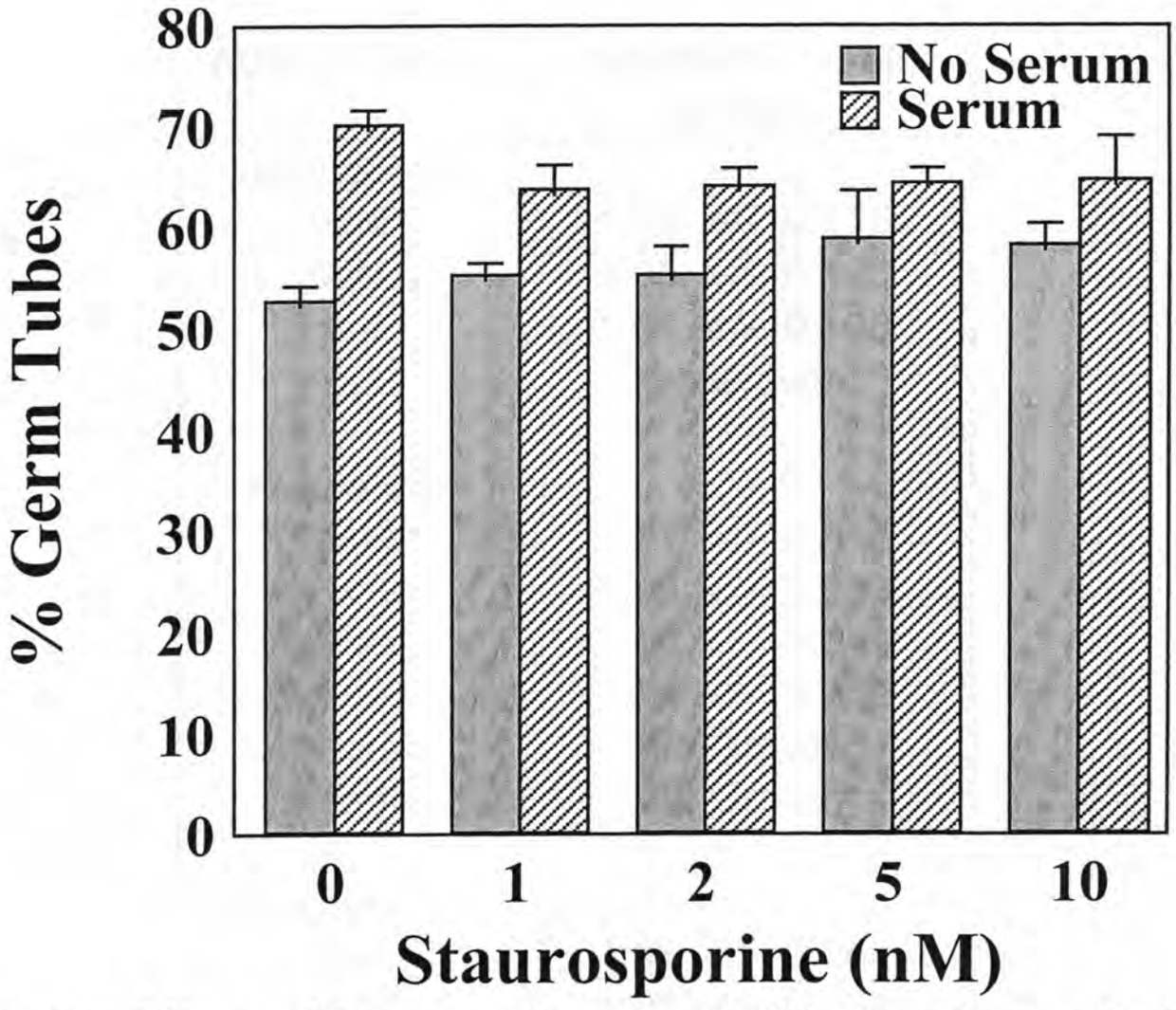

Figure 2.3: Effects of Staurosporine on *Candida* Morphogenesis. Cells were induced to undergo morphogenesis in the presence of the indicated concentrations of staurosporine. The percentage of cells with germ tubes was calculated. Each point represents the mean +/-standard error for 3 independent experiments with duplicate samples within each experiment.

AGENT/CONC.	DOUBLING TIME (HRS)
Sphinganine	
0μM	1.05 +/- 0.115
5μM	1.05 +/- 0.126
10μM	1.60 +/- 0.100
25μM	No Growth
Propranolol	
0mM	1.21 +/- 0.010
0.1mM	1.08 +/- 0.088
0.5mM	1.15 +/- 0.044
1.0mM	1.22 +/- 0.060
2.0mM	No Growth
5.0mM	No Growth
Staurosporine	
0nM	1.08 +/- 0.060
1nM	1.02 +/- 0.054
2nM	1.004 +/- 0.067
5nM	1.00 +/- 0.017
10nM	0.97 +/- 0.074

Table 2.2: Effects of Pharmacological Agents on Population Doubling Times. Data are shown as an average of 4 separate experiments +/- standard error of the mean.

Biochemical Assay of PLD1 Activity

The results obtained *in vivo* suggested that propranolol might inhibit the conversion of PLD1-derived PA to DAG. Figure 2.4 illustrates the effects of propranolol on the conversion of PA to DAG *in vitro*. The *in vitro* assays used BODIPY-PC as the substrate. In samples containing 1mM propranolol, PA amounts were 53.6% higher than in controls. In addition, the amount of DG in the 1 mM samples was 28.6% less than in samples lacking propranolol. P-values in both cases were less than 0.005, indicating statistical significance. Propranolol concentrations of 5 mM inhibited both DG and PA production. However, this concentration was concluded to be toxic to the cells, based on growth assays (Table 2.2). The data suggest that PLD1-derived DAG may be required for this morphogenesis.

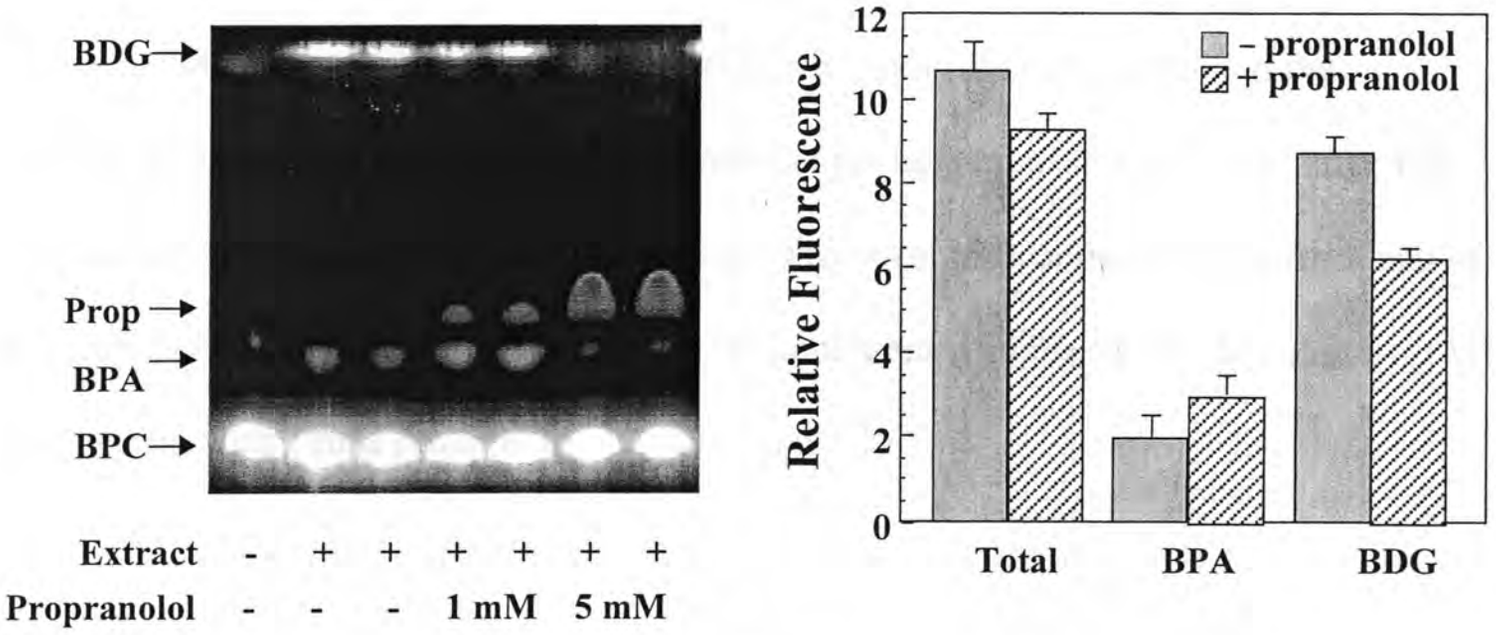

Figure 2.4: Propranolol Inhibits CaPLD1 Activity. The TLC plate scan (left) shows *Ca*PLD1 activity in response to propranolol. The data quantitated in the right panel are based on the mean +/- standard error of 6 independent experiments.

Dg Kinase Assays

DG kinase assays were used to determine whether propranolol inhibited the formation of DAG *in vivo*. The data showed that the amount of DAG present after 120 minutes of incubation with 2 mM propranolol was not significantly different from that of untreated cells (Figure 2.5). This result is not consistent with the 28.6% decrease in DAG levels seen with 1 mM propranolol *in vitro* (Figure 2.4).

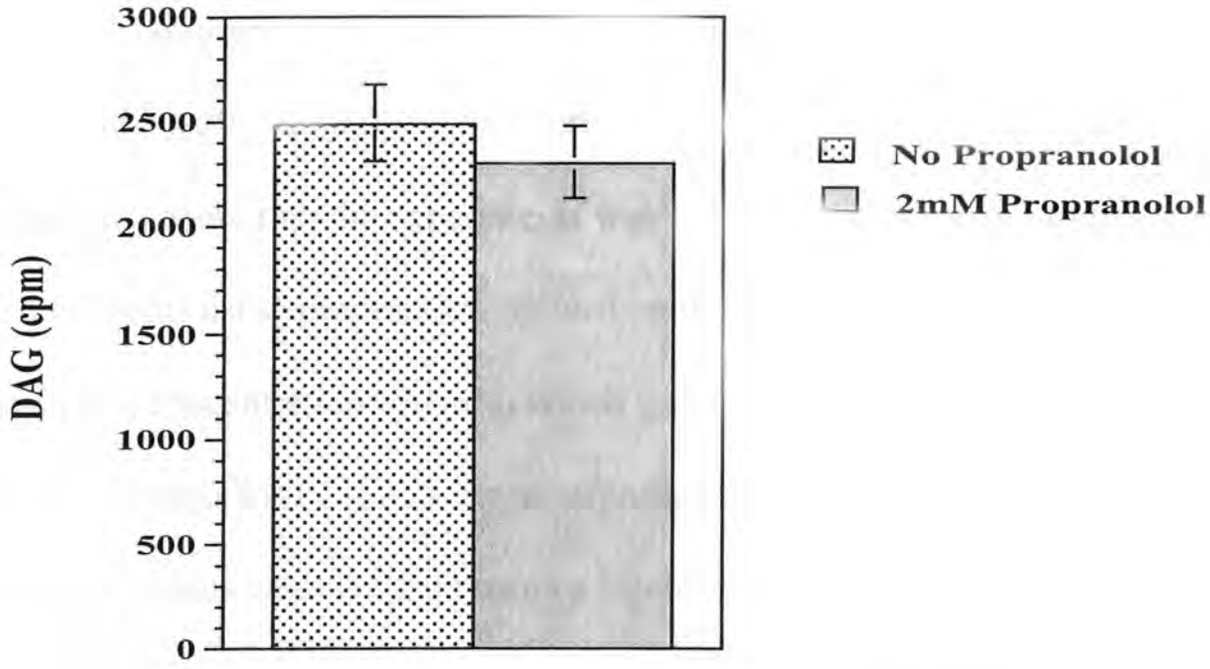

Figure 2.5: DAG Levels In Vivo Show No Change in Response to Propranolol. Lipid extracts from cell cultures were exposed to 2mM propranolol for 2 hours. DAG levels were assayed using a DG kinase assay, as described in the text. Each point represents the mean +/- standard error for 4 independent experiments with duplicate samples within each experiment.

Biochemical Assay for Phosphatidic Acid Phosphohydrolase Activity

A PAP assay was designed using BPA as the substrate. Time course assays for PAP activity using 50μM BPA were performed, prior to the BPA/propranolol experiments, to ensure that the experiments were within a linear range of kinetics (Figure 2.6). Experiments using propranolol showed no inhibition at any of the concentrations used. Even at a concentration of 1mM, which was sufficient to block the conversion of PA to DAG in assays for PLD activity, no significant change in PAP function was observed. The results were graphed using a Michaelis-Menten plot (Figure 2.7).

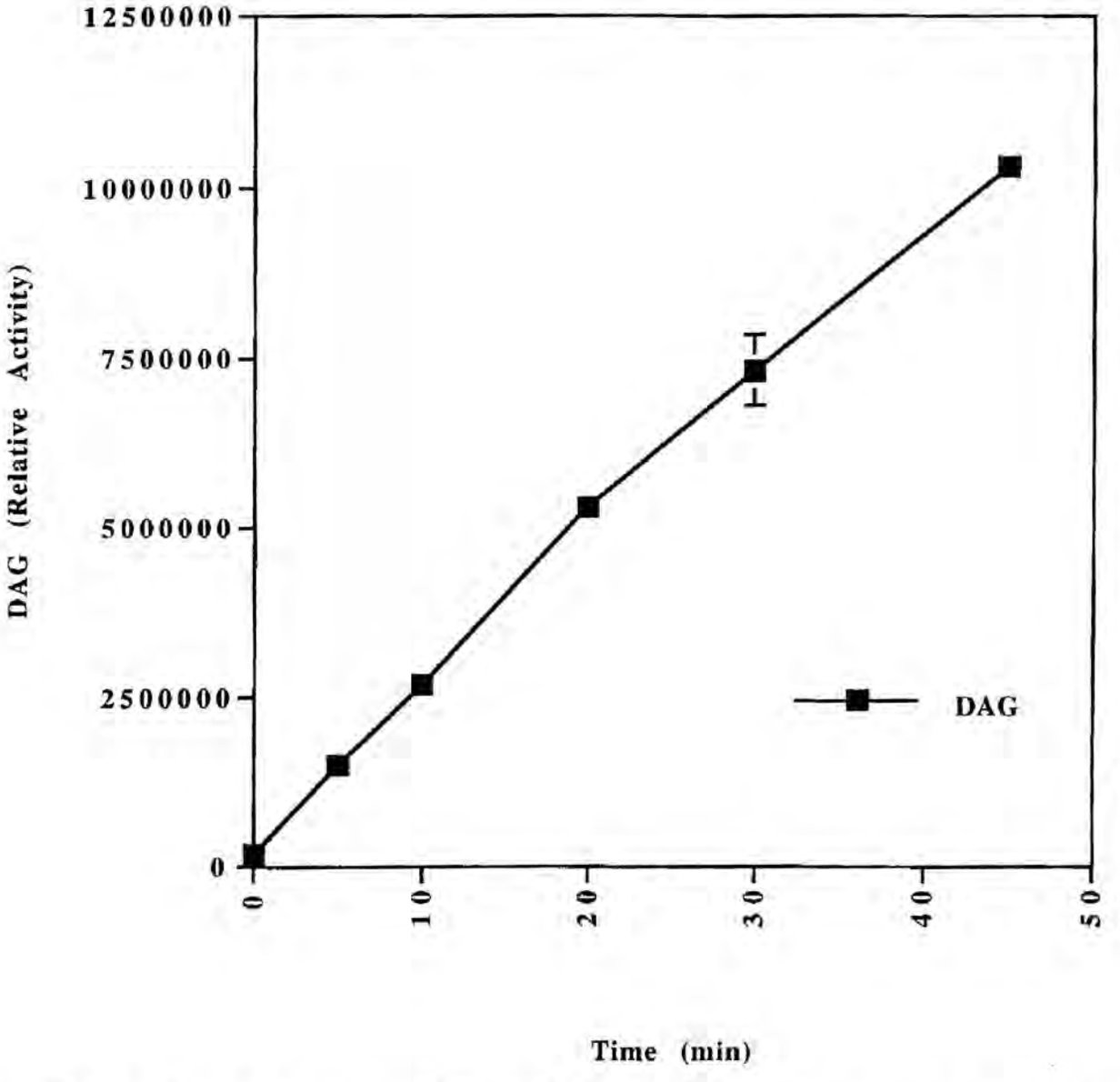

Figure 2.6: Time Course of BPA Hydrolysis. Time course assays for PAP activity were performed to establish linear kinetics. A Fluorimager (Molecular Dynamics) was used to measure fluorescence. Experimental fluorescence was calculated relative to that of the control sample of 50μM BPA at 0 minutes. Each point represents the mean +/- standard error for 4 independent experiments.

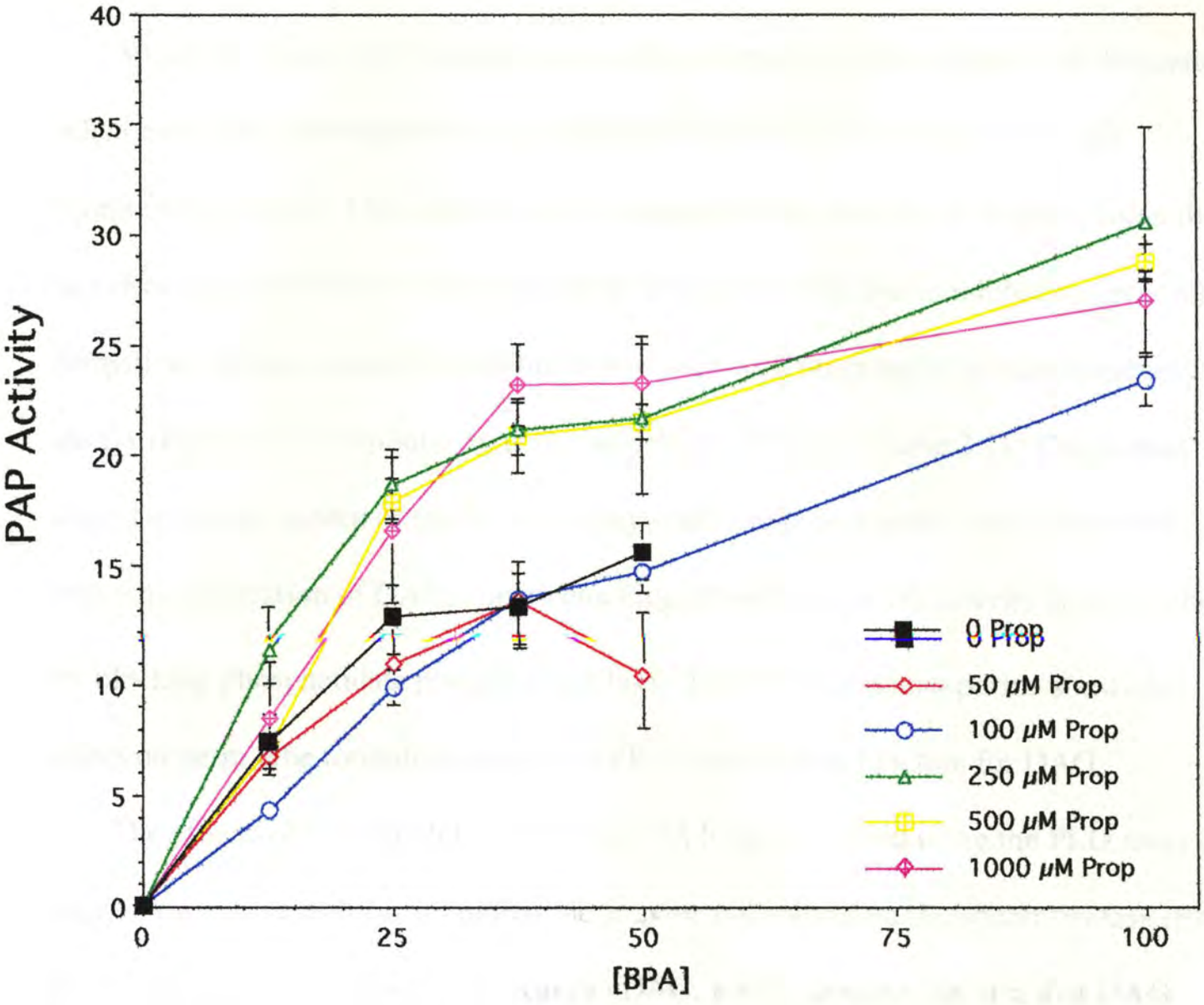

Figure 2.7: Effects of Propranolol on PA Phosphohydrolase Activity. Experiments were performed in which increasing concentrations of BPA and fixed concentrations of propranolol were incubated with yeast extracts, within each separate assay. The product produced from BPA hydrolysis was quantitated. Results were calculated from 4 independent experiments +/- standard error of the mean.

CONCLUSION

Wildtype strain 28367 showed a dose-dependent inhibition of germ tube formation when exposed to sphinganine, suggesting that PLD1 may play a role in the cell morphology switch. This observation is strengthened by that fact that sphinganine did not affect cell proliferation at a concentration that was effective in inhibiting germ tube formation. A dose-dependent inhibition was seen with propranolol in morphogenesis assays (Figure 2.2), without a reduction in cell proliferation (Table 2.2). One would expect a similar pattern of results for sphinganine and propranolol, since both potentially affect the generation of DAG, one by blocking phospholipase D1 activity and the other by blocking phosphatidate phosphohydrolase. The fact that staurosporine showed no effect on germ tube formation suggests a PKC-independent function for DAG.

The effects of propranolol on DAG and PA levels observed using the PLD assays suggest that propranolol is inhibiting the enzyme phosphatidate phosphohydrolase (PAP). *In vitro* assays demonstrated a decrease in DAG, which supports the idea that DAG derived from PLD1-produced PA is playing a role in morphogenesis. However, in testing the effect of propranolol *in vivo*, using DAG kinase assays, it was confirmed that there was no significant decrease in DAG concentration in strain 28367 at 2 mM concentrations of propranolol (Figure 2.5). These data suggest a potential for the cell to compensate for the decrease in the production of DAG by PLD1. Such compensation may occur by upregulation of PLC1 activity or inhibition of the CDP-choline pathway. Compensation could also occur through the use of the enzyme mon-acyl-glycerol-acyl transferase (64) or inositol-phosphoryl ceramide synthase (65). Additionally, *in vivo* data may also point to an alternative mechanism in which propranolol does not inhibit PAP.

This idea is supported by the *in vitro* assays performed using a fluorescent-labeled PA (BPA) to measure PAP activity. With these assays, it was shown that increasing concentrations of propranolol had limited inhibitory effect on PA phosphohydrolase activity (Figure 2.7). Alternatively, the lack of inhibition in the assays using BPA as the substrate may be due to the location of the BODIPY group. The BODIPY group of BPC, which is the substrate used for PLD activity assays, is located on Carbon 1. In contrast, the BODIPY group of BPA is located on Carbon 2 (Figure 2.8). The Carbon 2 location could block the binding of propranolol to the PA molecule. Furthermore, the high concentrations of propranolol needed to produce an effect also argues for a different mechanism. Inhibition of PLD activity in yeast is seen in the millimolar range (5), while in mammalian systems inhibition is seen in the micromolar range. Finally, it has been demonstrated that propranolol binds directly to acidic phospholipids such as PA and phosphatidylserine (66). Thus, propranolol may act by directly binding to PA, sequestering the PA from PAP and other downstream targets.

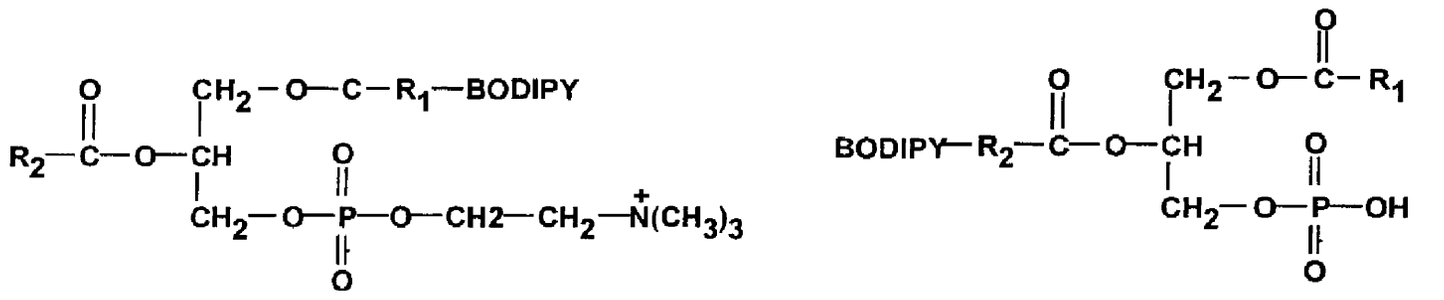

Figure 2.8: Structures of BODIPY-Labeled Substrates Used in Fluorescence Assays. The BODIPY group of BPC is located on Carbon 1, unlike the BODIPY group of BPA, which is located on Carbon 2. In the BPC structure, R_1 represents an undecyl group, and R_2 represents a decanoyl group. The R_1 of BPA represents a hexadecanoyl group, and R_2 represents a pentanoyl group.

CHAPTER 3

THE PHENOTYPE OF A *C. ALBICANS* PLD1 MUTANT (*pld*1Δ1)

In order to more effectively examine the physiological role of PLD1, a *C. albicans* *PLD1* mutant (*pld*1Δ1) was constructed by our collaborator, Dr. Bernhard Hube of the Robert Koch Institute, Berlin. This mutant was used in our lab to analyze the phenotypes of a PLD1 deficient *C. albicans* strain in order to establish a phenotype. PLD1 assays were performed on the mutant (*pld*1Δ1) and wildtype control (SC5314) to confirm the absence of PLD1 activity in the mutant. Plate assays with various types of media were then used to establish a phenotype of the knockout. The media used were selected based on their ability to promote hyphae formation by wildtype cells at 37°C.

MATERIALS AND METHODS

Biochemical Assay of PLD1 Activity

Assays were performed as described in Chapter 2. The assays were performed with extracts from strain SC5314 (wildtype) and *pld*1Δ1 (mutant).

Plate Assays to Examine Morphogenesis

A variety of solid media (Table 3.1) were inoculated with both wildtype (SC5314) and mutant (*pld*1Δ1) and incubated at 37°. Colonies were observed after 1 and 3 days. Colonies were photographed using a Zeiss MicroCam with 331 Polaroid film.

SOLID MEDIA	DESCRIPTION
Corn Meal (2% Tween)	Low nutrient medium; promotes chlamydospores
Lee's Synthetic	Minimal medium with amino acids; promotes hyphae
Saboraud Dextrose	Glucose medium sometimes used for strain storage
Spider (Mannitol)	Mannitol as the carbon source; promotes hyphae
Spider (Glucose)	Glucose as carbon source
YPD (Mannitol)	Strain storage medium in which mannitol replaces glucose
YPD (Glucose)-Control	Glucose medium used for daily storage; promotes yeast cells

Table 3.1: Media Used in Plate Assays. Each medium presents the cells with unique conditions that can alter morphology.

DG Kinase Assays

Assays were performed as described in Chapter 2, using strain SC5314 (wildtype) and *pld*1Δ1 (mutant).

Measurements of Cell Growth in the Presence of Primary Vs Secondary Carbon Sources

YEP cultures, containing either mannitol or glucose as the carbon source, were inoculated with SC5314 and *pld*1Δ1. All tubes were be incubated for 16-18 hours at 30°C. All cultures were then diluted to an OD of 0.150-0.200 and returned to the 30°C incubator. The absorbancy at 600 nm was measured hourly for each sample. Once log phase growth was reached, the experiment was terminated, and the absorbancies plotted against time to calculate a growth rate for each sample. This experiment was repeated three times with independent overnight cultures.

RESULTS

Biochemical Assay of PLD1 Activity

To confirm that the *pld*1Δ1 mutant lacked PLD1 activity, the isogenic wildtype and mutant strains were assayed for PLD1 activity *in vitro*. The wildtype strain sample contained all of the products indicative of PLD1 activity using this assay method: phosphatidic acid (PA), phosphatidylbutanol (PBu), and diacylglycerol (DAG). However, the mutant showed an absence of all of these products (67). These results showed a lack of PC-specific PLD activity, thereby confirming that *C. albicans* expresses a single PLD with this enzymatic activity.

Plate Assays to Examine Morphology

Mutant cells plated on Spider medium at 37°C for 3 days showed no visible hyphae using microscopic observation at 40x (Figure 3.1). Wild type cells on Spider medium showed numerous invasive hyphae. Data with the *pld*1Δ*1* mutant established a decrease in morphogenesis on various types of media with the exception of Corn Meal Agar (25 Tween) and Lee's Synthetic medium (Table 3.2).

The results obtained with the YPD control plates compared to the Spider medium plates prompted us to test the possibility that morphogenesis was regulated by a factor derived from the carbon source. The most distinct difference between Spider and YPD medium is the carbon source used. Glucose, which is the standard carbon source in YPD, is a rich six-carbon sugar that the cells can utilize readily. Mannitol, another six-carbon sugar, is used in Spider medium and is known to be a poor source of carbon for these cells. Spider medium plates in which glucose was substituted for mannitol yielded results similar to those of YPD for both wildtype and mutant.

SOLID MEDIA (at 37°C)	SC5314 (3 days) at 40x Magnitude	ΔPLD14-3-2 (3 days) at 40x magnitude
Corn Meal Agar (2% Tween)	Long hyphae with many lateral buds	Long hyphae with many lateral buds
Lee's Synthetic	Hyphal formation	Hyphal formation; wrinkled colonies
Saboraud Dextrose	No visible hyphae	No visible hyphae
Spider (Mannitol)	Extensive, invasive hyphae	No visible hyphae
Spider (Glucose)	No visible hyphae	No visible hyphae
YPD (Mannitol)	Visible hyphae	No visible hyphae
YPD (Glucose)-Control	Yeast cells; no hyphal formation	Yeast cells; no hyphal formation

Table 3.2: SC5314 and ΔPLD 14-3-2 Morphologies on Different Media. Various media were selected based on their effects on morphology (see Table 3.1).

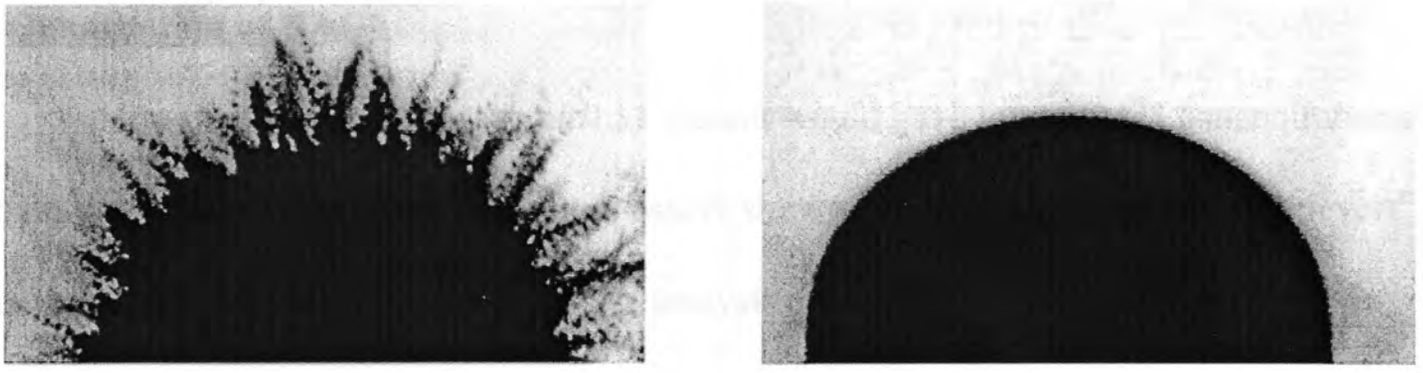

Figure 3.1: Wildtype (left) **and Mutant** (right) **Colonies on Spider Media.** A complete absence of extended hyphae was observed with the pld1 mutant on Spider medium. Photographs were taken with x40 magnification.

Quantitation of DAG

It was anticipated that the *pld*1Δ1 mutant would have lower DAG concentrations than the wildtype; however, DG kinase assays showed significantly higher DAG levels in the mutant (Figure 3.2). An ANOVA analysis calculated a p-value of 0.0462. This result suggests either that the cell can adapt to the loss of PLD1 activity and maintain its pool of DAG, or that PLD1 is not a major source of DAG.

Measurements of Cell Growth in the Presence of Primary Vs Secondary Carbon Sources

A lower growth rate was expected for *pld*1Δ1 cells as compared to wildtype when mannitol was used as the carbon source. This hypothesis was based on the fact that the use of a secondary carbon source (mannitol) in the plate assays resulted in increased hyphal formation (Figure 3.2). We hypothesized that cells may be using hyphal formation as a foraging mechanism when given mannitol. However, no difference in growth rate between wildtype and mutant was observed in the presence of mannitol (data not shown).

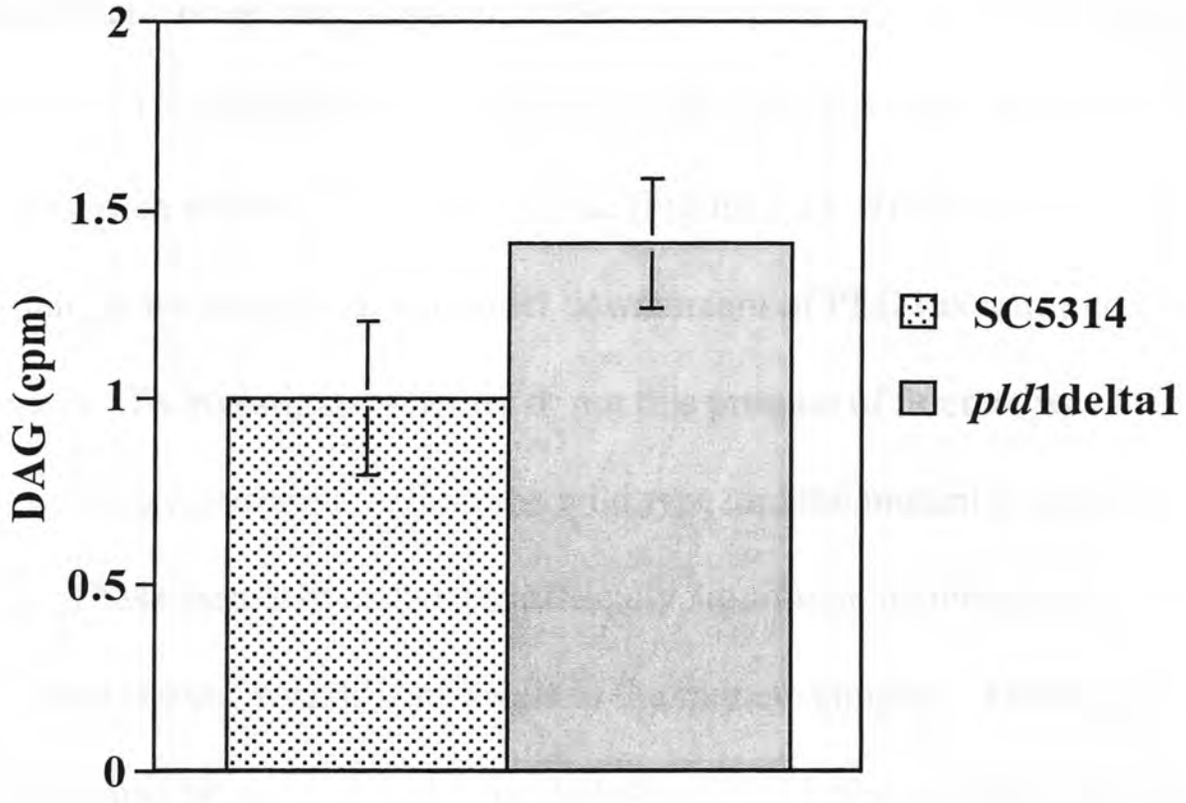

Figure 3.2: In Vivo DAG levels of SC5314 and *pld*1Δ1. Lipid extracts were assayed for DAG content using a DG kinase assay. Shown is the average of 4 separate experiments +/- the standard error of the mean.

CONCLUSION

Initial plate assays established a distinct phenotype for the *pld*1Δ1 mutant on solid Spider medium. The mutant showed a complete absence of visible hyphae, in contrast to the wild type which showed extensive hyphae (Figure 3.1). These observations suggested that, as hypothesized, a product downstream of PLD1 is required for morphogenesis. To investigate whether or not this product of interest was DAG, DG kinase assays were performed on both the wild type and the mutant to compare *in vivo* DAG levels. These assays revealed a statistically significant difference in DAG levels between the two strains, with higher levels in the mutant samples. This could suggest that the mutant may be compensating for its loss of PLD1-derived DAG by using PLC-derived DAG. This is presently being investigated in the lab, where preliminary data show that the mutant has increased PLC activity compared to the wild type. Compensation by PLC-derived DAG could occur based on the fact that the experiments discussed were carried out no longer than two hours, and it has been shown that PtdIns-PLC activity contributes to the initial rapid phase of AVP-dependent DAG in a rat skeletal muscle cell line L6 (59). Naro et al. showed that PC-PLD activity was entirely responsible for sustained DAG levels which were required for the differentiation of L6 myoblasts into multinucleated fibers (59). Fungi may also transfer phosphoryl-inositol from phosphatidyl inositol (PI) to phytoceramide to form inositol-phosphoryl ceramide through the use of the enzyme IPC synthase. This reaction is accompanied by the release of DAG (65). A final path which may be used to compensate for the loss of PLD-derived DAG is by the conversion of monoacyl-glycerol-3-phosphate to PA by the enzyme

monoacyl-glycerol-acyl-transferase. The PA that is produced can then be dephosphorylated by PAP to produce DAG (64).

CHAPTER 4

CONCLUSION

The objective of this research was to establish a role for the enzyme PLD1 in *C. albicans*, specifically with regard to its involvement in morphogenesis. The results of this study are consistent with the hypothesis that a lack of PLD1 activity results in reduced ability to form hyphae. Therefore, loss of PLD1 activity potentially results in a less virulent pathogen. However, the results were not sufficient to establish a precise location for PLD1 in the morphogenesis pathway, or to pinpoint which downstream products of PLD1 (e.g., PA or DAG) are required for the yeast-to-hyphae switch.

The experiments utlizing propranolol seemed to suggest the importance of DAG in the morphological switch. The mechanism by which propranolol is presumed to act is by inhibition of PA phosphohydrolase. This mechanism was tested using *in vitro* fluorescence assays with BODIPY-PA as the substrate. Interestingly, no inhibition was found with increasing concentrations of propranolol using this assay. One possible reason for the lack of inhibition is the structure of the substrate used in this assay. The 1-BODIPY- PC is cleaved to 1-BODIPY-PA by PLD1, and 1-BODIPY-PA is rapidly dephosphorylated by PAP (Figure 2.8). In the PAP assays for propranolol inhibition, 2-BODIPY-PA was used as a substrate (Figure 2.8). This form of PA may not be a substrate for the propranolol-sensitive PAP. This is supported by Figure 2.4 which illustrates the dephosphorylation of 1-BODIPY-PA to produce DAG.

Another point of interest is that derived from the comparison of *in vitro* propranolol fluorescence assay data with that of the *in vivo* DAG quantitation experiments in the presence of propranolol. The decrease in DAG shown using the *in vitro* biochemical assays (Figure 2.4) was not seen with *in vivo* assays, which demonstrated no significant decrease in DAG levels in the 28367 wildtype in the presence of 2 mM propranolol. This

could be due to the possible use of PLC1-derived DAG by the cells to compensate for the loss of DAG produced through PLD1 activity. This idea was shown to be the case with AVP-stimulated L6 cells in the presence of propranolol, where enhanced PA accumulation increased PLC activity (59). Compensation may also be due to the enzyme inositol-phosphoryl ceramide synthase (64) or mono-acyl-glycerol-acyl transferase (65). The data also suggests that loss of PA, rather than insufficient DAG, may be responsible for the inhibition of morphogenesis. Data from our laboratory (Desrosier and Dolan, in preparation) show that the fluorescence of propranolol is quenched by PA in a concentration-dependent manner, suggesting that propranolol binds directly to the PA molecule.

It can be concluded that a lack of PLD1 activity has an adverse effect on hyphal formation and produces a less virulent pathogen. In assays utilizing various types of solid media, and particularly with Spider medium, a reduced ability to form lateral, invasive hyphae was shown repeatedly with the *pld*1Δ1 mutant. Unpublished data from our laboratory (Bell et al.; manuscript in preparation) have established *C. albicans pld*1Δ1 as an avirulent strain in a mouse model system. It was determined that alimentary tract colonization was the same for strains B311 (wildtype, homozygous for *URA*3), CAF2-1 (wild type, heterozygous for *URA*3), and *pld*1Δ1 (mutant, heterozygous for *URA*3). However, when lethality experiments were performed, it was shown that at all time points up to 35-42 days, *pld*1Δ1-inoculated mice had a 100% survival rate in comparison to both wildtype strains, which demonstrated a 0% survival rate. Although it remains unclear exactly what role PLD1 and its products play in the signaling pathway leading to morphogenesis, it is clear that a role for this enzyme does exist.

References

1. **M. Liscovitch, M. Czarny, G. Fiucci, Y. Lavie and X. Tang.** 1999. "Localization and possible functions of phospholipase D isozymes." Biochim Biophys Acta **1439:**245-63.
2. **M. Heller.** 1978. "Phospholipase D." Adv Lipid Res **16:**267-326.
3. **S. A. Rudge and J. Engebrecht.** 1999. "Regulation and function of PLDs in yeast." Biochim Biophys Acta **1439:**167-74.
4. **M. Kobayashi and J. N. Kanfer.** 1987. "Phosphatidylethanol formation via transphosphatidylation by rat brain synaptosomal phospholipase D." J Neurochem **48:**1597-603.
5. **K. M. Ella, J. W. Dolan and K. E. Meier.** 1995. "Characterization of a regulated form of phospholipase D in the yeast *Saccharomyces cerevisiae*." Biochem J **307:**799-805.
6. **R. C. Franson, L. K. Harris, S. S. Ghosh and M. D. Rosenthal.** 1992. "Sphingolipid metabolism and signal transduction: inhibition of in vitro phospholipase activity by sphingosine." Biochim Biophys Acta **1136:**169-74.
7. **J. Stutchfield and S. Cockcroft.** 1993. "Correlation between secretion and phospholipase D activation in differentiated HL60 cells." Biochem J **293:**649-55.
8. **A. Carnero, A. Cuadrado, L. del Peso and J. C. Lacal.** 1994. "Activation of type D phospholipase by serum stimulation and ras-induced transformation in NIH3T3 cells." Oncogene **9:**1387-95.
9. **C. L. Yu, M. H. Tsai and D. W. Stacey.** 1988. "Cellular ras activity and phospholipid metabolism." Cell **52:**63-71.

10. **K. R. Morlock, J. J. McLaughlin, Y. P. Lin and G. M. Carman.** 1991. "Phosphatidate phosphatase from *Saccharomyces cerevisiae*. Isolation of 45- and 104-kDa forms of the enzyme that are differentially regulated by inositol." J Biol Chem **266:**3586-93.

11. **Y. Nishizuka.** 1995. "Protein kinase C and lipid signaling for sustained cellular responses." FASEB J **9:**484-96.

12. **D. S. Min, S. K. Park and J. H. Exton.** 1998. "Characterization of a rat brain phospholipase D isozyme." J Biol Chem **273:**7044-51.

13. **O. Kozawa, P. Blume-Jensen, C. H. Heldin and L. Ronnstrand.** 1997. "Involvement of phosphatidylinositol 3'-kinase in stem-cell-factor- induced phospholipase D activation and arachidonic acid release." Eur J Biochem **248:**149-55.

14. **J. H. Exton.** 1999. "Regulation of phospholipase D." Biochim Biophys Acta **1439:**121-33.

15. **E. J. Yeo, A. Kazlauskas and J. H. Exton.** 1994. "Activation of phospholipase C-gamma is necessary for stimulation of phospholipase D by platelet-derived growth factor." J Biol Chem **269:**27823-6.

16. **Y. H. Lee, H. S. Kim, J. K. Pai, S. H. Ryu and P. G. Suh.** 1994. "Activation of phospholipase D induced by platelet-derived growth factor is dependent upon the level of phospholipase C-gamma 1." J Biol Chem **269:**26842-7.

17. **W. D. Singer, H. A. Brown, X. Jiang and P. C. Sternweis.** 1996. "Regulation of phospholipase D by protein kinase C is synergistic with ADP-ribosylation factor and independent of protein kinase activity." J Biol Chem **271:**4504-10.

18. **U. Rumenapp, M. Schmidt, F. Wahn, E. Tapp, A. Grannass and K. H. Jakobs.** 1997. "Characteristics of protein-kinase-C- and ADP-ribosylation-factor- stimulated phospholipase D activities in human embryonic kidney cells." Eur J Biochem **248:**407-14.

19. **B. Geny, A. Fensome and S. Cockcroft.** 1993. "Rat brain cytosol contains a factor which reconstitutes guanine- nucleotide-binding-protein-regulated phospholipase-D activation in HL60 cells previously permeabilized with streptolysin O." Eur J Biochem **215:**389-96.

20. **S. Cockcroft, G. M. Thomas, A. Fensome, B. Geny, E. Cunningham, I. Gout, I. Hiles, N. F. Totty, O. Truong and J. J. Hsuan.** 1994. "Phospholipase D: a downstream effector of ARF in granulocytes." Science **263:**523-6.

21. **H. A. Brown, S. Gutowski, C. R. Moomaw, C. Slaughter and P. C. Sternweis.** 1993. "ADP-ribosylation factor, a small GTP-dependent regulatory protein, stimulates phospholipase D activity." Cell **75:**1137-44.

22. **A. R. Siddiqi, J. L. Smith, A. H. Ross, R. G. Qiu, M. Symons and J. H. Exton.** 1995. "Regulation of phospholipase D in HL60 cells. Evidence for a cytosolic phospholipase D." J Biol Chem **270:**8466-73.

23. **Y. Banno, K. Tamiya-Koizumi, H. Oshima, A. Morikawa, S. Yoshida and Y. Nozawa.** 1997. "Nuclear ADP-ribosylation factor (ARF)- and oleate-dependent phospholipase D (PLD) in rat liver cells. Increases of ARF-dependent PLD activity in regenerating liver cells." J Biol Chem **272:**5208-13.

24. **J. J. Provost, J. Fudge, S. Israelit, A. R. Siddiqi and J. H. Exton.** 1996. "Tissue-specific distribution and subcellular distribution of phospholipase D in rat: evidence

for distinct RhoA- and ADP- ribosylation factor (ARF)-regulated isoenzymes." Biochem J **319:**285-91.

25. **C. P. Morgan, H. Sengelov, J. Whatmore, N. Borregaard and S. Cockcroft.** 1997. "ADP-ribosylation-factor-regulated phospholipase D activity localizes to secretory vesicles and mobilizes to the plasma membrane following N-formylmethionyl-leucyl-phenylalanine stimulation of human neutrophils." Biochem J **325:**581-5.

26. **J. Whatmore, C. P. Morgan, E. Cunningham, K. S. Collison, K. R. Willison and S. Cockcroft.** 1996. "ADP-ribosylation factor 1-regulated phospholipase D activity is localized at the plasma membrane and intracellular organelles in HL60 cells." Biochem J **320:**785-94.

27. **A. M. Vinggaard, J. J. Provost, J. H. Exton and H. S. Hansen.** 1997. "Arf and RhoA regulate both the cytosolic and the membrane-bound phospholipase D from human placenta." Cell Signal **9:**189-96.

28. **S. C. Olson, E. P. Bowman and J. D. Lambeth.** 1991. "Phospholipase D activation in a cell-free system from human neutrophils by phorbol 12-myristate 13-acetate and guanosine 5'-O-(3- thiotriphosphate). Activation is calcium dependent and requires protein factors in both the plasma membrane and cytosol." J Biol Chem **266:**17236-42.

29. **J. Y. Kwak, I. Lopez, D. J. Uhlinger, S. H. Ryu and J. D. Lambeth.** 1995. "RhoA and a cytosolic 50-kDa factor reconstitute GTP gamma S-dependent phospholipase D activity in human neutrophil subcellular fractions." J Biol Chem **270:**27093-8.

30. **V. Natarajan, S. Vepa, R. S. Verma and W. M. Scribner.** 1996. "Role of protein tyrosine phosphorylation in H2O2-induced activation of endothelial cell phospholipase D." Am J Physiol **271:**L400-8.

31. **A. Ullrich and J. Schlessinger.** 1990. "Signal transduction by receptors with tyrosine kinase activity." Cell **61:**203-12.

32. **R. W. Randall, G. D. Spacey and R. W. Bonser.** 1993. "Phospholipase D activation in PDGF-stimulated vascular smooth muscle cells." Biochem Soc Trans **21:**352S.

33. **L. C. Wilkes, V. Patel, J. R. Purkiss and M. R. Boarder.** 1993. "Endothelin-1 stimulated phospholipase D in A10 vascular smooth muscle derived cells is dependent on tyrosine kinase. Evidence for involvement in stimulation of mitogenesis." FEBS Lett **322:**147-50.

34. **J. Song and D. A. Foster.** 1993. "v-Src activates a unique phospholipase D activity that can be distinguished from the phospholipase D activity activated by phorbol esters." Biochem J **294:**711-7.

35. **T. Hunter and J. A. Cooper.** 1985. "Protein-tyrosine kinases." Annu Rev Biochem **54:**897-930.

36. **S. A. Qureshi, C. K. Joseph, M. Rim, A. Maroney and D. A. Foster.** 1991. "v-Src activates both protein kinase C-dependent and independent signaling pathways in murine fibroblasts." Oncogene **6:**995-9.

37. **T. J. Martins, Y. Sugimoto and R. L. Erikson.** 1989. "Dissociation of inositol trisphosphate from diacylglycerol production in Rous sarcoma virus-transformed fibroblasts." J Cell Biol **108:**683-91.

38. **J. G. Song, L. M. Pfeffer and D. A. Foster.** 1991. "v-Src increases diacylglycerol levels via a type D phospholipase- mediated hydrolysis of phosphatidylcholine." Mol Cell Biol **11:**4903-8.

39. **D. N. Brindley.** 1984. "Intracellular translocation of phosphatidate phosphohydrolase and its possible role in the control of glycerolipid synthesis." Prog Lipid Res **23:**115-33.

40. **K. M. Ella, J. W. Dolan, C. Qi and K. E. Meier.** 1996. "Characterization of *Saccharomyces cerevisiae* deficient in expression of phospholipase D." Biochem J **314:**15-9.

41. **S. M. Honigberg, C. Conicella and R. E. Espositio.** 1992. "Commitment to meiosis in *Saccharomyces cerevisiae*: involvement of the SPO14 gene." Genetics **130:**703-16.

42. **K. Rose, S. A. Rudge, M. A. Frohman, A. J. Morris and J. Engebrecht.** 1995. "Phospholipase D signaling is essential for meiosis." Proc Natl Acad Sci U S A **92:**12151-5.

43. **N. McLain and J. W. Dolan.** 1997. "Phospholipase D activity is required for dimorphic transition in *Candida albicans*." Microbiol **143:**3521-6.

44. **C. G. Saltarelli.** 1989. "*Candida albicans*. The Pathogenic Fungus. New York: Hemisphere Pub. Corp.

45. **F. C. Odds.** 1988. "*Candida* and Candidosis. 2nd ed. London; Philadelphia; Bailliere Tindall.

46. **T. M. Arendorf and D. M. Walker.** 1979. "Oral *Candida*l populations in health and disease." Br Dent J **147:**267-72.

47. **T. M. Arendorf and D. M. Walker.** 1980. "The prevalence and intra-oral distribution of *Candida albicans* in man." Arch Oral Biol **25:**1-10.

48. **J. R. Kohler and G. R. Fink.** 1996. "*Candida albicans* strains heterozygous and homozygous for mutations in mitogen-activated protein kinase signaling components have defects in hyphal development." Proc Natl Acad Sci U S A **93:**13223-8.

49. **K. L. Clark, P. J. Feldmann, D. Dignard, R. Larocque, A. J. Brown, M. G. Lee, D. Y. Thomas and M. Whiteway.** 1995. "Constitutive activation of the *Saccharomyces cerevisiae* mating response pathway by a MAP kinase kinase from *Candida albicans*." Mol Gen Genet **249:**609-21.

50. **E. Leberer, D. Harcus, I. D. Broadbent, K. L. Clark, D. Dignard, K. Ziegelbauer, A. Schmidt, N. A. Gow, A. J. Brown and D. Y. Thomas.** 1996. "Signal transduction through homologs of the Ste20p and Ste7p protein kinases can trigger hyphal formation in the pathogenic fungus *Candida albicans*." Proc Natl Acad Sci U S A **93:**13217-22.

51. **H. Liu, J. Kohler and G. R. Fink.** 1994. "Suppression of hyphal formation in *Candida albicans* by mutation of a STE12 homolog." Science **266:**1723-6.

52. **P. Singh, S. Ghosh and A. Datta.** 1997. "A novel MAP-kinase kinase from *Candida albicans*." Gene **190:**99-104.

53. **H. J. Lo, J. R. Kohler, B. DiDomenico, D. Loebenberg, A. Cacciapuoti and G. R. Fink.** 1997. "Nonfilamentous *C. albicans* mutants are avirulent." Cell **90:**939-49.

54. **E. Leberer, K. Ziegelbauer, A. Schmidt, D. Harcus, D. Dignard, J. Ash, L. Johnson and D. Y. Thomas.** 1997. "Virulence and hyphal formation of *Candida albicans* require the Ste20p- like protein kinase CaCla4p." Curr Biol **7:**539-46.

55. **B. R. Braun and A. D. Johnson.** 1997. "Control of filament formation in *Candida albicans* by the transcriptional repressor TUP1." Science **277:**105-9.

56. **C. Csank, C. Makris, S. Meloche, K. Schröppel, M. Röllinghoff, D. Dignard, D. Y. Thomas and M. Whiteway.** 1997. "Derepressed Hyphal Growth and Reduced Virulence in a VH1 Family-related Protein Phosphatase Mutant of the Human Pathogen *Candida albicans*." Mol Biol Cell **8:**2539-51.

57. **M. M. Billah, S. Eckel, T. J. Mullmann, R. W. Egan and M. I. Siegel.** 1989. "Phosphatidylcholine hydrolysis by phospholipase D determines phosphatidate and diglyceride levels in chemotactic peptide-stimulated human neutrophils. Involvement of phosphatidate phosphohydrolase in signal transduction." J Biol Chem **264:**17069-77.

58. **T. Kurz, I. Schneider, R. Tolg and G. Richardt.** 1999. "Alpha 1-adrenergic receptor-mediated increase in the mass of phosphatidic acid and 1,2-diacylglycerol in ischemic rat heart." Cardiovasc Res **42:**48-56.

59. **F. Naro, V. Donchenko, S. Minotti, L. Zolla, M. Molinaro and S. Adamo.** 1997. "Role of phospholipase C and D signalling pathways in vasopressin- dependent myogenic differentiation." J Cell Physiol **171:**34-42.

60. **K. K. Griendling, S. E. Rittenhouse, T. A. Brock, L. S. Ekstein, M. A. Gimbrone, Jr. and R. W. Alexander.** 1986. "Sustained diacylglycerol formation from inositol phospholipids in angiotensin II-stimulated vascular smooth muscle cells." J Biol Chem **261:**5901-6.

61. **C. J. Molloy, D. S. Taylor and H. Weber.** 1993. "Angiotensin II stimulation of rapid protein tyrosine phosphorylation and protein kinase activation in rat aortic smooth muscle cells." J Biol Chem **268:**7338-45.

62. **E. J. Freeman.** 2000. "The Ang II-induced growth of vascular smooth muscle cells involves a phospholipase D-mediated signaling mechanism." Arch Biochem Biophys **374:**363-70.

63. **J. E. Cutler.** 1991. "Putative virulence factors of *Candida albicans*." Annu Rev Microbiol **45:**187-218.

64. **K. Athenstaedt and G. Daum.** 1997. "Biosynthesis of phosphatidic acid in lipid particles and endoplasmic reticulum of *Saccharomyces cerevisiae*." J Bacteriol **179:**7611-6.

65. **T. Hashida-Okado, A. Ogawa, M. Endo, R. Yasumoto, K. Takesako and I. Kato.** 1996. "AUR1, a novel gene conferring aureobasidin resistance on *Saccharomyces cerevisiae*: a study of defective morphologies in Aur1p-depleted cells." Mol Gen Genet **251:**236-44.

66. **W. K. Surewicz and W. Leyko.** 1981. "Interaction of propranolol with model phospholipid membranes. Monolayer, spin label and fluorescent spectroscopy studies." Biochim Biophys Acta **643:**387-97.

67. **B. Hube, D. Hess, C. A. Baker, M. Schaller, W. Schafer and J. W. Dolan.** 2001. "The role and relevance of phospholipase D1 during growth and dimorphism of *Candida albicans*." Microbiology **147:**879-89.