Transport and Metabolism of Glycerophosphodiesters by Candida albicans

Andrew C Bishop

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TRANSPORT AND METABOLISM OF GLYCEROPHOSPHODIESTERS BY

CANDIDA ALBICANS

A Dissertation
Submitted to the Bayer School of Natural and Environmental Sciences

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In partial fulfillment of the requirements for
the degree of Doctor of Philosophy

By
Andrew C. Bishop

May 2013
TRANSPORT AND METABOLISM OF GLYCEROPHOSPHODIESTERS BY

CANDIDA ALBICANS

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Approved March 6th, 2013

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ABSTRACT

TRANSPORT AND METABOLISM OF GLYCEROPHOSPHODIESTERS BY

CANDIDA ALBICANS

By

Andrew C. Bishop

May 2013

Dissertation supervised by Jana Patton-Vogt, Ph.D.

Glycerophosphodiesters are products of phospholipase B-mediated hydrolysis of phospholipids. Their transport and metabolism is elaborated in the fungal pathogen, Candida albicans, as compared to the non-pathogenic Saccharomyces cerevisiae. C. albicans contains four ORFs (Ca GIT1-4) predicted to encode transporters for glycerophosphodiesters, compared to one (Sc GIT1) in S. cerevisiae. Here I have identified the gene products responsible for glycerophosphoinositol (GroPIns) and glycerophosphocholine (GroPCho) transport. C. albicans strain lacking ORF 19.34, which codes for CaGit1, is unable to transport intact GroPIns. Transport activity can be rescued by reintegration of one copy of CaGIT1 back into the genome. Similarly, a strain lacking CaGIT3 (ORF 19.1979) and CaGIT4 (ORF 19.1980) is unable to transport intact GroPCho into the cell. Reintegrating one copy of either CaGIT3 or CaGIT4 can rescue
GroPCho transport activity. Initial transport assays and kinetic analyses indicate that CaGit3 is responsible for the majority of GroPCho transport activity. In addition, I present evidence that CaGDE1 (ORF 19.3936) codes for an enzyme with glycerophosphodiesterase activity against GroPCho. Homozygous deletion of CaGDE1 results in a buildup of internal GroPCho, which is restored to wild type accumulation by reintegration of one copy of CaGDE1 into the genome. The transcriptional regulator, CaPho4, was shown to positively regulate the expression of CaGIT1, CaGIT3, CaGIT4, and CaGDE1. Finally, glycerophosphodiester transport and metabolism was active under physiological relevant conditions that C. albicans may experience in the human host.
DEDICATION

My dissertation is dedicated to the late Dr. Mitchell E. Johnson. Mitch was my undergraduate mentor and allowed me to gain research experience in his analytical chemistry lab. Mitch was the first person to have a major impact on my scientific career. His bluntness and advice changed my views on life and initiated the path that I am continuing today. His love for the Three Stooges, Bob Dylan, and making extremely witty comments will stay with me for many years to come.
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TABLE OF CONTENTS

Page

Abstract .................................................................................................................................................... iv

Dedication .................................................................................................................................................... vi

Acknowledgement ......................................................................................................................................... vii

Table of Contents ....................................................................................................................................... ix

List of Tables ................................................................................................................................................ xiv

List of Figures ............................................................................................................................................ xv

Chapter 1: Introduction ............................................................................................................................... 1

1.1 The Candida genus and Candida albicans ......................................................................................... 1

1.2 Morphological Forms ............................................................................................................................ 2

1.3 Properties that Influence Virulence of C. albicans ............................................................................. 3

1.4 Phospholipids and Phospholipases ....................................................................................................... 4

1.5 Glycerophosphodiester Transport and Catabolism ............................................................................ 9

1.6 Objectives ............................................................................................................................................... 12

Chapter 2: Robust Utilization of Phospholipase-Generated Metabolites,
Glycerophosphodiesters by Candida albicans: Role of the CaGit1 Permease ................................. 13

Chapter 2 Attributions .................................................................................................................................. 13

2.1 Introduction ........................................................................................................................................... 13

2.2 Materials and Methods .......................................................................................................................... 15

2.2.1 Strains and media ............................................................................................................................... 15
2.2.2 Construction of homozygous insertion mutant ................................................................. 16
2.2.3 Construction of plasmid pDDB78GIT1 ............................................................................. 17
2.2.4 Insertional complementation of git1::UAU1/git1::URA3 ...................................................... 18
2.2.5 Screening of the transcriptional regulator deletion set ....................................................... 18
2.2.6 [3H]inositol-GroPIns and [3H]choline-GroPCho uptake assays ............................................. 18
2.2.7 LC-MS analysis of GroPIns uptake ....................................................................................... 20
2.2.8 Analysis of internal [3H]GroPIns metabolites ................................................................. 21
2.2.9 RNA Extraction and qRT-PCR gene expression analysis .................................................. 22
2.3 Results ................................................................................................................................. 23
  2.3.1 Potential ScGIT1 homologs found in C. albicans genome .................................................. 23
  2.3.2 Role of CaGit1 in the utilization of GroPIns and GroPCho as phosphate sources ...... 23
  2.3.3 Uptake of label from [3H]inositol-GroPIns requires CaGit1 and is regulated by
  phosphate availability .................................................................................................................. 26
  2.3.4 Glycerophosphodiester utilization at physiological pH .................................................. 28
  2.3.5 GroPIns kinetics, specificity, and proton dependence ....................................................... 32
  2.3.6 Depletion of GroPIns from the medium corresponds to the presence of CaGit1 and
  intact GroPIns detected intracellularly .................................................................................. 36
  2.2.7 CaPHO4 as required for GroPIns transport, utilization, and expression .................... 38
  2.4 Discussion ......................................................................................................................... 42
  2.5 Acknowledgments ............................................................................................................. 46

Chapter 3: Transport and Metabolism of Glycerophosphocholine by Candida albicans: Role of CaGit3, CaGit4, and CaGde1 .................................................................................. 49
Chapter 3 Attributions ........................................................................................................ 47
3.1 Introduction .................................................................................................................. 47
3.2 Materials and Methods .............................................................................................. 49
  3.2.1 Strains and media ..................................................................................................... 49
  3.2.2 Construction of homozygous mutants .................................................................... 50
  3.2.3 Construction of complementation plasmids .......................................................... 53
  3.2.4 Insertional complementation of deletion mutants .................................................... 53
  3.2.5 Short term [3H]choline-GroPCho transport assays ................................................. 54
  3.2.6 Preparation of external and internal cellular fractions and analysis of [3H] Choline-
      containing metabolites by ion-exchange chromatography ........................................... 55
  3.2.7 RNA extraction and gene expression analysis by quantitative Real Time-PCR ........ 56
  3.2.8 [3H]GroPCho uptake in serum and XTT cell proliferation analysis ...................... 57
3.3 Results ......................................................................................................................... 59
  3.3.1 Novel construction of a CaGIT2-4 mutant ............................................................. 59
  3.3.2 Role of CaGIT3 and CaGIT4 in the utilization of GroPCho as a phosphate source .... 61
  3.3.3 CaGit3 is the major GroPCho permease and is regulated by phosphate
      availability ....................................................................................................................... 61
  3.3.4 Loss of CaGDE1 alters GroPCho catabolism ......................................................... 64
  3.3.5 CaGit3 and CaGit4 independent utilization of GroPCho ......................................... 68
  3.3.6 CaPho4 Regulates the Expression of CaGIT3, CaGIT4, and CaGDE1 .................... 73
  3.3.7 Kinetics of GroPCho transport by CaGit3 and CaGit4 ........................................... 73
  3.3.8 GroPCho utilization under serum growth conditions ............................................. 75
3.4 Discussion ..................................................................................................................... 77
3.5 Acknowledgements .................................................................................................................. 82

Chapter 4: Conclusions .................................................................................................................. 83

4.1 Comparison of glycerophosphodiester metabolism in C. albicans and S. cerevisiae .................. 83
4.2 Phosphate Response in C. albicans and S. cerevisiae ................................................................. 85
  4.2.1 S. cerevisiae response ........................................................................................................... 85
  4.2.2 C. albicans response ............................................................................................................ 87
4.3 Putative glycerophosphodiesters across fungal species ............................................................ 88
4.4 Open Questions ......................................................................................................................... 93

References ........................................................................................................................................ 97

Appendix ......................................................................................................................................... 112
A.1 Putative glycerophosphodiester permeases of non-Candida fungal species ....................... 112
A.2 Phosphate starvation of C. albicans induces invasive growth ................................................. 114
A.3 GroPCho competition assay and pH dependence assay ......................................................... 115
A.5 Altered growth of C. albicans mutant zcf30Δ/Δ on GroPCho as sole phosphate source 117
A.6 GroPCho and GroPIns are able to support C. albicans biofilm growth .............................. 118
A.8. Phosphatidylcholine supports growth of C. albicans as a phosphate source and growth is partially dependent on CaGIT2-4 .................................................................................... 121
A.9 Loss of CaGDE1 alters GroPIns catabolism for *C. albicans* .................................................. 125
## LIST OF TABLES

<table>
<thead>
<tr>
<th>Table</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Table 2-1</td>
<td>Strain Table Chapter 2</td>
<td>16</td>
</tr>
<tr>
<td>Table 3-1</td>
<td>Strain Table Chapter 3</td>
<td>51</td>
</tr>
<tr>
<td>Table 3-2</td>
<td>Deletion Primer Sequences</td>
<td>52</td>
</tr>
<tr>
<td>Table 3-3</td>
<td>Complementation Primer Sequence</td>
<td>52</td>
</tr>
<tr>
<td>Table 3-4</td>
<td>Quantitative RT-PCR Primer Sequences</td>
<td>52</td>
</tr>
<tr>
<td>Table 3-5</td>
<td>Expression of <em>CaGIT3</em>, <em>CaGIT4</em>, and <em>CaGDE1</em> is regulated by phosphate availability</td>
<td>63</td>
</tr>
<tr>
<td>Table 4-1</td>
<td>Putative glycerophosphodiester permeases of common fungal species</td>
<td>90</td>
</tr>
</tbody>
</table>
# LIST OF FIGURES

## Chapter 1

| Figure 1-1. Potential cellular membrane phospholipids | 5 |
| Figure 1-2. Sites of phospholipid hydrolysis by four distinct types of Phospholipases | 6 |
| Figure 1-3. Phospholipase B mediated hydrolysis of a phospholipid into a glycerophosphodiester and free fatty acids | 6 |
| Figure 1-4. Phospholipid deacylation pathway in *S. cerevisiae* | 10 |

## Chapter 2

| Figure 2-1. *C. albicans* utilizes GroPIns and GroPCho as sources of phosphate | 25 |
| Figure 2-2. [*3H*]GroPIns uptake requires CaGit1 and is regulated by phosphate availability | 27 |
| Figure 2-3. *C. albicans* and *S. cerevisiae* vary in their ability to grow on glycerophosphodiesters at physiological pH | 29 |
| Figure 2-4. GroPIns and GroPCho utilization by *C. albicans* at pH 6.5 versus pH 7.5 | 30 |
| Figure 2-5. Kinetics of GroPIns transport | 33 |
| Figure 2-6. Specificity and proton dependence of GroPIns transport | 35 |
| Figure 2-7. Depletion of GroPIns from the medium as a function of growth | 37 |
| Figure 2-8. Effect of *PHO4* deletion on growth and transport activity | 40 |

## Chapter 3

| Figure 3-1. Both *CaGIT3* and *CaGIT4* can rescue growth of a *git2-4Δ/Δ* mutant utilizing GroPCho as sole phosphate source | 60 |
Figure 3-2. GroPCho transport is regulated by phosphate availability. CaGit3 displays a
greater initial GroPCho uptake than CaGit4........................................................................62
Figure 3-3. Deletion of CaGDE1 results in altered GroPCho catabolism .........................66
Figure 3-4. Internalization of intact $[^3]$HGroPCho does not occur in the absence of
CaGIT3 and CaGIT4, though some $[^3]$HGroPCho is hydrolyzed extracellularly ...............69
Figure 3-5. CaPho4 is partially responsible for regulating CaGIT3, CaGIT4 and CaGDE1
under low phosphate conditions........................................................................................72
Figure 3-6. Kinetics of GroPCho transport by CaGit3 and CaGit4 ....................................74
Figure 3-7. GroPCho is transported and metabolized in the presence of serum.................76

Chapter 4

Figure 4-1 Summary of glycerophosphodiester transport and metabolism in C. albicans ...94
Chapter 1

Introduction

1.1 The *Candida* genus and *Candida albicans*

The *Candida* genus contains over 200 different species and a small percentage of these species are known to pose a risk to humans. The non-human pathogen related species have been isolated from various environments and include: *C. materiae*, *C. amazonensis*, *C. cellulosicola*, *C. saraburiensis*, *C. prachuapensis* and *C. queiroziae* from decaying plant matter (1-5); *C. northwykensis*, *C. easanensis*, *C. pattaniensis*, *C. nakhonratchasimensis* and *C. ficus* from insect guts or excrements (6-8); and *C. thasaenesis* and *C. digboiensis* from hazardous wastes (9,10). Although these species pose no known threat to humans, they display the versatility of the *Candida* genus by their ability to colonize and survive in a variety of environments.

*C. albicans* is a human pathogen that resides in the gastrointestinal tract, oral cavity, and vaginal cavity of healthy individuals where it serves as a member of the natural microbial flora (11-13). Although other *Candida* species (*C. glabrata*, *C. parapsilosis*, *C. tropicalis*, and *C. krusei*) cause infections in a human host, *C. albicans*-related infections are believed to be the most common and have the most impact on human health (14). Of all acquired bloodstream infections, including those arising from bacteria, *Candida* infections are the 4th most common, costing roughly $40,000 extra per hospital patient that acquires candidemia (15). *C. albicans* is more likely to cause a major infection under conditions where the host loses its ability to combat the pathogen. Therefore, infection is most common in ICU patients and
individuals with compromised immune systems due to diseases like HIV, those receiving cancer treatments, or those on an immunosuppressant therapy (14,15). Due in part to its medical relevance, much research has focused on understanding *C. albicans* biology and pathogenicity.

### 1.2 Morphological Forms

*C. albicans* can exist in three distinct morphological forms (16): yeast, hyphal and pseudohyphal. These forms differentiate *C. albicans* from its distant relative, *Saccharomyces cerevisiae* (16,17), which mainly exists in the yeast form, but can be induced to the pseudohyphal form. The two species are believed to have diverged from each other more than 800 million years ago (18). *C. albicans* cells in the yeast morphology can be described as growing similarly to that of *S. cerevisiae*, where a daughter cell buds from a mother cell, producing two separate cells. In hyphal growth, the cells do not bud, but produce long filaments that protrude from the mother cell with no constrictions (16). The intermediate psuedohyphal form has both yeast and hyphal characteristics, but is never consistently in either form. It produces larger buds than the yeast form but with constricted filaments that do not completely detach from each other (16). *C. albicans* can readily switch between the three morphological forms depending on environmental conditions (16) and this ability to switching is essential for the organism’s virulence (17).

*C. albicans* has been studied in two well defined types of growth, planktonic and biofilm growth. Planktonic consists of free floating cells, grown in well aerated cultures. Biofilm growth involves adherence to a surface, creating a complex community of cells
and extracellular matrices (19). All three morphological forms of *C. albicans* are present and contribute to the formation of biofilms (19). To initiate biofilm formation, free floating yeast cells adhere to host surface proteins or to implanted devices (i.e. catheters, pace makers, prosthetics, etc.) (19,20). The cells then go through morphological changes, initiating hyphal formations and the production of an extracellular matrix as the biofilm grows and matures (20). Once the biofilm has fully matured, it will begin to disseminate yeast form cells that break away from the biofilm and travel to other locations in the host (19,20). The progressive nature of biofilm formation by *C. albicans* relies on certain adaptations that are driven by virulence factors. These factors are gene products that aid the organism's ability to survive in environments uninhabitable by non-pathogenic organisms.

### 1.3 Properties that Influence Virulence of *C. albicans*

Many *C. albicans* virulence factors are involved in the processes of phenotypic switching, morphological dimorphism, cell adhesion and the production of hydrolytic enzymes (17). One well studied example of phenotypic switching is a system involving the transition of cell growth from white round colonies consisting of normal yeast cells to opaque colonies that consist of irregular and elongated cells (21,22). This white to opaque transition can influence the ability of *C. albicans* to invade host tissue (22-24). Various environmental cues can influence phenotypic switching including oxidative stress, starvation, or growth in the presence of serum or N-Acetylglucosamine (23).

A morphological change is the ability to readily switch between yeast, hyphal and pseudohyphal growth (16,17). These changes in morphology can occur in response to
alterations in pH, temperature, CO₂ concentrations or other environmental cues (17). Morphological dimorphism can impact virulence in two different ways, \textit{i)} through the formation of pseudohyphae and hyphae, which are required for tissue invasion, and \textit{ii)} through the ability to disseminate yeast cells that can easily travel through the host's blood stream and within infected tissues to create new sites of infection (17).

Adherence, the ability of cells to bind to a surface, is the first step in the formation of biofilms (20) and is also required for tissue invasion (17). Once cells adhere to a surface they must acquire nutrients that may not be readily available from their current environment. Hydrolytic enzymes, which are known virulence factors, aid in this process by releasing nutrients that can be used by the cell (17). These enzymes include proteases, lipases, and phospholipases, which serve to attack proteins, lipids, and phospholipids (17). Many of these enzymes are active during infection (25), contributing to the pathogenicity of \textit{C. albicans}.

\textbf{1.4 Phospholipids and Phospholipases}

Phospholipases are universal, having been found in bacteria, plants, fungi, and mammals (26-28). Phospholipases catabolize phospholipids through the hydrolysis of specific bonds of phospholipids. Phospholipids are composed of four primary components: a polar head group, a phosphate group, a glycerol backbone, and two fatty acyl esters. In Figure 1-1, a schematic of a phospholipid is represented with the possible polar head groups. Various combinations of different phospholipids contributes to the heterogeneous character of cellular membranes and the functionality of membrane
Figure 1-1. Potential cellular membrane phospholipids. A generic structure of a phospholipid containing a phosphate group and two fatty acyl esters attached to a glycerol backbone. The X on the phosphate group can represent any of the potential polar head groups pictured to create the listed phospholipids. Modified with permission from Tao Sun.
Figure 1-2. Sites of phospholipid hydrolysis by four distinct types of phospholipases

Phospholipase (PL) A₁, A₂, B, C, and D hydrolyze different location on a phospholipid producing various phospholipid metabolites. \( R_1 \) and \( R_2 \) represent any length fatty acyl chain. \( R_3 \) represents any polar head group (Choline, Inositol, Ethanolamine, Serine, Glycerol).

Figure 1-3. Phospholipase B mediated hydrolysis of a phospholipid into a glycerophosphodiester and free fatty acids. \( R_1 \) and \( R_2 \) represent any length for the fatty acid tails. \( R_3 \) represents any potential polar head group.
proteins. The bond that the phospholipase attacks determines its categorization as either A, B, C, or D (29) (FIG1-2). Phospholipase A (PLA) enzymes are made up of two groups, PLA₁, which attacks the sn-1 acyl esters, and PLA₂, which attacks the sn-2 acyl esters on the glycerol backbone of phospholipids (29). If only one of these enzymes acts on a phospholipid, a lysophospholipid containing one acyl ester is produced. If both PLA₁ and PLA₂ act on a phospholipid, a glycerophosphodiester and free fatty acids are produced. Phospholipase B (PLB) enzymes attack both the sn-1 and sn-2 positions on the glycerol backbone producing glycerophosphodiesters and free fatty acids (26,29) (FIG1-3). The reaction in Figure 1-3 is a common occurrence in S. cerevisiae, where it can serve as the first step in the recycling of phospholipid metabolites of cellular membranes (30). From the phospholipids listed in Figure 1-1, this reaction can produce the following: glycerophosphoglycerol (GroPG), glycerol-3-phosphate (GroP), glycerophosphoethanolamine (GroPE), glycerophosphoserine (GroPS) and the two glycerophospdiesters relevant to this study, glycerophosphoinositol (GroPIns) and glycerophosphocholine (GroPCho). Some PLBs also have lysophospholipase transacylase activity to produce phospholipids from lysophospholipids (31). Phospholipase C (PLC) enzymes attack phosphodiester bonds located between the phosphate group and the glycerol backbone producing 1,2-diacylglycerol and a phosphate monoesters, the identity of which depends on the particular glycerophospholipids being attacked (26,32). Phospholipase D (PLD) enzymes attack the ester bond between the phosphate group and the polar head group of the glycerophospholipid, producing phosphatidic acid and the respective alcohol (26,29).
All four types of phospholipase activities are present in *C. albicans*, but only select PLBs and the sole PLD have been reported to be tied to virulence (29,31-34). The *C. albicans* genome contains multiple ORFs (*CaPLB1*-5) that are predicted to encode PLB enzymes based on having a catalytic domain that is common for phospholipases of the B type as indicated on the *Candida* Genome Database (CGD, www.candidagenome.org) using InterPro (IPR002642) (35). Two of these ORFs, *CaPLB1* (ORF19.689) and *CaPLB5* (ORF19.5102) have been implicated in the virulence of *C. albicans* (31,34). Disruption of *CaPLB1* resulted in attenuated virulence in an intravenous mouse model, and upon reintegration of one copy of *CaPLB1*, virulence was restored (34). Additionally, *CaPLB1* has been shown to be expressed under elevated temperature, pH and in the presence of both serum and phospholipids (36), conditions *C. albicans* may experience in a human host. Disruption of *CaPLB5* also attenuated virulence and reduced organ burden of the kidneys, liver, and brain in a mouse model (31). Reconstitution of one copy of *CaPLB5* restored virulence (31). *CaPLB2* (ORF19.690) is expressed during infection, but its importance during the infection process is unknown (25,37). *CaPLB3* (ORF19.6594) is predicted to be a GPI anchored protein (38,39) and has been detected extracellularly (40). *CaPLB3* expression is induced by treatment of cells with the antifungal drug fluconazole (40), but the gene has not been linked to virulence. In the current assembly of ORFs on CGD, there have been some changes to the putative *CaPLB4*. Originally ORF 19.1442 and ORF19.1443 were both recognized as putative PLB ORFs, but in the new assembly the two ORFs have been merged into *CaPLB4.5* (ORF 19.1442). A role of *CaPLB4.5* has yet to be determined, but it has been described as a putative GPI anchored protein (39). Several laboratories have
studied the PLBs of *C. albicans*, and in many cases, a link to virulence has been found. In contrast, these studies have not addressed the fate of glycerophosphodiesters, the products of PLB activity (FIG1-3).

**1.5 Glycerophosphodiester Transport and Catabolism**

Our current understanding of glycerophosphodiester transport and catabolism is based on work from bacteria, plants, mammalian cells and most importantly, *S. cerevisiae*. With *S. cerevisiae* and *C. albicans* diverged from each other over 800 million years ago (18), major difference exist between the two organisms. However, using the well-characterized *S. cerevisiae* as a foundation for studies in *C. albicans* is common. Although there are limitations to this process, it provides a starting point for the characterization of genes in *C. albicans*.

A schematic representation of phospholipid deacylation and glycerophosphodiester recycling in *S. cerevisiae* is detailed in Figure 1-4. Phospholipids (Phosphatidylinositol (PI) and Phosphatidylcholine (PC)) can be hydrolyzed at the cell membrane by PLBs (reaction in FIG 1-3) to release free fatty acids and glycerophosphodiesters (GroPIns and GroPCho). ScPlb1 has been shown to be specific for PC, while ScPlb3 is specific for PI (30). GroPIns and GroPCho are released into the periplasmic space, and subsequently transported into the cell by the permease ScGit1. ScGit1 is the only glycerophosphodiester permease in *S. cerevisiae*, and has a higher affinity for GroPIns than GroPCho (30). Sources of GroPIns and GroPCho are not limited to the plasma membrane phospholipids, but can also be obtained from the environment.
Figure 1-4. Phospholipid deacylation pathway in *S. cerevisiae*. Details are provided in the text. In this schematic, phospholipids, phosphatidylinositol (PI) and phosphatidylcholine (PC) are represented in blue. Proteins: Plb1-3, Git1, and Gde1 are represented in green. Phospholipid metabolites: GroPIns, GroPCho, free fatty acids, GroP and choline are represented in orange.
Once GroPCho and GroPIIns are transported into the cell, the protein Gde1, the only characterized glycerophosphodiesterase in *S. cerevisiae*, hydrolyzes GroPCho into GroP and free choline (30). Currently, the enzyme responsible for the hydrolysis of GroPIIns is unknown, but like choline, inositol can be reutilized for lipid biosynthesis to create new phospholipids (30).

In mammalian cells, a functional homolog to ScGit1 exists, Glut2, which is responsible for transporting GroPIIns (41). Expression of Glut2 was found to be tissue specific, localized to the liver, kidney and small intestine (42). In contrast to the eukaryotic transporters, *E. coli* deploys a multi-protein complex consisting of 5 proteins (UgpB, A, E, C, and Q) that work together to transport glycerophosphodiesters into the cell (43-45). Interestingly, it is believed that the glycerophosphodiesters are hydrolyzed by a glycerophosphodiesterase (UgpQ) as they are being transported into the cell (44). In the eukaryotic systems, glycerophosphodiesterase activity is independent of transport.

In general, a glycerophosphodiesterase hydrolyzes glycerophosphodiesters into GroP and their respective alcohols (30,46). Proteins thought to contain glycerophosphodiesterase domains can be identified using InterPro (IPR004129) (35), where they are categorized by a conserved catalytic domain. Glycerophosphodiesterase activity has been tied to a number different biological functions including the production of skeletal muscle (47), osmotic regulation in the kidneys (48) and phosphate utilization in *E. coli* (43), *Arabidopsis thaliana* (49), White Lupin (50) and *S. cerevisiae* (30).

Proteins with glycerophosphodiesterase motifs are assumed to hydrolyze only glycerophosphodiesters, but recently a mammalian glycerophosphodiesterase, (GDE2), has been implicated in neurogenesis through the cleavage of GPI anchors (51). Therefore
the enzymatic substrates may go beyond the hydrolysis of glycerophosphodiester bonds, at least for mammals.

In the preceding section (1.5), I have provided a general overview of glycerophosphodiester transport and catabolism using literature that predates the work performed for my dissertation project. More detailed information can be found in the introduction to each of my publications that constitute Chapters 2 and 3.

1.6 Objective

The overall goal of this work was to characterize the transport and metabolism of glycerophosphodiesters by the pathogen *C. albicans*. Several compelling facts led to my interest in pursuing this project. Firstly, *C. albicans* has four uncharacterized ORFs (*CaGIT1-4*) predicted to be involved in glycerophosphodiester transport, compared to one in the non-pathogenic *S. cerevisiae*. Secondly, GroPCho and GroPIns are found in tissues and fluids throughout the human body, where they can potentially be used by *C. albicans* as nutrient sources during infection. Lastly, PLBs, which produce glycerophosphodiesters, have been linked to virulence of *C. albicans*. The specific objectives of my work were *i*) to examine glycerophosphodiester transport and metabolism in a variety of conditions that *C. albicans* may encounter in the human host, *ii*) to identify and characterize gene product(s) responsible for the facilitating the transport of intact GroPCho and GroPIns into the cell and *iii*) to identify and characterize a gene product responsible for the internal catabolism of GroPCho.
Chapter 2

Robust Utilization of Phospholipase-Generated Metabolites, Glycerophosphodiester by Candida albicans: Role of the CaGit1 Permease

Andrew C. Bishop, Tao Sun, Mitchell E. Johnson, Vincent M. Bruno, and Jana Patton-Vogt

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Chapter 2 Attributions
I did all of the work in this chapter expect those noted here:

Claudia Almaguer, a technician in Dr. Jana Patton-Vogt’s laboratory, constructed strains git1-/ +pDDB78 (JPV526) and git1-/ +pDDB78GIT1 (JPV512).

Tao Sun, a graduate student in Dr. Mitch Johnson’s laboratory, performed the LC-MS analysis of extracellular levels of glycerophosphoinositol.

2.1 Introduction

Glycerophosphodiester result from the complete deacylation of glycerophospholipids via phospholipase-mediated hydrolysis. Most fungal cells, including C. albicans and S. cerevisiae, contain multiple phospholipase B (PLB)-encoding genes (29) that act on both fatty acyl ester groups to produce glycerophosphodiester, such as glycerophosphocholine (GroPCho) and glycerophosphoinositol (GroPIns) (30). The potential role of C. albicans PLBs as virulence factors has been explored by others (34,36,52-54). For example, C. albicans
strains exhibiting elevated PLB activity have been shown to be associated with increased virulence in mouse models of disseminated candidiasis (55). Disruption of PLB1 was subsequently shown to result in attenuated virulence in a mouse model (53), and reintroduction of a functional PLB1 into this mutant to restore virulence to levels observed for the parental strain (34). Also, inactivation of another PLB gene, PLB5, has been shown to result in attenuated virulence (31). Notably, the fate and potential function of the products of PLB turnover, the glycerophosphodiesters, have not been addressed.

Although glycerophosphodiesters are produced via C. albicans PLB activity, the organism, being an opportunistic commensal, is also likely exposed to sources of glycerophosphodiesters that are present in the host as a result of host phospholipase activity. Indeed, the literature indicates that glycerophosphodiesters, especially GroPIns and GroPCho, are present in serum, as well as other mammalian fluids and tissues. For example, GroPCho is an abundant organic osmolyte found in the renal medulla of the kidney (48,56) and both GroPIns and GroPCho are found in other parts of the urinary tract, including renal proximal tubules (57). GroPCho has also been found in organs of the gastrointestinal tract, including the small and large intestines (58-60). Serum, cerebral spinal fluid, and brain tissue contain GroPCho, in addition to lysophosphatidylcholine and phosphatidylcholine that can be converted to GroPCho via phospholipases B (61-63). GroPIns has also been noted in other cells and tissues, including brain, kidney, and others (64).

In S. cerevisiae, extracellular GroPIns and, with less affinity, GroPCho, are transported into the cell via the ScGit1 transporter. Once inside the cell, they are metabolized and used as sources of nutrients such as phosphate, inositol, and choline (65-
C. albicans contains four open reading frames (ORFs) (CaGIT1-4) predicted to encode transporters with a high degree of similarity to S. cerevisiae GIT1 (ScGIT1) product (68). ScGit1 and CaGit1-4 are classified as members of the major facilitator superfamily (MFS) (69). The MFS is present in all kingdoms of life. Most MFS proteins are between 400-600 amino acids in length and contain either 12 or 14 membrane-spanning segments. MFS proteins facilitate symport, antiport, or uniport of various substrates. Those substrates include nutrients, drugs, nucleotides, nucleosides, and other metabolites (70). Using the Transport Commission (TC) system (71), 95 potential MFS proteins clustering into 17 families have been predicted for C. albicans (69), but only a handful have been characterized. Like ScGit1, CaGit1-4 are predicted to belong to the phosphate: H+ symporter (PHS) family (TC no. 2.A.1.9) of the MFS (69). In total, the C. albicans genome is predicted to have 5 PHS family members: CaGit1-4, and the homolog of the S. cerevisiae high affinity phosphate transporter, Pho84. No member of this family has been characterized in C. albicans.

Here, we investigate the ability of C. albicans to transport GroPIns and GroPCho into the cell and to utilize those compounds as sources of phosphate. In addition, we identify CaGIT1 (orf19.34) as a GroPIns permease.

2.2 Materials and Methods

2.2.1 Strains and media: Strains were grown aerobically at either 30°C or 37°C with shaking. Turbidity was monitored by measurement of optical density at 600 nm (OD_{600}) on a Biomate 3 Thermo Spectronic spectrophotometer. Synthetic complete (yeast nitrogen base [YNB]) medium was prepared as described previously (72). High P, and
low P$_i$ media were made by replacing the KH$_2$PO$_4$ (1 gm/liter) in synthetic complete medium with KCl (1 gm/liter) and adding KH$_2$PO$_4$ to 10 mM (high P$_i$) or 0.2 mM (low P$_i$). All media for *C. albicans* was supplemented with 80 µg/ml of uridine. For some experiments, media lacking KH$_2$PO$_4$ (no-P$_i$ medium) contained GroPIns (Sigma #G1891), GroPCho (Sigma #G5291) or glycerol-3-phosphate (GroP) (Sigma #G7886) at the indicated concentrations. Where indicated, YNB was buffered to pH 7.5 using 150 mM HEPES. Strains were maintained on yeast extract-peptone-dextrose (YEPD) media consisting of 20 g glucose, 10 g yeast extract, and 20 g bacto peptone per liter. The genotypes of *C. albicans* strains are indicated in Table 2-1. The *S. cerevisiae* strain, BY4741, is MATa his3A1 leu2A0 met15A0 ura3A0.

**Table 2-1 Strain Table Chapter 2**

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<th>Strain</th>
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**2.2.2 Construction of homozygous insertion mutant.** A clone of the *GIT1* gene containing a Tn7-UAU transposon insertion produced via UAU1 methodology (73,74) was obtained from Aaron Mitchell, Carnegie Mellon University. Plasmid CAGFN83
(clone ID#29331) bears the GIT1 gene containing the UAU1 insertion at bp 536. CAGFN83 was digested with NotI to release the Tn7-UAU1 mutagenized GIT1 gene and transformed into strain BWP17 (ura3Δ::λimm434/ura3Δ::λimm434his1::hisG/his1::his arg4::hisG/arg4::hisG; (75). Several Arg+ transformants were isolated and subjected to Arg+ Ura+ selection as described (73,75). Genomic DNA was extracted from Arg+ Ura+ transformants and PCR was performed using forward primers Arg4det (5’-GGAATTGATCAATTATCTTTTGAAC-3’) (76), and GITF2 (5’-TTCGGACAAGTGATTATTGGATTAACCGCT-3’), and reverse primer GITR3 (5’-TATAACTGACAAGCAGAAGAAAGGGTTTA-3’). Heterozygous insertion mutants displayed two bands upon PCR: a 1.3 Kb fragment corresponding to GIT1 allele amplified with primers GITF2 and GITR3, and a 2.7 Kb fragment corresponding to the presence of the git1::Tn7-UAU1 allele amplified with primers Arg4det and GITR3. Homozygous insertion mutants, git1::UAU1/git1::URA3 (JPV484), displayed only the 2.7 kb band. The git1::UAU1/git1::URA3 mutant is also referred to here as git1-/-.

2.2.3 Construction of plasmid pDDB78GIT1. GIT1 was amplified from genomic DNA using a forward primer incorporating a NotI restriction site (bold) located 900 bp upstream of the start site (5’-AATGTTAAATGCGGCCGCCTGTACACGGCTTTATCGCA CGGGATATGAA-3’) and a reverse primer incorporating an EcoRI restriction site (bold) located 540 bp downstream of the stop site (5’-AATGTTAAAGGGGAATTCTCGAAATTTGGTTATGTAGGGTTTCAGTAAAAA-3’).
The resulting PCR product and plasmid pDDB78 (77) were digested with NotI and EcoRI and ligated together to obtain plasmid pDDB78GIT1.

**2.2.4 Insertional complementation of git1::UAU1/git1::URA3.** Plasmid pDDB78GIT1 was linearized by cutting within the HIS1 gene with NruI and the resulting product was transformed into git1::UAU1/git1::URA3 (JPV484) to produce JPV512, referred to here as git1::UAU1/git1::URA3 + pDDB78GIT1 (77). Several His+ transformants were selected and tested for complementation of the mutant phenotype. Empty plasmid pDDB78 was also linearized and transformed into git1::UAU1/git1::URA3 (JPV484) to produce JPV526, referred to here as git1::UAU1/git1::URA3 + pDDB78.

**2.2.5 Screening of the transcriptional regulator deletion set:** The deletion library (78) was purchased from the Fungal Genetics Stock Center (FGSC). The deletion strains were constructed in strain SN152 (arg4Δ/arg4Δ, leu2Δ/leu2Δ, his1Δ/his1Δ, URA3Δ/ura3Δ, IRO1Δ/iro1Δ). The auxotrophic markers HIS1 and LEU2 were used to delete the genes, as described (78). A wild type control strain in which HIS1 and LEU2 were reintroduced into the parent strain (WT-TF) was included in the set. The deletions strains were screened by growing in liquid YNB medium containing either 200 µM GroPIns or 200 µM KH₂PO₄ as the source of phosphate. Growth after 48 hours at 37°C was monitored.

**2.2.6 [³H]inositol-GroPIns and [³H]choline-GroPCho uptake assays:** Label uptake assays (also referred to as transport assays) were performed essentially as described previously (65). For the standard assay, aliquots of the cultures were harvested and
washed with sterile water. Each cell pellet was suspended in 100 mM sodium citrate buffer, pH 5.0 to \( OD_{600} = 5 \). Following 10 min incubation at 30°C with agitation, the reaction was started by the addition of 50 \( \mu l \) of 25 \( \mu M \) \[^3\text{H}\]GroPIns or 50 \( \mu l \) of 1 mM \[^3\text{H}\]GroPCho to 200 \( \mu l \) of the cell suspension to produce final concentrations of 5 \( \mu M \) \[^3\text{H}\]GroPIns and 200 \( \mu M \) \[^3\text{H}\]GroPCho. Following 10 min (for GroPIns transport) or 2 min (for GroPCho transport) incubation at 30°C, the reaction was stopped by the addition of 10 ml ice-cold H\(_2\)O. The samples were filtered through glass fiber (GF/C) filters, and the filters washed with ice-cold H\(_2\)O. Radioactivity on the filter was determined by liquid scintillation counting. Data are presented as pmol/min/optical density unit at OD\(_{600}\) nm (ODU). For \textit{S. cerevisiae}, transport assays were performed as described above for the standard \textit{C. albicans} assays, with the exception that for both \[^3\text{H}\]GroPIns and \[^3\text{H}\]GroPCho a final concentration of 5 \( \mu M \) was used in 10 min assays. Tritium labeled GroPIns (\[^3\text{H}\]inositol-GroPIns) and tritium labeled GroPCho (\[^3\text{H}\]choline-GroPCho) were produced through the deacylation of phosphatidyl-myoinositol-\[^2\text{H}\]inositol (American Radiolabeled Chemicals) and phosphatidyl-methyl-\[^3\text{H}\]choline (American Radiolabeled Chemicals) as described previously (79).

For the GroPIns transport competition assays, conditions were identical to those described above except that 25 mM HEPES buffer (pH 5) was used instead of citrate buffer and the transport assay mixtures included 1 mM GroPIns, 1 mM inositol, 1 mM KH\(_2\)PO\(_4\), or 1 mM GroP, as indicated. The pH of the assays did not change during the course of the experiment. For the protonophore experiment, 100 mM Carbonyl cyanide 3-chlorophenylhydrazone (CCCP) (Alfa Aesar #L06932) stocks in ethanol were added to 25 mM HEPES buffer for a final concentration of 50 \( \mu M \). For the experiments involving
alterations in transport assay pH, 25 mM HEPES buffer was adjusted to pH 6.5, pH 7.5 or pH 8.5, as indicated.

For the determination of GroPIns transport kinetics (FIG 2-5), assays were performed in 100 mM Citrate buffer, pH 5. Transport was started with the addition of 50 μl of [3H]GroPIns ranging in concentrations from 5 μM to 500 μM of GroPIns, as indicated. Transport activity was linear with time at each substrate concentration used. Assays were stopped after 5 min. Saturation kinetics data for GroPIns was analyzed using the Levenberg-Marquardt algorithm for nonlinear regression in Graphpad Prism (version 4.0) to determine the apparent $K_m$ and $V_{max}$. Values were determined by least squares fitting of the data to the Michaelis-Menten equation $V=V_{max}[S](K_m+[S])^{-1}$, where $S$ represents GroPIns. The saturation kinetics data was manipulated to show linearization by a Hanes plot transformation (80).

2.2.7 LC-MS analysis of GroPIns uptake: A 250 μl portion of each medium sample was diluted 5-fold in methanol-water (90:10) and centrifuged at 10,000 X g for 5 min. The supernatants were transferred into high-pressure liquid chromatography (HPLC) vials, dried down by nitrogen gas, resuspended in 250 μl of 75:25 acetonitrile-methanol, and placed in the autosampler of the LC system. Samples were analyzed using an Agilent 6460 triple-quadrupole mass spectrometer (MS) coupled to an Agilent 1200 LC system. The scan mode was set to multiple-reaction monitoring (MRM) targeted for fragmentation of GroPIns (333→153) in negative-ionization mode. The capillary voltage was set to -3.5 kV, the fragmentor voltage to 50 V, and the collision energy to 20 eV, with a dwell time of 200 ms. The drying gas flow rate was set to 8 liters/min, and the
nebulizer pressure was 50 lb/in². Hydrophilic interaction liquid chromatography (HILIC) was performed with a 5 μm XBridge column (150 by 4.60 mm, Waters, Milford, MA). The mobile phase was 50:50 acetonitrile-water containing 10 mM NH₄ Acetate (NH₄OAC), with a resulting pH of 7.19. The chromatography was performed using isocratic elution at a flow rate of 0.5 ml/min. An injection volume of 10 μl was used and each sample was run in triplicate. Data were analyzed by MassHunter workstation software (Agilent). The peak area of GroPIns was compared with the external calibration curve to calculate its relative concentration in the medium.

2.2.8 Analysis of internal [³H]GroPIns metabolites: Following a standard [³H]GroPIns transport assay, internal counts were isolated via trichloroacetic acid (TCA) extraction (81) and separated by ion-exchange chromatography essentially as described previously (72). Briefly, at the conclusion of a 5-min [³H]GroPIns transport assay, 750 μl of sterile water was added to the 250 μl cell suspension and the cells were pelleted. The cells were washed with 500 μl of sterile water and repelleted. The pelleted cells was then suspended in 100 μl of a 5% TCA solution and incubated on ice for 10 min. After the incubation, the cells were pelleted, the supernatant, containing the intracellular water-soluble metabolites, was removed to a fresh tube. An equal volume of 1M Tris (pH 8) was added to the supernatant to neutralize it. Neutralized samples were diluted and applied to 1 ml of Dowex 1X8-400 anion-exchange columns. Potential inositol containing metabolites (inositol, GroPIns, inositol-phosphate) were eluted from the column as described previously (72). Appropriate tritium-labeled standards were used to validate this procedure. Radioactivity was determined using scintillation counting.
2.2.9 RNA Extraction and qRT-PCR gene expression analysis. Cultures were grown in either low $P_i$ or high $P_i$ medium supplemented with 80$\mu$g/ml uridine to an OD$_{600}$ between 0.8 and 1.2. RNA was extracted using a hot phenol-chloroform extraction (82). RNA was DNase treated using the TURBO DNA-free Kit (Applied Biosystems). A 5$\mu$g sample of RNA was treated with 2 units of DNase and incubated at 37$^\circ$C for 30 min. Samples were stored at -80$^\circ$C until analysis. Primer 3 software (http://frodo.wi.mit.edu/primer3/) was used to design primers for CaGIT1 FOR: 5'-CGCATCTTTGTCAACTCAAG-3' and REV: 5'-TAGCAGCTTCACCTTGCTGTC-3'. Primer sequences for the endogenous control (CaTDH3) were: FOR: 5'-TGCTAAAGCGTTGGTAAGG and REV: 5'-AAATCGGTGGAGACAACAGC-3' (83). Real time RT-PCR was performed using Power SYBR Green RNA-to-C$_T$ 1-Step Kit (Applied Biosystems). Each reaction consisted of 0.2 $\mu$l of a 125X RT Enzyme mix, 12.5 $\mu$l of a 2X RT-PCR, 500 nM of primers, and 1.5 $\mu$l DNase treated RNA in a total volume of 25 $\mu$l. Experimental samples were analyzed in triplicate on an Applied Biosystems StepOnePlus instrument. Reverse transcription was carried out at 48$^\circ$C for 30 min, followed by 95$^\circ$C for 10 min for RT inactivation and polymerase activation, followed by 40 cycles for amplification at 95$^\circ$C for 15 s, 50$^\circ$C for 30 s, and 72$^\circ$C for 40 s, followed by the melt curve to check for primer specificity. A no-template and minus reverse transcriptase control reactions were performed to confirm lack of contamination in the RNA samples and/or the reagents. CaGIT1 expression was analyzed using the $\Delta\Delta$C$_T$ Method (84) and normalized to the endogenous control CaTDH3 expression. Wild type strain grown in low-$P_i$ was normalized to 1 and used as a comparison for fold change.
2.3 Results

2.3.1 Potential ScGIT1 homologs found in the C. albicans genome. Four ORFs with high similarity to ScGIT1, the gene that encodes the S. cerevisiae GroPIns and GroPCho transporter, have been identified in the C. albicans genome (68,69). CaGIT1 corresponds to orf19.34. Currently, the Candida genome Database (CGD, http://www.candidagenome.org) has CaGIT1 as the primary designation for orf19.34, but has noted a nomenclature conflict in that both orf19.34 and orf19.1979 have previously been called CaGIT1. A WU-BLAST2 search reveals that CaGit1 displays 53% identity and 69% similarity to ScGit1 over 504 amino acids (97% of the protein). CaGIT1 resides on chromosome 2, while CaGIT2-4 (orfs19.1978-19.1980) lie in a tandem repeat on the left arm of chromosome 5 and are highly similar to each other. CaGit2 (orf19.1978) displays 75% identity and 86% similarity with CaGit3 (orf19.1979) and 69% identity and 81% similarity to CaGit4 (orf19.1980) in regions covering at least 95% of the predicted proteins.

2.3.2 Role of CaGit1 in the utilization of GroPIns and GroPCho as phosphate sources. As shown in Fig. 2-1, a wild type C. albicans strain (DAY185) is able to utilize both GroPIns (Fig. 2-1A) and GroPCho (Fig. 2-1B) as sources of phosphate. As expected, the wild type strain grew when KH2PO4 was supplied (Fig. 2-1C), but did not grow when no phosphate source was supplied (Fig. 2-1D). To investigate the role of CaGIT1 in the utilization of GroPIns and GroPCho, a homozygous insertion mutant of CaGIT1 was constructed using UAU methodology (73,74) to produce strain
git1:UAU1/git1::URA3, as described in Materials and Methods. For insertional complementation, plasmid pDDB78 (77) containing CaGIT1 pDDB78GIT1 was linearized and transformed into git1:UAU1/git1::URA3 to produce git1:UAU1/git1::URA3 + pDDB78GIT1. As evident in Fig 2-1A, insertional mutagenesis of CaGIT1 abolished the utilization of GroPIns as a phosphate source on YNB plates, while reintegration of CaGIT1 restored that ability. The plates shown were grown at 37°C, but identical results were obtained at 30°C. Also evident is that CaGIT1 is not required for the utilization of GroPCho as a phosphate source.
Figure 2-1. *C. albicans* utilizes GroPIns and GroPCho as sources of phosphate.

Strains pre-grown in 200 μM KH₂PO₄ (low P_i) containing medium were harvested, washed, and spotted in a series of 10x dilutions onto plates containing 200 μM GroPIns (A), 200 μM GroPCho (B), 200 μM KH₂PO₄ (C), or no phosphate (D). Data are representative of multiple experiments.
2.3.3 Uptake of label from [³H]inositol-GroPIPs requires CaGit1 and is regulated by phosphate availability. Since GroPIPs and GroPCho can act as sources of phosphate, we next monitored the ability of cells to take up label from [³H]inositol-GroPIPs and [³H]choline-GroPCho. Label uptake was monitored in cells grown YNB media containing either high (10 mM KH₂PO₄) or low (0.2 mM KH₂PO₄) phosphate level (Fig. 2-2), as phosphate availability has been shown to regulate the expression of ScGIT1 (65). The transport assays (2 min for GroPCho, 10 min for GroPIPs) were optimized to ensure that uptake was linear with time at the given substrate concentration. As shown in Fig. 2-2A, [³H]GroPIPs uptake is completely absent in cells grown under high Pᵢ conditions, and the label uptake that occurs in cells grown in low Pᵢ medium requires CaGit1. In contrast, growth under high phosphate conditions decreased, but did not abolish, the uptake of label from [³H]GroPCho (Fig. 2-2B). As expected, CaGIT1 played no role in [³H]GroPCho uptake under high or low phosphate conditions.

Notably, uptake of label from [³H]GroPCho is quite robust, being roughly 50-fold greater than that from [³H]GroPIPs under low phosphate conditions. Furthermore, it is roughly 500-fold greater than that observed in S. cerevisiae cells grown under low phosphate conditions (compare Fig. 2-2B and 2-2C). In contrast, [³H]-GroPIPs transport is roughly the same in C. albicans as it is S. cerevisiae cells grown under low phosphate conditions (compare Fig. 2-2A and 2-2C).
Figure 2-2. $[^3]$HGroPIns uptake requires CaGit1 and is regulated by phosphate availability. (A and B) Strains grown in either 10 mM KH$_2$PO$_4$ (high P$_i$) or 0.2 mM KH$_2$PO$_4$ (low P$_i$) containing media were harvested, washed, and assayed for either (A) GroPIns transport in the presence of 5 μM $[^3]$HGroPIns or (B) GroPCho transport in the presence of 200 μM $[^3]$HGroPCho, as described for the standard transport assays. (C) S. cerevisiae transport was determined in the presence of 5 μM $[^3]$HGroPIns or $[^3]$HGroPCho, as described in material and methods. Values represent mean ± S.E. of triplicate determinations. Experiment was repeated with similar results.
2.3.4 Glycerophosphodiester utilization at physiological pH. In order to determine if the observed growth on GroPIns and GroPCho might also occur under nonacidic conditions that may be encountered in a human host, we monitored growth of the wild type \textit{C. albicans} strain at pH 7.5. We also compared the growth to that obtained for the nonpathogenic \textit{S. cerevisiae} (Fig. 2-3). As seen in Fig. 2-3A, \textit{C. albicans} grew when 200 \( \mu \text{M} \) of \( \text{KH}_2\text{PO}_4 \), GroPIns, or GroPCho were provided as the phosphate sources. Growth on GroPCho was just as robust as when \( \text{KH}_2\text{PO}_4 \) was supplied as the phosphate source after 24 and 48 h. Growth on GroPIns was somewhat slower, requiring 48 hours for the cells to reach \( \text{OD}_{600} \) between 3 and 4. In contrast, \textit{S. cerevisiae} grew at pH 7.5 when \( \text{KH}_2\text{PO}_4 \) was supplied, but displayed little or no growth when GroPIns or GroPCho was supplied (Fig. 2-3B). Thus, \textit{C. albicans} is clearly more adept than \textit{S. cerevisiae} at using glycerophosphodiesters as phosphate sources at pH 7.5. Serum pH, typically between 7.3-7.5, is often referred to as physiological pH.
Figure 2-3. *C. albicans* and *S. cerevisiae* vary in their ability to grow on glycerophosphodiesters at physiological pH. Wild type *C. albicans* (DAY185) (A) and *S. cerevisiae* (BY4741) (B) strains were grown in YNB medium buffered to pH 7.5, containing 200 μM GroPlns, 200 μM GroPCho, or 200 μM KH₂PO₄ as phosphate sources, or containing no phosphate source. Values represent mean ± S.E. of duplicate determinations. Experiment was repeated with similar results.
Figure 2-4. GroPIns and GroPCho utilization by *C. albicans* at pH 6.5 versus pH 7.5. (A and B) Strains were grown in either YNB medium at pH 6.5 or YNB medium buffered to pH 7.5 containing (A) 200 μM GroPIns or (B) 200 μM GroPCho as sole phosphate sources. (C and D) Strains grown in low P<sub>i</sub> YNB medium and low P<sub>i</sub> YNB medium buffered to pH 7.5 were harvested, washed, and assayed for either (C) GroPIns transport in the presence of 5 μM [<sup>3</sup>H]GroPIns or (D) GroPCho transport in the presence of 200 μM [<sup>3</sup>H]GroPCho, as described for standard transport assays. Values represent mean ± S.E. of duplicate determinations. Experiment was repeated with similar results.
A direct comparison of the utilization of glycerophosphodiesters by *C. albicans* at pH 6.5 versus pH 7.5 is shown in Fig. 2-4 A and B. These mildly acidic and mildly alkaline conditions could both be encountered by *C. albicans* in a mammalian host. For these experiments, cells were grown under low phosphate conditions. First, it is clear that *C. albicans* reaches a lower optical density at pH 7.5 as compared to pH 6.5 when grown on GroPIns (Fig. 2-4A). At the same time, [³H]GroPIns uptake at pH 7.5 is lessened by approximately two-thirds when compared to cells grown at pH 6.5 (Fig. 2-4C), and is dependent upon *CaGIT1*. Loss of *CaGIT1* also results in the inability of cells to utilize GroPIns as a phosphate source at pH 6.5 (Fig. 2-4A), as expected from the plate results seen in Fig. 2-1. At pH 7.5, however, the growth that occurs on GroPIns after 48 hours is primarily independent of *CaGIT1*. Thus, a CaGit1-independent mechanism exists for scavenging phosphate from GroPIns at pH 7.5.

Interestingly, Fig. 2-4B shows that *C. albicans* grows equally well at pH 6.5 as at pH 7.5 when GroPCho is supplied as the phosphate source. At both pH 6.5 and pH 7.5, growth on GroPCho is independent of *CaGIT1* (Fig. 2-4B). Although uptake of label from [³H]GroPCho into cells grown at pH 7.5 is decreased by about one-third compared to those grown at pH 6.5 (Fig. 2-4D), it is still quite high. These experiments were performed at 30°C, because the filamentation that occurs at pH 7.5 and 37°C causes the cells to flocculate and makes it difficult to get accurate optical density readings and repeatable transport assays. However, the following major findings were repeated at 37°C (data not shown): *i*) *CaGIT1* is required for liquid growth on GroPIns at pH 6.5. and *ii*) *CaGIT1* is not required for growth on GroPCho at either pH 6.5 or pH 7.5.
2.3.5 GroPIns kinetics, specificity, and proton dependence. Since our data indicated that CaGit1 is a transporter for GroPIns, but not GroPCho, we focused the remainder of our studies on characterizing GroPIns transport through CaGit1. For analyzing the kinetic parameters and specificity of $[^3]H$inositol-GroPIns uptake, cells were grown under low $P_i$ conditions on unbuffered YNB medium, the conditions under which GroPIns transport was shown to be greatest. Uptake of label from $[^3]H$inositol-GroPIns conformed to Michaelis-Menten kinetics (Fig. 2-5), as expected for saturable carrier-mediated transport. The apparent $V_{max}$ for GroPIns transport was 960 ± 70 pmoles/min/ODU and the apparent $K_m = 28 ± 6 \mu M$. This compares well with the apparent $K_m$ determined for GroPIns transport by ScGit1 (ca. 20 $\mu M$) (85). Transformation of the data (Fig. 2-5B) into a Hanes plot (80) results in a straight line, suggesting a single transport system under the conditions tested. These results provided added confidence that the observe label uptake from $[^3]H$GroPIns was indeed the result of “transport”.
Figure 2-5. Kinetics of GroPIns transport. (A) Strain grown in 0.2 mM KH$_2$PO$_4$ (low P$_i$) containing media were harvested, washed, and assayed for GroPIns transport in the presence of concentrations between 1 μM to 100 μM [$^3$H]GroPIns in 100 mM citrate buffer, pH 5.0, as described in text. Substrate uptake was plotted against initial concentrations of GroPIns. Data were linearized (B) using a Hanes plot transformation. Values represent mean ± S.E. of duplicate determinations. Experiment was repeated with similar results.
We next tested the transport specificity of CaGit1 by performing competition experiments. For these experiments, a 40-fold excess of unlabeled compounds was added to the transport assay and the effect upon GroPlns transport activity monitored (Fig. 2-6A). Compounds able to compete with GroPlns for transport through CaGit1 or to bind to the permease with some specificity should decrease transport activity. As expected, an excess of unlabelled GroPlns decreased the transport activity (Fig. 2-6A). Interestingly, glycerol-3-phosphate (GroP) also decreased transport activity. This finding suggested that GroP was transported by CaGit1, or that it bound to CaGit1 with enough affinity to inhibit GroPlns transport, but that it was not transported by CaGit1. To address these possibilities, we first performed $[^3]H$glycerol-GroP transport assays using our standard GroPlns transport conditions (data not shown), but were unable to detect any activity. Next, we monitored the ability of C. albicans to utilize GroP as a phosphate source, and found that it does, but that the growth is not dependent on CaGIT1 (Fig. 2-6C). Thus, the most likely interpretation of these experiments is that GroP can compete with GroPlns for binding to CaGit1, but that GroP is not transported to an appreciable extent by CaGit1.

Since GroP can clearly act as a phosphate source for C. albicans, there must be another mechanism for its utilization. Two possibilities are that GroP has a transporter distinct from CaGit1, or that GroP is hydrolyzed extracellularly to free phosphate and glycerol before being used as a phosphate source.

GroPlns transport was not affected by an excess of either unlabeled inositol or unlabeled phosphate, indicating that those compounds have little or no affinity for the permease. In particular, the fact that excess unlabeled inositol did not decrease label incorporation from $[^3]H$inositol-GroPlns is evidence that our transport assays measure
Figure 2-6. **Specificity and proton dependence of GroPIns transport.** (A and B) Wild type strain (DAY185) was grown in low Pᵢ YNB medium to logarithmic phase, harvested, washed and assayed for transport activity in the presence of the indicated compounds at 1 mM for GroPIns, GroP, inositol, sodium phosphate, and 50 μM for CCCP (A) or as a function of increasing pH (B), as described in text. (C) Strains grown in low Pᵢ media were harvested, washed and spotted in a series of 10x dilutions onto plates containing 200 μM GroP. Values for (A) and (B) represent mean ± S.E. of duplicate determinations. Experiments were repeated with similar results.
intact \(^3\text{H}\)inositol-GroPIns transport, and not the uptake of free \(^3\text{H}\)inositol following hydrolysis of \(^3\text{H}\)inositol-GroPIns outside of the cell.

If CaGit1 is a proton symporter, as predicted by in silico analysis (69), the protonophore, carbonyl cyanide 3-chlorophenylhydrazone (CCCP), should drastically reduce GroPIns transport activity, and it did (Fig. 2-6A). In addition, if proton-motive force is important to transport, altering the pH of the assay buffer should affect transport activity. As shown in Fig. 2-6B, GroPIns transport activity decreased with increasing pH, consistent with CaGit1 being a proton symporter.

2.3.6 Depletion of GroPIns from the medium corresponds to the presence of CaGit1 and intact GroPIns detected intracellularly. Further evidence for the transport of GroPIns across the plasma membrane was obtained by monitoring the levels of the metabolite in the medium by liquid chromatography-mass spectroscopy (LC-MS) as a function of growth. At time zero, cultures were spiked with 200 µM GroPIns, and after 6 and 24 h of growth, the GroPIns remaining in the medium was monitored by LC-MS (Fig. 2-7). The levels of GroPIns in the medium of the wild type strain and the homozygous insertion mutant bearing a reintegrated copy of CaGIT1 decreased with time (Fig. 2-7A) and cell growth (Fig. 2-7B). In contrast, the homozygous git1-/git1- grew very little, and there was no decrease in the GroPIns peak. The fact that the levels of GroPIns in the medium remained the same in the git1-/git1- mutant is another indication that extracellular hydrolysis of the compound does not occur under these conditions.
Figure 2-7. Depletion of GroPIns from the medium as a function of growth. (A) The indicated strains were inoculated in YNB medium lacking KH₂PO₄ that contained 200 μM GroPIns. The GroPIns concentration in the medium was determined by LC-MS after 0, 6, and 24 h. Values represent mean ± S.E. of triplicate determinations. (B) Growth curves of the strains.
As final confirmation that GroPIns is transported intact into the cell, we analyzed the labeled compounds in the intracellular fraction of the cell following a short-term transport assay with \(^3\text{H}\)inositol-GroPIns. The internal water-soluble counts were extracted, separated, and identified, and the amount of label present in the particulate membrane fraction of the cell was also determined. Importantly, we detected intact \(^3\text{H}\)GroPIns as 21% ± 6% of the internalized label. In addition, we detected free \(^3\text{H}\)inositol (69% ± 6%), as would be expected if GroPIns is rapidly hydrolyzed once it enters the cell. Finally, we also detected 11% ± 2% of the label in the membrane fraction, presumably in phosphatidylinositol or the inositol containing sphingolipids derived from phosphatidylinositol.

\textbf{2.3.7 \textit{CaPHO4} is required for GroPIns transport, utilization, and expression.} In order to gain insight into the transcriptional regulation of \textit{CaGIT1}, a library of transcription factor mutants (78) was screened. A total of 143 strains, each bearing homozygous deletions in a single transcriptional regulator, were assayed for growth when GroPIns was supplied as the phosphate source. Only one strain, that bearing a deletion in \textit{CaPHO4}, displayed a clear inability to grow on GroPIns (Fig. 2-8A). The deletion set contained two isolates of each strain, and both isolates behaved identically. The \textit{pho4Δ/Δ} mutants were able to grow on GroPCho, although slightly less well than the wild type (Fig. 2-8A). Note that little or no background growth was seen in the \textit{pho4Δ/Δ} mutant compared to the wild type strain when no phosphate source was provided. This result is likely due to the fact that \textit{CaPHO4} is required to induce genes involved in mobilization of internal stores of phosphate and phosphate scavenging when phosphate is limiting, as
is the case with *S. cerevisiae* (86,87). As expected, transport assays confirmed that little or no transport activity exists in either *pho4Δ/Δ* mutant isolate (Fig. 2-8B). RT-PCR gene expression analysis was also performed on both *pho4Δ/Δ* isolates and the reference wild type (Fig. 2-8C). These results confirm that *CaPho4* is required for *CaGIT1* expression.
A. GroPlns

WT

pho4Δ/pho4Δ

GroPCho

WT

pho4Δ/pho4Δ

KH₂PO₄
No Phosphate

B. 

C. Normalised Fold Expression

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<th>High P₁</th>
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<td>&lt;0.001</td>
</tr>
<tr>
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<td>&lt;0.001</td>
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<tr>
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<td>&lt;0.003</td>
<td>&lt;0.002</td>
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Figure 2-8. Effect of PHO4 deletion on growth and transport activity. (A) The wild type strain and a pho4Δ/Δ deletion mutant were spotted in a series of 10x dilutions onto plates containing the indicated sources of phosphate at a concentration of 200 μM. A second pho4Δ/Δ isolate gave identical results. (B) Uptake activity of the wild type and the two pho4Δ/Δ deletion mutant isolates. Values represent mean ± S.E. of triplicate determinations. Experiment was repeated with similar results. (C) Expression of CaGIT1 in the pho4Δ/Δ mutant and reference wild type. Strains were grown on synthetic YNB supplemented with 200μM (low P_i) or 10mM (high P_i) KH₂PO₄. RNA was extracted and expression measure by quantitative real time-PCR analysis. Values were normalized to the CaTDH3 control and to 1. Values represent the mean of triplicate determinations ± SE, where applicable.
2.4 Discussion

Fungal cells scavenge nutrients from the environment to support their cellular activities. Potential nutrients can arise through the organism’s own cellular activities or may be provided by the host environment. The glycerophosphodiesterases produced through phospholipase-mediated hydrolysis of phospholipids are utilized by the non-pathogenic *S. cerevisiae* as sources of phosphate, inositol, and choline (30,65,66). Here we have shown that the pathogenic organism, *C. albicans*, is also capable of transporting and utilizing glycerophosphodiesterases as nutrients, but a number of differences between the organisms are evident.

Whereas a single transporter is responsible for both GroPIns and GroPCho transport in *S. cerevisiae* (67), our data indicate that at least two glycerophosphodiester transporters exist in *C. albicans*, with CaGit1 being a GroPIns permease. CaGit1 is required for the utilization of GroPIns as a phosphate source on standard YNB solid and liquid medium at pH 6.5. In addition, CaGit1 is required for GroPIns transport when cells are grown at both acidic (pH 6.5) and physiological pH (pH 7.5). The increased transport activity seen at lower pH is consistent with a recent paper in which high throughput sequencing of cDNA revealed much greater expression of *CaGIT1* at pH 4 as compared to pH 8 (88). Although no GroPIns transport activity occurs in the absence of CaGit1, *C. albicans* is still able to use GroPIns as a phosphate source at pH 7.5, albeit slowly. Thus, a second, CaGit1-independent, mechanism must exist for utilizing GroPIns as a phosphate source at physiological pH. That mechanism may involve an unidentified transport activity with slow enough kinetics that it was not detected under the short-term transport assay employed here or, perhaps, external hydrolysis of GroPIns to liberate free
phosphate. Indeed, starvation for phosphate is known to induce the production of extracellular phosphatases and phosphoesterases for hydrolyzing and scavenging phosphate in *S. cerevisiae* (86,87), although none that hydrolyze GroPIns have been identified. Note that the extracellular liberation and subsequent transport of free phosphate that could theoretically occur at pH 7.5 would not be detected in our uptake assays, as the phosphate group of [³H]inositol-GroPIns is not labeled.

Several lines of evidence support the notion that GroPIns is transported intact across the plasma membrane via CaGit1, even though a second, CaGit1-independent, mechanism also exists for acquiring phosphate from GroPIns at pH 7.5. First, competition assays indicate that neither free inositol nor free phosphate, potential hydrolysis products of GroPIns, have significant affinity for the permease. Although GroP, another potential hydrolysis product of GroPIns, did compete for transport activity, we were unable to detect GroP transport (data not shown), and the ability of GroP to support growth was not dependent on CaGit1. Thus, GroP likely has affinity for the transporter, but is not a substrate for it. Second, the ability of cells to utilize GroPIns as a phosphate source correlates with the depletion of the compound from the medium as measured by LC-MS. Importantly, the LC-MS data also shows that GroPIns is stable in the medium at pH 6.5, since GroPIns levels did not change, even after 24 h of incubation in medium inoculated with a *git1/-git-* mutant. Finally, we detected intact GroPIns in the intracellular fraction of the cell following a short-term transport assay.

Several aspects of uptake of label from [³H]GroPCho by *C. albicans* are worth noting. First of all, uptake of label from [³H]GroPCho is much greater (approximately 50-fold) than that from [³H]GroPIns. Also, when comparing uptake between *S. cerevisiae*
and *C. albicans*, an enormous difference exists, with the activity of *C. albicans* being approximately 500-fold greater when the organisms are grown under identical growth conditions. The fact that *C. albicans* has three remaining ORFs with high similarity to CaGit1 leads to the possibility that one or more of those may be involved in GroPCho transport. It is also possible that other, unidentified, transporters are involved.

Not unexpectedly, phosphate levels regulate GroPIns transport activity, as they do in *S. cerevisiae* (89). In addition, we identified CaPHO4, a homolog of ScPHO4, as being required for the transport and utilization of GroPIns as a phosphate source. The *pho2Δ/Δ* mutant was not picked up in our screen, and it was not required for growth on GroPIns when tested individually (data not shown). In *S. cerevisiae*, ScPHO2 and ScPHO4 are both involved in the transcriptional regulation of a number of phosphate-responsive genes (86,87), including ScGIT1. However, others have shown that CgPHO2 is not important for the transcriptional response to low phosphate in *Candida glabrata* and, specifically, for the induction of the *C. glabrata* homolog of GIT1 (90). Interestingly, a paper in which the property differences of the major clades of *C. albicans* were investigated found an association between phosphate-related metabolism and virulence (91). Specifically, *CaGIT1* (numbered orf19.34, but not named in the paper) was one of 18 genes, 5 involved in phosphate metabolism, whose expression profile differed significantly between isolates of high, medium, and low virulence (91), being the highest in the most virulent strains.

Our results show that *C. albicans* has an expanded ability to transport and utilize glycerophosphodiesters, as compared to *S. cerevisiae*. Notably, *C. albicans* is able to use GroPIns and GroPCho as phosphate sources at both acidic and physiological pH (the
approximate pH of serum, pH 7.5), whereas *S. cerevisiae* does so only marginally at pH 7.5. This fact gains importance when considering that *C. albicans* may be exposed to a range of pHs, from acidic to alkaline, in its mammalian host (92,93). In fact, when considering the gastrointestinal tract alone, pH can vary from pH 2 in the stomach, to pH 6-7.4 in the intestine and terminal ileum, to pH 6.7 in the rectum (93). The vagina is also considered to be an acidic environment. Finally, human saliva can range from pH 6.5 - pH 7.5 (94). Thus, our focus on mildly acidic (pH 6.5) and mildly basic (pH 7.5) conditions is relevant to conditions that could be encountered during infection. Another expansion of abilities when comparing glycerophosphodiester utilization between the two organisms is not only that GroPIns and GroPCho utilization occurs via separate mechanisms in *C. albicans*, but that those mechanisms are regulated differentially by both pH and phosphate availability. Like pH, phosphate levels are likely to vary in the human host. For example, while phosphate levels in a healthy human serum are normally between 0.8-1.45 mM, those levels can drop to 0.3 mM and lower (the low phosphate range for the experiments performed here), depending upon diet and various disease states that induce hypophosphatemia (95). Taken together, our findings lead us to speculate that the expanded ability of *C. albicans* to utilize GroPIns and GroPCho results from the organism’s pathogenic nature and its need to occupy a variety of environments within its host organism. This possibility is buttressed by the fact that GroPIns and GroPCho are present and abundant in human fluids, as mentioned in the introduction (48,56-64).
Future studies will focus on identifying the transporter/s responsible for GroPCho utilization and on determining the importance of glycerophosphodiester metabolism on *C. albicans* virulence and survival in a mammalian host.

### 2.5. Acknowledgements

We thank Qi Zhao and William C. Nierman (TIGR), Frank J. Smith and Aaron P. Mitchell (Carnegie Mellon University), and NIH grant 1R01AI057804 for the gift of plasmid CAGFN83. We thank Claudia Almaguer and Beth Surlow for technical assistance. Strains *pho4ΔΔ*-X1 and *pho4ΔΔ*-Y1 (obtained from the FGSC) were prepared by Oliver Homann. We thank the National Science Foundation for providing support (MRIDBI-0821401) towards purchase of mass spectrometers.
Chapter 3

In preparation

Transport and Metabolism of Glycerophosphocholine by Candida albicans: Role of CaGit3, CaGit4, and CaGde1

Andrew C. Bishop, Shantanu Ganguly, Benjamin M. Cooley, Aaron P. Mitchell and Jana Patton-Vogt

Chapter 3 Attributions
I did all of the work in this chapter expect those noted here.

Shantanu Ganguly, a graduate student in Dr. Aaron Mitchell’s laboratory, constructed the strains git3Δ/Δ (JPV 662/SGH319) and git4 (JPV 665/SGH320). He also constructed the strain git2-4Δ/Δ (JPV692/SGH338), which I used to constructed strains git2-4Δ/Δ+pDDB78 (JPV707), git2-4Δ/Δ +pDDB78GIT2 (JPV754), git2-4Δ/Δ +pDDB78GIT3 (JPV737), and git2-4Δ/Δ+pDDB78GIT4 (JPV756).

Benjamin Cooley, an undergraduate student in Dr. Patton-Vogt’s laboratory, aided me in sample processing of [3H]Choline-metabolites by ion-exchange chromatography.

3.1 Introduction

Candida albicans is an opportunistic fungal pathogen that is part of the natural flora of healthy human beings (96). C. albicans is also the most common cause of hospital related fungal infections. ICU patients and individuals with compromised immune systems are most likely to be affected due to cancer treatments, immunosuppressant therapy, or diseases like HIV (14,15). It is well known that pathogenic organisms develop strategies to survive and proliferate in the host. One strategy known to influence fungal virulence is the secretion of phospholipases (26,29). It is thought that phospholipases may enhance virulence by damaging the host membranes (26), but their importance may also be related to their ability to release metabolites that
can be used as nutrients. *C. albicans* contains five phospholipase B (PLB) encoding genes (29) and disruption in *CaPLB1* or *CaPLB5* has been shown to attenuate virulence (31,53). PLBs catalyze the hydrolysis of both fatty acyl esters on phospholipids to produce both free fatty acids and water-soluble lipid metabolites, called a glycerophosphodiester (26,30).

We have reported that *C. albicans* transports glycerophosphodiester, specifically GroPIns and GroPCho, into the cell where they are hydrolyzed and used as a source of phosphate, choline, and inositol (97). *CaGIT1*, identified based on sequence similarity to *S. cerevisiae* GIT1, was shown to be responsible for GroPIns transport. Initial GroPCho transport in *C. albicans* is roughly 50 times greater than that of *S. cerevisiae* and 3 other ORFs (*CaGIT2-4*; 19.1978-19.1980) with similarity to *CaGIT1* exist in the *C. albicans* genome (97). *CaGIT2-4* lie in a tandem repeat on chromosome 5 and, like *CaGIT1*, are members of the H\(^+\) phosphate family of the major facilitators superfamily (MSF) (69). While *S. cerevisiae* has two members of this family, ScGit1 and ScPho84, *C. albicans* has five members, CaPho84 and CaGit1-4. The elaboration of this transport family in *C. albicans* may be related to the presence of glycerophosphodiester in the human host.

Phospholipid metabolites, including glycerophosphodiester, have been located throughout mammalian tissues and fluids (48,56-63,98). GroPCho and its precursor, phosphatidylcholine, are prevalent in serum (61,98). They have also been located in the gastrointestinal tract and the urinary tract (57-60), two places where *C. albicans* is known to colonize (96,99). GroPCho is present in renal medullar of the kidney, where it serves as a protective osmolyte against high concentration of NaCl and Urea (56), and where
there is risk of *C. albicans* infection in critically ill patients (96). Additionally GroPCho is found in the brain and spinal fluid (62,63).

In this study, we have further characterized GroPCho transport and metabolism in *C. albicans*. We have identified CaGit3 and CaGit4 as GroPCho transporters, with CaGit3 exhibiting the major activity. In addition, we have identified CaGde1 as a glycerophosphodiesterase involved in intracellular GroPCho catabolism. Our findings also provide evidence that GroPCho utilization by *C. albicans* occurs under conditions that may be encountered in the human host.

**3.2. Material and Methods**

**3.2.1 Strains and media.** *C. albicans* strains used in this study can be found in Table 3-1. Strains were grown aerobically at 30°C unless otherwise noted. Turbidity was monitored by measurement of absorbance at 600nm ($A_{600}$) on a Biomate 3 Thermo Spectronic spectrophotometer. Media used for this study was synthetic complete (yeast nitrogen base [YNB]) containing 2% glucose, which has previously been described (72). Media phosphate concentrations were controlled by removing KH$_2$PO$_4$ (1g/L) from the synthetic mix and replacing it with KCl (1 g/L). KH$_2$PO$_4$ was then added back into media at high (10mM) or low (200μM) concentrations as indicated. In some experiments, media contained GroPCho (200μM) (Sigma no. G5291). For all experiments, media was supplemented with 80 μg/ml uridine. For serum experiments, bovine serum (Sigma no. B9433) was added to synthetic complete YNB media containing KH$_2$PO$_4$ (1mM) at 10% of the total volume. Strains were maintained on YEPD agar plates (yeast extract 10g, peptone 20g, dextrose 10g per liter) at room temperature.
### 3.2.2 Construction of homozygous mutants.

Primers used in this study are listed in Table 3-2. The wildtype strain (DAY185) and methodology for creating homozygous deletion mutants have been previously described (75,100). In general, a forward deletion primer consisted of 100 nt homologous to the upstream region of the gene's start site plus an adaptor sequence (5' - TTTCCGTCACGACGTT-3') that flanked the 5' end of the *URA3* gene in plasmid pGEM-URA3 (JVE 278) (75). A reverse deletion primer consists of 100 nt of homology downstream of a gene's stop site plus an adaptor sequence (5' - GTGGAATTGTGAGCGGATA-3') that flanked the 3' end of the *URA3* gene in plasmid pGEM-URA3. The 1.4 kb PCR product, was transformed into strain BWP17 and Ura\(^+\) transformants were selected. Identical primers were used to PCR amplify the *ARG4* gene from plasmid pRS-ARG4 (JVE279) (75) for deletion of the second allele since the same adaptor sequence was contained in both plasmids pGEM-URA3 and pRS-ARG4. The approximately 2.5 kb PCR product containing the *ARG4* gene, was transformed into the heterozygous mutant strain and Ura\(^+\) Arg\(^+\) transformants were selected. A homozygous deletion mutant would produce PCR products for both the inserted *URA3* gene (1.2 kb) and inserted *ARG4* gene (2.3 kb), but not the native locus. For two strains (*git3\(\Delta\)/\(\Delta\) and *git4\(\Delta\)/\(\Delta\)*), homozygous deletions were made utilizing the *URA3* cassette described above, but following heterozygous mutant verification, 5-fluoroorotic acid (5-FOA) counter selection was performed (100). The *URA3* cassette was again transformed into the heterozygous mutant strain, deletion of the second native allele was verified, and counter-selection on 5-FOA was repeated producing a Ura\(^-\) homozygous deletion mutant.
Table 3-1. Strain Table Chapter 3

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...
Table 3-2. Deletion Primer Sequences

Table 3-3. Complementation Primer Sequences

Table 3-4. Quantitative RT-PCR Primer Sequences
3.2.3 Construction of complementation plasmids. Complementation plasmids were constructed for *CaGIT2, CaGIT3, CaGIT4*, and *CaGDE1* using plasmid pDDB78 (100). Each target gene was amplified from genomic DNA using primers that can be found in Table 2B. Each forward primer was located approximately 1500bp upstream from the start site and contained a 5' Not I restriction site plus 100 nt of homology to the genomic DNA. The reverse primer was located approximately 300 bp downstream of the stop site and contained a 5' EcoRI restriction site plus 100 nt of homology to the genomic DNA. Upstream and downstream locations varied depending on intergenic distances and neighboring ORFs. The vector pDDB78 was linearized with restriction enzymes Not I and EcoRI. The linear pDDB78 and amplified target gene were transformed into *S. cerevisiae* BY4741 strain (trp1Δ63, his3Δ200, ura3−52, leu2Δ1) (JPV1) (100) for in-vivo recombination and Trp+ colonies were selected. Plasmids were extracted from Trp+ colonies using the acid washed beads and a Zyppy Mini prep kit (Zymo). Extracted plasmids were then transformed into *E. coli* and Amp+ plasmids were selected. The plasmids were extracted from E. coli and verified by PCR and agarose gel electrophoresis.

3.2.4 Insertional complementation of deletion mutants. Plasmids pDDB78GIT2, pDDB78GIT3, pDDB78GIT4, and pDDB78GDE1 were linearized with Nru I, which cut the plasmids within the HIS1 gene. Linearized pDDB78GIT2, pDDB78GIT3 and pDDB78GIT4 plasmids were then transformed into *git2-4Δ/Δ* (JPV 692/SGH338) and linearized pDDB78GDE1 was transformed into *gde1Δ/Δ* (JPV 733) for recombination at the HIS1 locus. His+ transformants were tested for complementation of the mutant
phenotype when possible. Complementation was also verified by PCR. Empty plasmid pDDB78 was also linearized and transformed into both git2-4Δ/Δ and gde1Δ/Δ strains.

3.2.5 Short term [3H]GroPCho transport assays. Short-term transport assays were performed as previously described (97). Briefly, cultures were grown to log phase at 30°C. Aliquots were harvested, washed 1X in sterile DI H2O and suspended in 100 mM citrate buffer (pH 5) to an A600 of 5. Cell suspensions were incubated at 30°C for 10 minutes with agitation to allow for equilibration to buffer conditions. The assay was started by the addition of 50 μl of 1 mM [3H]GroPCho to 200 μl of cell suspension making a final concentration of 200 μM [3H]GroPCho. After 2 minutes of incubation at 30°C, the assay was stopped by the addition of 10 ml ice-cold DI H2O. The samples were immediately filtered through glass fiber (GF/C) filters (Whatman no 1822-025). The filters were washed 1X with ice-cold DI H2O and suspended in 10 ml of scintillation fluid. Radioactivity was measured by liquid scintillation counting. Tritium-labeled GroPCho ([3H]choline-GroPCho) was produced through the chemical deacylation of phosphatidyl-methyl-[3H]choline (American Radiolabeled Chemicals) as previously described (79).

To determine GroPCho transport kinetics, transport assays were performed as described above. Assays were started with the addition of 50 μl [3H]GroPCho ranging in concentrations from either 20 μM to 1 mM or 5 μM to 200 μM depending on which GroPCho transporter was being assayed (See Figure 3-6). Saturation kinetics data for GroPCho were analyzed to determine estimated Km and Vmax using Levenberg-Marquardt algorithm for nonlinear regression in GraphPad Prism (version 4.0). Values were determined by least squares fitting of the data to the Michaelis-Menten equation (V =
\[ V_{\text{max}}[S] \cdot (K_m + [S])^{-1} \], where S represents GroPCho. Linearization of kinetics data was performed using Hanes plot transformations (80).

### 3.2.6 Preparation of external and internal cellular fractions and analysis of \[^3\text{H}\]choline-containing metabolites by ion-exchange chromatography.

\[^3\text{H}\]choline-containing metabolites were analyzed in both intracellular and extracellular fractions of the cell following short-term transport assays and long-term incorporation studies. For the short term studies, a standard 2 minute transport assay was performed except that the assay was not stopped with 10 ml of DI H\(_2\)O. Rather, 750 \(\mu\)l of H\(_2\)O was added to the 250 \(\mu\)l cell suspension. The cells were pelleted and the supernatant was removed as the extracellular fraction. The pellet was washed 1X in 500 \(\mu\)l of DI H\(_2\)O and pelleted. The supernatant was again removed to the extracellular fraction.

To isolate an internal fraction (81) the cell pellet was suspended 100 \(\mu\)l of 5% TCA solution followed by 10-minute incubation on ice. Cells were again pelleted and the supernatant was removed as the internal fraction. To neutralize the TCA, equal volume of 1 M Tris (pH 8) was added to the TCA fraction. The cell pellet was washed 1X with 300 \(\mu\)l of DI H\(_2\)O and the supernatant was added to the internal fraction.

Long-term incorporation studies were designed to analyze label uptake incrementally over 24 hours. The assay was started by inoculating 2.2 ml cultures of synthetic complete YNB media containing 200 \(\mu\)M KH\(_2\)PO\(_4\) and 50 \(\mu\)M \[^3\text{H}\]GroPCho to an \(A_{600}\) of 0.05. At 6, 12, 18, and 24 hours after inoculation, 400 \(\mu\)l of culture was removed. Cells were pelleted and the extracellular fraction was removed. When necessary, non-radioactive cells were added to aid in the pelleting of radioactive cells.
Pellets were washed with 600 µl of DI H₂O and again pelleted. The pellet was suspended 100 µl of 5% TCA solution followed by 10 min incubation on ice. Cells were again pelleted and the TCA fraction was removed as the internal fraction. To neutralize the TCA, equal volume of 1M Tris (pH 8) was added to the TCA fractions. The cells were washed 1X with 300 µl DI H₂O, pelleted and the supernatant was removed to the internal fraction. Internal and external samples were diluted 4X in DI H₂O, applied to 250 µl Dowex 50W8, 200-400 anion exchange columns, and eluted as described previously (101). Briefly, [³H]GroPCho was eluted with 2X 1 ml H₂O washes. [³H]Choline-phosphate, if present, was eluted with a subsequent 1 ml H₂O wash. [³H]Choline was eluted with 5 ml of 1 M HCl. Standards were used to verify that columns were functioning correctly. Radioactivity in each sample was determined using liquid scintillation counting.

3.2.7 RNA extraction and gene expression analysis by quantitative Real Time-PCR.

RNA extraction and quantitative RT-PCR procedures were performed as described previously (97). Briefly, strains were grown in low (200 µM) or high (10 mM) phosphate media described above. Cultures were grown to A₆₀₀ between 0.8 and 1.2, harvested, and RNA was extracted using a hot-phenol chloroform extraction (82). RNA was DNase treated using the Turbo DNA- Free kit (Applied Biosystems) and stored at -80°C until analysis. Primer 3 software (http://frodo.wi.mit.edu/primer3/) was used to design forward and reverse qRT-PCR primers for CaTDH3, CaGIT3, CaGIT4, and CaGDE1 (Table 3-2C). Quantitative RT PCR was carried out on an Applied Biosystems StepOnePlus instrument using Power SYBR green RNA to C_T 1 Step Kit (Applied Biosystems). Each
reaction consisted of 0.2 μl of a 125X RT enzyme mix, 12.5 μl of 2X RT-PCR mix, 1 μM primers, and 1.5 μl of DNase treated RNA brought up to a volume of 25 μl with DEPC treated H₂O. Experimental samples were performed in triplicate and are representative of 3 independent determinations. Reverse transcription was performed at 50°C for 15 min, followed by 95°C for 15 min for RT inactivation and polymerase activation. This was followed by 40 cycles of 95°C for 15 s, 50°C for 30 s for primer annealing and 72°C for 40 s for amplification. Melt curve analysis was used to check for product specificity. Control reactions without reverse transcriptase were also performed. Expression levels were analyzed using the ΔΔCT method (84) and normalized to the endogenous control, CaTDH3. For experiments determining the change in expression due to phosphate concentrations, wild type (DAY185) low phosphate expression was normalized to 1 and used to compare fold change in high phosphate expression. For experiments determining the change in expression due to CaPHO4, wild type (WT-TF) low phosphate expression was normalized to 1 and used to compare fold change in the pho4Δ/Δ strain under low phosphate conditions.

3.2.8 [3H] GroPCho uptake in serum and XTT cell proliferation assay. Parallel radioactive and non-radioactive experiments were performed. Non-radioactive cultures were used to assess cell growth using the XTT cell proliferations assay kit (Abnova). This assay measures the reduction of XTT (2,3-bis-(2-methoxy-4-nitro-5-sulfophenyl)-5-[(phenylamino) carbonyl]-2H-tetrazolium hydroxide) through the production of a colorimetric product and was previously used to monitor cell proliferation in C. albicans biofilm growth (102). Cultures (1ml) of synthetic complete media (1mM KH₂PO₄)
containing 10% bovine serum were inoculated to an A$_{600}$ of 0.1. For the radioactive samples the media was supplemented with 200 µM [$^3$H] GroPCho, while 200 µM GroPCho was used in the non-radioactive samples. Cultures were grown at 37°C with agitation for 6 hours. Following the incubation period, the radioactive samples were centrifuged at 3500 rpm for 4 min to pellet the cellular mass. The 1ml supernatant was carefully removed as the extracellular fraction. Pellets were washed with 500 µl of DI H$_2$O and centrifuged again removing the supernatant to the extracellular fraction. The pellet was suspended 100 µl of 5% TCA solution followed by 10 min incubation on ice. Cells were again pelleted and the TCA fraction was removed as the internal fraction. To neutralize the TCA, equal volume of 1M Tris (pH 8) was added to the TCA fractions. The cells were washed 1X with 300 µl DI H$_2$O, pelleted, and the supernatant was removed to the internal fraction. Internal [$^3$H]choline-containing metabolites were separated by ion-exchange chromatography as described in materials and methods.

Cell growth was assessed in non-radioactive cultures using the XTT kit since the presence of serum induced hyphae formation and clumpy cells whose growth could not be monitored by measuring optical density. After the 6 hours of growth, 10 µl of XTT reagent mix was added to each 1ml culture and carefully mixed with a pipette. Tubes were incubated upright at 37°C for 4 hours. Tubes were centrifuged at 3500 rpm for 4 min to pellet cells and 50 µl of supernatant was diluted 10 fold in DI H$_2$O. Absorbance was measured at 450 nm and this number was used to normalize radioactive uptake of [$^3$H] GroPCho to approximate cellular growth across the different strains.
3.3 Results

3.3.1 Novel construction of a CaGIT2-4 mutant. *C. albicans* contains 4 ORFs (*CaGIT1-4*) that are predicted to be involved in glycerophosphodiester transport.

Previously we reported that *CaGIT1* (ORF19.34) codes for a permease, CaGit1, that is solely responsible for the uptake of glycerophosphoinositol (GroPIns), but does not have specificity for GroPCho, a highly transported metabolite in *C. albicans* (97). Of the four ORFs, *CaGIT1* is the most similar to *ScGIT1* and lies on chromosome 2. The remaining three ORFs, *CaGIT2-4*, lie in a tandem repeat on chromosome 5 and are the focus of this work. We compared *CaGIT2-4* to each other using NCBI BLAST (103) on the *Candida* Genome Database (CGD, www.candidagenome.org). Pairwise alignment of CaGit3 with CaGit2 results in a 75.3% identity match and 86.8% positive match with no gaps. Alignment of CaGit3 with CaGit4 results in a 73.7% identity match and 83.3% positive match with no gaps. Alignment of CaGit2 with CaGit4 results in a 70.3% identity match and 81.5% positive match with no gaps. Since all three ORFs are very similar to each other, we reasoned that single mutation phenotypes might be difficult to detect because of compensation by the other remaining ORFs. Using methodology described in materials and methods, the region from *CaGIT2* to *CaGIT4* spanning roughly 8500 bp was deleted from the genome. To the best of our knowledge this is the first report of a triple mutant created by knocking out 3 genes in tandem.
Figure 3-1. Both CaGIT3 and CaGIT4 can rescue growth of a git2-4Δ/Δ mutant utilizing GroPCho as sole phosphate source. Strains were grown in synthetic YNB liquid media lacking a phosphate source (C), supplemented with 200μM KH2PO4 (B), or with 200μM GroPCho (A). Cultures were incubated at 30°C with shaking and A600 readings taken incrementally over 48 hours. Values represent mean ± standard error (SE) of triplicate determinations. Strains are WT-DAY185 or git2-4Δ/Δ, which was reintegrated with an empty vector (pDDB78), CaGIT2 (pDDB78GIT2), CaGIT3 (pDDB78GIT3), or CaGIT4 (pDDB78GIT4).
3.3.2 Role of *CaGIT3* and *CaGIT4* in the utilization of GroPCho as a phosphate source. A strain bearing a homozygous deletion of *CaGIT2-4* displayed altered growth when GroPCho was provided as the sole phosphate source (FIG 3-1A). Early growth (<12 hours post inoculation) was abolished, although growth did resume roughly 12-18 hours post inoculation by a mechanism independent of *CaGIT2-4*. Reintegration of either *CaGIT3* or *CaGIT4* into the genome of *git2-4Δ/Δ* rescued this early growth defect on GroPCho, while reintegration of *CaGIT2* and the empty vector (pDDB78) did not. All strains grew uniformly when KH$_2$PO$_4$ was supplied (FIG 3-1B) and only background growth was observed in the absence of a phosphate source (FIG 3-1C).

3.3.3. *CaGit3* is the major GroPCho permease and is regulated by phosphate availability. Since *CaGIT3* and *CaGIT4* rescued the growth defect observed in a *git2-4Δ/Δ* strain, we next monitored the initial uptake of [$^3$H]GroPCho in short-term assays. We showed previously that [$^3$H]GroPCho uptake was regulated by phosphate availability (97), therefore cells were pre-grown in synthetic media containing either 200 μM (Low) or 10 mM (High) KH$_2$PO$_4$. Short-term (2 min) initial transport assays were performed. Reintegration of *CaGIT3* rescued a majority of the activity lost by the deletion of *CaGIT2-4* (FIG 3-2A). Reintegration of *CaGIT4* also rescued initial [$^3$H]GroPCho transport, albeit at a much lower level than *CaGIT3*. However, on an adjusted scale (FIG 3-2B) it is evident that [$^3$H]GroPCho uptake through CaGit4 is not trivial, but is comparable to [$^3$H]GroPIns uptake by CaGit1 that is observed in a wild type strain (97). As shown in Table 3-5, growth in high phosphate reduces the mRNA levels of *CaGIT3* and *CaGIT4* as determined by quantitative RT-PCR.
Figure 3-2. GroPCho transport is regulated by phosphate availability. CaGit3 displays a greater initial GroPCho uptake than CaGit4. A) Strains grown to log phase in synthetic YNB media containing either 200μM KH$_2$PO$_4$ (L) or 10mM KH$_2$PO$_4$ (H) were harvested and assayed for their ability to transport [$^3$H]GroPCho in 2 min assays. B) Enlargement of $git2$-$4\Delta/\Delta$ + pDDB78GIT4 (L) from A compared to uptake of [$^3$H] GroPIns by WT-DAY185 strain grown under low phosphate conditions. Values represent mean ± standard error (SE) of triplicate determinations. Strains are WT-DAY185 or $git2$-$4\Delta/\Delta$, which was reintegrated with an empty vector (pDDB78), $CaGIT2$ (pDDB78GIT2), $CaGIT3$ (pDDB78GIT3), or $CaGIT4$ (pDDB78GIT).
Table 3-5. Expression of CaGIT3, CaGIT4, and CaGDE1 is regulated by phosphate availability. WT-DAY185 was grown in synthetic YNB liquid media supplemented with Low P_i (200μM) or High P_i (10mM) KH₂PO₄ to log phase. Cells were harvested, RNA was extracted and expression of CaGIT3, CaGIT4 and CaGDE1 was measured by quantitative real-time PCR. CaTDH3 was used as an endogenous control. Fold change in expression was calculated between WT-DAY185 Low P_i and High P_i. Values were normalized to 1. Values represent mean ± standard error (SE) of triplicate determinations.
3.3.4 Loss of *CaGDE1* alters GroPCho catabolism. Since deletion of *CaGIT2-4* did not completely abolish the ability of *C. albicans* to utilize GroPCho as a phosphate source, we next deleted a gene predicted to be involved in GroPCho catabolism. A single ORF in the *C. albicans* genome has sequence similarity to *S. cerevisiae* *GDE1* (YPL110C), which codes for a protein with glycerophosphodiesterase activity specific for GroPCho (66,104). Using NCBI BLAST (103) (default settings, CGD) ORF 19.3936, here named CaGde1, was found to have 42% identity match and 58% positive match to ScGde1 with 15% gaps. *ScGDE1* is predicted to encode a cytosolic protein of 1223 amino acids, while *CaGDE1* is predicted to encode a cytosolic protein of 1162 amino acids. Homozygous deletion of *CaGDE1* (*gde1ΔΔ+pDDB78*) did not abolish the cells ability to utilize GroPCho as sole phosphate source, although there was a measurable difference between wild type and the *gde1ΔΔ+pDDB78* strain (FIG 3-3A). The difference in growth was rescued when a copy of *CaGDE1* (pDDB78GDE1) was reintegrated into the genome of the *gde1ΔΔ* strain (FIG 3-3A). The difference in growth was rescued when a copy of *CaGDE1* (pDDB78GDE1) was reintegrated into the genome of the *gde1ΔΔ* strain (FIG 3-3A). We next performed short-term (2 min) transport assays followed by the analysis of the internal metabolites. As shown in Figure 3-3B, internal [^3]HGroPCho as well as [^3]Hcholine were present in a wild-type strain. In the homozygous deletion mutant, we observed a build up of internal [^3]HGroPCho and minimal levels of [^3]Hcholine. Reintegration of one copy of *CaGDE1* resulted in wild-type levels of [^3]HGroPCho and [^3]Hcholine, suggesting that *CaGDE1* codes for a glycerophosphodiesterase that acts on GroPCho. As a control, *git2-4ΔΔ+pDDB78* was also included to show that GroPCho is
not internalized in the absence of a transporter. Similar to CaGIT3 and CaGIT4, growth in the presence of high phosphate reduced the expression of CaGDE1 (Table 3-5).
Figure 3-3. Deletion of CaGDE1 results in altered GroPCho catabolism. (A) Strains were grown in synthetic YNB liquid media lacking phosphate and supplemented with 200µM GroPCho. Cultures were incubated at 30°C with shaking and A₆₀₀ readings taken incrementally over 48 hours. (B) Strains were grown to log phase in synthetic YNB Low phosphate media, harvested and incubated with [³H] GroPCho for 2 minutes. Internal pools of [³H]GroPCho and [³H]Choline were determined by ion-exchange chromatography. Values represent mean ± standard error (SE) of triplicate determinations. Strains are WT- DAY185 or gde1Δ/Δ, which was reintegrated with an empty vector (pDDB78) or CaGDE1 (pDDB78GDE1).
3.3.5 CaGit3 and CaGit4 independent utilization of GroPCho. To explore the growth observed in a git2-4Δ/Δ strain that began to occur roughly 12 hours after inoculation (FIG 3-1A), we monitored the incorporation and catabolism of $[^3H]$GroPCho by monitoring both intracellular and extracellular water-soluble fractions at incremental time points over 24 hours. For these assays, the medium was supplemented with 200μM KH$_2$PO$_4$ in addition to $[^3H]$GroPCho to supply a phosphate source for initial growth of the git2-4Δ/Δ mutant. Focusing on the internal fraction of the wild-type strain (FIG 3-4A), the level of $[^3H]$GroPCho seen at 6 hours decreased thereafter, and a parallel increase of internal free $[^3H]$choline occurred. This data suggests that the internal GroPCho was being hydrolyzed to $[^3H]$choline and GroP. The lack of free $[^3H]$choline at the 24 hour time point can be explained by the fact that it had been incorporated into phosphatidylcholine and thus was not present in the water-soluble fractions (data not shown). Outside of the cell, the level of $[^3H]$GroPCho decreased with time as it was transported into the cell, but reached a plateau at approximately 12 hours (FIG 3-4B). Notably, if $[^3H]$choline appeared in the extracellular fraction starting at approximately 6 hours, suggesting that external hydrolysis was occurring parallel to GroPCho transport (FIG 3-4B).

In the git2-4Δ/Δ mutant we were unable to detect any significant levels of internal $[^3H]$GroPCho over 24 hours of growth (FIG 3-4C), indicating that deletion of CaGIT2-4 abolished the cells’ ability to transport intact GroPCho even over 24 hours.
Figure 3-4. Internalization of intact $[^3]$HGroPCho does not occur in the absence of CaGIT3 and CaGIT4, though some $[^3]$HGroPCho is hydrolyzed extracellularly.

Strains were grown in liquid YNB synthetic media containing 200$\mu$M KH$_2$PO$_4$ in the presence of $[^3]$H GroPCho. At each time point, samplings were harvested and separated into extracellular and intracellular fractions. Each fraction was analyzed by ion-exchange chromatography to determine the distribution of counts into either $[^3]$HGroPCho or $[^3]$HCholine. Values represent means ± standard error (SE) of duplicate determinations. A) Intracellular and B) Extracellular fraction of WT-DAY185. C) Intracellular and D) Extracellular fraction of git2-4Δ/Δ with an empty vector (pDDB78) reintegrated. E) Intracellular and F) Extracellular fraction of git2-4Δ/Δ with CaGIT3 (pDDB78GIT3) reintegrated.
Importantly, after 12 hours of growth we were able to detect internal $[^3\text{H}]$choline in the
$\text{git2-4}\Delta\Delta$ mutant (FIG 3-4C), as well as a decrease in extracellular $[^3\text{H}]\text{GroPCho}$ (FIG 3-4D) and the appearance of external $[^3\text{H}]$choline. These data suggests that $[^3\text{H}]\text{GroPCho}$ was being hydrolyzed outside of the cell to release free choline, as was the case for wild type (FIG 3-4B).

Since CaGit3 is the primary permease for GroPCho, we analyzed the $\text{git2-4}\Delta\Delta$ mutant with $\text{CaGIT3}$ reintegrated into the genome to verify that intracellular and extracellular levels of metabolites were returned to that of wild type (FIG 3-4E &F) in the presence of a GroPCho permease. Radioactivity not accounted for in the extracellular and intracellular fractions was detected in the membrane fraction by way of free choline incorporation into newly synthesized phosphatidylcholine (data not shown).

To summarize the results of Figure 3-4, the data suggest that during the first roughly 12 hours post-inoculation, wild type cells were utilizing GroPCho by transporting it in the cell (primarily via CaGit3) and hydrolyzing it to release free choline. After 12 hours of growth, when there was little continued uptake of intact GroPCho, the most likely scenario was that GroPCho was being hydrolyzed extracellularly and the resulting metabolites transported into the cell as needed. The hydrolysis of GroPCho into free choline and GroP is the most likely pathway given that we did not detect choline-phosphate in internal or external fractions.
Figure 3-5. CaPho4 is partially responsible for regulating CaGIT3 CaGIT4 and CaGDE1 under low phosphate conditions. A) Wild type (WT-TF) and pho4Δ/Δ strains were grown in synthetic YNB liquid media lacking phosphate (No P$_i$), supplemented with 200μM KH$_2$PO$_4$ (Low P$_i$) or 200μM GroPCho. Cultures were incubated at 30°C with shaking and A$_{600}$ readings taken incrementally over 24 hours. B) Strains were grown to log phase in Low P$_i$ medium. Cells were harvested, washed and analyzed for their ability to transport [³H] GroPCho over 2 min. C) Strains were grown to log phase in Low P$_i$ medium. Cells were harvested, RNA was extracted and expression of CaGIT3, CaGIT4 and CaGDE1 was measured by quantitative real-time PCR. CaTDH3 was used as an endogenous control. Fold change expression was calculated compared to WT-TF and values were normalized to 1. Values represent mean ± standard error (SE) of triplicate determinations. Experiments were repeated with similar results.
3.3.6 CaPHO4 Regulates the Expression of CaGIT3, CaGIT4 and CaGDE1. CaPho4 regulates the expression of CaGIT1 and thereby regulates the transport of GroPIns by C. albicans (97). In FIG 3-5A, a wild type strain and a pho4Δ/Δ mutant were grown on synthetic media either lacking phosphate supplemented with KH₂PO₄, or supplemented with GroPCho. Although no differences in growth were observed (FIG 3-5A), initial [³H]GroPCho transport activity was greatly reduced in the pho4Δ/Δ mutant (FIG 3-5B). It is not surprising that the activity remaining in the pho4Δ/Δ mutant (250 pmol/min/ODU) was enough to support full growth on GroPCho (Fig 3-5A), as this level of activity is enough to support full growth on GroPIns as a phosphate source (97). Quantitative RT-PCR analysis of gene expression was performed for CaGIT3, CaGIT4, and CaGDE1 in WT-TF and the pho4Δ/Δ mutant grown under low Pᵢ conditions (FIG 3-5C). Normalizing the WT-TF to 1, an approximate 10-fold reduction in mRNA levels was evident for both CaGIT3 and CaGDE1 in the pho4Δ/Δ strain. CaGIT4 showed a roughly 4-fold decrease in message levels in a pho4Δ/Δ strain as compared to WT-TF.

3.3.7 Kinetics of GroPCho transport by CaGit3 and CaGit4. Given that our results indicated that both CaGit3 and CaGit4 act as GroPCho transporters, we used a git4Δ/Δ mutant to analyze CaGit3 transport kinetics and a git3Δ/Δ mutant to analyze CaGit4 transport kinetics. For analyzing kinetics, strains were grown in synthetic media containing low phosphate. Initial transport of [³H]GroPCho by both CaGit3 and CaGit4 conformed to Michaelis-Menton kinetics
Figure 3-6. Kinetics of GroPCho transport by CaGit3 and CaGit4. Strains were grown to log phase in synthetic YNB Low phosphate media, harvest, washed and assayed for initial transport of [³H] GroPCho at varying concentrations of GroPCho substrate. A) \textit{git4Δ/Δ} mutant is assayed to analyze transport activity of CaGit3 from 20μM to 1mM GroPCho. B) \textit{git3Δ/Δ} mutant is assayed to analyze transport activity of CaGit4 from 5μM to 200μM GroPCho. [³H] GroPCho transport activity is plotted against initial concentrations of GroPCho. Data were linearized using Hanes plot transformations (Inset A & B).
Under the given conditions, CaGit3 exhibited a $V_{\text{max}}$ of $3940\pm150$ pmol/min/ODU and a $K_m$ of $45\pm6$ µM. CaGit4 exhibited a $V_{\text{max}}$ of $340\pm40$ pmol/min/ODU and a $K_m$ of $16\pm7$ µM. Data was transformed into a Hanes plot (Inset FIG 3-6A&B) to verify linearity and the presence of a single transporter for each analysis. The error bars observed for the minor transporter, CaGit4, are attributed to the fact that transport was quite low compared to CaGit3 and that it was greatly affected by small changes in the cell density at inoculation as well as cell density at the time of harvesting cells for the assay.

3.3.8 GroPCho utilization under serum growth conditions. In an attempt to explore the relevance of the GroPCho transport under conditions similar to those experienced in a human host, we performed GroPCho incorporation experiments in the presence of 10% serum and 1 mM KH$_2$PO$_4$, which is within the phosphate concentration range reported for serum (105). Cultures were allowed to grow for 6 hours at 37°C at which point the internal metabolites were analyzed (FIG 3-7). Both $[^3]$HGroPCho and $[^3]$Hcholine were detected internally in a wild-type strain. When CaGIT2-4 were deleted, no internal radiolabeled metabolites were detected. Upon reintegration of CaGIT3, wild-type levels of metabolites were observed. A minimal amount of $[^3]$HGroPCho was detected when CaGIT4 was reintegrated. A strain containing a homozygous deletion of CaGDE1 exhibited a build up of $[^3]$HGroPCho and less $[^3]$Hcholine compared to wild-type levels. Upon reintegration of CaGDE1, internal metabolites returned to levels similar to wild type.
Figure 3-7. GroPCho is transported and metabolized in the presence of serum.

Strains were pre-grown in synthetic YNB media containing 1 mM KH₂PO₄ (YNB 1 mM P_i). The assay was initiated by inoculating cultures of synthetic YNB 1 mM P_i medium containing 10% Bovine Serum and 200 μM [³H]GroPCho to A₆₀₀ 0.1. Cultures were incubated at 37°C for 6 hours with agitation. After 6 hours, cultures were separated into extracellular, intracellular and membrane fractions. Fractions were analyzed by liquid scintillation counting. [³H]Choline metabolite ratios were determined by ion exchange chromatography. Cell growth between the hyphae forming strains, was normalized using XTT reduction analysis (Abnova) performed parallel to the radioactive assay. Results shown represent mean ± SE of duplications. Experiment was repeated with similar results. Strains are WT-DAY185, git2-4Δ/Δ, which was reintegrated with an empty vector (pDDB78), CaGIT3 (pDDB78GIT3), or CaGIT4 (pDDB78GIT) and gde1Δ/Δ, which was reintegrated with an empty vector (pDDB78) or CaGDE1 (pDDB78GDE1).
These results indicate that both CaGit3 and CaGde1 are active in the presence of serum, which is known to contain GroPCho (61,98,106). Cell growth was monitored by XTT reduction assay in order to analyze growth across strains that are hyphae forming and clumping in the presence of serum.

3.4 Discussion

Glycerophosphodiesters are produced through the activity of deacylating phospholipases, such as those of the A1, A2 and B-type. B-type phospholipases (PLBs), enzymes that can remove both fatty acyl esters, are found in many organisms including bacteria (107,108), humans (109), and fungi (29). PLBs are considered virulence factors for several pathogenic fungi (26,110). Not surprisingly, glycerophosphodiesters are common metabolites, having been found in many cell types and, importantly, are reported to occur in several locations in the human body, including serum (56-59,61-63,98). Our work and that of others has shown that extracellular and intracellular glycerophosphodiesters are recycled into biological molecules through some combination of transport and hydrolysis (44,49,50,66,97,104). C. albicans' ability to transport glycerophosphodiesters is expanded as compared to the non-pathogenic S. cerevisiae (97). While S. cerevisiae has one transporter (ScGit1) responsible for GroPIns and GroPCho transport, C. albicans has four ORFs with similarity to ScGIT1 and much greater GroPCho transport activity (97). Additionally, we reported that while CaGit1 is responsible for GroPIns transport, GroPCho is utilized by a different mechanism (97). The data presented here indicate that there are two transporters specific for GroPCho uptake in C. albicans, CaGit3 and CaGit4. Evidence to support this conclusion is two-
i) either CaGit3 or CaGit4 is required for early post-inoculation growth when GroPCho is supplied as sole phosphate source (FIG 3-1A) and ii) measurable initial transport of GroPCho requires either CaGit3 or CaGit4, with CaGit3 displaying much greater initial transport activity (FIG 3-2A&B). Nonetheless, cells lacking CaGIT3 and CaGIT4 are able to initiate growth on GroPCho 12-18 hours post inoculation (FIG 3-1A), suggesting that an alternate mechanism for GroPCho utilization is initiated in that time frame. Our data, obtained using [3H]GroPCho in which the choline molecule was labeled, indicate that this alternate mechanism involves the extracellular hydrolysis of [3H]GroPCho to [3H]choline and GroP, as we observe the production of extracellular [3H]choline (FIG 3-4D). Presumably the unlabeled GroP is hydrolyzed to release free phosphate that is utilized by the cell. The hydrolysis of [3H]GroPCho into [3H]choline and GroP is the most likely pathway given that we did not detect [3H]choline-phosphate in internal or external fractions. It is worth noting that this transporter-independent pathway for GroPCho utilization does not exist in S. cerevisiae, where deletion of ScGIT1 completely abolishes the ability of cells to utilize GroPCho as a phosphate source (66).

Glycerophosphodiesters are hydrolyzed to GroP and their respective alcohol by glycerophosphodiesterases (30,46). In order to better understand GroPCho catabolism in C. albicans, we deleted CaGDE1 (ORF 19.3936), which is predicted to be a cytosolic protein with a glycerophosphodiester phosphodiesterase domain. CaGde1 is highly similar to the only known GroPCho glycerophosphodiesterase in S. cerevisiae (ScGde1) and is annotated CGD as a glycerophosphodiester glycerophosphodiesterase using InterPro (IPR004129) (35). Loss of CaGDE1 results in a build up of internal GroPCho
(FIG 3-3B), providing evidence that CaGde1 acts upon GroPCho. However, loss of CaGDE1 does not abolish GroPCho utilization (FIG 3-3A), so other gene products must exist that are capable of hydrolyzing GroPCho, but that are less similar to a standard glycerophosphodiesterase (IPR004129). This result also stands in contrast to that which occurs in S. cerevisiae, where deletion of ScGDE1 renders the cells unable to grow on GroPCho as a phosphate source (66).

Glycerophosphodiesterase activity is present in a wide range of organisms, including mammals, plants, and bacteria (30). In mammalian cells, several putative glycerophosphodiesterase domains have been identified (46). For example, GDE5 selectively hydrolyzes GroPCho and has been implicated in skeletal muscle development (47). In E. coli, a multi protein complex, the Ugp system (consisting of UgpB,A,E,C, and Q), exists to utilize GroPCho and is regulated by phosphate availability (43-45). UgpB binds GroPCho and brings it to 3 membrane proteins, UgpA, UgpE and UgpC, which are required for GroPCho transport. As GroPCho is transported, it is hydrolyzed by a cytosolic glycerophosphodiester phosphodiesterase, UgpQ (44). In two separate plant models, glycerophosphodiesterase activity has been linked to phosphate starvation (49,50). In Arabidopsis thaliana, phosphate starvation induces the expression of glycerophosphodiesterases and plants lacking the corresponding genes exhibit decreased growth rates (49). In White Lupin (Lupinus albus), two glycerophosphodiesterases (GPX-PDE1 and GPX-PDE2) have been characterized (50). Upon phosphate starvation, expression of GPX-PDE1 and GPX-PDE2 is up-regulated and both enzymes are linked to the growth of root clusters (50). Interestingly, GPX-PDE1 complements the S. cerevisiae gde1Δ mutant (50), in terms of growth on GroPCho as a phosphate source (66).
As has been reported for plants and bacteria, we show here that GroPCho utilization in *C. albicans* is linked to phosphate limitation. *CaPHO4* and *CaGRF10* are orthologs to *ScPHO4* and *ScPHO2* (68), genes that encode transcription factors that regulate phosphate responsive genes in *S. cerevisiae* (86,87). As was the case for *CaGit1* (97), the transcriptional regulator *CaPho4* regulates the expression of *CaGIT3, GIT4*, and *CaGDE1* (FIG 3-5C). However, loss of *CaGRF10* does not affect the ability of *C. albicans* to utilize GroPCho (data not shown). Additionally, *CaGRF10* does not regulate the expression of *CaGIT1* (97) and others have noted that it does not appear to be involved in the phosphate response in *C. albicans* (99). Interestingly, a recent report indicates that phosphate limitation might have implications for *C. albicans* virulence (99). In this study, *C. albicans* strains isolated from the stool of critically ill patients underwent filamentation and expressed a lethal phenotype against mice and *C. elegans* upon phosphate limitation (99). It is tempting to speculate that the phosphate-regulated genes involved in GroPIns (*CaGit1*) (97) and GroPCho (*CaGit3, CaGit4, and CaGde1*) utilization may play a role in these phenotypes.

Besides acting as a nutrient source, other functions have been ascribed to GroPCho. A well-known function involves osmotic regulation in renal medullary tissue of mammals, where GroPCho counteracts the effects of high NaCl and urea concentrations (48,56). In the fungal pathogen, *Cryptococcus neoformans*, GroPCho appears to be required for capsule enlargement (110). Capsule enlargement is necessary for this organism's virulence, as it provides protection from phagocytosis as well as oxidation (110). A *C. neoformans* strain lacking phospholipase B1 (PLB1) loses its ability to enlarge its capsule, but supplementation of GroPCho rescues this phenotype.
Interestingly, *C. neoformans* contains a putative protein that is 42% similar (NCBI-Blast) to CaGit3 suggesting that it too may transport intact GroPCho.

Although a GroPIns permease (GLUT2) has been identified in mammalian cells (41) and GroPCho transport has been described in *E. coli* (43,45), to the best of our knowledge, this is the first identification of a GroPCho permease in a eukaryotic organism. Interestingly, proteins with high similarity to the major GroPCho permease, CaGit3, appear to be widespread in fungi. A NCBI blast (103) search of protein sequences from fungal organisms revealed 11 organisms that contain one or more predicted proteins with at least 50% identity match to CaGit3. Within the *Candida* species, *C. dubliniensis, C. parapsilosis, C. tropicalis* and *C. orthopsilosis* all contain one or more predicted proteins that range between 73-98% identical to CaGit3.

We have shown previously that *C. albicans* is able to utilize GroPCho under conditions of elevated temperature and pH found in the human body (97). Here we show that *C. albicans* employs CaGit3 to transport and CaGde1 to metabolize GroPCho in the presence of serum and the concentration of phosphate found in serum, roughly 1 mM (FIG 3-7). Serum contains not only GroPCho, but also its precursor, phosphatidylcholine (61,98). Thus, our results offer evidence that glycerophosphodiester transport and metabolism are physiological relevant cellular functions under conditions of human infection. Obvious future experiments to pursue include those aimed at determining the role, if any, that these gene products play in pathogenicity or the ability of the organism to colonize the human host.
3.5 Acknowledgements

Strains WT-TF and \textit{pho4Δ/Δ} (obtained from the FGSC) were prepared by Oliver Homann. Partial funding support from the NIH (R15GM104876 awarded to Dr. Jana Patton-Vogt)
Chapter 4

Conclusions

My work has resulted in several clear contributions to our understanding of glycerophosphodiester transport and metabolism in \textit{C. albicans}. I have identified CaGit1 as the permease responsible for the transport of GroPIns into the cell. I have identified CaGit3 and CaGit4 as two novel permeases responsible for the transport of GroPCho into the cell. This is the first report of an eukaryotic transporter with high specificity for GroPCho. I have shown that CaGde1 is a glycerophosphodiesterase involved in internal GroPCho metabolism. Also my work has demonstrated that \textit{CaGIT1, CaGIT3, CaGIT4,} and \textit{CaGDE1} expression is regulated by the CaPho4 transcription factor. Finally, I have shown that glycerophosphodiester transport and metabolism occurs under conditions that may be experienced in a human host.

4.1 Comparison of glycerophosphodiester metabolism in \textit{C. albicans} and \textit{S. cerevisiae}.

Many noteworthy similarities and differences between \textit{S. cerevisiae} and \textit{C. albicans} have been demonstrated in this study. Based on the results presented in the study, CaGit1 is the ortholog of ScGit1. Their protein sequences are also similar (FIG 4-1A). Both of these proteins transport intact GroPIns into the cell with similar estimated \(K_m\) and \(V_{max}\) values under the conditions studied. In contrast to ScGit1, CaGit1 does not transport GroPCho. Another difference between the two proteins is that CaGit1 functions at physiological pH, but ScGit1 does not.
The addition of ORFS CaGIT2-4 as a tandem repeat on chromosome 5 has increased ability of C. albicans to utilize GroPCho. I have determined that CaGit3 is a GroPCho permease that is responsible for the majority of GroPCho transport. Although CaGit4 does have minimal level of transport activity, the $K_m$ and $V_{max}$ for CaGit3 are much higher. Interestingly, the $K_m$ and $V_{max}$ of CaGit3 are very similar the $K_m$ and $V_{max}$ reported for the inorganic phosphate transporter in S. cerevisiae, ScPho84 (111). Thus, not only can C. albicans utilize glycerophosphodiesters more robustly than S. cerevisiae, it can transport GroPCho at a rate similar to that by which S. cerevisiae transports phosphate (under the given conditions). Both CaGit3 and CaGit4 are also active at alkaline pH (FIG 2-4, Appendix A-3).

Another difference between the two organisms is that C. albicans can utilize glycerophosphodiesters in the absence of the permease that transports the metabolite into the cell, while S. cerevisiae cannot. S. cerevisiae strains lacking the only glycerophosphodiester permease, ScGit1, are unable to utilize GroPIns or GroPCho for growth a phosphate source (66,67). In contrast, C. albicans can utilize GroPIns and GroPCho in the absence of an appropriate permease. In the absence of CaGit1, C. albicans is able to utilize GroPIns as a phosphate source for growth when the pH of the growth media is elevated (7.5). Similarly, in the absence of CaGit3 and CaGit4, GroPCho can be utilized as a phosphate source 12-18 hours post inoculation at all pHs tested. In both cases, the data indicates that glycerophosphodiesters are being hydrolyzed extracellularly to release components that can be taken up by other transporters. Thus, C. albicans has also increased its ability to catalyze glycerophosphodiesters compared to S. cerevisiae. In S. cerevisiae, deletion of ScGde1 eliminates the cells ability to utilize
GroPCho (66,104). Since elimination of CaGde1 reduces, but does not abolish the cell’s ability to utilize GroPCho, there must be one or more enzymes having specificity for GroPCho. Additionally, I have evidence that CaGde1 has activity for internalized GroPIns (Appendix A.8). In contrast, the ScGde1 does not hydrolyze GroPIns and a glycerophosphodiesterase specific for GroPIns in S. cerevisiae is unknown (30). In the current ORF assembly, C. albicans does not contain any proteins with sequence similarities to ScPho5, the major phosphatase in S. cerevisiae (44,112). Interestingly, C. albicans does contain at least 10 putative phosphatase proteins, most of which are uncharacterized and may have the ability to hydrolyze glycerophosphodiesters.

4.2 Phosphate Response in C. albicans and S. cerevisiae

The proteins investigated in this study are a part of a larger story, which is the phosphate response in C. albicans. This response is understudied, but the initial work here and by the others (99), has shown some interesting results. More work needs to be performed to fully understand the importance of the phosphate response in C. albicans and relate it to the overall pathogenicity of the organism.

4.2.1 S. cerevisiae response

The phosphate (P_i) response in S. cerevisiae has been extensively studied, and was reviewed in Ljungdahl and Daignan-Fornier 2012. This process involves a complex network of sensing, transporting, and signaling in response to intracellular and extracellular P_i levels. Studies have been carried out in a number of different P_i conditions including transitions from high P_i to low P_i, cells adapted to low P_i, and low P_i vs. P_i
starvation (113). The phosphate response is regulated by PHO-regulatory pathway, which includes positive regulators ScPho2, ScPho4, and ScPho81 and negative regulators ScPho80 and ScPho85 (113). Within the phosphate response cascade, ScPho2 and ScPho4 are transcription factors whose interaction is required for a phosphate response under low phosphate conditions (114). ScPho80 and ScPho85, which are active when phosphate conditions are high (115), form a protein kinase complex that interacts with ScPho4 (116). This interaction results in the phosphorylation of ScPho4, its export from the nucleus (117), and prevents it from activating phosphate response genes when P_i is plentiful (115). ScPho81 has been shown to be a positive regulator of phosphate response genes through its ability to negatively regulate ScPho80-ScPho85 complex (118), allowing ScPho4 to remain in the nucleus. ScPho4 and ScPho2 have also been shown to be important in the utilization of alternate phosphate sources (89). Both ScPho4 and ScPho2 regulate ScGit1 transcription, essentially regulating transport of GroPIns and GroPCho (89,119).

Phosphate uptake and sensing plays a major role in the regulation of phosphate response genes (113). *S. cerevisiae* has two high affinity P_i transports Pho84 and Pho89 (120,121) and three low affinity P_i transports Pho87, Pho90, and Pho91 (111). Pho84 and Pho89 are both under control of the PHO-regulatory pathway and contribute to internal levels of P_i (120,121). The high affinity transporters are not essential proteins because low affinity transporters, function independently of internal phosphate concentrations (122). Evidence also suggests that the low affinity transporters may be involved in extracellular phosphate sensing contributing to the internal signaling (113).
The PHO-regulatory network and $P_i$ levels influence at least 6 proteins (ScPho3, ScPho5, ScPho8 and ScPho10-12) with phosphatase activity in *S. cerevisiae* (112,123,124). CaPho4 and CaPho2 have been shown to bind to the promoter region of CaPho5 (125), the major secreted acid phosphatase in *S. cerevisiae* (112,124). CaPho5 expression is impacted by internal concentrations of $P_i$, which can be greatly influenced by phosphate transport (111,112). None of these known phosphatases in *S. cerevisiae* are able to act on extracellular glycerophosphodiesters.

### 4.2.2 C. albicans response

Very few studies have reported on phosphate responsive genes in *C. albicans*. Most of the ORFs are uncharacterized and named based on sequence similarity to *S. cerevisiae* ORFs. In all but a few cases, the ORFs have merely been incorporated into more global studies looking at transcriptional profiling of biofilms (126-128), morphology changes (129-131), and other various stressors including changes in osmotic pressure, oxidation state, pH, and the presence of antifungal drugs (132-135).

Interestingly, one expression study analyzing message levels from *C. albicans* strains isolated from a mouse virulence model shows upregulation of *CaPHO84, CaPHO87,* and *CaPHO100* (homolog of *ScPHO3*) which are predicted to code for a high affinity phosphate transporter, a low affinity phosphate transporter, and an acid phosphatase, respectively (91). A NCBI BLAST search reveals that CaPho84 is 66% identity match with 4% gaps to ScPho84, the primary high affinity phosphate permease in *S. cerevisiae* (120). Additionally, *CaPHO84* and *CaGIT3* expression has also been shown to be upregulated in biofilms (126).
The only *C. albicans* PHO-related ORFs to be characterized is *CaPHO4*, which encodes the transcription factor CaPho4. Aside from work in this dissertation, one study to date has reported specifically on the phosphate response in *C. albicans* involving CaPho4 (99). This study showed that *CaPHO4* expression was upregulated under phosphate limiting conditions (99). This report complements my findings that show CaPho4 regulates the expression of *CaGIT1, CaGIT3, CaGIT4*, and *CaGDE1*. In contrast to *S. cerevisiae*, interactions between transcription factors ScPho4 and ScPho2 are not required for the activation of phosphate responsive genes. Our data and others (99) were unable to find a connection between CaPho2 and a response to PHO-regulatory pathway. This is not entirely surprising, as others have reported on the differences between the regulatory circuits between *S. cerevisiae* and *C. albicans* (78,136).

### 4.3 Putative glycerophosphodiester permeases across fungal species

To gain a perspective on the potential proteins with glycerophosphodiester transport activity in other fungal species besides *C. albicans* and *S. cerevisiae*, a NCBI BLAST search was performed using ORF translations of both CaGit1 and CaGit3 obtained from CGD. The purpose was to determine if there are homologous GIT-related proteins in other fungal pathogens. Data from this search are compiled in Table 4-1 and Appendix A-1. This search resulted primarily in human and plant pathogens from the Ascomycota phylum with the exception of two species, *Cryptococcus neoformans* and *Cryptococcus gattii*, from the Basidiomycota phylum. For comparison purposes CaPol3, a protein coding for DNA polymerase 3 in *C. albicans*, was also included in this search to show similarities of an essential protein among these species. Species that contained GIT-
related proteins that are very similar (> 70% identity match) to C. albicans GIT-related proteins, also contained Pol3 proteins that are very similar (> 70% identity match) to CaPol3. Most of these proteins were in the Candida genus, with the exception of Lodderomyces elongisporus, a relative of the Candida Genus. L. elongisporus, by comparative sequence analysis, is roughly 97.5 % similar to C. albicans (137). In the more divergent genera, Apergillus and Coccidioides, the protein with the highest similarity to GIT-related proteins are between 50-60% identity match to CaGit1 and CaGit3, but their respective Pol3 proteins are also less similar (~60% identity match). It is tempting to speculate that these putative GIT-related proteins may experience similar evolutionary pressure as an essential protein, such as DNA polymerase 3, due to the environments in which they colonize.

The presence of one or more proteins with similarity to CaGit1 or CaGit3 occurred in species other than C. albicans (C. dubliniensis, C. orthopsilosis, C. parapsilosis, C. tenuis, and C. tropicalis) (Table 4-1). Many contained proteins ranging between 74 and 98 % identity match to CaGit1 and CaGit3, covering over 90% of the protein. It is worth mentioning that Candida species that are not known to impact human health were not prevalent in this search, though roughly 190 exist. This observation is most likely due to the lack of sequence data for these species that pose little risk to humans. This may be result from a greater emphasis on sequencing the genomes of species that are human pathogens.

Other fungal species diverged from the Candida genus contain GIT-related proteins (Appendix A-1). Aspergillus fumigatus, like C. albicans, is considered to be a common cause of fungal infection in humans (138). A. fumigatus, found commonly in
Table 4-1 Putative glycerophosphodiester permeases of common fungal species

<table>
<thead>
<tr>
<th>Species</th>
<th>Count</th>
</tr>
</thead>
<tbody>
<tr>
<td>cerevisiae</td>
<td>1</td>
</tr>
<tr>
<td>albicans</td>
<td>5</td>
</tr>
<tr>
<td>dubliniensis</td>
<td>4</td>
</tr>
<tr>
<td>orthopsilosis</td>
<td>5</td>
</tr>
<tr>
<td>parapsilosis</td>
<td>5</td>
</tr>
<tr>
<td>tenuis</td>
<td>5</td>
</tr>
<tr>
<td>tropicalis</td>
<td>5</td>
</tr>
</tbody>
</table>
Table 4-1 Putative glycerophosphodiester permeases of common fungal species.

NCBI BLAST search of three *C. albicans* proteins, CaGit1, CaGit3, and CaPol3 against pathogenic and non-pathogenic fungal species. Column 1 indicates the genus (bold) followed by one or more species. Columns 2 and 3 indicate the fungal strains that were sequenced to obtain the putative protein sequence and the NCBI reference sequence number for each protein. Columns 4-6 indicate the percent identity match for each putative protein against CaGit1, CaGit3, and CaPol3. Proteins with greater than 50% identity match to CaGit1 are bolded in column 4 and the species is highlighted blue in column 1. Proteins with greater than 50% identity match to CaGit3 are bolded in column 5 and the species highlighted orange in column 1. The percent coverage for each protein was 90% or greater.
soil, exist as a spore that can potentially enter humans through inhalation (138). Similar to *C. albicans*, *A. fumigatus* primarily causes issues for individuals with compromised immune systems (138). The *Aspergillus* genus contains many different species (Appendix A-1), but only a few have been associated with human infection (138).

Interestingly, many of the species, *A. fumigatus*, *A. terreus*, *A. nidulan*, *A. clavatus*, *A. flavus*, and *A. oryzae* contain putative proteins that are greater than 50% identity match to CaGit3 and the species *A. niger* and *A. kawachii* contain putative proteins that are greater than 50% identity match to CaGit1. Another fungal pathogen, *Coccidioides immitis*, normally found in soil, but also has the potential to infect humans (139), has one putative protein that is greater than 50% identity to CaGit3.

The common fungal species, *Cryotococcus neoformans*, contains 2 GIT-related proteins, although they are less similar to CaGit1 and CaGit3 (<50% identity match) (Appendix A-1). This is not surprising given that this species belongs to different phylum, Basidiomycota (140), but still interesting because a recent report tied GroPCho to the virulence of *C. neoformans* (110).

Of all the species indentified in this search, only one was not a human pathogen. This species, *Magnaporthe oryzae*, is a plant pathogen affecting rice (141). *M. oryzae* has three putative GIT-related proteins, all of which are less than 50% identity match to CaGit1 and CaGit3 (Appendix A-1). Evidence of GIT-related proteins in these species that have diverged from *C. albicans* provides evidence that these types of proteins have survived and in many cases expanded within particular species.

Pathogenic organisms have increased their frequency of genes involved in pathogenicity and nutrient acquisition as compared to non-pathogenic organisms
In particular, cell wall associated proteins and secreted hydrolytic enzymes have been investigated (139,144). Not surprisingly these gene duplications are associated with the proteins products of recognized virulence factors. Still, gene duplications relevant to nutrient transport have been reported (142,143). *C. albicans* contains a family of six polyamine transporters, compared to one in *S. cerevisiae*, which play a role in transporting polycationic molecules necessary for protein and nucleic acid synthesis (143). Additionally, a family of nine oligopeptide transporters exists in the *C. albicans* genome compared to three in *S. cerevisiae*, with *CaOPT7* being involved glutathion utilization (142). The duplication of putative glycerophosphodiester transport proteins in *C. albicans* compared to *S. cerevisiae* may be another instance of *C. albicans* increasing its ability to utilize potential nutrient sources.

### 4.4 Open Questions

The summary of glycerophosphodiester metabolism in *C. albicans* (FIG 4-1) is based primarily on the work described in my dissertation. However, several open questions still remain. First is the characterization of the CaGit1-CaGit3-CaGit4 independent utilization of GroPIns and GroPCho. This could be addressed through *i*) the identification of enzymes that are hydrolyzing extracellular GroPIns and GroPCho, *ii*) determining to what extent these enzymes are regulated by phosphate and *iii*) analyzing the role, if any, CaGit3 or CaGde1 play the activation of this novel scavenging pathway. This alternate pathway may in fact be a major contributor to ability of *C. albicans* to invade host tissue, especially under low phosphate environments. An experiment to identify potential proteins involved in extracellular hydrolysis of GroPIns and GroPCho
Figure 4-1 Summary of glycerophosphodiester transport and metabolism in *C. albicans*. This schematic offers a summary of what is currently known for glycerophosphodiester transport and metabolism in *C. albicans*. Extracellular GroPIns and GroPCho can be obtained from the environment. Presumably, PLB-mediated hydrolysis of phospholipids could also result in extracellular GroPIns and GroPCho, as it does in *S. cerevisiae*, but this has not been documented for *C. albicans*. GroPIns is transported through the permease Git1, while GroPCho is transported primarily by the permease Git3 and minimally by the permease Git4. Once internalized, GroPCho and GroPIns are hydrolyzed by the glycerophosphodiesterase, Gde1, producing GroP and either free choline or inositol. Both choline and inositol can then be reincorporated into newly synthesized phospholipids.
would be to analyze mRNA levels of putative phosphatases on CGD. Since I have shown that extracellular hydrolysis of GroPCho occurs 12-18 hours post inoculation (Fig 3-4), mRNA could be analyzed incrementally over 24 hours of growth. Furthermore, if *CaGIT2* are not present and the strains are forced to utilize GroPCho as a phosphate source, potential candidates may show increase levels of mRNA expression compared to strains grown on a different phosphate source (KH$_2$PO$_4$). Additionally, analyzing mRNA expression of putative phosphatases in strains lacking *CaGIT3* or *CaGDE1* would provide information on the role, if any, these ORFs play in the regulation of this alternate pathway from GroPCho utilization.

Another question that would be interesting to pursue is the role of CaGit2, which is currently unknown. From work performed here, CaGit2 is unable to transport GroPIns or GroPCho, but this does not rule out specificity for other glycerophosphodiester (GroPS or GroPE). Interestingly, the precursors of these glycerophosphodiesters, phosphatidylserine and phosphatidylethanolamine, are also present in the human host (145). An experiment to determine if CaGit2 has specificity for GroPS or GroPE would be to utilize a *git2*-Δ/Δ strain with either an empty vector (pDDB78) or *CaGIT2* (pDDB78GIT2) reintegrated into the genome. Along with a wild type strain (WTaday185), these two strains can be tested for their ability to utilize GroPS and GroPE as phosphate sources.

Based on work in this dissertation, the permease CaGit4 is able to transport low levels of GroPCho. This activity appears to be insignificant to CaGit3 transport activity, but may have a secondary role. It is interesting to speculate that CaGit4 and CaGit2 may also play a role in sensing alternate phosphate sources. *S. cerevisiae* has a developed
systems for sensing glucose (146) and its believed that its low affinity P\textsubscript{i} transporters may also play a role in sensing phosphate availability (113). Given that \textit{C. albicans} has increased its ability to transport alternate phosphate sources, it is not out of the questions to think that this pathogen may have developed strategies for sensing extracellular phosphate sources.

Lastly, the involvement the glycerophosphodiester utilization by the CaGit1/3/4 dependent and independent pathways has in the pathogenicity and commensal growth in a mammalian host should be pursued. Results from my dissertation show that these mechanisms are active under conditions that \textit{C. albicans} may experience in a human host, suggesting that they may play a role in nutrient acquisition during infection.
REFERENCES:


*Critical Reviews in Microbiology* **35**, 340-355


*PLoS pathogens* **6**, e1000806


Appendix

The appendix contains preliminary experiments of interest that were not pursued further and were not included in the publications that make up chapter 2 and 3. Some of the data included in this appendix is relevant for discussion purposes for Chapter 4.

A.1
A.1 Putative glycerophosphodiester permeases of non-*Candida* fungal species. NCBI BLAST search of three *C. albicans* proteins, CaGit1, CaGit3, and CaPol3 against pathogenic fungal species. Column 1 indicates the genus (bold) followed by one or more species. Columns 2 and 3 indicate the fungal strains that were sequenced to obtain the putative protein sequence and the NCBI reference sequence number for each protein. Columns 4-6 indicate the percent identity match for each putative protein against CaGit1, CaGit3, and CaPol3. Proteins with greater than 50% Identity match to CaGit1 are bolded in column 4 and the species is highlighted blue in column 1. Proteins with greater than 50% Identity match to CaGit3 are bolded in column 5 and the species highlighted orange in column 1. All of the genera listed in this figure are members of the Ascomycota phylum with the exception of *Cryptococcus*, which is a member of the Basidiomycota Phylum.
A.2. **Phosphate starvation of C. albicans induces invasive growth.** Over night culture of *C. albicans* WT (DAY185) was grown over in synthetic YNB (2% glucose) media containing 80 μg/ml uridine and 200 μM KH₂PO₄. In H₂O, cultures were diluted to OD₆₀₀ of 0.4 and then diluted 1000 fold. 50 μL of diluted culture was spread onto synthetic YNB (2% glucose) agar plates with ranging initial KH₂PO₄ concentrations. After 7 days of incubation at 30 °C, cell colonies were washed off with DI H₂O and the resulting cells that had invaded the agar were visualized under 30x magnification. Colonies grown on 0.4 mM KH₂PO₄ (A) experience increased invasion into the agar plates compared to 1mM (B) or 5mM (C) KH₂PO₄. Enlarged picture of a colony on 0.4 mM KH₂PO₄ shows a colony pre-washed (D) and post-washed (E).
A.3. GroPCho competition assay and pH dependence assay. Transport assays were performed on a *C. albicans* WT (DAY185) strain as described in Material and Methods:

(2.2.6) (A) \[^3\text{H}\]GroPCho uptake in the presence of 40 mM GroP (G3P), 40 mM Choline, 40 mM sodium phosphate (Na-P) and 50 \(\mu\)M Carbonyl cyanide 3-chlorophenylhydrazone (CCCP). (B) \[^3\text{H}\text{transport} \]GroPCho uptake in the presence of altering pH (5-8.5). GroP competes with \[^3\text{H}\]GroPCho uptake while choline and sodium phosphate do not (A). The protonophore CCCP eliminates the proton gradient, eliminating \[^3\text{H}\]GroPCho uptake (A). \[^3\text{H}\]GroPCho uptake is pH dependent (B) although a large amount of activity remains at pH 8.5.
A.4 Uptake of [³H]GroPCho by *C. albicans* clinical isolates, ICU1, ICU12, and SC5314. Transport assays were performed on a *C. albicans* WT (DAY185) strain and 3 clinical *C. albicans* isolates as described in Materials and Methods: (3.2.5) Isolates ICU1 and ICU12 were generously provided by Alverdy Lab at the University of Chicago, Chicago IL (99) and SC5314 was generously provided by the Mitchell Lab at Carnegie Mellon University, Pittsburgh PA (147). All three clinical isolates ICU1, ICU 12 (A) and SC5314 (B) were able to transport [³H]GroPCho.
A.5 Altered growth of *C. albicans* mutant *zcf30Δ/Δ* on GroPCho as sole phosphate source. *C. albicans* strains WT-TF and *zcf30Δ/Δ* were grown as described in Materials and Methods: (3.2.1). Transport assays were performed as described in Materials and Methods: (3.2.5). A strain lacking *CaZCF30* (ORF19.5251) exhibited a decrease in growth on GroPCho as the sole phosphate source in the first 12 hours post inoculation (A). Both \[^3\text{H}\] GroPCho transport activity (B) and internal \[^3\text{H}\]choline metabolites (data not shown) were unaffected in strains lacking *CaZCF30*. 
WT DAY 185

*git2-4Δ/Δ+Empty*

*git2-4Δ/Δ+ pDDB78GIT3*

*gde1Δ/Δ+Empty*
A.6 GroPCho and GroPIns are able to support *C. albicans* biofilm growth.

*C. albicans* strains were grown overnight in synthetic YNB (2% glucose) medium containing 80 µg/ml uridine and 200 µM KH₂PO₄. Four ml of bovine serum (Sigma B9433) was added to 6 well culture plates (Cellstar Cat. No. 657 185) and incubated for 24 hours. The bovine serum was replaced with 4 ml of synthetic YNB (2% glucose) media contain either 200 µM KH₂PO₄ (P), 200 µM GroPCho or 200 µM GroPIns. Overnight cultures were used to inoculate each well with the labeled strains to A₆₀₀ of 0.5 and incubated at 37°C with agitation (35 rpm) for 90 min to allow for adherence. After incubation, the cultures were removed from each well and 4 ml DI H₂O was carefully used to wash of cells that were not adhered to the plastic surface. Fresh media (4 ml) was added to each well and the plates were incubated at 37°C with agitation (35 rpm) for 24 hours. GroPCho and GroPIns were able to support biofilm growth. No differences were observed between WT(DAY185) and the various mutant strains. This suggests that the CaGit3-CaGit4 independent pathway of GroPCho utilization is sufficient to support biofilm growth. Strains are *C. albicans* WT(DAY185), git2-4Δ/Δ with an empty vector reintegrated (pDDB78) or *CaGIT3* reintegrated (pDDB78GIT3) and gde1Δ/Δ with an empty vector reintegrated (pDDB78).
A.7 Initial $[^3]$HGroPCho uptake by 24 hour C. albicans biofilms. Biofilms were grown for 24 hours as described in A-6 C. albicans WT (DAY286) in YNB (2% glucose) medium containing 80 µg/ml uridine and 200µM KH$_2$PO$_4$. Biofilms were disrupted and removed to 15 ml centrifuge tubes. Cultures were pelleted, washed and resuspended in 500 µl of 25 mM Hepes buffer (pH 5) or 25 mM HEPES buffer (pH 5) with 50 µM Carbonyl cyanide 3-chlorophenylhydrazone (CCCP) by vortexing. Samples were incubated at 37°C for 10 min with agitation and 200 µl of each sample were aliquoted. Transport assays were started by the addition of 50 µl of 1 mM $[^3]$HGroPCho and stopped after 2 min with 10 ml of cold DI H$_2$O. Samples were filtered through glass filters (GF/C) and radioactivity was determined by liquid scintillation counting. Cells from a 24 hour biofilm are able to actively transport $[^3]$HGroPCho while transport in the presence of protonophore CCCP eliminated transport activity.
A.8.1

A. Phosphatidylcholine

B. GroPCho

1. WT(DAY185)
2. git2-4Δ/Δ + pDDB78
3. git2-4Δ/Δ + pDDB78GIT3
4. gde1Δ/Δ + pDDB78

C. KH₂PO₄

D. No Phosphate
A.8.2 Growth Day 2
A.8.3 Growth Day 4
**A.8 Phosphatidylcholine supports growth of C. albicans as a phosphate source and growth is partially dependent on CaGIT2-4.**

### A.8.1 C. albicans strains (1)

WT(DAY185), (2) git2-4Δ/Δ+pDDB78, (3) git2-4Δ/Δ+pDDB78GIT3, and (4) gde1Δ/Δ+pDDB78 were grown overnight in YNB (2% glucose) media containing 80 μg/ml uridine and 200 μM KH₂PO₄. Cultures were then diluted to A₆₀₀ 0.1, 0.01, 0.001, and 0.0001 and 5 μl spotted onto YNB (2% glucose) agar plates with no phosphate (D) containing 200 μM phosphatidylcholine (A), 200 μM GroPCho (B) or 200 μM KH₂PO₄ (C). As indicated in **A.8.1**, plates were incubated at 30°C for 2 Days (**A.8.2**) and 4 Days (**A.8.3**). An observed decrease in growth is observed (**indicated by the arrows**) for git2-4Δ/Δ+pDDB78 when GroPCho is the sole phosphate source at day 2 (**A.8.2**) and when phosphatidylcholine is the sole phosphate source day 4 (**A.8.3**).
A.9 Loss of CaGDE1 alters GroPIns catabolism for C. albicans. Internal $[^3]$Hinositol metabolite were prepared as described Material and Methods: (2.2.8). Assays were incubated with 5 μM $[^3]$HGroPIns for 5 min. In a WT(DAY185) strain, $[^3]$HGroPIns and $[^3]$HInositol are detected internally. In strains lacking CaGDE1 a build up of $[^3]$HGroPIns and a decrease of $[^3]$HInositol is observed. When one copy of CaGDE1 is reintegrated back into the gde1Δ/Δ genome, $[^3]$HGroPIns and $[^3]$HInositol levels are rescued to wild type. Strain git2-4Δ/Δ+pDDB78 has similar levels as WT (DAY185), as $[^3]$HGroPIns enters through CaGit1.