CHARACTERIZATION OF THE ROLES OF PHOSPHOLIPASE B1 AND THE CYCLIC-AMP (CAMP)-PROTEIN KINASE A (PKA) PATHWAY IN NUTRITIONAL AND OSMOTIC STRESS RESPONSE IN SCHIZOSACCHAROMYCES POMBE

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ABSTRACT

Physiological stress is a reality faced by all cells. Even though these stresses are often disruptive of normal cellular function, cells must adapt to the stressor or perish. This study utilizes the model organism *Schizosaccharomyces pombe*, commonly known as fission yeast, to study a novel stress response pathway for adaptation to nutrient deprivation and hyperosmotic stress involving phospholipase B1 (Plb1) and components of the cyclic-AMP (cAMP)-protein kinase A (PKA) pathway. A previous study showed that deletion of *plb1* confers sensitivity to hyperosmotic stress in the form of potassium chloride (KCl). This phenotype could be rescued by over-expression of components of the cAMP-PKA pathway, and likewise, deletion of these components also lead to KCl-sensitivity phenotypes. This study shows that deletion of these genes also correlates with increased fragmentation of mitochondria under conditions of hyperosmotic stress. Interestingly, addition of rotenone or loss of mitochondrial PE synthesis – conditions that have previously been associated with increased mitochondrial fragmentation – exacerbated phenotypes of the *plb1Δ* mutant. Mitochondrial fragmentation was also accompanied by increased mitophagy in KCl-treated *plb1Δ* cells. Cell cycle arrest in G2/M and cytokinesis was observed in KCl-treated cells in which the gene encoding the PKA catalytic subunit *pka1* had been deleted. These phenotypes – mitochondrial fragmentation and failure to complete cytokinesis – may be due to deregulation of PS and PE synthesis and cellular distribution.
In addition to participating in hyperosmotic stress response, Plb1 and the cAMP-PKA pathway cooperate to respond to nutrient deprivation. The function of the cAMP-PKA pathway in glucose sensing is well established in yeast. Previous studies have suggested a role for phospholipases B (PLBs) in nutrient scavenging, though a limited number of studies have examined how nutrient content affects secretion of these PLBs. In this study, we have found that the secretion of Plb1 is increased in nutrient-poor media. In particular, glucose content greatly affects Plb1 secretion, since incubation in low-glucose media highly increases secretion whereas addition of glucose reduces secretion. Since the cAMP-PKA pathway is responsible for detecting glucose in the media, we predicted that deletion of \(pka1\) would lead to increased Plb1 secretion. However, Plb1 secretion was not appreciably altered in a \(pka1\Delta\) mutant. Interestingly, \(pka1\Delta\) cells did have increased levels of Plb1 protein, and the localization of Plb1 in these cells resembled that seen in glucose-starved cells, suggesting that Plb1 and the cAMP-PKA pathway may interact with regards to glucose sensing.
LIST OF ABBREVIATIONS AND SYMBOLS

<table>
<thead>
<tr>
<th>Symbol</th>
<th>Description</th>
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<tbody>
<tr>
<td>$\Delta\Psi_m$</td>
<td>mitochondrial electric membrane potential</td>
</tr>
<tr>
<td>cAMP</td>
<td>Cyclic adenosine monophosphate</td>
</tr>
<tr>
<td>CDK</td>
<td>Cyclin-dependent kinase</td>
</tr>
<tr>
<td>CHO</td>
<td>Chinese hamster ovary cells</td>
</tr>
<tr>
<td>DMSO</td>
<td>Dimethyl sulfoxide</td>
</tr>
<tr>
<td>EA</td>
<td>Ethanolamine</td>
</tr>
<tr>
<td>ER</td>
<td>Endoplasmic reticulum</td>
</tr>
<tr>
<td>ERAD</td>
<td>Endoplasmic Reticulum-Associated Degradation</td>
</tr>
<tr>
<td>GFP</td>
<td>Green Fluorescent Protein</td>
</tr>
<tr>
<td>GPI</td>
<td>glycosylphosphatidylinositol</td>
</tr>
<tr>
<td>GroPC</td>
<td>Glycerophosphocholine</td>
</tr>
<tr>
<td>GroPE</td>
<td>Glycerophosphoethanolamine</td>
</tr>
<tr>
<td>GroPI</td>
<td>Glycerophosphoinositol</td>
</tr>
<tr>
<td>KCl</td>
<td>Potassium Chloride</td>
</tr>
<tr>
<td>kDa</td>
<td>Kilodalton</td>
</tr>
<tr>
<td>MAM</td>
<td>Mitochondria-associated membrane</td>
</tr>
<tr>
<td>MAPK</td>
<td>Mitogen-activated protein kinase</td>
</tr>
<tr>
<td>PC</td>
<td>Phosphatidylcholine</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase Chain Reaction</td>
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<tr>
<td>PDK</td>
<td>Phosphoinositide-dependent kinase</td>
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<tr>
<td>Abbreviation</td>
<td>Description</td>
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<td>--------------</td>
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<tr>
<td>PE</td>
<td>Phosphatidylethanolamine</td>
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<td>PI</td>
<td>Phosphatidylinositol</td>
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<tr>
<td>PKA</td>
<td>Protein Kinase A</td>
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<tr>
<td>PLB</td>
<td>Phospholipase B</td>
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<tr>
<td>PLC</td>
<td>Phospholipase C</td>
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<tr>
<td>PLD</td>
<td>Phospholipase D</td>
</tr>
<tr>
<td>PS</td>
<td>Phosphatidylserine</td>
</tr>
<tr>
<td>SDS-PAGE</td>
<td>Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis</td>
</tr>
<tr>
<td>TLC</td>
<td>Thin Layer Chromatography</td>
</tr>
<tr>
<td>WT</td>
<td>Wild type</td>
</tr>
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</table>
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CHAPTER 1
INTRODUCTION

All living organisms, from single cells up to more complex, multicellular organisms, must maintain metabolic and structural homeostasis in order to survive and proliferate. This is a difficult enough task for a cell under normal circumstances, and is made even more difficult by perturbations from the cell’s environment. Environmental stress can come in many forms, such as the presence of a toxin or lack of an important nutrient. A cell must not only mount an immediate, temporary response to the stressor, but also initiate cellular programs that prepare the cell for long-term exposure to the stressor. These adaptations to stress are affected by signaling pathways that activate downstream players and enact cellular changes. These studies presented here elucidate a cooperative role for the cyclic-AMP (cAMP) protein kinase A (PKA) signaling pathway and the phospholipase B 1 (Plb1) in the cellular response to hyperosmotic stress and nutrient deprivation in the fission yeast *Schizosaccharomyces pombe*.

*General cellular response to hyperosmotic stress*

Cells experience hyperosmotic stress when the concentration of osmolytes, such as salt, is higher outside of the cell than that inside the cell. Hyperosmotic stress is experienced regularly in certain cell types within multicellular organisms, such as cells of the kidney and other excretory organs. Hyperosmotic stress is also experienced by single-
celled organisms, which must survive in an environment that is highly changeable with regards to hydration status. Likewise, the mechanisms by which both single- and multi-cellular organisms respond and adapt to hyperosmotic surroundings are highly conserved.

Cells rapidly expel water from the cell and subsequently shrink as an immediate consequence of increased external solute. Though this action serves to temporarily restore isotonic balance, the rapid water loss and reduction of cell size cause mechanical stress due to compaction of biomolecules. This molecular crowding, coupled with increased intracellular levels of charged molecules, severely impairs the functions of many of these biomolecules (Garner and Burg 1994). In the budding yeast, *Saccharomyces cerevisiae*, the transmembrane proteins Sho1 and Sln1 are responsible for directly sensing hyperosmotic stress, most likely via cytoskeletal perturbation caused by osmotic shock (Hohmann 2002). These osmo-sensing proteins are upstream activators of the HOG pathway, a MAP kinase pathway that is essential for growth in hyperosmotic media in *S. cerevisiae* (Brewster and Gustin 1994; Hohmann 2002). The homolog of Hog1 in the fission yeast *Schizosaccharomyces pombe*, Spc1/Sty1, is also necessary for growth in hyperosmotic media (Millar et al. 1995). Activation of these MAP kinases causes them to be translocated to the nucleus, where they regulate a number of transcription factors (Shüller et al. 1994; Shiozaki and Russell 1996; Hohmann 2002). Among the genes that are transcribed are those responsible for producing organic osmolytes that allow cells to regain a portion of their original volume without upsetting osmotic balance. This is a conserved response to hyperosmotic stress, with a variety of organic osmolytes being produced in different organisms. The principal osmolyte produced in budding and fission yeasts is glycerol, which is produced by the enzyme glycerol-3-phosphate dehydrogenase.
Another universal response to hyperosmotic stress is transient, multi-phase cell cycle arrest (Kultz et al. 1998; Michea et al. 2000; Dmitrieva and Burg 2005). Osmotic stress-induced G1, G2, and S phase arrests in *S. cerevisiae* are dependent on Hog1 activity, which controls the levels of cyclins and activates cyclin-dependent kinase (CDK)-inhibitor proteins, such as Swe1 (Clotet et al. 2006; Yaakov et al. 2009). *S. pombe* cells exposed to hyperosmotic stress arrest in G2 phase via phosphorylation and nuclear export of Cdc25, an activator of the mitotic CDK, Cdc2 (López-Avilés et al. 2005). After addition of salt stress, a certain percentage of *S. pombe* cells are stimulated to undergo anaphase followed by a temporary block in cytokinesis (Kishimoto and Yamashita 2000; Alao et al. 2010). It has been suggested that a possible role for these cell cycle blocks is to allow time for the cell to adapt to the stress.

If cellular damage from osmotic stress cannot be repaired, these damaged components must be degraded and recycled. Autophagy is an evolutionarily conserved process by which cytoplasmic materials are delivered to the vacuole for degradation and recycling. Hyperosmotic stress has been shown to induce autophagy in CHO cells, as indicated by accumulation of the autophagic marker LC3-II (Han et al. 2010). Autophagy has also been shown to be important for drought resistance in wild emmer wheat (Kuzuoglu-Ozturk et al. 2012). A transcriptomic study found that desiccation stress leads to induction of autophagy genes (Ratnakumar et al. 2010). Cells that fail to adapt to hyperosmotic conditions initiate apoptosis, a cell death program that is characterized by numerous cellular markers, including activation of caspase-3, increased permeability and
fragmentation of mitochondria, and fragmentation of chromosomal DNA (Zhang et al. 2000; Copp et al. 2005).

*General cellular response to nutrient deprivation with a focus on glucose deprivation*

Glucose is the primary energy source for the majority of unicellular eukaryotes, and in its absence, cells must alter their metabolic program. Motile cells deprived of nutrients have the option to travel to different locations to acquire new food sources. However non-motile species, such as yeasts, are dependent on nutrients available in their immediate environment. In the presence of sufficient glucose, yeast cells undergo glycolysis and divide by mitosis. But when glucose is limiting, yeasts must rely on alternate carbon sources or undergo meiosis to produce spores in the absence of a suitable carbon source (van Werven and Amon 2011).

In *S. pombe*, glucose in the environment is detected by means of the transmembrane protein Git3, which transmits a signal to a heterotrimeric G-protein complex consisting of the Gα subunit, Gpa2 and the Gβ subunit, Git5 (Hoffman and Winston 1990; Hoffman and Winston 1991; Welton and Hoffman 2000). The Gα subunit subsequently activates adenylate cyclase, Cyr1, which catalyzes the production of cyclic AMP (cAMP). cAMP binds the protein kinase A (PKA) regulatory subunit, Cgs1, causing it to dissociate from the catalytic subunit, Pka1 (Hoffman and Winston 1990; Hoffman and Winston 1991; Welton and Hoffman 2000). This pathway is responsible for repressing the transcription of genes involved in gluconeogenesis, and deletion of any of these components leads to derepression of the gluconeogenic gene *fbp1*, which encodes fructose 1,6-bisphosphatase (Hoffman and Winston 1990; Hoffman and Winston 1991;
Welton and Hoffman 2000). A similar glucose-sensing pathway is found in S. cerevisiae, except that Ras1 and Ras2 monomeric G-proteins can also activate adenylate cyclase (Santangelo 2006; Busti et al. 2010).

Adaptation to nutrient limitation in fission yeast involves switching from catabolic to anabolic pathways, increased recycling of cellular compounds, or entry into a dormant stage with reduced metabolic activity. In S. pombe, deficiency in fermentable carbon sources, such as glucose, and nitrogen, coupled with addition of a non-fermentable carbon source initiates mating, meiosis program and subsequent sporulation (Piekarska et al. 2010). Removal of nitrogen from the media induces extended cell cycle arrest in G1, also known as quiescence, which is also accompanied by an increase in autophagy (Mukaiyama et al. 2009).

Contrary to conventional logic, it seems that glucose deficiency can be beneficial to many organisms. Glucose has a pro-aging effect, since deletion of git3 and pka1 increases chronological lifespan, and over-expression of gpa2 decreases lifespan (Roux et al. 2009; Roux et al. 2006). The same effect is seen in cells without the regulatory PKA subunit cgs1, which have decreased survival in stationary phase, a growth phase in which yeast cells experience chronological aging (DeVoti et al. 1991). Similarly, deletion of HXK2, a gene involved in glycolysis in S. cerevisiae, mimics glucose deprivation and extends lifespan (Lin et al. 2002). Glucose deprivation or incubation in non-fermentable carbon sources leads to oxidative stress response in S. pombe (Kim et al. 2006; Palabiyik et al. 2012) and S. cerevisiae (Jamieson 1998). In S. pombe, glucose deprivation leads to increased transcription of stress-responsive genes through activation of Atf1 and Pap1 by the MAP kinase Sty1/Spc1 (Madrid et al. 2004; Zuin et al. 2010). Increased oxidative
stress response in glucose-deprived cells likely increases resistance to further oxidative assaults and accounts for the increased longevity observed in cells deprived of glucose.

*Changes in lipid composition and membrane structure in response to hyperosmotic stress and nutrient deprivation*

Cellular response to various stresses often involves reorganization of phospholipid content to serve a number of purposes, including the *de novo* production of lipid signaling molecules. For instance, budding and fission yeast rapidly accumulate the second messenger phosphatidylinositol-3,5-bisphosphate (PtdIns(3,5)P$_2$) following NaCl-induced hyperosmotic stress, which is dependent upon the phosphatidylinositol 3-OH kinase vps34 (Dove et al. 1997). The production of the signaling molecules inositol 1,4,5-triphosphate (InsP$_3$) and diacylglycerol (DAG) by cleavage of phosphatidylinositol 4,5-bisphosphate (PI(4,5)P$_2$) by phospholipase C (PLC) is implicated in cell shrinkage (Côté 1995). Although DAG has signaling functions, it is also converted to phosphatidic acid (PA) through the action of diacylglycerol kinase (DGK) (Munnik et al. 1998; Munnik et al. 2000; Munnik 2001). PA levels are also increased via action of phospholipase D (PLD), levels of which become elevated in response to salt stress (Munnik et al. 2000; Meijer et al. 2002). In *Chlamydomonas moewusii*, PLD-dependent accumulation of PA in response to NaCl is due to PLD’s hydrolysis of its preferred substrate dioleoyl-phosphatidylethanolmine (Arisz et al. 2003). In addition to acting as a precursor for other phospholipids, PA also has important signaling functions for several processes, including membrane trafficking and cell proliferation (English 1996). In *Chlamydomonas moewusii*, salt stress in the form of NaCl leads to transient formation of lysophosphatidic
acid (LPA) that is, in part, produced from hydrolysis of PA by PLA₂ (Meijer et al. 2001). LPA also plays a role in signaling by binding to G-protein-coupled LPA receptors (Noguchi et al. 2009). Osmotic stress associated with dehydration and hydration in plants has been accompanied by an increase in N-acylphosphatidylethanolamine (NAPE), produced from phosphatidylethanolamine precursor in the microsomes, which may play a role in signaling or in recycling of fatty acids (Chapman 2000).

Phospholipid reorganization in response to hyperosmotic stress is not limited to the production of signaling molecules. Hyperosmotic stress can also lead to changes in phospholipid composition and subsequent changes in membrane structure. Salt stress-induced changes in phospholipid composition can vary greatly, depending on the organism. For instance, the salt-sensitive yeast *S. cerevisiae* have increased levels of phosphatidylcholine (PC) and phosphatidylinositol (PI) and decreased phosphatidylserine (PS) and phosphatidylethanolamine (PE), while the halotolerant species *Candida membranefaciens* shows increased levels of PE and PI following salt stress (Khware et al. 1995; Sharma et al. 1995). Increased membrane fluidity seems to correlate with increased osmotolerance, since halophilic and halotolerant yeast species experience an increase in the levels of unsaturated fatty acids coupled with increased membrane fluidity as measured by fluorescence anisotropy (Khware et al. 1995; Turk et al. 2004). In addition, over-expression of sunflower oleate desaturases, which results in the increased prevalence of unsaturated fatty acid species and subsequent increased membrane fluidity, leads to increased salt tolerance in the salt-sensitive species *S. cerevisiae* (Rodríguez-Vargas 2007). The phospholipid PC is degraded in renal cells by the phospholipase B neuropathy target esterase (NTE) to produce the compatible osmolyte.
glycerophosphocholine (GroPC) (Zablocki et al. 1991; Gallazzini et al. 2006; Fernández-Murray and McMaster 2007). Hyperosmotic stress has also been reported to cause turnover of PC to synthesize GroPC in budding yeast.

Nutrient deprivation can also lead to changes in phospholipid composition, both to internal and external phospholipids. One way that yeast cells can compensate for loss of a nutrient is by scavenging the environment for an alternative nutrient source. Reduction of exogenous inositol or phosphate induces production of glycerolphosphoinositol (GroPI) from the hydrolysis of extracellular phosphatidylinositol (PI) (Patton et al. 1995, Merkel et al. 2005a; Patton-Vogt 2007). Glycerophosphates, such as GroPI, are then taken up by the cell via glycerophosphate transporters (Patton-Vogt 2007). Yeast cells can also change the intracellular phospholipid composition in response to nutrient deprivation. The plasma membrane of Saccharomyces cerevisiae is greatly enriched for PS when glucose is the carbon source, but PS levels are reduced when the cells are switched to media with non-fermentable carbon sources. Incubation in non-fermentable carbon sources also leads to increased cardiolipin levels in the mitochondria (Tuller et al. 1999). Cultured tobacco cells deprived of sucrose degraded membrane phospholipids, especially PC, by an autophagy-independent process (Inoue and Moriyasu 2006a; Inoue and Moriyasu 2006b).

**Phospholipases B and their known roles in stress response**

Fungal Phospholipases B (PLBs) are remarkably similar. Many fungal species have more than one gene encoding PLBs. The *S. cerevisiae* genome encodes three PLBs (Lee et al. 1994; Fyrst et al. 1999; Merkel et al. 1999), while the genome of Candida
*albicans* encodes five PLBs (Braun et al. 2005; Theiss et al. 2006), and the genome of *S. pombe* encodes six PLBs (Yang et al. 2003). Several characterized PLBs from different fungal species are either secreted into the media or retained in the cell wall or periplasmic space (Lee et al. 1994; Santangelo et al. 1999; Chen et al. 2000; Merkel et al. 2005a; Turner et al. 2006). *Cryptococcus neoformans* Plb1 is concentrated into lipid rafts in the cell membrane by means of a C-terminal glycosylphosphatidylinositol (GPI) anchor (Saifakas et al. 2006). Secretion of the protein is achieved by cleavage of the GPI anchor by an unidentified enzyme (Djordjevic et al. 2005). Fungal PLBs are also highly glycosylated, and this glycosylation is necessary for secretion of the protein (Okumura et al. 1981; Lee et al. 1994; Turner et al. 2006). Another characteristic of fungal PLBs is the extremely low pH at which they function, with optimal pH values as low as 2.5 (Sugatani et al. 1978; Chen et al. 2000; Fujino et al. 2006; Merkel et al. 2005a). *Cryptococcus* Plb1 has several important motifs that are found in PLA2 enzymes, including a lipase motif (GXSXG), a subtilisin protease aspartate motif (IXVVDSGLXXXN), and a phospholipase motif (SGGGXRAM) (Jones et al. 2007). Deletion of essential residues within these motifs reduces all three enzymatic activities of Plb1, and results of homology modeling revealed that these residues are arranged in a catalytic dyad that is similar to that of PLA2 enzymes (Jones et al. 2007). PLB enzymes hydrolyze a variety of phospholipid substrates, though they often have preferences for specific phospholipids. Purified phospholipase B from *Penicillium notatum* showed that lysophosphatidylcholine (LysoPC) was hydrolyzed 15 times faster than diacyl-phosphatidylcholine, indicating that PLB action does not likely produce lysophospholipid products (Sugatani et al. 1978). PLB1 from the pathogenic fungus *Cryptococcus neoformans* was able to hydrolyze all
phospholipids except PA, though PC and phosphatidylglycerol (PG) were preferred substrates (Santangelo et al. 1999; Chen et al. 2000). Substrate preference for PLBs is changeable based on conditions in the media. For S. cerevisiae Plb1 and Plb2, PS is preferred as a substrate at the optimum pH range of 2.5-3.5, whereas PE and PC are preferred by Plb1 and PS is preferred by Plb2 at pH of 5 and above (Merkel et al. 2005a).

Secreted PLBs have been shown to be virulence factors in pathogenic fungal strains. High phospholipase B-producing strains of Cryptococcus neoformans were better able to invade cells of the brain and lungs when injected into mice (Chen et al. 1997). Likewise, strains in which the cnplb1 gene had been knocked out were less virulent than wild type strains (Cox et al. 2001). Major components of host lung surfactant were found to be the preferred substrates of Cryptococcus Plb1, suggesting that Plb1 contributes to virulence by hydrolyzing host membranes (Santangelo et al. 1999; Chen et al. 2000). In Cryptococcus neoformans, Plb1 is necessary for evasion of host macrophages by participating in eicosinoid production (Noverr et al. 2003). Similarly, Cryptococcus Plb1 also contributes to host evasion by incorporating arachidonic acid released from macrophages into Cryptococcus membranes (Wright et al. 2007). Plb1 contributes to the process of adhesion to the host lung epithelium by producing fatty acids through hydrolysis of host plasma membranes (Ganendren et al. 2006).

Besides contributing to virulence in pathogenic fungi, PLBs also have additional functions not related to host invasion in non-pathogenic yeasts. Over-expression of Plb2 in S. cerevisiae confers resistance to lysophosphatidylcholine (Fyrst et al. 1999). In addition, deletion of all three plb genes in S. cerevisiae leads to sensitivity to excess lysoPC produced by ectopic expression of PLA1 or PLA2 genes from Arabidopsis.
These studies suggest that fungal PLBs may function to remove excess lysophospholipids from the cell. PLBs may also play a role in the response to different environmental stresses. Plb1 from *Cryptococcus neoformans* was also shown to be necessary for processing of exogenous LysoPC under conditions of hyperosmotic stress in the form of 5% glucose (Wright et al. 2007). In mammalian renal medullary cells, the organic osmolyte glycerophosphocholine (GroPC) is produced from the hydrolysis of PC by a phospholipase B called neuropathy target esterase (NTE) (Zablocki et al. 1991; Gallazzini et al. 2006; Fernández-Murray and McMaster 2007). *S. cerevisiae* has also been shown to produce GroPC from PC in response to hyperosmotic stress, though PLB activity was not implicated in this process (Kiewietdejonge et al. 2006). Plants exposed to various types of stress, including dehydration stress, produce N-acylphosphatidylethanolamine (NAPE) (Chapman 2000). In *S. cerevisiae*, NAPE production is reduced by 45% at exponential phase and 60% at stationary phase in a *plb1Δ plb2Δ plb3Δ* triple mutant, suggesting that the transacylase activity of PLB is necessary to synthesize NAPE (Merkel et al. 2005b). Our lab has previously shown that the gene encoding *plb1* in *S. pombe* is necessary for growth in hyperosmotic conditions in the form of potassium chloride (KCl) (Yang et al. 2003). In addition, we have identified several multicopy suppressors that, when over-expressed, can rescue the KCl-sensitivity phenotype of *plb1Δ*, and a summary of these genetic interactions is shown in Figure 1.1.

PLBs may also respond to stress induced by nutrient deprivation. In *S. cerevisiae*, removal of phosphate or inositol from the culture media results in increased production of GroPI, mostly likely due to secreted Plb3 activity (Patton et al. 1995, Merkel et al. 2005a; Patton-Vogt 2007). In *Cryptococcus neoformans*, Plb1 is necessary for growth when
lysoPC is the only carbon source and is also necessary for LysoPC uptake (Wright et al. 2007). Several of the genes identified in the multicopy-suppressor screen for rescue of KCl-sensitivity of plb1Δ were components of the cyclic-AMP (cAMP)-protein kinase A (PKA) signaling pathway, which responds to exogenous glucose levels (Figure 1.1; Welton and Hoffman 2000; Hoffman and Winston 1990; Hoffman and Winston 1991). Likewise, plb1Δ cells mate on rich media, a phenotype that is shared by mutants of the cAMP-PKA pathway (Yang et al. 2003).

Current studies

The goal of the current study is to elucidate mechanisms by which Plb1 responds to hyperosmotic and nutritional stresses and how it interacts with the cAMP-PKA pathway. A pivotal study done by our lab showed that deletion of S. pombe plb1 leads to growth defects on hyperosmotic media and mating on nutrient-rich media, indicating that Plb1 plays a role in hyperosmotic stress response and glucose sensing (Yang et al. 2003). Indeed, components of the cAMP-PKA pathway – the major glucose sensing pathway in fission yeast – were found to rescue the KCl-sensitivity phenotype of plb1Δ when over-expressed and conferred sensitivity to KCl when deleted (Yang et al. 2003). Components of the cAMP-PKA pathway were also identified in a multicopy suppressor screen for genes that rescued the KCl-sensitivity phenotype of plb1Δ when over-expressed (Fig. 1.1). Plb1 and several of its multicopy suppressors were found to interact genetically with Pps1, a phosphatidylserine synthase homolog that catalyzes the formation of PS (unpublished results, Figure 1.2). Many of the phenotypes of a pps1Δ mutant can be
rescued by addition of ethanolamine to the culture media, suggesting that an important role for PS is to serve as a precursor for PE synthesis (Matsuo et al. 2007).

In Chapter 2, we show that, similar to other fungal PLBs, Plb1 from *S. pombe* is secreted. This secretion is dependent upon the presence of an N-terminal signal peptide as well as N-glycosylation of the protein. The glucose content of the media was found to control the amount of Plb1 secretion, with Plb1 secretion being inversely proportional to glucose content. Deletion of *pka1*, a key component of the glucose-sensing pathway in *S. pombe*, did not significantly alter the amount of Plb1 secretion. However, Plb1 protein levels were increased in *pka1Δ* cells, and the localization of Plb1 was similar to that seen in low glucose media. These results suggest that Plb1 and the cAMP-PKA pathway cooperate to respond to reduced environmental glucose, though the cAMP-PKA pathway does not directly control Plb1 secretion.

Also in Chapter 2, we show that non-secreted Plb1 is sufficient for growth on media containing KCl, suggesting that action of intracellular Plb1 is important for response to hyperosmotic stress. Interestingly, Plb1 was found to localize to the vacuole under normal conditions as well as conditions of hyperosmotic stress. Plb1 was also found to localize to the septa in cells grown in minimal media and treated with KCl. Additionally, *plb1Δ* was hypersensitive to rotenone, a drug that has been shown to induce mitochondrial fragmentation in *S. pombe* (Wang et al. 2010). Several studies have linked loss of mitochondrial PE synthesis with mitochondrial fragmentation (Signorell et al. 2009; Joshi et al. 2012). Similarly, deletion of mitochondrial PSDs exacerbated the KCl-sensitivity phenotype of *plb1Δ* cells. In line with these data, treatment with KCl lead to prolonged mitochondrial fragmentation in *plb1Δ* cells. KCl-treated *plb1Δ* cells also had a
higher rate of mitophagy, as measured by degradation and vacuolar localization of the mitochondrial protein Sdh2. These data suggest that Plb1 may play a role in maintenance of mitochondrial structure, though the mechanism by which Plb1 participates in this process was not elucidated.

In Chapter 3, we show that pka1 is necessary for growth, not survival in hyperosmotic conditions. Further analysis showed that pka1Δ cells indefinitely arrest in G2 and cytokinesis following treatment with KCl. Previous studies have shown that PKA activity negatively regulates G2/M phase progression through interaction with Cdc25 and Wee1 (Duckworth et al. 2002; Shibuya 2003; Stanford and Ruderman 2005; Han and Conti 2006). We hypothesized that loss of pka1 lead to misregulation of Cdc25 under conditions of hyperosmotic stress. However, over-expression of wee1 did not rescue the growth of pka1Δ on hyperosmotic media. The cytokinesis arrest seen in KCl-treated pka1Δ cells was interesting, since cytokinesis defects are also observed in KCl-treated plb1Δ cells. It could be speculated that Plb1 and Pka1 play a role in proper positioning of PE to the site of cytokinesis under conditions of hyperosmotic stress.

In Chapter 4, we show that cells lacking the gene encoding adenylate cyclase, cyr1, have increased levels of the catalytic subunit Pka1, approximately half of which is hyperphosphorylated. Interestingly, cyr1Δ cells grown in hyperosmotic conditions - in which PKA activity has been shown to be essential - as well as in glucose starved conditions - in which PKA activity is thought to be downregulated - show increased proportions of Pka1 protein in the hyperphosphorylated state. Combination of hyperosmotic stress and glucose starvation also lead to increased proportion of Pka1 protein in the hyperphosphorylated state in cyr1Δ cells, suggesting that the purpose of
this phosphorylation is to maintain Pka1 activity under conditions that have reduced cAMP signaling. Interestingly, hyperphosphorylation of Pka1 was not observed in cells lacking the gene for the PKA regulatory subunit, \( cgsl \), suggesting that Cgs1 physically participates in hyperphosphorylation of Pka1. It is also possible that increased Pka1 activity seen in \( cgsl^\Delta \) cells negatively regulates hyperphosphorylation of Pka1. Interestingly, conditions that lead to hyperphosphorylation of Pka1 in WT cells were associated with reduced Pka1 protein levels in cells with the \( cgsl^\Delta \) mutation. This suggests that a possible role of this hyperphosphorylation is to increase the stability of the Pka1 protein and prevent its degradation.
REFERENCES


Figure 1.1
FIGURE LEGEND

Figure 1.1. Summary of multicopy suppressor screen to find genes that rescue the KCl sensitivity phenotype of \( plb1\Delta \) when over-expressed.

Summary of the multicopy suppressor screen to find genes that rescue the KCl-sensitivity phenotype of \( plb1\Delta \) cells when over-expressed. Gene products identified from the screen are colored in green, while the proteins colored in blue are intended to fill in components of the cAMP-PKA pathway. The catalytic PKA subunit, Pka1, and the arrestin-related protein, Pbs4, are placed parallel to Plb1, since genetic studies have indicated that these protein work in parallel pathways to regulate hyperosmotic stress response.

Figure 1.2. Summary of major phospholipid biosynthesis pathways.

In \( S. \ pombe \), CDP-diacylglycerol (CDP-DAG) is a shared precursor for synthesis of PS and PI. PS synthesis is catalyzed by phosphatidylserine synthase, Pps1, while PI is synthesized via phosphatidylinositol synthase, Pis1. PS can be converted to PE by action of phosphatidylserine decarboxylases, Psd1 and Psd2 in the mitochondria, or Psd3 in the Golgi/vacuole. PE produced in the mitochondria or Golgi is transported to the ER, where it is converted to PC by phosphatidylethanolamine methyltransferases Cho1 and Cho2. PE and PC can also be synthesized from ethanolamine and choline precursors, respectively, via enzymes of the Kennedy pathway. Reactions in the Kennedy pathway take place in the ER.
CHAPTER 2

SECRETED PHOSPHOLIPASE B1 (PLB1) RESPONDS TO GLUCOSE DEPRIVATION WHILE NONSECRETED PLB1 IS NECESSARY FOR OSMOTIC STRESS RESPONSE AND MAINTENANCE OF MITOCHONDRIAL STRUCTURE

McInnis, B., Mitchell, J., Wang, Y., Bishop, A., Patton-Vogt, J., Marcus, S. Stevan Marcus prepared figure 4.1. Brittney McInnis collected and analyzed the data for figure 2.2A-B, 2.3A-D, 2.5, 2.6, and 2.7A, D-E. Brittney McInnis and Jessica Mitchell collected and analyzed data for figure 2.2C. Andrew Bishop collected and analyzed the data for figure 2.3E. Jessica Mitchell collected and analyzed the data for figure 2.4. Yiwei Wang collected and analyzed data for figure 2.7B-C. Brittney McInnis, Stevan Marcus, and Jana Patton-Vogt co-wrote the manuscript.
ABSTRACT

Fungal phospholipases B (PLBs) possess several enzymatic activities and have been shown to hydrolyze all classes of phospholipids, giving these enzymes the potential to function in various phospholipid-remodeling processes within the cell. Several studies have alluded to a function for secreted PLBs in environmental phospholipid scavenging, but few studies have explored how nutrient levels affect PLB secretion. Similar to other fungal PLBs, we show that Plb1 from *Schizosaccharomyces pombe* is secreted, and the presence of an N-terminal signal peptide and N-glycosylation is necessary for this secretion. Interestingly, we found that the rate of Plb1 secretion is influenced by the quality of the cells’ culture media, with secretion being increased in lower nutrient media. Plb1 was also found to localize internally to vacuoles, perhaps due to endocytosis of secreted Plb1 or diversion of the protein from the secretory pathway. Addition of glucose to low nutrient media substantially reduced Plb1 secretion, indicating a role for glucose as a signaling molecule to control Plb1 secretion. Deletion of *pka1*, which is part of the primary glucose response pathway in *S. pombe*, did not increase secretion of Plb1. However, intracellular Plb1 protein levels were increased in *pka1Δ* cells, and the localization of Plb1-GFP was similar to that seen in glucose starved WT cells. These results indicate that, though the rate of Plb1 secretion is affected by exogenous glucose availability, modulation of secretion is due to the action of some signaling pathway other than the cAMP-PKA pathway. In a previous study, we reported that Plb1 was necessary for growth on hyperosmotic media. We show here that a non-secreted form of Plb1 is sufficient for growth on hyperosmotic media, indicating that Plb1 affects phospholipid composition within the cell to respond to hyperosmotic stress. Phenotypes of *plb1Δ* are
exacerbated by addition of the drug rotenone and loss of mitochondrial phosphatidylethanolamine synthesis, both of which have been associated with mitochondrial fragmentation. Similarly, plb1Δ cells treated with 1.2 M KCl show prolonged mitochondrial fragmentation. Cells lacking plb1 were also associated with increased degradation of the mitochondrial protein Sdh2 after extended periods of KCl stress, suggesting that mitophagy is increased in these cells. These results suggest that Plb1 is necessary to maintain mitochondrial integrity following treatment with hyperosmotic stress.
INTRODUCTION

Many fungal species produce phospholipase B (PLB) enzymes, which possess a number of enzymatic activities. Fungal PLBs have phospholipase B and lysophospholipase activities that enable PLB enzymes to hydrolyze fatty acids from both sn1 and sn2 positions almost simultaneously. Fungal PLB’s also possess lysophospholipid acyltransferase activity in which a fatty acid is transferred to a lysophospholipid to produce a new phospholipid (Köhler et al. 2006; Ramrakhiani and Chand 2011). Most studied PLBs are secreted proteins that have been found to be highly glycosylated (Lee et al. 1994; Chen et al. 2000; Fujino et al. 2006). PLB enzymes have been found to be important virulence factors for several pathogenic fungi, including Cryptococcus neoformans and Candida albicans. High phospholipase B-producing strains of Cryptococcus neoformans and Candida albicans were more virulent, which correlated with increased host mortality (Ibrahim et al. 1995; Chen et al. 1997). Secreted PLBs enhance adhesion to lung epithelium and promote evasion of host macrophages in Cryptococcal infections (Ganendren et al. 2006; Noverr et al. 2003; Wright et al. 2007). However, >85% of phospholipase activity in Cryptococcus is cell-associated, suggesting that non-secreted PLB is also important for cellular function (Djordjevic et al. 2005; Santangelo et al. 1999).

The function of PLBs in non-pathogenic yeast is somewhat unclear. This is complicated by the fact that deletion or over-expression of all three PLBs in S. cerevisiae does not affect viability (Merkel et al. 2005a). Secreted PLB has been found to play a role in detoxification of exogenous lysophosphatidylcholine (LPC) (Fyrst et al. 1999; Merkel et al. 1999). PLBs in S. cerevisiae were also found to confer resistance to LysoPC
produced intracellularly by ectopic expression of PLA$_2$ from *Arabidopsis thaliana* (Zhang et al. 2009). PLBs may play a role in hyperosmotic stress response in the fission yeast *Schizosaccharomyces pombe*, since loss of the *plb1* gene confers sensitivity to media containing high concentrations of salt (Yang et al. 2003). In renal medullary cells, the phospholipase B neuropathy target esterase 1 (Nte1) produces the osmolyte glycerophosphocholine (GroPC) from phosphatidylcholine (PC) in response to hyperosmotic stress (Zablocki et al. 1991; Gallazzini et al. 2006; Fernández-Murray and McMaster 2007). *S. cerevisiae* cells exposed to osmotic stress also turn over PC to produce GroPC, though PLB activity was not implicated in this process (Kiewietdejonge et al. 2006). Several types of cellular stress have induced the production of N-acylphosphatidylethanolamines (NAPEs) in plants and yeast (Chapman 2000; Merkel et al. 2005b). NAPEs were found to be reduced by 45% at exponential phase and 60% at stationary phase in *S. cerevisiae* strains lacking all three PLBs, suggesting that the transacylase activity of one of these PLBs is necessary for NAPE synthesis (Merkel et al. 2005b). However, loss of all three *plbs* in *S. cerevisiae* does not confer sensitivity to hyperosmotic stress (unpublished observation).

Secreted PLBs may function to scavenge phospholipids from the environment. *S. cerevisiae* Plb1 and Plb3 are responsible for production of extracellular GroPC/GroPE and GroPI, respectively (Lee et al. 1994; Merkel et al. 2005a). In *S. cerevisiae*, GroPI production is increased when cells are grown in media lacking inositol or containing low amounts of phosphate (Patton et al. 1995, Patton-Vogt 2007). *Cryptococcus neoformans* Plb1 is necessary for growth when LysoPC is the sole carbon source (Wright et al. 2007). Likewise, altering the composition of culture media can affect the rate of PLB secretion.
Introduction of the pathogenic yeast *Cryptococcus neoformans* into media that resembles a host environment increases the rate of secretion of phospholipase B (PLB), lysophospholipase (LPL), and lysophospholipase transacylase (LPTA) activities (Wright et al. 2002). Addition of glucose from a concentration of 0.1% to 1% did not affect secretion of PLB, LPL or LPTA activities in *Cryptococcus*, though increasing glucose to hyperosmotic levels (5%) did increase secretion (Wright et al. 2002; Wright et al. 2007). Re-feeding glucose to stationary *S. cerevisiae* cultures increases the rate of GroPI production (Hawkins et al. 1993). These results suggest that addition of glucose to the media stimulates PLB secretion.

In this study, we show that, similar to PLBs in other fungi, Plb1 from *Schizosaccharomyces pombe* is secreted and highly N-glycosylated. N-glycosylation and the N-terminal signal peptide are both needed for secretion of the protein. Secretion of Plb1 is remarkably increased in media with reduced nutrient content, and the amount of secretion was inversely proportional to the amount of glucose in the media. In fact, addition of glucose to the media decreased Plb1 secretion. However, this glucose-controlled secretion may be independent of the cAMP-PKA signaling pathway, since Plb1 secretion is barely increased in a *pka1Δ* mutant. However, the levels of Plb1-GFP protein is increased in a *pka1Δ* mutant, and the localization of Plb1-GFP in *pka1Δ* cells resembles that seen in wild type cells grown in low glucose media. These data suggest that secreted Plb1 plays a role in nutrient scavenging, especially under conditions of nutrient deprivation. In contrast, non-secreted Plb1 function is important for growth on hypertonic media, since expression of the non-secreted GFP-Plb1 can rescue growth of *plb1Δ* on hyperosmotic media.
One function of Plb1 in hyperosmotic media is the maintenance of mitochondria structure, though it is unclear how Plb1 participates in this process.
MATERIALS AND METHODS

Strains and growth media

*S. pombe* strains used in this study were derived from the *SP870* wild type strain, which are summarized in Table 4.1. The construction of *psd* mutant strains was described previously (Luo et al. 2009). The SPPLBIGFP strain was constructed using the 2-step PCR method described by Krawchuk and Wahls (1999). SP870 genomic DNA and the pFA6a-GFP (S65T)-kanMX6 plasmid were used as template with the oligonucleotide primer sets F1 5’- TGTCCAAAACCTTATGAGCGTG-3’ and R1 5’-GGGGATCCGTCGACCTGACCGTACGAAATGCGCTTACCAAGACGGC-3’, and F2 5’- GTTTAAACGAGCTCGAATTCATCGATTGAATCAATCCAATTACGCG-3’ and R2 5’- CACGTAAACTCACAGTCATCG-3’. Transformants were selected on YES + 0.1g/L G418, and the *plb1-GFP* fusion was detected using colony PCR. The *plb1::kanMX6* (SPPLB1K), *plb1::hphMX6* (SPPLB1H) and *plb1::ura4::ADE2* strains were constructed in a similar manner, except that the pFA6a-kanMX6, pFA6-hphMX6, and pFA6-ura4 plasmids were used as template with the oligonucleotide primers F1 5’–CTTAATCCATCCAAGCGAGG-3’ and R1 5’–GGGGATCCGTCGACCTGACCGTACGAAATGCGCTTACCAAGACGGC-3’, and F2 5’–GTTTAAACGAGCTCGAATTCATCGATTGAATCAATCCAATTACGCG-3’ and R2 5’–CACGTAAACTCACAGTCATCG-3’. The *ADE2* gene was inserted into the *ura4* gene in the *plb1::ura4* construct using the pVIN1 plasmid.

*S. pombe* cells were grown in either YES media (0.5% yeast extract, 3% glucose, 250 mg/L adenine, 250 mg/L uracil, 250 mg/L leucine, 250 mg/L lysine, 250 mg/L histidine), YES-G (0.5% yeast extract, 0.1% glucose, 3% glycerol, 250 mg/L adenine, 250 mg/L uracil, leucine, 250 mg/L lysine, 250 mg/L histidine), or synthetic minimal
medium (EMM) containing required auxotrophic supplements at a concentration of 250 mg/ml each.

**Plasmid construction**

To construct the pREP41GFP-Plb1, the *plb1* gene was amplified using the forward primer 5’-GGAAGGTGACACTGCTTTTCCGGAGATACAG-3’ and the reverse primer 5”-CATTGGATCTTTAAATGCTTCACCAAGAC-3’ to add *Bam* HI and a *Sal* I recognition sites to the 5’ and 3’ ends of the *plb1* protein coding sequence, respectively. The PCR product was digested with *Bam* HI and *Sal* I and cloned into the corresponding sites of pREP41GFP and pAAUCMGFP.

**Cell lysate preparation and immunoblotting**

*S. pombe* whole-cell lysates were prepared from boiled cells as described (Correa-Bordes and Nurse 1995) to inactivate the majority of protease activity prior to lysing the cells with glass beads. Proteins were separated by SDS-PAGE, transferred to nitrocellulose membranes, and Plb1-GFP fusion proteins were detected using anti-GFP monoclonal antibody (Roche, catalog no. 11814460) and the SuperSignal West Dura chemiluminescence detection system (Pierce). The colony immunoblotting assay for detecting secreted proteins was performed as described (Codlin and Mole 2009).

**Fluorescence microscopy and vacuolar and mitochondrial staining**

Staining of vacuolar membranes was performed as described (Iwaki et al. 2004). Ten ml of cells grown to 2×10^6 cells/ml were centrifuged and resuspended in 2 ml media plus 20 μM SynaptosRed (Calbiochem, catalog no. 574799). The cells were stained for 15 minutes at 30°C with shaking, washed once with 10 ml of media, and resuspended in 10 ml of media. The cultures were incubated at 30°C with shaking for 90 minutes. Mitochondria were stained with either 0.05 μM MitoTracker-Red or 0.1 μM MitoTracker
Green for 20 minutes at 30°C. All stained cells were visualized by epifluorescence microscopy using a Nikon 90i epifluorescence microscope system equipped with a CoolSNAP HQ2 monochrome CCD camera (Photometrics).

**Isolation of secreted, periplasmic, membrane, and cytoplasmic fractions**

For isolation of secreted proteins, 1x10⁸ cells grown in YES-G, EMM ± 1.2 M KCl, or YES ± 1.2 M KCl were spotted onto PDVF membranes laid onto respective media and incubated at room temperature for 18 hours. Cells were washed from the membranes, and proteins were eluted by vortexing in elution buffer (2% SDS, 1% Triton X-100, 50 mM Tris-HCl, pH 9.5) at 4°C for 10 minutes. Periplasmic space was isolated as described (Merkel et al. 2005). Cells grown to 1x10⁷ cells/ml were pelleted, washed with digestion buffer (1.2 M sorbitol, 10 mM KH₂PO₄, pH 7.4), and resuspended in digestion buffer containing 3 mg/ml zymolyase 20T and protease inhibitors to a final density of 0.3 g of wet cells per milliliter. The cells were incubated at 30°C with gentle agitation for 30 minutes and monitored for cell wall digestion. Spheroplasts were pelleted by centrifugation at 700 ×g for 20 minutes. The supernatant containing periplasmic extracts was collected and centrifuged at 15,000 ×g for 15 minutes to remove particulate matter. The remaining spheroplasts were washed with digestion buffer, resuspended in lysis buffer (0.6 M sorbitol, 10 mM imidazol-HCl, pH 6.4, 2 mM EDTA) containing protease inhibitors, and homogenized using a glass Dounce homogenizer. Cell debris and unbroken cells were removed by centrifugation at 1000 ×g for 10 minutes at 4°C, and the supernatant was centrifuged at 100,000 ×g for 1 hour at 4°C to separate the membrane fraction (pellet) and the cytoplasmic fraction (supernatant). Protein concentration was quantified by BCA, and a total of 17.5 µg of protein was loaded onto duplicate SDS-PAGE gels for subsequent immunoblotting and Coomassie staining.
Detection of extracellular phospholipase B activity

Extracellular PLB activity was measured as described (Michinaki et al. 2003). Briefly, cells were grown in EMM to log phase and diluted to $A_{600} = 0.2$ in fresh medium. 2.5 µg/ml of lysopalmitoyl phosphatidylcholine-L-1-[palmitoyl-1-14C] (Perkin Elmer) was added to the medium. Following 2 minutes of incubation at room temperature with agitation, the medium was separated from the cells by centrifugation. Lipids in the medium were extracted by adding 0.3 volumes of 0.1M citric acid and 4 volumes of CHCl₃: MeOH (2:1, v/v). The organic phase was removed to a fresh glass tube and dried under nitrogen gas. The lipids were then suspended in CHCl₃: MeOH (2:1, v/v) and spotted on a thin-layer chromatography (TLC) silica gel plate (Whatman) and separated by the solvent hexane:diethylether:acetic acid (80:30:1, v/v). The TLC plate was exposed to a phosphor storage screen and visualized by a Typhoon 8600 variable mode imager (Amersham Pharmacia Biotech). The lipids were quantified using Image-Quant software.

Lipid Analyses

Lipids were extracted from spheroplasted cells or organelles using chloroform-methanol as described (Bligh and Dyer 1959). For whole cell lipid analysis, phospholipids were separated on Whatman LK-5 silica plates using previously described methods (Fine and Sprecher 1982). Plates were sprayed with 0.05% primuline in 80% acetone, and phospholipids were visualized by exposure to UV light. Phospholipids were then quantified by densitometry.
RESULTS

*N-glycosylation and proteolytic cleavage of Plb1 are required for its secretion*

Similar to other fungal PLBs, the amino acid sequence of Plb1 has several asparagine residues in the consensus sequence (X-N-X-S/T) that are predicted to be N-glycosylated as well as a predicted N-terminal signal peptide that traffics the protein into the secretory pathway (Fig. 2.1). To test whether Plb1 protein is secreted, a colony dot blot was performed using cells expressing Plb1 that had been tagged on the C- or N-terminus with GFP. We predicted that tagging the N-terminus of Plb1 would block the signal peptide and prevent secretion of the protein. As shown in Fig 2.2A, GFP-tagged protein was found to be secreted from cells expressing Plb1-GFP, but not GFP-Plb1, demonstrating that Plb1 is processed through the secretory pathway via the N-terminal signal sequence. Treatment with the drug tunicamycin, which inhibits N-glycosylation, abolishes secretion of Plb1 (Fig. 2.2A). Interestingly, immunoblotting of protein secreted into the media revealed only the GFP tag (~26 kDa), which was most likely produced via proteolytic cleavage of Plb1-GFP (Fig. 2.2B). Within the cell, Plb1 is found primarily in the membrane fraction, with the major band being ~120 kDa, which may correspond to a glycosylated form (Fig. 2.2B). To test whether this higher molecular weight band was due to glycosylation of Plb1 protein, we treated cells expressing Plb1-GFP with tunicamycin, a drug known to inhibit N-glycosylation. Treatment with tunicamycin reduced the molecular weight of Plb1-GFP to that of GFP-Plb1, which does not enter the secretory pathway and is not expected to be glycosylated (Fig. 2.2C). These results indicate that Plb1 undergoes N-glycosylation, and that this glycosylation is required for secretion of Plb1.
Effect of nutrient quality of media on apparent Plb1 secretion

Interestingly, we observed that the degree of Plb1 secretion depends on the nutritional quality of the media, assuming that the GFP protein detected in the media is due to hydrolysis of secreted Plb1-GFP. For instance, Plb1 secretion is barely detectable in cells cultured in nutrient-rich media (YES), but is extremely robust in cells cultured in minimal media (EMM), which contains less glucose (Fig. 2.3A). Additionally, cells cultured in media with reduced glucose (YES-G) display a remarkable increase in Plb1 secretion (Fig. 2.3B). However, this differential secretion is not due to a difference in Plb1 glycosylation, as Plb1 has similar molecular weight in each different type of media (Fig. 2.3C). Interestingly, loss of plb1 only conferred a slight growth defect on EMM media, but not on YES or YES-G media (Fig. 2.3D). The apparent dispensability of Plb1 function on low nutrient media may be due to the fact that Plb1 shares function with additional PLBs and accounts for a small portion of total secreted PLB activity (Fig. 2.3E). Plb1-GFP was also shown to localize intracellularly to the vacuole in YES and YES-G, and to a lesser degree EMM (Fig. 2.4A-C). This vacuolar localization could be due to diversion of the Plb1 protein from the secretory pathway directly to the vacuole, or it could be due to endocytosis of secreted Plb1-GFP protein. In addition to vacuolar localization, Plb1-GFP is also shown to localize to the septum in EMM-cultured cells (Fig. 2.4B) and to the cell periphery in cells cultured in YES-G (Fig. 2.4C).
Secretion of Plb1-GFP is controlled by environmental glucose

We have observed that Plb1 secretion is increased in cells cultured in EMM and YES-G, as compared to cells cultured in YES. One primary difference among these media types is the concentration of glucose, with YES having 3% glucose, YES-G 0.1% glucose, and EMM 2% glucose. To test whether glucose acts as a signaling molecule to control Plb1 secretion, we performed a colony dot blot of cells expressing plb1-GFP on EMM media with the normal 2% glucose as well as EMM containing 3% glucose. As shown in figure 2.5A, Plb1 secretion is reduced in minimal media that has been supplemented with additional glucose, indicating that environmental glucose content can regulate the amount of Plb1 secretion. Based on this result, we next asked whether Plb1 secretion is regulated by signaling pathways that detect and respond to glucose availability. Fission yeast cells detect glucose using the transmembrane protein Git3, which activates the cyclic AMP (cAMP)-protein kinase A (PKA) signaling cascade (Hoffman and Winston 1990; Hoffman and Winston 1991). To determine if this pathway negatively regulates Plb1 secretion, we monitored Plb1 secretion in a strain lacking the gene pka1, which encodes the PKA catalytic subunit. As shown in figure 2.5B, Plb1 secretion was increased only slightly in pka1Δ cells. Further molecular analysis revealed that pka1Δ cells have increased levels of intracellular Plb1 protein (Fig. 2.5C). Interestingly, the localization of Plb1-GFP resembles that found in cells grown in YES-G media, with localization mostly to the vacuole lumen and the cell periphery (Fig. 2.5D).
Intracellular Plb1 activity is necessary for growth in hyperosmotic media

Our lab has previous shown that the \textit{plb1} gene is required for growth of \textit{S. pombe} cells in media containing high concentrations of KCl (Fig. 2.6A; Yang et al. 2003). Since we have found that secreted Plb1 is important for response to reduced extracellular glucose, we wondered whether secreted or non-secreted Plb1 is important for response to hypertonic stress. To answer this question, we tested whether non-secreted GFP-Plb1 could rescue the hyperosmotic growth defect of \textit{plb1}Δ cells. Indeed, non-secreted Plb1 is sufficient for growth on hyperosmotic media (Fig. 2.6A), indicating that intracellular Plb1 activity is important for hypertonic stress response. However, analysis of whole cell phospholipid levels has revealed no major differences between WT and \textit{plb1}Δ (Fig. 2.6B). We next wondered whether Plb1 was needed to affect phospholipid composition in a specific intracellular compartment during hyperosmotic stress response. We then observed the localization of GFP-tagged Plb1 protein. In hyperosmotic conditions, both Plb1-GFP and GFP-Plb1 were observed to colocalize to vacuoles, though many cells expressing GFP-Plb1 also showed localization to the nuclear periphery (2.6C). Plb1-GFP was also observed to localize to the septum in cells grown in EMM and treated with 1.2 M KCl.

Interestingly, \textit{plb1}Δ cells also show increased sensitivity to rotenone, a mitochondrial toxin that has been shown to induce fragmentation of mitochondria in \textit{S. pombe} (Fig. 2.7A; Wang et al. 2010). Interestingly, loss of mitochondrial phosphatidylserine decarboxylases Psd1 and Psd2, which catalyze the formation of phosphatidylethanolamine from phosphatidylserine (Matsuyama et al. 2006; Luo et al. 2009), exacerbated the KCl-sensitive phenotype of \textit{plb1}Δ (Table 2.2). Defective synthesis
of mitochondrial PE has been associated with mitochondrial defects, including mitochondrial fragmentation (Steenbergen et al. 2005; Signorell et al. 2009; Joshi et al. 2012). Interestingly, loss of Golgi/vacuolar Psd3 confers resistance to KCl stress in WT cells and partially rescues \textit{plb1}\textsuperscript{Δ} cells (Table 2.2). It should be noted that the loss of \textit{psd3} does not provide rescue from KCl-induced stress unless \textit{psd1} is also present. Similarly, treatment with 1.2 M KCl induces mitochondrial fragmentation at early timepoints in both WT and \textit{plb1}\textsuperscript{Δ} cells (Fig. 2.7B). However, WT cells regain mitochondrial organization at later timepoints, while \textit{plb1}\textsuperscript{Δ} mitochondria remain fragmented (Fig. 2.7B). In addition, \textit{plb1}\textsuperscript{Δ} cells have reduced mitochondrial membrane potential (\(\Delta\Psi_m\)) after 24 hours of KCl treatment, compared to WT cells (Figure 2.7C).

Fragmentation of the mitochondrial network results in refusion of healthy mitochondrial fragments, whereas depolarized fragments are unable to re-fuse and are subsequently degraded via mitophagy (Okamoto and Kondo-Okamoto 2012). The observation that \textit{plb1}\textsuperscript{Δ} cells exhibit fragmented mitochondrial with reduced \(\Delta\Psi_m\) after prolonged treatment with KCL lead us to think that Plb1 is involved in a stress-induced mitophagic process that removes defective mitochondria to the vacuole for subsequent degradation. To test this, we monitored the degradation of a GFP-tagged mitochondrial protein Sdh2. We predicted that, if Plb1 promotes mitophagy under conditions of hyperosmotic stress, we would see decreased degradation of Sdh2-GFP in a \textit{plb1}\textsuperscript{Δ} mutant. Converse to our hypothesis, the \textit{plb1}\textsuperscript{Δ} mutant showed increased degradation of Sdh2-GFP as well as colocalization of Sdh2-GFP to vacuolar compartments following KCl treatment, indicating that these cells have increased rates of mitophagy compared to WT cells (Figure 2.7D).
However, the rate of autophagy, as measured by degradation of the autophagy protein Atg8, is not appreciably greater in plb1Δ cells compared to WT cells treated with 1.2 M KCl (Figure 2.7E).
DISCUSSION

In this study, we have elucidated various roles for secreted and non-secreted phospholipase B1 (Plb1) in fission yeast. Similar to other characterized fungal PLBs, *S. pombe* Plb1 is a highly glycosylated protein that is processed through the secretory pathway. This glycosylation is necessary for secretion of Plb1, since the non-glycosylated protein would be misfolded and prevented from exiting the ER. Though GFP-tagged product was found to be secreted from cells expressing Plb1-GFP, SDS-PAGE analysis of secreted protein revealed only GFP protein. This is likely the result of cleavage of the C-terminus of Plb1-GFP, since GFP protein is not predicted to be secreted. In *Cryptococcus neoformans*, secreted Plb1 appears to be modified by proteolytic cleavage, which is necessary for release of Plb1 from the cell wall into the media (Djordjevic et al. 2005). Interestingly, the rate of Plb1-GFP secretion depended on the nutrient quality of the media. This suggests a phospholipid-scavenging role for Plb1 under conditions of nutrient limitation that is not unprecedented. For instance, phosphate limitation in budding yeast increases transcription of Git3, a permease that transports glycerophosphoinositol (GroPIns), a product of PI cleavage, into the cell (Almaguer et al. 2004). This is accompanied by increased production of GroPI, presumable produced by hydrolysis of extracellular PI by Plb3 (Patton et al. 1995, Patton-Vogt 2007). We observed that glucose content in the culture media was a key regulator of Plb1-GFP secretion. Cells grown in minimal media (EMM) and media with reduced glucose (YES-G) showed increased secretion of Plb1-GFP product compared to cells grown in rich media (YES) and minimal media supplemented with 3% glucose, which showed reduced secretion of Plb1-GFP product. Studies in other fungi also point to a role for PLBs in
response to glucose limitation. For instance, Plb1 is required for growth on glucose-limited media in which LysoPC is the only carbon source in *Cryptococcus neoformans* (Wright et al. 2007). Plb1 may play a role in hydrolyzing external phospholipids into glycerol phosphate and fatty acid components that can be internalized via specific transporters and integrated into the gluconeogenic pathway. Interestingly, Plb1 secretion was not increased when *pka1*, a component of a major glucose-sensing pathway in *pombe*, was deleted. However, *pka1Δ* cells have more intracellular Plb1 protein, which shows localization similar to that seen in wild type cells starved of glucose. While the cAMP-PKA pathway does not appear to directly regulate Plb1 secretion, there does seem to be some interaction between Plb1 and the cAMP-PKA pathway pertaining to response to nutrient deprivation.

Although secreted Plb1 activity seem to be important for responding to nutritional deficiency, non-secreted Plb1 appears to be more important for adaptation to hypersaline conditions. This conclusion is based on the experiment in which non-secreted GFP-Plb1 is able to rescue the KCl-sensitivity phenotype of *plb1Δ*. Our results suggest that at least one intracellular compartment for which Plb1 activity is important is the mitochondria. The worsened KCl-sensitive phenotype of *plb1Δ* due to loss of mitochondrial PE production by deletion of *psd1* and *psd2* may be due to excessive mitochondrial fragmentation brought on by the loss of mitochondrial PSDs (Signorell et al. 2009; Joshi et al. 2012). This may also explain the growth sensitivity of *plb1Δ* cells to rotenone, a drug that has been shown to induce mitochondrial fragmentation (Wang et al. 2010). Treatment with KCl leads to prolonged mitochondrial fragmentation and increased degradation of mitochondrial protein. This suggests that Plb1 is necessary to restore the
mitochondrial network following KCl stress and prevent excessive damage to mitochondria. This leads us to wonder as to the origin of the mitochondrial fragmentation observed in KCl-treated plb1Δ cells. The genetic interaction of plb1 with mitochondrial PE biosynthetic genes suggests that Plb1 may play a role in maintaining mitochondrial PE levels. Sufficient mitochondrial PE levels are important for maintenance of the mitochondrial network (Steenbergen et al. 2005; Signorell et al. 2009; Joshi et al. 2012). In addition, PE must be balanced with PS in order to stabilize a bilayer structure (Lewis and McElhaney 2000; Li and Schick 2000).
REFERENCES


Table 2.1. *S. pombe* strains used in this study.

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Table 2.2. *plb1* interacts with genes involved in PE synthesis.\(^a\)

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\(^a\)1 mM ethanolamine was added to the media.

\(^b\)(+) indicates relative cell growth
### Figure 2.1.

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Figure 2.2.
Figure 2.3.
Figure 2.4.
Figure 2.5.
Figure 2.6.
Figure 2.7.
FIGURE LEGENDS

Figure 2.1. Plb1 is predicted to be N-glycosylated and secreted.
Amino acid sequence of Plb1 showing putative N-terminal signal peptide required for entry into the secretory pathway (underlined residues) and asparagine residues predicted to be N-glycosylated (highlighted residues).

Figure 2.2. Secretion of Plb1 depends on N-glycosylation.
(A) Colony dot blot of cells expressing GFP-tagged Plb1 treated with tunicamycin. Cells expressing plb1-GFP, treated (+T) or untreated (-T) with 10 µM tunicamycin for 4 hours, and plb1Δ cells expressing GFPPlb1 were spotted onto nitrocellulose laid over minimal media. Cells were incubated at 25°C for 18 hours. Cells were washed off, and GFP-tagged protein was detected using anti-GFP antibody. Purified GFP was spotted onto the membrane as a positive control. (B) Immunoblot of secreted (S), periplasmic (P), membrane (M), and cytoplasmic (C) fractions from cells expressing Plb1-GFP. These fractions were isolated as described in the Materials and Methods, and protein was separated by SDS-PAGE. Proteins were transferred to nitrocellulose and immunoblotted against GFP. (C) Immunoblot of GFP-tagged Plb1 protein from cells treated with tunicamycin. Lysates from cells expressing Plb1-GFP and GFP-Plb1 were subjected to SDS-PAGE and immunoblotting against GFP and tubulin. Cells were treated with DMSO (+D), treated with 10 µM tunicamycin (+T), or not treated (-).
Figure 2.3. The rate of Plb1 secretion is inversely related to nutrient quality of media.

(A) Colony dot blot of cells expressing plb1-GFP grown in nutrient rich (YES) or nutrient-poor (EMM) media. Cells grown in either EMM or YES were spotted onto nitrocellulose laid over EMM or YES solid media. Cells were incubated at 25°C for 18 hours. Cells were washed off, and GFP-tagged protein was detected using anti-GFP antibody. Purified GFP was spotted onto the membrane as a positive control. SP870 cells (without GFP-tagged Plb1) were used as a negative control (NC). (B) Colony dot blot of cells expressing Plb1-GFP and grown in YES or YES-G (media with reduced glucose content). Cells were precultured in YES, washed 3X with YES-G, resuspended in YES-G, and grown for 24 hours. Cells were spotted onto nitrocellulose laid over YES or YES-G solid media. Cells were incubated at 25°C for 18 hours. Cells were washed off, and GFP-tagged protein was detected using anti-GFP antibody. Purified GFP was spotted onto the membrane as a positive control. SP870 cells (without GFP-tagged Plb1) were used as a negative control (NC). (C) Immunoblot of whole-cell lysates from cells expressing plb1-GFP and grown in YES, EMM, or YES-G media. Proteins from these cells were separated by SDS-PAGE, transferred to nitrocellulose, and immunoblotted against GFP. (D) Growth assay of WT and plb1Δ cells on YES, EMM, and YES-G media. Cells precultured in respective media were adjusted to a cell density of 5x10⁶ cells/ml, which was used to make serial dilutions. Each dilution was spotted onto YES, EMM, or YES-G solid media. (E) Exogenous PLB activity was determined by adding ¹⁴C-fatty acid-labeled lysophosphatidylcholine (lyso-PC) to wild type, plb1Δ, and plb1-6Δ cell suspensions. Following a 2-minute incubation, counts in the extracellular medium
were separated by TLC. At the conclusion of the assay 90% of counts remained in the medium outside of the cell. Approximately 50% of the counts were shown to be hydrolyzed to fatty acid from Lyso-PC in WT and \textit{plb1}\(\Delta\). Experiment was repeated with similar results.

**Figure 2.4. Plb1 localizes to vacuoles, septa, and cell periphery.**

Fluorescence micrographs of wild type (WT) cells and cells expressing \textit{plb1-GFP} and grown in (A) YES, (B) EMM, and (C) YES-G media, and stained with FM4-64 to visualize vacuole membranes. In (B) and (C), white arrows indicate septum localization of Plb1-GFP.

**Figure 2.5. Plb1 secretion is negatively regulated by glucose signaling.**

(A) Colony immunoblot of cells expressing \textit{plb1-GFP} and grown in EMM with 2% glucose or EMM with 3% glucose. SP870 cells without the \textit{plb1-GFP} construct are used as a negative control (NC) while purified GFP protein is used as a positive control. (B) Colony immunoblot of WT and \textit{pka1}\(\Delta\) cells expressing \textit{plb1-GFP} grown in YES media. SP870 cells without the \textit{plb1-gfp} construct are used as a negative control (NC) while purified GFP protein is used as a positive control. (C) Immunoblot of cell lysates from WT and \textit{pka1}\(\Delta\) cells expressing \textit{plb1-GFP}. SP870 cells without the \textit{plb1-gfp} construct were used as a negative control (NC). (D) Fluorescence micrographs of WT and \textit{pka1}\(\Delta\) cells expressing \textit{plb1-GFP}. SP870 cells without the \textit{plb1-GFP} construct were used as a negative control (NC).
Figure 2.6. Intracellular Plb1 activity is sufficient for response to hyperosmotic stress.

(A) Growth assay of plb1Δ cells with or without pREP41GFPplb1. plb1Δ cells with pREP41GFPplb1 plasmid were precultured in EMM + 50 µM thiamine, washed 3X with EMM, and cultured in EMM for 18 hours. WT, plb1Δ, and plb1Δ + pREP41GFPplb1 cells were adjusted to a cell density of 5x10^6 cells/ml, which was used to make serial dilutions. Each dilution was spotted onto EMM or EMM + 1 M KCl solid media. (B) Phospholipid analysis of WT and plb1Δ cells grown in YES or YES + 1.2 M KCl for 8 hours. Phospholipids were extracted as described in the Materials and Methods, separated by TLC, visualized with primuline, and quantified using densiometry. (C) GFP-tagged Plb1 localizes to the vacuolar lumen under hyperosmotic conditions. Cells expressing plb1-GFP were grown in YES or EMM. One volume of YES/EMM + 2.4 M KCl was added, and the cultures were incubated for 8 hours. Additionally, plb1Δ + pREP41GFPplb1 were precultured in EMM + 50 µM thiamine, washed 3X with EMM, and cultured in EMM for 18 hours. One volume of EMM + 2.4 M KCl was added, and the cultures were incubated for 8 hours. The vacuole lumen was stained with CMAC. GFP-tagged protein and CMAC were visualized by fluorescence microscopy.

Figure 2.7. Plb1 activity is needed for maintenance of mitochondrial structure under conditions of hyperosmotic stress.

(A) Growth assay of WT and plb1Δ cells on media containing rotenone. Cells were grown in YES media, and the cell density was adjusted to 5x10^6 cells/ml, which was used
to make serial dilutions. Each dilution was spotted onto YES or YES + 6.4 \mu g/ml rotenone. (B) Plb1 is necessary for maintenance of mitochondrial structure under hyperosmotic conditions. The mitochondria of WT and plb1\Delta cells stained with MitoTracker Green (Mito) (B) or MitoTrackerRed (C) after treatment with 1.2 M KCl for 0, 3, and 24 hours. (D) Degradation of the mitochondrial protein Sdh2-GFP is increased in KCl-treated plb1\Delta cells. WT and plb1\Delta cells expressing Shd2-GFP were treated with 1.2 M KCl and protein was harvested at the indicated timepoints. Protein was separated by SDS-PAGE and subjected to western blotting against GFP. (E) Autophagy is not upregulated in KCl-treated plb1\Delta cells. WT and plb1\Delta cells expressing GFP-Atg8 were treated with 1.2 M KCl and protein was harvested at the indicated timepoints. Protein was separated by SDS-PAGE and subjected to western blotting against GFP.
CHAPTER THREE

PROTEIN KINASE A (PKA1) IS NECESSARY FOR ESCAPE FROM G2 AND CYTOKINESIS CHECKPOINTS FOLLOWING HYPEROSMOTIC STRESS

McInnis, B.M., and Marcus, S. Britney McInnis analyzed and collected data presented in Figures 3.1-3.4 and Table 3.1. Yasuhiro Matsuo contributed to Figure 3.1B. Britney McInnis wrote the manuscript.
ABSTRACT

Organisms experience hyperosmotic stress when the external concentration of solutes becomes greater than that inside of the cell. An initial response to exposure to hyperosmotic stress is transient, multi-phase cell cycle arrest, which allows cells time to adjust to hyperosmotic conditions before resuming normal cell cycle progression. A previous study showed that pka1, the gene encoding the protein kinase A (PKA) catalytic subunit, is necessary for growth on high concentrations of potassium chloride (KCl) in the fission yeast Schizosaccharomyces pombe. In this study, we found that pka1 is necessary for growth, not survival, on hyperosmotic media. Unsynchronized pka1Δ cells were unable to resume mitosis following hyperosmotic stress, but gradually accumulated septated cells. Analysis of cells synchronized in G2 phase before treating with KCl revealed that pka1Δ cells were arrested in G2 and cytokinesis. PKA has previously been shown to control cell cycle progression in G2 phase by activating Wee1 and deactivating Cdc25. However, over-expression of wee1 did not rescue the KCl-sensitive growth phenotype of pka1Δ cells. These results demonstrate that Pka1 is necessary to lift G2 and cytokinesis checkpoints induced by hyperosmotic stress that is independent of Wee1.
INTRODUCTION

In order to grow and proliferate, all eukaryotic cells must execute phases of the cell cycle in the proper order and must coordinate these events with other cellular processes. In the fission yeast *S. pombe*, cells spend the majority of the cell cycle in G2, with the transition from G2 to M-phase being the point at which multiple regulatory pathways converge (Humphrey and Pearce 2005). This transition is driven by the cyclin-dependent kinase (CDK) Cdc2 and its cyclin partner Cdc13. Activation of Cdc2, and subsequent G2/M progression, depends on binding of Cdc13 to Cdc2, phosphorylation of T168 in the active site of Cdc2, and removal of inhibitory phosphorylation of Y15. The phosphorylation state of Y15 is the primary determinant of mitotic progression, and is therefore the target of multiple regulatory mechanisms (Gould 2004). Phosphorylation of Y15 by the kinase Wee1 is inhibitory to Cdc2 function, while dephosphorylation of Y15 by the phosphatase Cdc25 activates the CDK (Russell and Nurse 1986; Moreno et al. 1990; Featherstone and Russell 1991; Lundgren et al. 1991; Parker et al. 1992; McGowan and Russell 1993).

The events in the cell cycle occur at regular intervals unless the cell is challenged with some sort of physiological stress, in which case the cell must cease cell cycle progression in order to adapt to the stress before resuming the cell cycle. This is especially apparent during hyperosmotic stress, which induces transient cell cycle arrest in multiple phases of the cell cycle to allow cells to undergo various adaptations, including activation of signaling cascades, production of internal organic osmolytes, increased expression of heat shock proteins, and repair of cellular damage caused by initial osmotic shock (Kultz et al. 1998; Michea et al. 2000; Dmitrieva and Burg 2005;
Burg et al. 2007). Osmotic stress induces arrest in multiple points in the cell cycle in renal medullary cells, but the cells tend to accumulate in G2 since the G1 and S phase arrests are much shorter. The extent of the arrest is also directly proportional to the osmolality (Michea et al. 2000). In S. cerevisiae, temporary arrest in G1 and G2 depends on the MAP kinase Hog1, which acts by inducing cyclin degradation and activating CDK-inhibitor proteins, such as Swe1, the budding yeast homolog of Wee1. The G2 arrest induced in budding yeast under hyperosmotic stress is necessary for survival in these conditions, and this arrest is mediated by Swe1 (Clotet et al. 2006). In fission yeast, Srk1, a kinase in the p38 MPAK signaling pathway, phosphorylates Cdc25, which results in its binding to Rad24, a 14-3-3 protein, and export from the nucleus in response to hyperosmotic stress (López-Avilés et al. 2005). It has been reported that hyperosmotic stress leads to an increase in Cdc25 levels and therefore induces mitotic stimulation as measured by the percent binucleate cells (Kishimoto and Yamashita 2000b). A complement study showed that salt stress could induce a temporary arrest in cytokinesis due to increased levels of Cdc25 (Alao et al. 2010).

The cyclic-AMP (cAMP) protein kinase A (PKA) pathway is known to participate in stress response and cell cycle control. In its inactive form, the PKA heterodimer consists of two catalytic subunits bound to two regulatory subunits. Upstream activation of the PKA signaling pathway leads to formation of cyclic-AMP (cAMP), catalyzed by adenylate cyclase. cAMP binds the regulatory subunit, allowing the catalytic subunit to phosphorylate its targets (Taylor et al. 2008). The cAMP-PKA pathway is important for response to several types of stress, including osmotic stress. Osmotic stress in the form of sorbitol rapidly activates PKA in CHO cells (Mao et al.
In fission yeast, transcription of the gene encoding the catalytic subunit, \textit{pka1}, was increased in response to osmotic stress in the form of sorbitol (Chen et al. 2003). Additionally, components of the PKA pathway are necessary for growth and increased \textit{gpd1} expression in response to hyperosmotic stress in fission yeast (Yang et al. 2003). PKA has also been shown to function in cell cycle control. PKA prevents oocyte maturation in \textit{Xenopus} and mouse by inhibiting G2/M progression. This is accomplished by phosphorylation of specific residues on Cdc25, leading to its binding of a 14-3-3 protein and subsequent export from the nucleus (Duckworth et al. 2002; Shibuya 2003; Pirino et al. 2009). PKA also phosphorylates Wee1 in \textit{Xenopus} and mouse oocytes, thereby increasing Wee1 activity (Shibuya 2003; Stanford and Ruderman 2005; Han and Conti 2006). It is likely that PKA also inhibits G2/M progression in fission yeast, as over-expression of the catalytic subunit \textit{pka1} leads to growth arrest with elongated cells, which is indicative of arrest in G2 phase (Tallada et al. 2002). Increasing the levels of cAMP leads to decreased protein levels of Cdc25 (Kishimoto and Yamashita 2000a). PKA also plays a role in inhibiting anaphase progression by inhibiting assembly of the APC/cyclosome complex in fission yeast (Yamashita et al. 1999; Yanagida et al. 1999). PKA also cooperates with the kinase Chk1 to inhibit anaphase progression by phosphorylating and inhibiting Cdc20 following DNA damage (Searle et al. 2004). All of these studies point to a role for PKA as an inhibitor of cell cycle progression.

In this study, we show that fission yeast cells experience transient cell cycle arrest in G2 phase and cytokinesis following hyperosmotic stress. Interestingly, the PKA catalytic subunit Pka1 was found to be necessary to resume cell cycle control in both of these phases of the cell cycle.
MATERIALS AND METHODS

Strains, plasmids, and media

The strains used in this study were SP870 (from D. Beach), CHP453 (pka1Δ) (from C. Hoffmann), *cdc25-22* (from P. Russell), and *pka1Δ cdc25-22*, which was made by mating CHP453 and *cdc25-22*. The temperature-sensitive strains were maintained at the permissive temperature of 25°C, while remaining strains were maintained at 30°C. The *wee1* gene was over-expressed using the thiamine-repressible pREP3X vector. Transformants were selected and maintained on minimal media (EMM) lacking leucine and containing 15 µM thiamine. Non-transformed cells were grown in YEAU media. To induce hyperosmotic shock, one volume of YEAU + 2.4 M KCl was added to YEAU liquid cultures.

Stress survival assay

Cells were grown in YEAU to mid-log phase, and one volume of pre-warmed YEAU + 2.4 M KCl was added to each culture. Cells were incubated in 1.2 M KCl for 4 days, with the cell density being adjusted to maintain mid-log phase levels in wild type cells. Cells were washed three times with YEAU media, and serial dilutions of the cells were spotted onto YEAU plates that were incubated at 30°C for 3 days.

Hyperosmotic stress induced cell cycle arrest in synchronized cells

To synchronize *cdc25-22* and *pka1Δ cdc25-22* cells in G2 phase, cells were grown in YEAU to mid-log phase at 25°C, then shifted to 36°C for 4 hours (approximately 1 generation time). One volume of pre-warmed YEAU or YEAU + 2.4 M KCl was added
to split cultures, which were incubated at 36°C for another 1.5 hours. The cultures were released from cell cycle arrest at the permissive temperature of 25°C. One milliliter aliquots were removed from each culture and fixed with ethanol at the timepoints indicated, starting from the release point. Cells were washed, treated with RNase, and stained with propidium iodide to stain the nucleus and calcofluor to stain the septa and visualized using fluorescence microscopy.
RESULTS

*Pka1 is necessary for growth and cell cycle progression, but not survival, under hyperosmotic conditions*

Stress response depends on several kinases to phosphorylate targets that activate signaling cascades and elicit cellular changes. In *S. pombe*, the gene for the PKA catalytic subunit, *pka1*, is necessary for response to hyperosmotic stress, since *pka1Δ* cells are not able to grow on hyperosmotic media in the form of 1 M KCl (Fig. 3.1A, Yang et al. 2003). This may mean that Pka1 is needed for survival in hyperosmotic conditions, similar to the MAP kinase Spc1 (Degols et al. 1996). Another possibility is that Pka1 is necessary to resume growth from transient cell cycle arrest induced by hyperosmotic stress. To test this, we incubated wild type and *pka1Δ* cells in 1.2 M KCl for 96 hours. KCl was washed from the cells, which were spotted onto normal YEAM media. As shown in figure 3.1B, *pka1Δ* cells were able to survive prolonged incubation in hyperosmotic media and resume growth once the stress was removed, indicating that Pka1 is needed for growth, not survival, in hyperosmotic media. To observe cell cycle progression in wild type and *pka1Δ* cells, we measured the percentage of mitotic indicators, namely percent binucleate and septated cells, at timepoints immediately following the addition of hyperosmotic stress. Wild type cells showed two mitotic peaks, as measured by percent binucleate cells at 1 hour and another at 4 hours (Figure 3.1C). These mitotic peaks are not observed in cells under normal conditions (Figure 3.1C). Each peak in percent binucleate cells is followed by an increase in the percentage of septated cells, indicating that an increasing number of cells become blocked in cytokinesis (Fig. 3.1D). The first peak in binucleate cells is seen in *pka1Δ* cells, and this
peak is followed by an increase in septated cells similar to that seen in wild type (Fig. 3.1C). However, \(pka1\Delta\) cells do not exhibit a second mitotic peak, though the septation index does gradually increase to 22.6 ± 1.1% septated cells at the 5 hour timepoint (Fig. 3.1C and 3.1D). Other experiments measuring the septation index of unsynchronized \(pka1\Delta\) cells after prolonged exposure (~24 hours) to KCl have consistently yielded values of 20-25%, whereas wild type cells have septation indices resembling unstressed cells (data not shown). This indicates that a second mitotic peak and resulting accumulation of septated cells does not occur after the last measured timepoint in \(pka1\Delta\) cells. These results also suggest that \(pka1\Delta\) cells may remain arrested in G2 phase and cytokinesis for extended periods of time following hyperosmotic stress.

**Pka1 is necessary for release G2 phase and cytokinesis checkpoints following hyperosmotic stress**

The previous set of experiments suggested that \(pka1\) may be needed to lift transient cell cycle arrest in G2 and cytokinesis following KCl stress in order to allow cells to grow in hyperosmotic media. To test whether \(pka1\Delta\) cells experienced prolonged G2 and cytokinesis arrest in hyperosmotic media, cells were synchronized in G2 phase using the \(cdc25\)-22 temperature sensitive mutant, which produces subfunctional Cdc25 at 36°C but normally functioning Cdc25 at 25°C. To ensure that both \(cdc25\)-22 and \(cdc25\)-22 \(pka1\Delta\) constructs could be synchronized, both cells type were incubated at 36°C for 4 hours, then released in normal osmotic media (YEAU) at 25°C. We were able to synchronize both \(cdc25\)-22 and \(cdc25\)-22 \(pka1\Delta\) cells, which showed peaks in septation at similar timepoints (Fig. 3.2A). Interestingly, when these cells were grown on YEAU...
plates at various temperatures, loss of pka1 was able to rescue the growth of cdc25-22 at 36°C (Fig. 3.2B). This result suggests that Pka1 may negatively regulate G2 progression under normal conditions by antagonizing Cdc25 function as seen in Xenopus and mouse oocytes (Duckworth et al. 2002; Pirino et al. 2009; Shibuya 2003).

To verify whether pka1Δ cells experience prolonged arrest in G2 and cytokinesis upon extended incubation in hyperosmotic media, cdc25-22 and cdc25-22 pka1Δ cells were synchronized in G2 by incubation at 36°C for 4 hours. The cells were treated with 1.2 M KCl before being released at 25°C. As expected, cdc25-22 cells were able to proliferate in hyperosmotic media, whereas cdc25-22 pka1Δ cells were not (Fig. 3.3A). Both cdc25-22 and cdc25-22 pka1Δ cells had low septation indices following release at 25°C, indicating that the cells remained arrested in G2 phase even after 1.5 hours incubation in 1.2 M KCl at 36°C (Fig. 3.3B). cdc25-22 cells rapidly underwent septation following temperature release at 25°C, with peaks between 4 and 8 hours (Fig. 3.3B). This septation peak was accompanied by a peak in binucleate cells at 8 hours (Fig. 3.3C), and cell division occurred around 8 hours, as indicated by the reduction in cell size (Fig. 3.3D). The percentage of binucleate and septated cells gradually decreased to normal levels (Fig. 3.3B and 3.3C). cdc25-22 pka1Δ cells experience a modest increase in binucleate cells, followed by a gradual decline (Fig. 3.3C), which was accompanied by a gradual increase in septated cells (Fig. 3.3B). However, these septated cells do not divide, since the cell size remains constant through the KCl treatment (Fig. 3.3D). The septation index only reaches about 50 percent septated cells, indicating that roughly half of the cells are arrested in G2 while the other half are arrested in cytokinesis.
**Wee1-overexpression does not rescue the KCl-sensitive phenotype of pka1Δ**

We have previously seen evidence that \textit{pka1} is a negative regulator of Cdc25 (Fig. 3.2B), and that \textit{pka1} is also necessary to lift G2 and cytokinesis checkpoints for cell growth in hyperosmotic stress conditions. A possible explanation for these extended G2 and cytokinesis blocks is that Wee1 and Cdc25 activity may be misregulated in the absence of Pka1 phosphorylation. To test this, the \textit{wee1} coding sequence was over-expressed in WT and \textit{pka1Δ} strains. Additionally, we over-expressed \textit{wee1} in a \textit{cgs1Δ} mutant in which the PKA regulatory subunit has been deleted, leading to over-active Pka1 catalytic subunit. Cells in which the \textit{wee1} gene was over-expressed were inhibited for growth under non-stressed and KCl-stressed conditions, most likely due to Wee1’s negative effects on G2 progression (Table 3.1, Fig. 3.4). Interestingly, this growth arrest seemed to be more severe in \textit{cgs1Δ} cells, which is not unexpected since both \textit{wee1} and \textit{pka1} negatively regulate G2/M progression. However, \textit{wee1} over-expression did not rescue the osmosensitive growth defect of \textit{pka1Δ} cells (Table 3.1).
DISCUSSION

In this study, we have shown that the gene encoding the PKA catalytic subunit, \textit{pka1}, is necessary for growth, not survival, on hyperosmotic media. Wild type cells stressed with KCl experience two peaks in binucleate cells, followed by an accumulation of septated cells. The first mitotic peak, but not the second, is seen in \textit{pka1Δ} cells. The first mitotic peaks are likely caused by cells that were already prepared for anaphase upon addition of KCl. These cells were allowed to progress through anaphase while other cells were prevented from entering anaphase. The presence of this first peak in \textit{pka1Δ} cells points to the absence of a mitotic block in these cells. The second mitotic peak seen in wild type cells is likely a result of resumption of the cell cycle, which is not seen in \textit{pka1Δ} cells. These mitotic peaks were also seen at similar timepoints in wild type cells treated with 0.6 M KCl (Kishimoto and Yamashita 2000b). However, that study only measured the change in the percentage of binucleate cells, and did not detect the transient cytokinesis checkpoint that was seen even in wild type cells treated with KCl. However, Alao et al. (2010) observed an accumulation of septated cells following treatment with KCl.

The lack of a second mitotic peak in \textit{pka1Δ} cells suggest that these cells experience cell cycle arrest prior to the second round of mitosis. The maintenance of a septation index of \(~20\%\) suggests that these cells also arrest in cytokinesis. To further elucidate the phase in which arrest occurs, we tested whether wild type and \textit{pka1Δ} cells synchronized in G2 could escape checkpoints in this phase following KCl stress. Though wild type cells escape G2 arrest and carry on mitosis and cell division, \textit{pka1Δ} cells do not divide and gradually accumulate as septated cells. Since only about 50 percent of the
cells become septated, it is likely that the non-septated cells remain in G2 phase. These data suggest that *pka1* is necessary for escape from G2 and cytokinesis checkpoints following KCl stress. The presence of a slight peak in binucleate cells and the gradual increase in the septation index indicates that the G2 arrest in KCl-stressed *pka1Δ* cells is somewhat “leaky,” allowing some cells to escape into mitosis, after which they are blocked in cytokinesis. The cytokinesis arrest seems to be much more stringent and long lasting than G2 arrest in these cells. Similarly, *S. pombe* cells that are missing the gene *mkh1*, which encodes a MEK kinase homolog, are unable to complete cytokinesis when grown in nutrient-limited media containing 0.6 M KCl (Sengar et al. 1997), suggesting that *pka1* and *mkh1* may share functional roles in regulating cytokinesis under hyperosmotic conditions. However, unlike *mkh1Δ* cells, *pka1Δ* cells arrested in cytokinesis do not resume cell cycle progression to produce multiseptated cells, indicating that *pka1*, but not *mkh1*, is needed to lift KCl-induced blocks in G2. Additional studies have shown that *S. pombe* cells treated with KCl experience delayed cytokinesis, presumably brought on by increased levels of Cdc25 and Cdc13 (Alao et al. 2010). It is possible that Pka1 functions to alleviate checkpoint arrest by participating in the down-regulation of Cdc25 or Cdc13 levels.

In *S. pombe*, G2 phase is a period of longitudinal cell growth, which is why G2-arrested cells become longer. It was surprising to find that KCl-treated *pka1Δ* cells did not increase in cell length over time, even though a large portion of these cells are arrested in G2 or M-phase. It is plausible that Pka1 is necessary for increased growth during G2 phase, which may explain why G2-arrested *cdc25-22 pka1Δ* cells are much
shorter than arrested *cdc25-22* cells after initial temperature release to 25°C (Figure 3.3D).

Interestingly, loss of *pka1* partially rescued the growth defect of *cdc25-22* cells at the restrictive temperature. This result was not entirely unexpected, since the PKA catalytic subunit has been shown to negatively regulate G2/M phase progression by phosphorylating and inhibiting Cdc25 activity (Kishimoto and Yamashita 2000b; Duckworth et al. 2002; Tallada et al. 2002; Shibuya 2003; Pirino et al. 2009). However, we were able to synchronize *cdc25-22 pka1Δ* cells to the same degree as *cdc25-22* cells, suggesting that rescue occurs over longer timepoints and does not affect short-term synchronization. We hypothesized that the extended G2 and cytokinesis blocks seen in *pka1Δ* cells treated with 1.2 M KCl may be due to over-active Cdc25. However, over-expression of Wee1, which antagonizes Cdc25 activity, did not rescue the osmosensitive growth defect of *pka1Δ* cells. Another fission yeast study has shown that cytokinesis arrest induced by hyperosmotic stress is also accompanied by increased levels of Cdc25 protein (Alao el al. 2010). It would be interesting to see whether Cdc25 levels are increased in KCl-treated *pka1Δ* cells, even at extended timepoints. Though Wee1 over-expression does not rescue growth of *pka1Δ* cells on hyperosmotic media, it is possible that Wee1 over-expression may relieve the cytokinesis arrest by counteracting Cdc25 activity.
REFERENCES


Table 3.1. The effect of *wee1* over-expression on the KCl-sensitivity phenotype of *pka1Δ*.

<table>
<thead>
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<th>-KCl</th>
<th>+0.75 M KCl</th>
<th>+ 1 M KCl</th>
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<td>WT + pREP3X</td>
<td>+++a</td>
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<tr>
<td>WT + pREP3X <em>wee1</em></td>
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<tr>
<td><em>pka1Δ</em> + pREP3X</td>
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<td><em>pka1Δ</em> + pREP3X <em>wee1</em></td>
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<td><em>cgs1Δ</em> + pREP3X</td>
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<td><em>cgs1Δ</em> + pREP3X <em>wee1</em></td>
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</table>

*a* (+) indicates relative cell growth
Figure 3.1

A

\begin{align*}
\text{WT} & -\text{KCl} & +\text{KCl} \\
\text{pka1}\Delta & -\text{KCl} & +\text{KCl} \\
\end{align*}

B

\begin{align*}
\text{WT} & -\text{KCl 96 hr} & +\text{KCl 96 hr} \\
\text{pka1}\Delta & -\text{KCl 96 hr} & +\text{KCl 96 hr} \\
\end{align*}

C

\begin{align*}
\text{% binucleate cells} & \text{time (hours)} \\
\end{align*}

D

\begin{align*}
\text{% separted cells} & \text{time (hours)} \\
\end{align*}
Figure 3.2
Figure 3.3

A

B

C

D

\text{cell density (cells/mL)}

\text{% binucleate cells}

\text{% sepa rated cells}

\text{cell size (\mu m)}

\text{time after release (hours)}
Figure 3.4
FIGURE LEGENDS

Figure 3.1. Pka1 is necessary for growth and cell cycle progression, but not survival, under hyperosmotic conditions.

(A) Growth assay of wild type and pka1Δ on YEAU ± 1.2 M KCl. Cells precultured in YEAU were adjusted to a cell density of $10^7$ cells/ml, which was used to make serial dilutions. Each dilution was spotted onto YEAU or YEAU + 1 M KCl. (B) Survival assay of wild type and pka1Δ cells in YEAU + 1.2 M KCl. After incubation in YEAU + 1.2 M KCl for 4 days, cells were washed with YEAU and spotted on YEAU plates. (C) Mitotic stimulation of wild type (open diamonds) and pka1Δ (open circles) cells induced by hyperosmotic stress. Wild type and pka1Δ cells were grown in YEAU to mid-log phase. One volume of YEAU + 2.4 M KCl was added for a final concentration of 1.2 M KCl. Cells treated with 1.2 M KCl were compared to WT (closed diamonds) and pka1Δ (closed circles) treated with culture media (YEAU). Cells were removed and fixed with ethanol at the indicated timepoints, treated with RNase, then stained with propidium iodide and calcofluor to visualize binucleate septated cells.

Figure 3.2. Pka1 is a negative regulator of Cdc25.

(A) cdc25-22 and pka1Δ cdc25-22 cells were grown to mid-log phase in YEAU, synchronized in G2 by incubation at 36°C for 4 hours, and released at 25°C. The number of septated cells was assessed at the indicated timepoints. (B) Growth assay of wild type, pka1Δ, cdc25-22, and pka1Δ cdc25-22 on YEAU at 25°C and 36°C.
Figure 3.3. Pka1 is necessary for release of G2 phase and cytokinesis checkpoints following hyperosmotic stress.

cdc25-22 (open diamonds) and cdc25-22 pka1Δ (open circles) cells were synchronized in G2 phase by incubating at 36°C for 4 hours. One volume of YEAU + 2.4 M KCl was added, and the cell cycle arrest was removed by incubating at 25°C. Cells were removed and fixed with ethanol at the indicated timepoints, treated with RNase, then stained with propidium iodide and calcofluor. The cell growth (A), septation index (B), binucleate cells (C), and cell length (D) was quantified.

Figure 3.4. Cell cycle arrest of KCl-treated pka1Δ cells is not due to dysregulation of Cdc25. WT, pka1Δ, and cgs1Δ cells transformed with empty vector (pREP3X) or vector expressing wee1 (pREP3Xwee1) were streaked onto EMM plates without thiamine and containing 0 M, 0.75 M, or 1 M KCl.
CHAPTER 4

CHARACTERIZATION OF A CYCLIC AMP-INDEPENDENT MECHANISM FOR PROTEIN KINASE A REGULATION IN SCHIZOSACCHAROMYCES POMBE

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ABSTRACT

Cyclic AMP (cAMP)-dependent protein kinase A (PKA) has been found to be involved in various processes in eukaryotic organisms. In fission yeast, the principal mechanism for regulation of the catalytic subunit of PKA, Pka1, is by the regulatory subunit Cgs1, which is released from Pka1 following interaction with cAMP produced by adenylate cyclase (Cyr1). Cells lacking adenylate cyclase express more Pka1 protein, most of which is in a hyperphosphorylated state. Increased prevalence of the hyperphosphorylated protein was observed under conditions in which PKA activity is downregulated, such as stationary phase and glucose deprivation, as well as under conditions in which PKA activity is necessary, such as hyperosmotic stress. These results suggest an adaptive mechanism to maintain protein kinase A activity in the absence of cAMP signaling. The hyperphosphorylated protein is not observed under any conditions in cells lacking cgs1, suggesting that the regulatory subunit may be physically necessary for the hyperphosphorylation of Pka1 to occur, or that increased Pka1 activity negatively regulates this hyperphosphorylation.
INTRODUCTION

Cyclic AMP (cAMP)-dependent protein kinase A is a serine/threonine kinase that functions in a well-characterized signaling cascade. The PKA holoenzyme consists of two regulatory subunits bound to two inactive, catalytic subunits. Adenylate cyclase is an upstream component of the pathway that produces the secondary messenger cAMP, which binds to the regulatory subunit and causes the release of the activated catalytic subunits. The catalytic subunit then phosphorylates various cellular targets, including those involved in nutrient sensing and cell growth, stress response, conjugation and meiosis, sporulation, apoptosis, and phospholipid metabolism (Stettler et al. 1997; Kim and Carman 1999; Hatanaka and Shimoda 2001; Shabb 2001; Chen et al. 2003; Yang et al. 2003; Hoffman 2005; Santangello 2006; Dechant and Peter 2008; Taylor et al. 2008). In the fission yeast *Schizosaccharomyces pombe*, research concerning protein kinase A has focused primarily on its role in nutrient sensing. For example, Pka1 inhibits the transcription of gluconeogenic genes, such as *fbp1*, in the presence of glucose (Hoffman and Winston 1990; Hoffman and Winston 1991; Welton and Hoffman 2000;). Pka1 is also responsible for inhibiting *mei2* transcription, which is required for initiation of the meiotic program, when nitrogen sources are sufficient (DeVoti et al. 1991). It has recently been demonstrated that PKA plays a role in apoptosis and chronological aging in yeast (Roux et al. 2006; Pereira et al. 2008).

Although research has typically focused on the identification and description of targets of the PKA pathway, many studies are beginning to shed light on additional regulatory mechanisms for components of this pathway, most notably the catalytic subunit PKA. It has become clear that regulation of PKA activity is much more complex.
than the linear pathway that has traditionally been described. Mammalian systems use A-kinase anchoring proteins (AKAPs) and A-kinase interacting proteins (AKIPs), which interact with the regulatory subunit to localize the holoenzyme to subcellular compartments (Michel and Scott 2002). These AKAPs often form protein complexes with other regulatory molecules, including adenylate cyclase and phosphodiesterase (PDE), to create different regions of compartmentalized signaling within the cell (Cooper 2005). Although no open reading frames for AKAPs or AKIPs have been identified in yeast, Zds1 has been shown to participate in the relocalization of the regulatory subunit Bcy1 in *Saccharomyces cerevisiae* (Griffioen et al. 2001). Recent studies using GFP-tagged proteins have shown that the subcellular localization of the catalytic and regulatory subunits changes dramatically in response to various conditions in both budding and fission yeast (Griffioen et al. 2000; Matsuo et al. 2008).

In addition to changes in subcellular localization, the catalytic subunit can also be regulated by post-translational modification. The PKA catalytic subunit has been shown to undergo several modifications, including myristoylation of the N-terminus, which may function in membrane targeting of the protein, and phosphorylation (Gesellchen et al. 2006). Two-dimensional gel electrophoresis of the catalytic subunit from *S. cerevisiae* has revealed multiple phosphorylation sites, though the locations and functions of these phosphorylations have not been described (Levin and Zoller 1990). The post-translational modification of PKA that has been the best characterized is the phosphorylation of a conserved threonine residue in the activation loop by a phosphoinositide-dependent protein kinase (PDK1) (Taylor et al. 2008). This phosphorylation has been shown to
occur on T197 in mammals and on T356 in fission yeast by the kinase Ksg1 (Tang and McLeod 2004; Taylor et al. 2008).

In *S. pombe*, the catalytic subunits of protein kinase A are encoded by a single gene, *pka1*, whereas *S. cerevisiae* has three *tpk* genes that encode catalytic subunits (Toda et al. 1987; Maeda et al. 2004). Additionally, *S. pombe* has single genes that encode each for adenylate cyclase and the regulatory subunit, *cyr1* and *cgs1*, respectively. Having single genes that encode for components of this pathway greatly aids in genetic studies. More importantly, these genes are not necessary for viability under normal growth conditions, which is not the case in *S. cerevisiae* (Matsumoto et al. 1982; Toda et al. 1987; Maeda et al. 2004). This makes fission yeast an important model organism for studying characteristics of this pathway. In this study, we describe various modifications to the Pka1 protein in adenylate cyclase mutants, including increased protein levels and the presence of a hyperphosphorylated portion of the protein. This phosphorylation can be reversed by addition of cAMP and is dependent on the presence of the regulatory subunit. An increased proportion of Pka1 protein is found in the hyperphosphorylated form upon numerous treatments, including glucose, stationary phase, and exposure to osmotic stress, suggesting that this phosphorylation functions to maintain PKA activity in all of these conditions, which may be associated with decreased cAMP signaling. Additionally, this hyperphosphorylation was not observed in cells containing the *cgs1Δ* mutation, suggesting that Cgs1 may recruit kinases to Pka1, or that increased Pka1 activity may negatively regulate this hyperphosphorylation. Pka1 protein levels were decreased in *cgs1Δ* cells under conditions in which Pka1 was hyperphosphorylated in WT cells, suggesting that this hyperphosphorylation may serve to stabilize the protein.
MATERIALS AND METHODS

Strains and media

The strains used in this study are SP870 (h90 ade6-210 leu1-32 ura4-D18) (from D. Beach), YMSM101 (h90 ade6-210 leu1-32 ura4-D18 pka1-GFP (S65T)-kanMX6), YMSM102 (h90 ade6-210 leu1-32 ura4-D18 cyr1::ura4 pka1-GFP (S65T)-kanMX6), YMSM103 (h90 ade6-210 leu1-32 ura4-D18 cgs1::ura4 pka1-GFP (S65T)-kanMX6), YMSM104 (h90 ade6-210 leu1-32 ura4-D18 cyr1::LEU2 cgs1::ura4 pka1-GFP (S65T)-kanMX6), which have been described previously (Matsuo et al. 2008). Strains were grown in YES (0.5% yeast extract, 3% dextrose, adenine (250 mg/liter), uracil (250 mg/liter), leucine (250 mg/liter), lysine (250 mg/liter), histidine (250 mg/liter)), or YES-G (YES media with only 0.1% glucose and 3% glycerol), or synthetic minimal medium (EMM). To induce hyperosmotic stress, one volume of pre-warmed YES + 2.4 M KCl was added to split cultures grown in YES.

Lysate preparation and Immunoblotting

Cell lysates were prepared by the boiled lysis method as previously described (Correa-Bordes and Nurse 1995). The protein concentration was quantified by BCA assay, and proteins were separated by SDS-PAGE on 7.5% polyacrylamide gels. GFP-tagged proteins were detected using anti-GFP antibody (Roche catalog no. 11814460001) and the SuperSignal West Dura chemiluminescence detection system (Pierce). Alpha tubulin was visualized using anti-α-tubulin (Sigma catalog no. T5168).
Immunoprecipitation and phosphatase treatment of Pka1-GFP

Cell lysates from cyr1Δ mutants were prepared by breaking cells with glass beads in NP40 buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1% Nonidet P-40, 0.5% deoxycholate), and 2 mg of total protein was incubated with Protein-G Agarose beads that had been pre-conjugated with anti-GFP antibody. After washing with NP40 buffer, beads were treated with alkaline phosphatase (New England Biolabs catalog no. M0290S) in NEB Buffer 3 (New England Biolabs) at 36°C for 1 hour. A portion of the beads was also used for negative controls, which included reaction without phosphatase enzyme and reaction with phosphatase enzyme plus phosphatase inhibitor (10 mM sodium orthovanadate). Beads were mixed with loading buffer and heated at 95°C for 5 minutes. The resulting supernatant was loaded onto a 7.5% polyacrylamide gel and immunoblotted as described above.
RESULTS

cAMP deficiency leads to increased expression and phosphorylation of the catalytic subunit Pka1

We have previously described the localization of GFP-tagged Pka1 protein in wild type and cyr1Δ cells (Matsuo et al. 2008), and we have observed that the Pka1-GFP signal is noticeably brighter in cyr1Δ cells than in wild type cells (Fig. 4.1A). To determine if the increased GFP signal was due to increased amounts of protein rather than autofluorescence, immunoblots of cell lysates from wild type and cyr1Δ cells were prepared using an antibody against the GFP tag. In fact, Pka1 protein levels are twice as abundant in cyr1Δ cells than in wild type cells (Fig. 4.1B). Interestingly, a significant proportion of Pka1 protein from cyr1Δ cells is present as a heavier band (Fig. 4.1B). To test whether the changes in Pka1 protein levels and mobility were due solely to the absence of cyclic AMP (cAMP), the enzymatic product of Cyr1, rather than loss of a downstream target of Cyr1, we treated cyr1Δ cells with 5 mM cAMP and isolated cell lysates at the indicated timepoints. Pka1 protein levels returned to the lower mobility form after only 10 minutes of treatment with cAMP (Fig. 4.1C).

Post-translational modifications can involve various chemical constituents and can modify the protein’s activity as well as its subcellular localization. To test whether this post-translational modification of Pka1 protein in cyr1Δ cells is due to phosphorylation, Pka1-GFP was immunoprecipitated from cyr1Δ cell lysates using anti-GFP antibody and treated with alkaline phosphatase. Treatment with phosphatase resulted in loss of the heavier band, indicating that this post-translational modification is due to phosphorylation by an unknown kinase (Fig. 4.1D).
A previous study has shown that localization of the Pka1 catalytic subunit depends on the presence of the regulatory subunit (Matsuo et al. 2008), prompting us to test whether the regulatory subunit was necessary for the modifications to Pka1 observed in the cyr1Δ mutant. Deletion of the regulatory subunit returns Pka1 protein to wild type expression levels and mobility in cyr1Δ cells (Fig. 4.2), suggesting that the regulatory subunit is required for these changes to occur.

Effects of glucose deprivation and hyperosmotic stress on Pka1 phosphorylation

Once it was determined that Pka1 was being modified via phosphorylation as a result of loss of cyr1, we wanted to determine whether this phosphorylation affected Pka1 activity in cyr1Δ cells. To attempt to answer this, we monitored Pka1 phosphorylation in cyr1Δ cells that had been grown in conditions known to involve upregulated or downregulated Pka1 activity. It is well known that the cAMP-PKA pathway regulates nutrient sensing in both budding and fission yeast, and removal of nutrients leads to transcriptional activation of genes that are normally repressed by Pka1 during vegetative growth (Santangelo 2006; Stettler et al. 1997; Hoffman 2005; Hoffman and Winston 1990; Hoffman and Winston 1991; DeVoti et al. 1991; Welton and Hoffman 2000; Thevelein and de Winde 1999). Most recently, it has been shown that removal of nutrients from culture media can induce changes in the localization of the PKA catalytic subunit (Matsuo et al. 2008; Griffioen et al. 2000). To further characterize the effect of this phosphorylation of Pka1 in cyr1Δ mutants, Pka1 protein was analyzed in wild type, cyr1Δ, cgs1Δ, and cyr1Δ cgs1Δ cells cultured for extended periods of time in glucose-deficient media. Glucose starvation induced a shift to the phosphorylated protein in cyr1Δ
mutants (Fig. 4.3A). Not surprisingly, the hyperphosphorylated band was not observed in the \textit{cgs1}\textDelta or \textit{cyr1}\textDelta \textit{cgs1}\textDelta cells (Fig. 4.3A).

Analysis of Pka1 protein from \textit{cyr1}\textDelta cells grown in glucose-deficient conditions seems to indicate that the function of this phosphorylation is to inhibit Pka1 activity. To test this further, Pka1 protein from wild type and \textit{cyr1}\textDelta cells was monitored in conditions in which Pka1 activity is essential. It has recently been shown that many components of the cAMP-PKA pathway are necessary for growth in hyperosmotic media (Yang et al. 2003). It can therefore be concluded from this that Pka1 is generally active under these conditions. If the phosphorylation observed in \textit{cyr1}\textDelta is inhibitory to Pka1 activity, we predict that exposure of these cells to conditions of hyperosmotic stress will result in loss of the upper band. To induce hyperosmotic stress, cells were treated with 1.2 M KCl for 8 hours. Interestingly, KCl treatment lead to the prevalence of the upper band in \textit{cyr1}\textDelta cells as well as increased Pka1 protein levels (Fig. 4.3B). Also, KCl treatment induced the intermediate mobility bands in wild type Pka1 protein, but heavier bands were not seen in \textit{cgs1}\textDelta or \textit{cyr1}\textDelta \textit{cgs1}\textDelta (Fig. 4.3B).

Since \textit{cyr1}\textDelta cells treated with KCl also exhibit accumulation of the phosphorylated form of Pka1 in a fashion similar to cells starved of nutrients, it is not likely that this phosphorylation serves to negatively regulate Pka1 activity. Instead, it may be that the purpose of this phosphorylation is to preserve Pka1 activity in cells that are unable to regulate intracellular levels of cAMP. It then follows that Pka1 function is needed for response to conditions of nutrient deprivation and osmotic stress. Interestingly, a hyperphosphorylated band similar to that seen in \textit{cyr1}\textDelta cells was
observed in wild type cells exposed to hyperosmotic stress and glucose starvation simultaneously. As usual, hyperphosphorylated bands were not seen in cgs1Δ cells.

*Analysis of Pka1 protein from stationary phase cultures*

To determine whether the catalytic subunit undergoes modification in response to stationary phase, Pka1 protein was analyzed from cells grown to early stationary phase (0 h) and 24 hours post-stationary phase entry. Similar to glucose starvation and treatment with hyperosmotic stress, Pka1 protein accumulated as the hyperphosphorylated form in cyr1Δ cells that had been incubated in stationary phase for 24 hours (Fig. 4.4A). Interestingly, the hyperphosphorylated form was also seen in wild type cells incubated in stationary phase for 24 hours (Fig. 4.4A). Interestingly, cgs1Δ and cyr1Δ cgs1Δ cells do not display any higher mobility forms, and Pka1 protein levels are diminished in cgs1Δ and cyr1Δ cgs1Δ cells cultured 24 hours post-stationary phase entry (Fig. 4.4B).
DISCUSSION

We have shown in this study that loss of the adenylate cyclase gene *cyr1* results in increased Pka1 protein levels. This may be a mechanism to increase PKA activity in cells that do not have cAMP signaling capabilities. This result is supported by an earlier study that found that *cyr1Δ* mutants had higher levels of *pka1* transcript than WT cells (Stiefel et al. 2004). In addition to increased Pka1 protein levels, we also observed that roughly half of the Pka1 protein was hyperphosphorylated in *cyr1Δ* cells. Ksg1, a functional homolog of phosphoinositide-dependent protein kinase (PDK1), is the only kinase in *S. pombe* that has been demonstrated to phosphorylate the Pka1 catalytic subunit on its activation loop *in vivo* (Tang and McLeod 2004). Though Ksg1 is a possible candidate kinase for the observed phosphorylation, this kinase has only been shown to phosphorylate T356 (Tang and McLeod 2004), whereas the extent of phosphorylation observed in the *cyr1Δ* mutant appears to be due to multiple phosphorylations. Further biochemical studies will be needed in order to identify the sites of phosphorylation as well as the kinase responsible for these phosphorylations. It is tempting to think that the primary function of this hyperphosphorylation in *cyr1Δ* cells is to induce nuclear export of Pka1 protein. However, only half of the Pka1 protein in *cyr1Δ* cells is in the hyperphosphorylated form, whereas all of it shows cytoplasmic localization. This hyperphosphorylated form is rapidly dephosphorylated upon addition of exogenous cAMP. It is likely that the cAMP signal activates a phosphatase that rapidly targets and dephosphorylates hyperphosphorylated Pka1.

Interestingly, this hyperphosphorylated form is not observed in *cyr1Δ* cells in which the gene for the regulatory gene *cgs1* is also missing. Gupta et al. observed that, in
glucose-starved conditions, Pka1 forms a complex with Cgs1 prior to being phosphorylated, and Cgs1 is necessary for this phosphorylation (2011a). Since cyr1Δ cgs1Δ cells have wild type levels of Pka1 protein, this implies that pka1 expression may be negatively regulated by Pka1 itself. These results parallel those from a previous study showing that pka1 transcripts are upregulated in a cyr1Δ mutant, while deletion of cgs1 leads to reduced transcript in a cyr1 mutant (Stiefel et al. 2004). These results also suggest that the phosphorylation of Pka1 observed in cyr1Δ cells is not due to autophosphorylation, since deletion of cgs1 leads to hyperactivation of Pka1. A study by Gupta et al. showed that Cgs1 formed a complex with Pka1 prior to phosphorylation of Pka1 (2011b). It is quite possible that the regulatory subunit plays a similar role in yeast as it does in mammals and functions to recruit kinases and other regulatory molecules to the catalytic subunit.

To determine if the purpose of hyperphosphorylation of Pka1 in cyr1Δ was to regulate Pka1 activity, we examined whether glucose deprivation or KCl stress induced accumulation of the hyperphosphorylated band. Accumulation of the phosphorylated band in cyr1Δ cells in glucose starved-cells seems to imply that this phosphorylation negatively regulates Pka1 catalytic activity, since these conditions have been associated with decreased Pka1 activity (Hoffman and Winston 1990; DeVoti et al. 1991; Hoffman and Winston 1991). However, these studies have focused on the upregulation of transcriptional targets that are repressed by Pka1 under conditions of sufficient nutrients. In the case of glucose starvation, this can be explained by the observation that Pka1 is relocalized to the cytoplasm, thereby separating it from its transcriptional targets (Matsuo et al. 2008). It should be noted that in wild type cells deprived of glucose, Pka1 does not
experience the mobility shift seen in cyr1Δ cells, suggesting that cAMP is still present in the cell under these conditions. This view is supported by a study in S. cerevisiae showing that cAMP levels are not significantly reduced in response to catabolite repression (Eraso and Gancedo 1984).

Further supporting the claim that cAMP-independent hyperphosphorylation of Pka1 does not down-regulate Pka1 activity is the fact that treatment with KCl also leads to accumulation of the hyperphosphorylated protein in cyr1Δ cells. Components of the cAMP-PKA pathway have been shown to be necessary for growth on media containing high concentrations of KCl (Yang et al. 2003). Some heavier Pka1 bands are observed in wild type cells treated with KCl, which may correlate with export of Pka1 from the nucleus (Matsuo et al. 2008). The combination of glucose starvation and hyperosmotic leads to accumulation of wild type Pka1 in a form that resembles hyperphosphorylated Pka1 in cyr1Δ cells, suggesting that this may be a mechanism to preserve Pka1 activity in conditions in which cAMP signaling is reduced.

Similar to the combined glucose starvation and KCl stress experiment, extensive time in stationary phase induces accumulation of hyperphosphorylated Pka1 not only in cyr1Δ cells but also wild type cells. Once again, this hyperphosphorylated band is not observed in cgs1Δ or cyr1Δ cgs1Δ cells, and perhaps as a consequence, Pka1 protein levels are diminished in these cells. These results reinforce earlier observations showing that Cgs1 is necessary for this phosphorylation to occur, and that this phosphorylation is not due to autophosphorylation. Since cgs1Δ and cgs1Δ cyr1Δ cells do not display the hyperphosphorylated form of Pka1 in late stationary phase and also experience decreased levels of Pka1, it is possible that the presence of this phosphorylation contributes to the
stability of the protein. It is possible that the hyperphosphorylated form of Pka1 may have a functionally distinct role that is necessary for stationary phase. Unphosphorylated Pka1, on the other hand, may be responsible for upregulating a different set of subfunctions, all of which may be counterproductive to stationary phase viability. Therefore, as a consequence of not being able to induce the hyperphosphorylated form of Pka1, $cgs1\Delta$ cells are forced to decrease their levels of unphosphorylated Pka1. These results seem to contradict those reporting that Pka1 activity leads to decreased survivability in stationary phase. $S.\ pombe$ mutants for the regulatory subunit were originally described as having delayed entry into stationary phase as well as decreased viability in stationary phase (DeVoti et al. 1991). It has also been demonstrated that $pka1\Delta$ cells are more viable in stationary phase than wild type cells, suggesting that Pka1 activity negatively correlates with survival in stationary phase (Roux et al. 2006). However, another study showed that $cyr1\Delta$ mutants do not survive well in stationary phase (Stiefel et al. 2004). Our results suggest that cAMP signaling is decreased in stationary phase, but Pka1 activity is still needed, since Pka1 is hyperphosphorylated in WT cells to the same degree as in $cyr1\Delta$ cells.
REFERENCES


Figure 4.1

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Figure 4.3

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C  KCl Stress ± Glucose Starvation

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Figure 4.4

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*Stationary Phase Stress*

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**B**  

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FIGURE LEGENDS

Figure 4.1. Analysis of GFP-tagged Pka1 protein from wild type and adenylate cyclase mutants.

(A) Fluorescence microscopy of pka1-GFP (WT) and cyr1Δ pka1-GFP (cyr1Δ) cells grown in YES media. (B) Protein extracts from pka1-GFP (WT) and cyr1Δ pka1-GFP (cyr1Δ) cells cultured in YES were separated by SDS-PAGE on a 7.5% polyacrylamide gel, and Pka1-GFP protein was detected by immunoblotting using anti-GFP antibody. SP870 was used as a negative control (Con). (C) Protein was extracted from cyr1Δ pka1-GFP (cyr1Δ) cells treated with 5 mM cAMP at several timepoints and visualized by SDS-PAGE (7.5% polyacrylamide) followed by immunoblotting using anti-GFP antibody. (D) Pka1-GFP protein was immunoprecipitated from cyr1Δ pka1-GFP cells and treated with alkaline phosphatase. Controls included reaction without phosphatase enzyme (Lane 1) and reaction with enzyme plus 2.5 mM of phosphatase inhibitor, sodium orthovanadate (Lane 3). The black arrows denote the hyperphosphorylated band, while the white arrows denote the dephosphorylated band.

Figure 4.2. Analysis of GFP-tagged Pka1 protein in strains lacking regulatory components of the cAMP/PKA pathway.

Protein extracts from pka1-GFP (WT), cyr1Δ pka1-GFP (cyr1Δ) cgs1Δ pka1-GFP (cgs1Δ), and cyr1Δ cgs1Δ pka1-GFP (cyr1Δ cgs1Δ) cells cultured in YES were separated by SDS-PAGE on a 7.5% polyacrylamide gel, and Pka1-GFP protein was detected by immunoblotting using anti-GFP antibody.
Figure 4.3. Effects of glucose deprivation and hyperosmotic stress on Pka1 phosphorylation.

(A) Effects of glucose starvation. pka1-GFP (WT), cyr1Δ pka1-GFP (cyr1Δ), cgs1Δ pka1-GFP (cgs1Δ), and cyr1Δ cgs1Δ pka1-GFP (cyr1Δ cgs1Δ) cells were grown to log phase in YES media, washed three times with YES-G (3% glycerol, 0.1% glucose), and resuspended in YES-G. Cell lysates were prepared and analyzed at 8 hours after incubation in YES (-) or YES-G (+) media. (B) Effects of hyperosmotic stress. pka1-GFP (WT), cyr1Δ pka1-GFP (cyr1Δ), cgs1Δ pka1-GFP (cgs1Δ), and cyr1Δ cgs1Δ pka1-GFP (cyr1Δ cgs1Δ) cells were grown to mid-log phase in YES media, and one volume of YES + 2.4 M KCl for a final KCl concentration of 1.2 M. Cell lysates were isolated from cultures grown in YES media (-) or treated with YES + 1.2 M KCl (+) for 8 hours.(C) Combined effects of glucose deprivation and hyperosmotic stress. pka1-GFP (WT) and cgs1Δ pka1-GFP (cgs1Δ) cells were cultured in YES + 1.2 M KCl overnight, washed 3X with YES-G + 1.2 M KCl (YES-G + KCl), and resuspended at 5x10⁶ cells/ml in YES-G + KCl. Cells were incubated for an additional 8 hours before immunoblotting. Shown is protein from cells untreated (-) or treated with YES-G + 1.2 M KCl (+). For (A), (B) and (C), protein was separated by SDS-PAGE (7.5% polyacrylamide) and immunoblotted using anti-GFP antibody. Black arrows denote the hyperphosphorylated band, while white arrows denote the dephosphorylated band.

Figure 4.4. Analysis of Pka1 in stationary phase cultures.

Proteins were isolated from pka1-GFP (WT), cyr1Δ pka1-GFP (cyr1Δ), cgs1Δ pka1-GFP (cgs1Δ), and cyr1Δ cgs1Δ pka1-GFP (cyr1Δ cgs1Δ) cells grown to log phase, 0
hours after stationary phase entry, and 24 hours after stationary phase entry and separated by SDS-PAGE (7.5% polyacrylamide). Pka1-GFP protein was visualized by immunoblotting using anti-GFP antibody. Black arrows denote hyperphosphorylated bands.
Response to physiological stress is achieved by coordinated action of various signaling pathways, which ultimately bring about physical changes within the cell, including changes in lipid composition and membrane structure. The results of these studies have demonstrated previously undescribed roles for the phospholipase B1 (Plb1) and the cAMP-PKA pathway in cellular response to hyperosmotic and nutritional stress in the fission yeast *Schizosaccharomyces pombe*.

*Plb1 and the cAMP-PKA pathway respond to glucose limitation*

The role of the cAMP-PKA pathway in glucose sensing in yeast is well known. External glucose is detected by way of the transmembrane protein Git3, which initiates a signaling cascade that ultimately triggers production of the second messenger cAMP by adenylate cyclase and subsequent activation of the PKA catalytic subunit Pka1. When glucose is present in the culture media, this pathway represses transcription of gluconeogenic genes, namely *fbp1* (Hoffman and Winston 1990; Hoffman and Winston 1991; Welton and Hoffman 2000). Stationary phase is also associated with nutrient limitation and reduced Pka1 activity, since loss of *pka1* leads to increased survival in stationary phase (Roux et al. 2006). However, *cgs1Δ* cells have reduced stationary phase survival (Stiefel et al. 2004). Glucose limitation and stationary phase both induce export
of the Pka1 catalytic subunit from the nucleus to the cytoplasm and vacuole (Matsuo et al. 2008; Gupta et al. 2011a). Glucose starvation has been shown to induce increased phosphorylation and nuclear export of the Pka1 catalytic subunit, pointing to phosphorylation as a regulatory mechanism for Pka1 localization (Matsuo et al. 2008; McInnis et al. 2010; Gupta et al. 2011a). The phosphorylation seen in response to glucose starvation is dependent on T356 phosphorylation by the kinase Ksg1 (Gupta et al. 2011b). We have found that Pka1 is also phosphorylated in cells lacking the gene cyr1, which encodes adenylate cyclase. The Pka1 phosphorylation seen in cyr1Δ cells appears to be more extensive than that observed in wild type cells deprived of glucose, suggesting that Pka1 is either phosphorylated at multiple sites by Ksg1 or that another kinase is responsible for the extra phosphorylation seen in the absence of cyr1 (McInnis et al. 2010). Glucose starvation and stationary phase entry both lead to increased proportion of hyperphosphorylated Pka1 in cyr1Δ cells. A similar degree of phosphorylation is only observed in wild type cells grown to stationary phase. These results suggest that one possible function of this hyperphosphorylation is to preserve Pka1 activity in conditions of reduced cAMP signaling. These results also suggest that cAMP signaling is completely turned off in stationary phase, whereas some cAMP signaling remains during glucose deprivation, since this hyperphosphorylated form is seen in WT cells in stationary phase, but not in glucose-deprived conditions. A previous study has shown that cAMP levels are not substantially reduced in response to glucose deprivation (Eraso and Gancedo 1984). These results also suggest that, contrary to previous studies (DeVoti et al. 1991; Lin et al. 2002; Roux et al. 2009; Roux et al. 2010), Pka1 is active during
stationary phase, since Pka1 protein is hyperphosphorylated in wild type cells to the same degree as in cyr1Δ cells.

Interestingly, the hyperphosphorylated Pka1 band that is seen in cyr1Δ cells is not observed when cgs1 is also absent (McInnis et al. 2010). It has also been reported that the Cgs1-Pka1 complex is formed prior to hyperphosphorylation of Pka1, and Pka1 association with the regulatory subunit was required for Pka1 phosphorylation (Gupta et al. 2011b). These results suggest that interaction of Cgs1 and Pka1 is required for Pka1 phosphorylation. It is possible that the fission yeast PKA regulatory subunit plays a similar role to its counterpart in mammals and recruits kinases to the catalytic subunit (Cooper 2005). The results with cgs1Δ cells also shed light for an additional function of Pka1 hyperphosphorylation. Conditions that induce the hyperphosphorylation of Pka1 in wild type cells, such as stationary phase and combined glucose starvation with osmotic stress, also lead to reduced levels of Pka1 protein in cgs1Δ cells (McInnis et al. 2010). This suggests that hyperphosphorylation may serve to increase the stability of the Pka1 protein. Another possible role for this hyperphosphorylation might be to block Cgs1 binding sites on the Pka1 protein.

Studies examining the contribution of phospholipase B to nutritional stress response are not quite as exhaustive as those involving the cAMP-PKA pathway. In S. cerevisiae, inositol and phosphate depletion are associated with increased production of GroPI, presumably via hydrolysis of PI by Plb3 (Patton et al. 1995; Merkel et al. 2005a; Patton-Vogt 2007). PLB1 from Cryptococcus neoformans was shown to be required for growth when LysoPC was the only carbon source (Wright et al. 2007). Several studies have documented various environmental conditions that affect secretion of PLB activity.
In *Cryptococcus neoformans*, conditions that replicate the host environment, such as serum and 37°C incubation temperature, increase secretion of PLB, LPL, and LPTA activities associated with PLB1 (Wright et al. 2002). Glucose does not seem to affect PLB1 secretion in this organism, although increasing the glucose concentration to hyperosmotic levels does increase secretion (Wright et al. 2007). In *S. cerevisiae*, re-feeding glucose to stationary phase cultures increases GroPI production (Hawkins et al. 1993). These studies would suggest that glucose is a positive effector for secretion of PLB activity. However, our results suggest that glucose is a negative regulator of Plb1 secretion in log-phase *S. pombe* cells. However, addition of glucose to cells grown in minimal media did not completely abrogate Plb1 secretion, indicating that additional media components affect the rate of Plb1 secretion. While Plb1 secretion is increased on media with low glucose, Plb1 is not required for growth on glucose, perhaps due to redundant function by other PLBs. In contrast, *plb1Δ* cells grew more slowly on minimal media than wild type cells, suggesting that Plb1 is needed for growth when other nutrients are deficient. It was surprising to find that Plb1 secretion was not increased in the absence of Pka1, as this kinase functions in a glucose-sensing pathway. However, the protein levels of Plb1 were increased in *pka1Δ* cells, and Plb1 localization in *pka1Δ* cells was similar to that seen in wild type cells incubated in low glucose media. This suggests that Pka1 may regulate Plb1 in response to glucose deprivation, though Plb1 secretion is not regulated by Pka1.
**Plb1 and the cAMP-PKA pathway are necessary for maintenance of mitochondrial structure under conditions of hyperosmotic stress**

One of the challenges faced by yeast researchers attempting to assign a functional role for PLBs in non-pathogenic yeasts is the absence of a discernable phenotype for strains in which all of the genes encoding PLBs have been deleted (Merkel et al. 2005a). The only role ascribed to PLB in a non-pathogenic context is for the removal of excess lysophospholipids that have been supplied to the cell by artificial means (Fyrst et al. 1999, Merkel et al. 1999; Zhang et al. 2009). Plb1 from *S. pombe* is unique, in that its deletion leads to several phenotypes, the key one being growth and morphological defects in hyperosmotic media (Yang et al. 2003). Hyperosmotic stress in *S. cerevisiae* was associated with intracellular production of GroPC from the turnover of PC (Kiewietdejonge et al. 2006). Though we did determine that intracellular Plb1 activity is sufficient for hyperosmotic stress response, we did not observe reduction in PC levels in either wild type or plb1Δ treated with 1.2 M KCl. In fact, differences in whole-cell phospholipid levels were not detected between wild type and plb1Δ cells treated with 1.2 M KCl. However, plb1Δ cells did display aberrant mitochondrial morphology when treated with 1.2 M KCl for extended periods of time. Mitochondrial fragmentation in the plb1Δ cells was accompanied by mitochondrial depolarization, as indicated by MitoTracker-Red staining. Interestingly, conditions that have been shown to induce mitochondrial fragmentation, such as rotenone and loss of mitochondrial PE synthesis (Wang et al. 2010; Joshi et al. 2012; Signorell et al. 2009; Kuroda et al. 2011), also lead to worsened phenotypes of plb1Δ cells.
It is not unlikely that hyperosmotic stress induces mitochondrial damage, and that these damaged mitochondria would need to be removed by mitophagy, a type of autophagy specific for mitochondria. Autophagy is induced by the addition of osmotic and desiccation stresses in several organisms (Han et al. 2010; Ratnakumar et al. 2010; Kuzuoglu-Ozturk et al. 2012). In addition, treatment of renal medullary with the salt NaCl induces depolarization of mitochondria, as indicated by reduced electric membrane potential ($\Delta \Psi_m$) (Michea et al. 2002). Following fragmentation of the mitochondrial network, healthy mitochondria fuse together to reform the network, whereas depolarized mitochondria are incapable of fusion and must be degraded by mitophagy (Okamoto and Kondo-Okamoto 2012). It is therefore likely that mitophagy is increased under hyperosmotic conditions in order to remove depolarized mitochondria. The observation that $plb1\Delta$ cells incubated in 1.2 M KCl for 24 hours exhibited depolarized mitochondria with a fragmented morphology suggested that Plb1 might promote mitophagy of damaged mitochondria. However, degradation of the mitochondrial protein Sdh2 was increased in KCl-treated $plb1\Delta$ cells, suggesting that mitophagy is increased in these cells. Interestingly, our lab has found that overexpression of the proteasome subunit, Pra7, can rescue the KCl-sensitivity of $plb1\Delta$ (unpublished observation). It is possible that overexpression of Pra7 ameliorates the KCl-sensitivity phenotype of $plb1\Delta$ by working in tandem with the autophagy pathway to remove damaged mitochondrial proteins.

The fact that KCl-treated $plb1\Delta$ cells experience increased rates of mitophagy indicates that these cells have more instances of damaged mitochondria, perhaps due to an inherent mitochondrial defect that makes the mitochondria of these cells more prone to
depolarization and fragmentation. One possibility is that Plb1 participates in a process that transports PS, which is synthesized in the ER, to the mitochondria. PS is transported from the ER to the mitochondria via the mitochondria-associated membrane (MAM), which is formed by close contact between the membranes of the two organelles (Ardail et al. 1991; Voelker 1991; Daum and Vance 1997; Kuge and Nishijima 2003; Voelker 2003). These membrane contacts are maintained by protein complexes that act as molecular tethers (Kornmann et al. 2009). Mitochondrial fragmentation is likely to occur as a result of reduction of PS, which is necessary to promote lamellar phase formation by PE. One of the most abundant mitochondrial phospholipids is PE, which has an innate ability to make reverse hexagonal structures in aqueous solution (Figure 5.1; Israelachvili et al. 1975; Zimmerburg 2000). Stabilization of PE in a lamellar phase (bilayer) structure requires the presence of PS in the membrane (Lewis and McElhaney 2000; Li and Schick 2000). It is possible that the prolonged mitochondrial fragmentation seen in KCl-treated plb1Δ cells is due to reduced mitochondrial PS.

The hypothesis made in the previous paragraph may also explain why GFPPlb1, which does not enter the secretory pathway and therefore defaults to the cytoplasm, is able to compensate for the loss of Plb1, which is normally expected to pass through the ER lumen via the secretory pathway. The majority of PS is synthesized in the MAM (Vance 1990), and PS is transported to the mitochondria via close association of the MAM and mitochondrial outer membranes (Achleitner, et al. 1999). However, only PS species with shorter, more unsaturated fatty acids are able to traverse from the MAM into the mitochondria, perhaps due to the increased ability of these phospholipid species to diffuse between membranes (Nichols 1988; Heikinheimo and Somerharju 1998;
Heikinheimo and Somerharju 2002). One possibility is that Plb1 ameliorates the process of PS translocation from the MAM to the mitochondria under conditions of hyperosmotic stress by replacing hydrophobic fatty acids of PS with more hydrophilic fatty acids. It is plausible that this can be done from the MAM lumen, which would target the inner MAM membrane, or the cytosol, which would target the outer MAM membrane. However, in the case of Plb1 affecting fatty acid identity of PS in the inner membrane of MAM, a PS flippase may also be required for translocation of modified PS from the inner to the outer MAM membrane.

This study has also revealed a genetic connection between Plb1 and PE biosynthesis genes in the KCl stress response. This connection may explain cytokinesis defects seen in KCl-treated \( plb1\Delta \) mutant in a previous study (Yang et al. 2003) and in the \( pka1\Delta \) mutant in this study. Components of the cAMP-PKA pathway were found to rescue the KCl-sensitive phenotype of \( plb1\Delta \), and likewise, share the KCl-sensitivity phenotype of \( plb1\Delta \) when they are deleted (Yang et al. 2003). Cells in which \( pka1 \) has been deleted are able to survive but are not able to grow on hyperosmotic media. Analysis of \( pka1\Delta \) cells arrested in G2/M phase prior to KCl treatment revealed that these cells are unable to complete cytokinesis. Additionally, \( plb1\Delta \) cells treated with KCl display defects in cytokinesis, such as cells with mispositioned septa and multiseptated cells (Yang et al. 2003). The redistribution of PE to the outer leaflet of the plasma membrane has been shown to be important for the completion of cytokinesis. Specifically, re-internalization of PE at the cleavage furrow is necessary for disassembly of the contractile ring and inactivation of RhoA (Emoto et al. 1996; Emoto and Umeda 2000; Emoto and Umeda 2001; Emoto et al. 2005). Additionally, reducing PE levels by
deleting all three PSDs in *S. pombe* leads to defects in cytokinesis (Luo et al. 2009). A mutant CHO cell line in which PE content was reduced by 50% was also inhibited for cytokinesis (Emoto and Umeda 2000). It is possible that Plb1 and the cAMP-PKA pathway cooperate to properly localize PE to the site of cell division under conditions of hyperosmotic stress.

*Concluding remarks*

While the enzymatic functions of PLBs have been thoroughly studied, and the function of these proteins as virulence factors in pathogenic fungi has been well established, the cellular functions of PLBs in non-pathogenic organisms is somewhat unclear. Studies in *S. cerevisiae* suggest a role for PLBs in clearing excess lysophospholipids that had been introduced by artificial means (Fyrst et al. 1999; Zhang et al. 2009). Multiple studies have pointed to a role for PLBs in environmental phospholipid scavenging, though few studies have examined environmental conditions that affect secretion of PLB. In this study we have found that secretion of Plb1 is influence by the amount of environmental glucose. However, the major glucose-sensing pathway in *S. pombe* was not found to influence Plb1 secretion. It would be interesting to study the mechanism by which glucose content affects Plb1 secretion, and which cellular players are involved. Also, secretion of Plb1 was not completely abrogated with addition of glucose to minimal media, suggesting that there are additional nutrients that control Plb1 secretion.

Interestingly, the cytokinesis defects of *plb1Δ* and *pka1Δ* in hyperosmotic conditions may be due to improper localization of PE at the site of cell division. We
speculated that Plb1 and the cAMP-PKA pathway participate in proper localization of PE to ensure completion of. However, further testing is necessary to verify this hypothesis. Proper localization to the cell division site can easily be monitored using a biotinylated cinnamycin construct (Emoto et al. 1996; Emoto and Umeda 2000; Emoto and Umeda 2001; Emoto et al. 2005). Cinnamycin is a peptide produced by several Streptomyces species that has the ability to bind PE (Makino et al. 2003). We would predict that KCl-treated plb1Δ and pka1Δ would have reduced cinnamycin staining at the site of cell division.

We also saw in Chapter 4 that a portion of Pka1 is hyperphosphorylated in cyr1Δ cells. It would be interesting to study which residues are being phosphorylated as well as the kinase responsible for this phosphorylation. Another area that could be studied would be the implications of this hyperphosphorylation on cellular function of Pka1. One possible role would be to increase the stability of the protein, since Pka1 protein levels are decreased in cgs1Δ cells under conditions in which WT Pka1 is hyperphosphorylated. To verify this hypothesis, we would need to monitor Pka1 protein in WT and cyr1Δ cells treated with cyclohexamide in order to compare the half-life of hyperphosphorylated Pka1.
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Figure 5.1.
FIGURE LEGEND

Figure 5.1. Structure of different liquid crystalline phases of membranes in aqueous solution.

Shown are lamellar (A) and reverse hexagonal (B) phases.