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Snf1 Mediated Phosphorylation and Activation of PAS Kinase

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ABSTRACT

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Nutrient sensing kinases sense available nutrients and regulate cell activity accordingly. Three of these enzymes are AMP regulated kinase (AMPK, or Snf1 in yeast), PAS kinase, and target of rapamycin (TOR), are conserved from yeast to man and have overlapping function. AMPK and Snf1 are important in sensing when nutrient status in the cell is low and down regulating energy consuming pathways. PAS kinase is required for glucose homeostasis in the cell, and responds to glucose levels. TOR senses nutrients such as amino acids and upregulates cell growth pathways primarily through protein synthesis. Due to the varying nature of these enzymes, cross talk is expected in order for the cell to properly regulate cellular metabolism and growth in response to energy and nutrient availability. Previous studies have shown that activation of yeast PAS kinase under nutrient stress conditions requires the presence of Snf1. The aim of this thesis is to determine whether Snf1 directly phosphorylates and activates PAS kinase through both in vivo and in vitro approaches. PAS kinase was found to require Snf1 for both activation and phosphorylation in vivo. In vitro kinase assays were also performed to confirm a direct phosphorylation event. The results from this study support the direct phosphorylation and activation of PAS kinase by Snf1, linking cellular energy status to glucose allocation.

Keywords: nutrient sensing kinases, target of rapamycin (TOR), AMP regulated kinase (AMPK), PAS kinase (PSK or PASK), Osh7, cellular metabolis

Table of Contents

List of Tables

List of Figures

Specific Aims

Aim 1: Find In vivo evidence for Snf1 phosphorylation of PAS kinase

A. Determine the sites critical for the Snf1-dependent gel shift seen in purified PAS kinase samples
B. Provide evidence for a cellular interaction between PAS kinase and Snf1

Aim 2: Determine if Snf1 directly phosphorylates and activates PAS kinase

A. Determine whether purified Snf1 directly phosphorylates purified PAS kinase
B. Determine whether phosphorylated PAS kinase is hyperactivated compared to unphosphorylated PAS kinase

Aim 3: Determine if Snf1 directly phosphorylates Osh7

CHAPTER 1: Introduction

Organisms have evolved the ability to sense nutrient availability in their environment. This allows the organism to adapt to the circumstances of the environment, being able to switch between situations of energy consumption to energy production as circumstances require. For example, humans and other animals have developed endocrine systems to regulate energy distribution throughout the body. Deregulation of the endocrine system and nutrient distribution has been associated with metabolic diseases such as diabetes.

Much like multicellular organisms have developed systems for regulating energy consumption and distribution of nutrients, single cells also have highly developed systems for sensing and regulating nutrient availability. Three of these nutrient sensing pathways are the AMP-regulated kinase (AMPK or Snf1), PAS kinase, and the target of rapamycin (TOR) each playing a unique role in sensing and utilizing nutrients in the cell.

AMPK

AMPK directly senses the energy status of the cell and upregulates energy producing pathways when cellular energy is low. AMPK was first known for its role in regulating fatty acid and cholesterol synthesis. AMPK was discovered as an inhibitor of Acetyl CoA carboxylase, the rate limiting enzyme in long chain fatty acid synthesis (Carlson and Kim, 1973). This inhibition of Acetyl CoA carboxylase occurs through direct phosphorylation. AMPK activity was also found to be responsible for the inhibition of hydroxymethylglutaryl-CoA (HMG-CoA), a key enzyme in cholesterol synthesis (Beg et al., 1978). It was later found that this inhibition occurred with low ATP availability (Carling et al., 1987).

AMPK is activated when the AMP to ATP ratio is high, responding to decreased energy status of the cell. AMP directly binds to the AMPK complex and allosterically activates it (Cheung et al., 2000). Along with allosteric activation, AMP (as well as ADP) inhibits inactivation of AMPK by binding the gamma subunit and preventing dephosphorylation of the activation loop (Suter et al., 2006; Xiao et al., 2011). AMPK is also activated by upstream kinases, primarily by LKB1 (Hawley et al., 2003; Woods et al., 2003). Another upstream activating kinase is calmodulin-dependent protein kinase kinase (CaMKK), which phosphorylates AMPK in response to a Ca^{2+} influx (Hawley et al., 2005; Woods et al., 2005)

Once AMPK is activated, it turns on energy production pathways and downregulates energy consumption processes. In addition to inhibiting fatty acid and cholesterol biosynthesis (discussed above), AMPK increases cellular energy status is to allow for more glucose to enter the cell. Activated AMPK leads to the translocation of GLUT4 vesicles to the cell membrane (Kurth-Kraczek et al., 1999), and upregulates the rate of GLUT4 transcription (Zheng et al., 2001). This provides an insulin independent mechanism of promoting GLUT4 translocation to the membrane (Hayashi et al., 1998). Similarly, AMPK has been found to promote GLUT1 translocation in cells that use GLUT1 transporters (Abbud et al., 2000).

Along with allowing for more glucose to enter the cell, AMPK regulates how the glucose is used upon entry. AMPK activation results in an increase in phosphofructokinase 2 activity, an important regulator in glycolysis, resulting in increased glycolysis and increased ATP synthesis (Marsin et al., 2000). AMPK also leads to decreased glycogen accumulation through the inactivation of glycogen synthase (Halse et al., 2003).

YEAST AMPK HOMOLOG, SNF1

Yeast Snf1 is a homologue of mammalian AMPK and is structurally similar to AMPK in both catalytic and non-catalytic domains (Mitchelhill et al., 1994; Stapleton et al., 1994; Woods et al., 1994). In addition to structural similarity, Snf1 functions similar to AMPK in that it activates respiration, and decreases fatty acid biosynthesis in yeast. Snf1 activates respiration in response to glucose deprivation in a process referred to as glucose derepression (Celenza and Carlson, 1986). Snf1 kinase activity allows for the expression of genes, including those required for respiration, normally repressed when glucose is readily available. Thus, yeast with a Snf1 mutation lose the ability to grow on non-fermenting carbon sources such as sucrose (Carlson et al., 1981).

Structure of the Snf1 Complex

The Snf1 complex is composed of three subunits, the catalytic Snf1 alpha subunit, the Snf4 gamma subunit and one of three beta subunits. The Snf1 catalytic subunit has both a kinase domain and a regulatory inhibitory domain (Celenza and Carlson, 1986). When glucose concentration is high in the cell the inhibitory domain binds to and inhibits the catalytic domain. However, when the glucose concentration becomes low the Snf1 inhibitory domain binds to the Snf4 subunits, relieving autoinhibition (Jiang and Carlson, 1996).

The Snf4 gamma subunit was found to physically interact with the Snf1 catalytic unit and is required for full Snf1 activity (Celenza and Carlson, 1989; Celenza et al., 1989). Snf4 also associates with each of the three beta subunits Sip1, Sip2, and Gal83 (Jiang and Carlson, 1997). The Snf1 complex is not activated allosterically, such as seen with AMPK, however it is activated in conditions that raise the AMP:ATP ratio (Wilson et al., 1996). The inability of the Snf1 complex to be allosterically activated by AMP may be explained by differences in the Snf4

subunit compared to the AMPK gamma subunit. When a His151Gly substitution, such as seen in the Snf4 subunit, is introduced into the AMPK gamma subunit, AMPK loses the ability to be allosterically activated by AMP (Adams et al., 2004).

The beta subunits (Sip1, Sip2, and Gal83) help direct the Snf1 complex to substrates. When glucose levels are high each of the three beta subunits are found in the cytosol. However, when glucose concentrations are low, the beta subunits localize to different parts of the cell, with Gal83 localizing to the nucleus, Sip2 remaining in the cytoplasm, and Sip1 localizing to the vacuole (Vincent et al., 2001). Since Gal83 is the beta subunit that localizes to the nucleus, it would appear that the Snf1-Gal83 is the complex is critical for the derepression of glucose-repressed genes.

Regulation of Snf1 Activity

Like AMPK, Snf1 is phosphorylated and activated by upstream kinases, which may explain how it responds to a high AMP:ATP ratio despite its inability to directly bind AMP (McCartney and Schmidt, 2001). Sak1, Elm1, and Tos3 phosphorylate and activate Snf1. A triple *sak1elm1tos3* deletion results in a *snf1* phenotype of impaired growth on a non-glucose carbon source (Hong et al., 2003). Of these enzymes only Elm1 is able to phosphorylate Snf1 in vitro, however an Elm1 deletion alone is not sufficient to produce a *snf1* phenotype (Sutherland et al., 2003). This suggests that all three enzymes are capable of activating Snf1.

While all three kinases are involved in activating Snf1, it does appear that each enzyme has a slight preference for the Snf1-β subunit complex they phosphorylate (Hedbacker et al., 2004; McCartney et al., 2005). Sak1 appears to be the major kinase that activates the Snf1-Gal83 complex, which is the only complex that is nuclear (Hedbacker et al., 2004). This would suggest that Sak1 is the major upstream kinase of Snf1 that affects glucose derepression. While

each kinase shows a preference for the Snf1 complex it phosphorylates, they are not limited to one Snf1-β subunit complex (McCartney et al., 2005). Snf1 is also activated under environmental stresses, such as sodium ion or alkaline pH stress, and this activation is dependent on the same three upstream kinases that are involved in metabolic stress (Hong and Carlson, 2007). It appears that under environmental stress Sak1 is again the major activating kinase for Snf1 (Hong and Carlson, 2007).

The inactivation of Snf1 is mainly through a Reg1-Glyc7 protein complex that dephosphorylates Snf1 when glucose is high (Huang et al., 1996a). Glyc7 harbors the phosphatase activity, and Reg1 is responsible for targeting Glyc7 to the Snf1 complex (Ludin et al., 1998; Tu and Carlson, 1995). In *reg1* cells Snf1 remains active regardless of the glucose status of the cell. When glucose concentrations become low in the cell, Snf1 phosphorylates Reg1, inactivating the Reg1-Glyc7 complex. (Sanz et al., 2000).

Downstream Effects of Snf1

Like mammalian AMPK, Snf1 influences many cellular functions with the aim of increasing energy production, and allowing the cell to adapt to a non-fermentable carbon source. As mentioned above, one of the major functions of Snf1 is to relieve inhibition of glucose repressible genes; this is done through the direct phosphorylation of repressor protein Mig1 (Treitel et al., 1998). When Mig1 is phosphorylated it is exported from the nucleus and no longer represses genes required for respiration, allowing the cell to utilize non-glucose carbon sources (DeVit and Johnston, 1999; Ostling and Ronne, 1998). Snf1 also phosphorylates and activates transcriptional activators such as Sip4 and Cat8, which also aids the yeast's ability to grow on alternative carbon sources (Lesage et al., 1996; Randez-Gil et al., 1997).

Along with the control of transcription factors, Snf1 is involved in the phosphorylation and regulation of many metabolic enzymes. Snf1, like AMPK, phosphorylates and inactivates acetyl-CoA carboxylase (Woods et al., 1994), inhibiting fatty acid biosynthesis. In contrast to mammalian cells where AMPK represses glycogen synthesis, yeast require Snf1 in order to accumulate glycogen and Snf1 activity is needed in order to activate glycogen synthase (Huang et al., 1996b).

PAS KINASE

PAS kinase is a newly discovered protein that is highly conserved from yeast to man and is an important enzyme involved in glucose allocation (Rutter et al., 2001). PAS kinase has both a regulatory N-terminal PAS domain and a serine/threonine kinase domain, both of which are highly conserved between species (Rutter et al., 2001). The PAS domain binds the kinase domain inhibits catalytic activity (Amezcua et al., 2002), with PAS kinase becoming more active when lacking the PAS domain (Rutter et al., 2001). The PAS domain adopts a typical structure comparable to other PAS domains with a β structure flanked by α helices (Amezcua et al., 2002). In an NMR screen using over 750 small organic molecules, the PAS domain was able to bind 9 hydrophobic compounds within its hydrophobic core and adopts a conformation similar to other ligand-bound PAS domains (Amezcua et al., 2002). In addition, these ligands appear to activate PAS kinase in vitro, suggesting that ligand binding inhibits kinase domain binding. While the biologically relevant molecule is yet to be determined, it is likely that the PAS domain binds a small molecule which allows for PAS kinase to self-regulate.

PAS kinase is important in the allocation of glucose in the cell. A *psk1psk2* mutant yeast strain accumulates excess carbohydrate and shows a reduction in glucans necessary for cell wall biosynthesis (Rutter et al., 2002; Smith and Rutter, 2007). In addition, the cells have an impaired

ability to grow on galactose (Rutter et al., 2002). Rutter et al. found that PAS kinase was able to phosphorylate transcription factors Caf20, Tif11, and Sro9, as well as UDP-glucose pyrophosphorylase (Ugp1) and glycogen synthase (Rutter et al., 2002).

Ugp1 at this time remains the most well understood PAS kinase substrate. Ugp1 is the major producer of UDP-glucose, which can be used for glycogen synthesis or cell wall beta-glucan synthesis (Daran et al., 1995). When PAS kinase phosphorylates Ugp1 it changes its cellular location, from the cytoplasm to cell periphery, which results in the donation of glucose to cell wall components at the expense of glycogen formation (Smith and Rutter, 2007). Human PAS kinase was also found to phosphorylate mammalian glycogen synthase at a known phosphosite in vitro, and inhibit glycogen synthase activity (Wilson et al., 2005).

While PAS kinase plays an important role in regulating metabolic events at a cellular level, its function affects the metabolic response of the whole organism as well. Glucose was found to activate PAS kinase in Min6 pancreatic β-cells (da Silva Xavier et al., 2004). Activated PAS kinase led to the activation of the preproinsulin promoter and inactivation of PAS kinase inhibited the preproinsulin promoter (da Silva Xavier et al., 2004). PAS kinase was found to regulate insulin transcription through the regulation of the transcription factor pancreatic duodenal homeobox-1 (PDX-1). It was found that when PAS kinase is activated by glucose, PDX-1 is less phosphorylated (Semache et al., 2013). This phosphorylation is likely to be indirect because PAS kinase has been shown to phosphorylate GSK3β , a known PDX-1 kinase. The phosphorylation of GSK3β occurs at Ser9, a known phosphorylation site that leads to the inactivation of GSK3β. Activated GSK3β leads to the phosphorylation and degradation of PDX-1 (Boucher et al., 2006). Thus, inactivation of GSK3β by PAS kinase phosphorylation would lead to increased stability of the PDX-1 protein (Semache et al., 2013). Supporting these results,

PAS kinase deficient mice were found to have impaired glucose-stimulated insulin secretion (Hao et al., 2007).

When the PAS kinase deficient mice were placed on a high fat diet they were resistant to weight gain, liver triglyceride accumulation, and were more sensitive to insulin (Hao et al., 2007). There is no significant difference between PAS kinase deficient mice and wild type in their food intake or activity levels; instead they appear to be hyper-metabolic, with more energy being released as heat (Hao et al., 2007). The PAS kinase deficiency in mice appears to confer a protective effect against the development of type II diabetes, and human mutations in PAS kinase (hPASK) have been found that lead to development of maturity-onset diabetes in the young (MODY). These L1051V and G1171E mutations of hPASK were found to be more active (able to autophosphorylate and phosphorylate substrates at greater specific activity) (Semplici et al., 2011), consistent with the protective role of a PAS kinas knockout. Thus we see that PAS kinase plays an important role in coordinating a metabolic response in yeast, mice and humans.

PAS Kinase is Activated by Metabolic and Cell Integrity Stress

While PAS kinase is an important enzyme in the regulation of glucose in the cell there is still much to learn about its regulation and function within the cell. There are two known stimuli that have been found to activate PAS kinase, growth on a non-fermantative carbon source and cell integrity stress (Grose et al., 2007). Under cell integrity stress conditions the Wsc proteins, membrane proteins that sense cell integrity stress, are required for activation. Interestingly, Snf1 was found to be required for activation of PAS kinase under nutrient stressing conditions, with PAS kinase becoming active under glucose deprivation, the same conditions as Snf1 activation (Grose et al., 2007). Another interesting observation was that the yeast orthologs Psk1 and Psk2

play a different role in response to the two stimuli, with Psk1 having the major role in response to metabolic stress.

TOR

Tor is named as such because it is the physical Target of Rapamycin (Sabatini et al., 1994), a commonly used drug. In yeast it was identified that the effect of rapamycin, G1 arrest, was the same as a knockout of the TOR genes (Kunz et al., 1993). The mammalian TOR protein forms two different complexes TORC1 and TORC2 with different functions (Loewith et al., 2002). In yeast there are two homologs of the TOR gene, TOR1 and TOR2, both of which have similar function and structure (Helliwell et al., 1994). While TOR1 is only in complex 1, TOR2 can be part of either complex 1 or complex 2.

TORC1

TORC1 is activated during times of nutrient abundance, such as high amino acid concentration, and activates autophagy as well as ribosomal translation. TORC1 dependent autophagy and phosphorylation of S6 protein were reported when amino acid concentration is high (Blommaart et al., 1995). When amino acids are present TOR binds with RAPTOR, which is required for the phosphorylation of the TORC1 substrates p70 SK6 protein and 4EBP1 (Hara et al., 2002). When 4EBP1 is phosphorylated it prevents the formation of the eIF4E and eIF4G complex (Sonenberg and Gingras, 1998), necessary for translation to begin (Moerke et al., 2007). TORC1 also upregulates lipid biosynthesis to allow for cell growth (Laplante and Sabatini, 2009). In summary TORC1 is activated in the presence of amino acids and promotes cell growth and proliferation via protein, ribosome and lipid biosynthesis.

TORC2

TORC2 in contrast to TORC1 is rapamycin insensitive, however it can still be disrupted by rapamycin, leading to a lethal phenotype (Loewith et al., 2002). Prolonged treatment of rapamycin prevents formation of TORC2, and as a consequence it is unable to maintain activation of downstream elements (Sarbassov et al., 2006). While both TORC1 and TORC2 function in protein biosynthesis, a loss of function of the TORC2 complex specifically leads to disorganization of the actin cytoskeleton (Schmidt et al., 1996). The yeast TOR1 protein is only found in TORC1 while TOR2 can be part of both TORC1 or TORC2, the actin cytoskeleton organization is associated only with TORC2 (Wullschleger et al., 2005). These differential roles of TORC1 and TORC2 are conserved in mammalian cells as well, with mammalian TORC2 (mTORC2) also being rapamycin insensitive and involved in the actin cytoskeleton organization (Jacinto et al., 2004). TORC2 dependent actin organization occurs through the Rho1 GTPase (Helliwell et al., 1998; Jacinto et al., 2004). Much like its acitivity, little is known about the regulation of TORC2 activity. One of the ways that TORC2 may be activated is through the physical association with the ribosome. TORC2 has been found to physically associates with the ribosome after activation of the insulin responsive kinase PI3K (Zinzalla et al., 2011).

CROSSTALK BETWEEN NUTRIENT SENSING ENZYMES

The activities of AMPK/Snf1, PAS kinase and Target of Rapamycin (TOR) are tightly regulated in order to execute an efficient response to changes in metabolic conditions. That is, the cell needs to partition glucose appropriately between ATP production pathways, or growth and proliferation pathways, suggesting that these pathways overlap. In fact, the activation of one of these kinases has been reported to lead to the activation or inactivation of another. There is evidence that activation of AMPK leads to the inactivation of TORC1 (Bolster et al., 2002) by

directly phosphorylating the regulatory-associated protein of mTOR (RAPTOR) (Gwinn et al., 2008). Phosphorylation and activation of TSC2 by AMPK during nutrient stress can also lead to the inactivation of mTOR, however TSC2 is not present in yeast. While AMPK has been found to regulate TORC1 activity, PAS kinase has been found to suppress TORC2 activity. Overexpression of PAS kinase was found to suppress a growth defect from a TORC2 temperature sensitive mutation (Cardon et al., 2012). The suppression of this growth defect was through the activation of the GTPase Rho1 and required Ugp1 phosphorylation. The downstream events of Rho1 that rescue the *tor2*^{ts} mutation remain unknown.

Nutrient sensing kinases have long been the target of many drug therapies. TOR is a target of anti-cancer therapies (Oshiro et al., 2004). AMPK is also the target of metformin, a major treatment for diabetes (Long and Zierath, 2006). While TOR and AMPK have been used as treatments for metabolic disorders, PAS kinase could prove to be another potential target for treatment of metabolic related diseases. In support of this human mutations in PAS kinase have been found to cause hypersecretion of insulin and lead to early onset of diabetes (Semplici et al., 2011). Also, as previously mentioned, PAS kinase knockout mice were found to be resistant to weight gain and had increased insulin sensitivity (Hao et al., 2007).

Studying these nutrient sensing enzymes and their interactions will not only further our understanding of basic cellular processes, but allow us to understand complex metabolic diseases. The remainder of this work will explore a new level of cross talk between the nutrient sensing kinase PAS kinase and Snf1.

CHAPTER 2: In Vivo Evidence for Snf1 Phosphoryation of PAS Kinase

Both Snf1 and PAS kinase 1 (Psk1) are activated in yeast when cells are grown on a non-fermentable carbon source such as raffinose. In addition, the presence of Snf1 is required for this activation (Figure 2.1) and activation occurs post-translationally (Grose et al., 2007). **We hypothesize that Snf1 directly phosphorylates and activates PAS kinase under non-fermenting conditions.** In vivo activation assays in order to determine how quickly PAS kinase is activated, which may support direct phosphorylation or transcriptional regulation. The results of this assay showed that PAS kinase is activated quickly when switched from glucose to raffinose containing media (Figure 2.1, Dr. Julianne Grose, unpublished data). This quick activation (less than 5 minutes) supports direct Snf1-dependent phosphorylation of PAS kinase.

In order to further test this hypothesis, various in vivo assays were performed to detect both Snf1-dependent phosphorylation of Psk1 and a direct protein-protein interaction between Snf1 and Psk1. For in vivo phosphorylation, PAS kinase was purified from wild type, *snf1,* and *reg1* cells (Snf1 hyperactive cells) and the phosphorylation status was monitored by both gel mobility shift assays and mass spectrometry. Co-purification and yeast-2-hybrid assays were also performed in order to determine if there is a direct Snf1/Psk1 interaction in the cell.

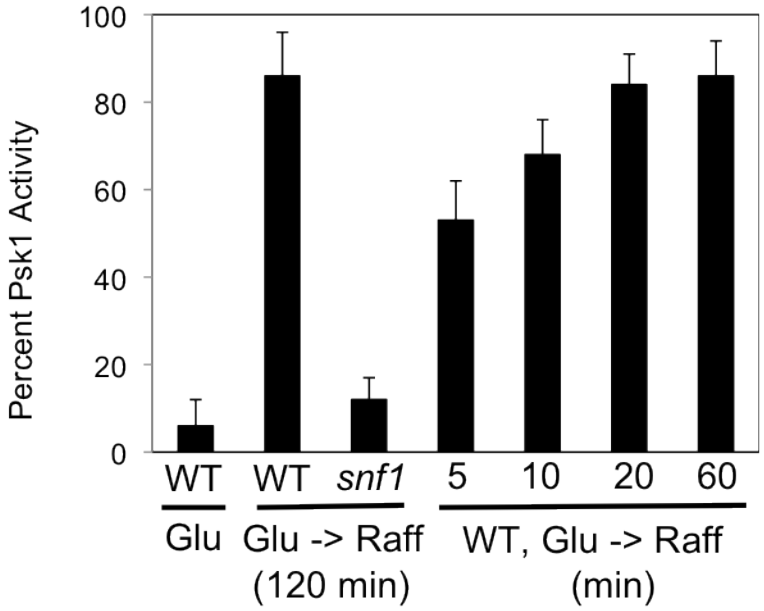

Figure 2.1: Psk1 is activated quickly and in a Snf1 dependent manner. Psk1 activity was measured based on percent phosphorylation of its substrate Ugp1. Yeast overnights were diluted 1:100 and were grown in YPAD (containing glucose) for three hours, filtered, and then resuspended. Percent Ugp1 phosphorylated was assayed as previously described (Smith et al., 2006).

Methods

Psk1 purification

His-tagged Psk1 was purified from *snf1* (JGY 91)*, reg1* (JGY 95)*,* and wild-type (JGY 1) yeast cells harboring a plasmid (pJG 858) containing the *PSK1*-HIS gene fusion expressed under the gal1-10 promoter (a strong, galactose-dependent promoter). The cells were grown overnight in 5 ml of SD-URA (synthetic media lacking uracil and supplemented with dextrose as the carbon source) to select for the plasmid, then transferred to 500 ml of SD-URA where they were grown for 16 hours at 30°C. After growth in SD-URA, the cells were centrifuged and resuspended in SGal-URA (synthetic media with galactose as carbon source) in order to promote expression of Psk1. The cells were grown in SGal-URA for 48 hours and were then centrifuged and flash-frozen at -80°C. To purify Psk1, cells were suspended in His-lysis buffer (50 mM HEPES, 300 mM NaCl, 20 mM KCl, 1:300 mammalian PICS (Sigma Aldrich, St. Louis, MA), 50 mM NaF, 50 mM glycerophosphate, pH 7.8). Cells were lysed using a M-110P homogenizer (Microfluidics, Westwood, MA), and the cell debris was separated by centrifugation. The cell lysate was then incubated with nickel-agarose beads for 3 hours. The nickel beads were then loaded onto a micro column and washed with 50 ml of His-lysis buffer. After washing, the protein was then eluted from the nickel beads by using elution buffer (HIS lysis buffer plus 250 mM imidazole and 100 mM NaCl). After purification, Psk1 was visualized using a Pierce Silver Stain Kit, Thermo Scientific, Rockford, IL. All chemicals were purchased from VWR, Radnor, Pennsylvania.

Mass Spectrometry

His-tagged Psk1 was purified from WT, *snf1,* and *reg1* yeast cells as described above. After 8% SDS-PAGE, the full-length protein was cut from protein gel using a sterile razorblade. The gel

slices were flash frozen at -80 °C and sent to Andrew Mathis of the Prince lab, BYU Chemistry & Biochemistry Department, for phosphosite mapping.

Yeast-2-hybrid Analysis

A yeast-2-hybrid analysis was performed with Psk1 and Snf1 subunits: Snf1, Sip2, and Gal83. The yeast-2-hybrid system is a method of looking at protein-protein interactions in the cell that utilizes a modified GAL4 promoter. The Gal4 transcription factor that recognizes the GAL4 promoter has both a DNA binding domain and an activation domain that recruits RNA polymerase. In the yeast-2-hybrid system, these two domains are separated and the proteins of interest are fused to either the bait (DNA binding domain) or the prey (activation domain). If the two bound proteins directly interact with each other in the cell, the fused activation domain is able to interact with the fused binding domain and recruit RNA polymerase at the GAL4 promoter. Reporter genes, *ADE2* and *HIS3*, are down-stream of the GAL4 promoter in the yeast chromosome (Y2H Gold, Clontech) and their expression is indicative of a protein-protein interaction.

PSK1, *SNF1*, *GAL83*, and *SIP2* were cloned into plasmids containing either the activation domain (pGAD) or the binding domain (pGBD). Plasmids containing *PSK1* were transformed into Y2H Gold cells (Clontech) along with *SNF1*, *GAL83*, or *SIP2* plasmids. Yeast transformed with the bait and prey plasmids were selected on plates lacking –TRP and –LEU (to select for the bait and prey plasmids respectively). The yeast were then streaked on SD-LEU-TRP-HIS-ADE selective media in order to determine if there was an interaction.

Results

When PSK1 was purified from wild type, *snf1,* and *reg1* (snf1 hyperactive) yeast cells, SDS-PAGE revealed a shift in the migration of the full-length protein when samples were

directly compared (Figure 2.2). Full length PAS kinase migrated higher in the gel from cells expressing Snf1 (wild-type and *reg1*). A gel shift can be noticed when proteins are phosphorylated or undergo some other type of modification. Due to the dramatic shift seen in the full length PAS kinase it is possible that there are either multiple phosphorylation events or there may be another type of modification taking place as well.

The full-length protein was analyzed by mass spectrometry to reveal putative phosphorylation sites. Mass spectrometry revealed that Psk1 phosphorylation occurred in a Snf1 dependent manner, and 7 phosphorylation sites of high and medium confidence were revealed: S1020, S1035, S1094, S10, T1021, T1079, and S255. No other modifications were observed. These data support that PAS kinase is being phosphorylated in vivo in a Snf1 dependent manner and that multiple phosphorylation event are occurring.

In order to determine if Snf1 directly phosphorylates and activates PAS kinase it is important to find out whether Snf1 and PAS kinase interact in the cell. DeMille et al., previously performed co-purification mass spectrometry screens (LC/MS/MS) performed in order to find potential PAS kinase interactors and identified Snf1 as well as two of its beta subunits (Sip2 and Gal83) as potential interactors (Table 2.1, DeMille et al., 2014). A co-purification mass spectrometry analysis is likely to reveal interacting complexes as well as direct binding partners. In order to determine which subunit was directly interacting with PAS kinase, a yeast-2-hybrid (Y2H) screen was performed using Snf1, Gal83, and Sip2. The results of the Y2H screen revealed that Gal83 was the main interactor with PAS kinase, with Sip2 and Snf1 not interacting with PAS kinase. The interaction was not observed when Gal83 was used as the prey (only when it was the bait), which may mean that this specific construct is non-functional. Gal83 interacted strongly with both the full-length PAS kinase and a ΔN692 (DeMille et al., 2014)

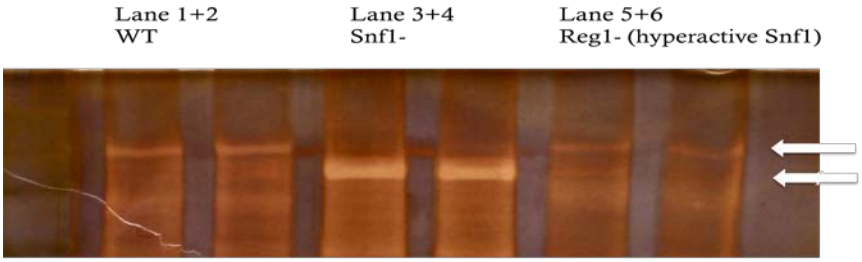

Figure 2.2: Psk1 undergoes a gel shift when purified from cells containing wild-type or hyperactive Snf1. Psk1 was HIS-tagged and purified from wild type, snf1, and reg1 (snf1 hyperactive) yeast. The purified Psk1 protein was analyzed by 8% SDS-PAGE and silver stain.

Table 2.1: PAS kinase co-purifies with Snf1, and subunits of the Snf1 complex. Psk1 was purified by His-tag purification and analyzed by LC/MS/MS to identify co-purified proteins (DeMille et al., 2014) Mass spectrometry revealed Snf1 and Snf1 β-subunits as possible Psk1 binding proteins.

Gene	**Name Description**	**Location**
SNF1	Sucrose Non-Fermenting	Cytoplasm
GAL83	Galactose Metabolism	Nucleus/Cytoplasm
SIP2	Snf1-Interacting Protein	Nucleus/Cytoplasm

truncation of PAS kinase (Figure 2.3 A.). The ΔN692 truncation removes the inhibitory PAS domain, and is more active than the full-length protein. The truncated PAS kinase interacts with substrates stronger than full-length PAS kinase, which interacts extremely weakly (DeMille et al., 2014). That Gal83 was able to bind full-length PAS kinase as strongly as the ΔN692Psk1 truncation suggests that Gal83 is not a substrate of PAS kinase.

In order to determine which specific Gal83 domains were required for interaction, various truncations of Gal83 were made and cloned into Y2H plasmids. Gal83 has a glycogen binding domain (aa1-141), Snf1 interacting domain (aa141-343), and Snf4 binding domain (343-417) (Figure 2.3 B.). An N-terminal truncation of Gal83 (aa1-342) had the strongest interaction with PAS kinase (Figure 2.3 C.). There was also weak interactions observed in truncations that contained only the Snf1 interacting domain. These interactions suggest that PAS kinase interacts with Gal83 at the N-terminus of the protein, with the Snf1 interacting domain being an important part of that interaction.

Conclusion

The results from these experiments support the hypothesis that Snf1 directly phosphorylates and activates PAS kinase. We see that PAS kinase is activated quickly under the same conditions that activate Snf1, and that activation under non-fermenting conditions requires Snf1. We would expect that if Snf1 directly phosphorylates and activates Psk1, that this would occur quickly. If activation of Psk1 was due to Snf1 dependent transcriptional regulation we would expect that activation would take much longer. Mass spectrometry analysis showed that Psk1 is phosphorylated at multiple sites in a Snf1 dependent manner. Co-purification and yeast-2-hybrid data also provided evidence that the Snf1 complex directly interacts with Psk1 in the

cell, specifically through beta subunit Gal83. The in vivo evidence gathered thus far shows that PAS kinase directly interacts with Snf1, is activated quickly, and is phosphorylated in a Snf1 dependent manner. These results support our hypothesis that PAS kinase is directly phosphorylated and activated by Snf1.

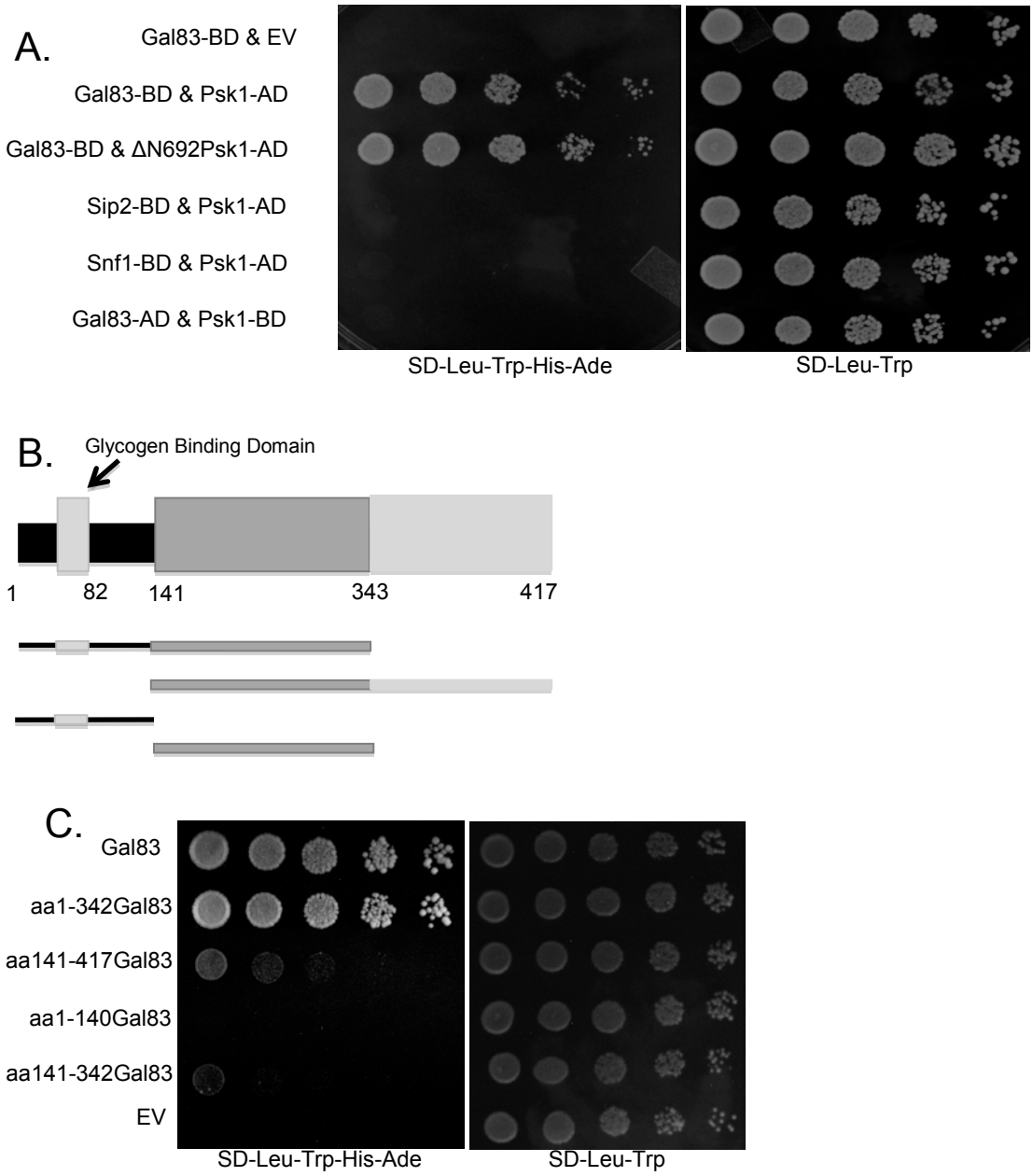

Figure 2.3: The Snf1 substrate targeting subunit Gal83 interacts with Psk1 in the Yeast-2-hybrid (Y2H) assay. (A) Gal83 but not Sip2 or Snf1 interact with Psk1 in the Y2H assay. Gal83, Sip2, and Snf1 were cloned into both the Y2H bait and prey plasmids, and co-transformed into yeast containing either the full-length or truncated Psk1 bait or prey plasmids. Transformed yeast were plated on SD-Leu-Trp-His-Ade to select for reporter genes. (B) Diagram of Gal83 truncations used to identify regions critical for interacting with Psk1. (C) Truncations with the Gal83 N-terminus and Snf1 binding domain interact with Psk1 in the Y2H assay. Different truncations of GAL83 were cloned into the Y2H bait plasmid with Y2H, and transformed into Y2H gold cells along with plasmids harboring Psk1 prey plasmids. The yeast were then plated on SD-Leu-Trp-His-Ade to select for reporter genes.

CHAPTER 3: In Vitro Evidence for Snf1 Phosphorylation of PAS Kinase

After finding in vivo evidence to support Snf1 mediated phosphorylation of PAS kinase, it was important to find supporting evidence in vitro. In vitro kinase assays remove many of the variables that are present in the cell, and provide further evidence for a direct Snf1-dependent phosphorylation of PAS kinase.

Methods

In Vitro Kinase Assays

For in vitro kinase assays Myc-tagged Snf1 was purified in a similar manner to His-purification (see Chapter 2), using XWA buffer (20 mM HEPES, 10 mM KCl, 1 mM EDTA, 1 mM EGTA, 50 mM NaCl, pH 7.4 and 10% glycerol) with proteinase (complete Protease Inhibitor Cocktail tablets, Roche) and phosphatase inhibitor (glycerophosphate and NaF) added to prevent proteolysis and dephosphorylation of Snf1. After the cells were lysed, cell lysates were incubated with anti-Myc conjugated beads (CellSignaling, Danvers, MA). After incubation the Snf1-bound beads were washed five times in XWA buffer, and resuspended in kinase buffer (50 mM Tris-HCl, 10 mM $MgCl_2$, 1mM dithiothreitol, 10 uM ATP, pH 7.5). Each 30 uL reaction contained 10 uL Snf1 beads in buffer, 8 uL His-tagged kinase dead Psk1 (Psk1-D1230A), truncated Psk1 (ΔN931Psk1), or Rod1, and kinase buffer. The reactions were incubated with 7.5 μCi P^{32}-ATP at 30 °C for 35 minutes. After the incubation period the reactions were stopped with SDS sample buffer, and reaction mixes were run on 8% SDS-PAGE. The incorporation of ^{32}P was measured by exposing the protein gel on GeneMate, Blue Autoradiology Film for 8-12 hours at -80 °C using a Kodak BioMax TransScreen-HE Intensifying Screen to enhance the signal.

In Vitro Activation Assays

In vitro activation assays were performed to determine if we could detect a difference in Psk1 activity Snf1 phosphorylation. In this assay His-tagged Psk1 was purified from *snf1* cells in order to prevent previous phosphorylation and activation of Psk1. The assay was performed by pre-incubating His-tagged Psk1 and Myc-tagged Snf1 in kinase buffer with ATP (50 mM Tris-HCl, 10 mM $MgCl_2$, 1mM dithiothreitol, 10 uM ATP, pH 7.5) for 10 minutes. After incubation, Psk1 substrate Ugp1 was added along with 0.2 mM ATP and 5 uCi ^{32}P-ATP, and incubated for an additional 10 minutes. The reactions then underwent SDS-PAGE and were stained with Coomassie-Blue. The protein gel was then dried and exposed on film to observe incorporation of ^{32}P.

Phosphosite Mutagenesis

In order to determine critical phosphorylation sites, two of the previously identified high confidence phosphosites S1020, and S1035 were mutated to alanine. Alanine does not have a hydroxyl group, and is unable to be phosphorylated. A plasmid (pJG 858) harboring *PSK1* was used as a template in the Quikchange II mutagenesis protocol (Agilent Technologies, Santa Clara, CA 95051). Primers were designed to replace the desired serine codon with an alanine codon and were used in PCR with pJG 858 as template. To remove the template plasmid, a DpnI digest was performed (the DpnI is a restriction endonuclease that digests methylated DNA, thus digesting any DNA that has been inside an E. coli cell). After the DpnI digest, the PCR product was then transformed into E. coli and colonies were sequenced to confirm the mutation (BYU DNA Sequencing Center). All enzymes, buffers, and E. Coli cells came from a QuickChange Lightning Site Directed Mutagenesis Kit (Agilent Technologies, Santa Clara, CA 95051).

Results

In order to determine if Snf1 directly phosphorylates PAS kinase in vitro, kinase assays were performed as described in Methods. A kinase dead Psk1 (Psk1-D1230A, DeMille et al., 2014) was used as a substrate to control for autophosphorylation. The results showed that the kinase dead Psk1 was phosphorylated in vitro in a Snf1-dependent manner (Figure 3.1). Rod1 has previously been shown to be a Snf1 substrate and was used as a control for Snf1 activity. Rod1 was also phosphorylated in vitro in a Snf1-dependent manner. It was observed that not only was full length PAS kinase phosphorylated in vitro, but also many of the Psk1 degradation products. To determine the primary region of the protein that is phosphorylated by Snf1, further in vitro kinase assays were performed using a kinase domain only Psk1 truncation (ΔN931PSK1, DeMille et al., 2014). The kinase only truncation was also phosphorylated in a Snf1 dependent manner suggesting that the primary region of phosphorylation of Psk1, occurs in the kinase region of the protein. If the critical phosphorylation site was in another region of the protein, we would not expect the kinase only truncation to be phosphorylated. These results should aid in mapping the Snf1-depedent Psk1 phosphorylation site.

From the putative Psk1 phosphorylation sites mapped by LC/MS/MS, two of these sites S1020, and S1035 were found to be high confidence on multiple runs. In order to determine if either of these sites were the critical phosphorylation site, each site was mutated to alanine.

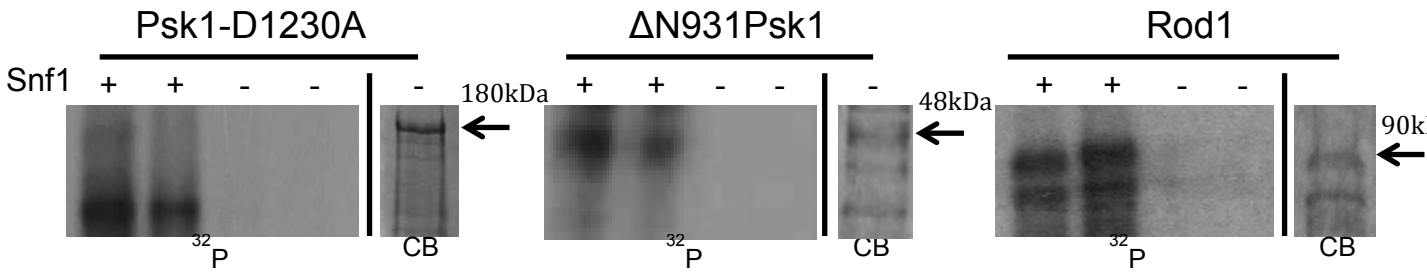

Figure 3.1: Snf1 phosphorylates Psk1 in vitro. His-tagged kinase dead Psk1 (D1330A) and kinase domain truncation of Psk1 (ΔN931) were incubated with Snf1-Myc in 1X kinase buffer and ^{32}P-ATP for 35 minutes. Incorporation of ^{32}P-ATP was assessed by exposing reactions on gel after 8% SDS-PAGE. Rod1 was used as a control to determine activity of Snf1.

When kinase assays were performed using S1020A, and S1035A Psk1 mutants, none of these mutants showed inhibited ability to be phosphorylated by Snf1 in vitro (data not shown). The difficulty in determining the critical phosphorylation sites is in part due to the large size of Psk1 (1,356 aa), and the multiple phosphorylation events. In order to narrow down the critical phosphorylation sites, an in vitro kinase assay of kinase dead Psk1 (both full length and truncated) will be analyzed by LC/MS/MS and phosphosites will be mapped. This assay will remove the potential for autophosphorylation of Psk1, as well as remove any potential alternative upstream kinases, thus the results from this assay should include only those sites that are due to direct Snf1 phosphorylation. The results from this assay are still pending, but the putative phosphosites will be similarly mutated and subjected to in vitro phosphorylation assays as has previously been done.

In order to support the hypothesis that phosphorylated Psk1 would be more active, in vitro Psk1 activation assays were performed. The assay measures the ability of Psk1 to phosphorylate the known substrate Ugp1. The assay was performed with or without Snf1. However, the assays consistently showed no difference in the ability of Psk1 to phosphorylate Ugp1 in vitro, whether Snf1 was present or not (data not shown). While we were able to find that activation of Psk1 required Snf1 in vivo, the in vitro results have not supported this hypothesis so far. This may be due to the assay conditions in vitro, which may not be able to detect differences in activity due to the abundance of substrate or ATP, or missing accessory factors. In addition, the apparent activation in vivo could be due to changes in localization or protein complexes.

Conclusion

The results from the in vitro phosphorylation assays support the hypothesis that Snf1 directly phosphorylates and activates PAS kinase. The in vitro assays remove the multiple

variables that are seen in the cell, and we see that Snf1 is able to directly phosphorylate PAS kinase. Thus far we have not been able to determine the critical PAS kinase phosphorylation site due to multiple phosphorylation events (17), the large size of the protein (1356 aa), and the low abundance of the protein. Snf1 is able to phosphorylate a truncated version of PAS kinase containing the kinase only domain in vitro. This evidence suggests that the critical phosphorylation event would occur in that segment of the enzyme. The in vitro assay should exclude any competing phosphorylation events that are occurring in the cell and give more specific phosphosites. We are currently using a kinase dead mutant (D1230A) in combination with the kinase only truncation in order to map the critical site. Further phosphosite mutants will then be made in order to determine which is the critical site. This should provide insight into the molecular mechanisms by which PAS kinase is activated.

CHAPTER 4: Snf1 phosphorylates Osh7

From co-purification screens performed by DeMille et al., Osh7 was retrieved as a possible interactor with Psk1 (DeMille et al., 2014). However, when Osh7 was subjected to kinase assays using Psk1 as the kinase, Osh7 was phosphorylated in samples containing either wild type Psk1 or kinase dead Psk1-D1230A (Figure 4.1, DeMille et al., 2014). This suggested that some contaminating kinase was phosphorylating the Osh7 sample since the kinase dead Psk1 completely eliminates phosphorylation of all known Psk1 substrates. Psk1 was purified using a His-tag purification and Snf1 has been found to both co-purify with Psk1 (DeMille et al., 2014), as well as bind to the nickel beads used in the His-tag purification. Thus, we hypothesized that Snf1 could be the kinase in these samples that was phosphorylating Osh7.

In order to determine if Snf1 was the kinase phosphorylating Osh7, in vitro kinase assays were performed (as described in chapter 3). The results of these kinase assays showed that Osh7 was phosphorylated in a Snf1-dependent manner (Figure 4.2). These data suggest that Osh7 is a novel substrate of Snf1. To determine the critical Osh7 phosphorylation site, in vitro kinase assays with Snf1 and Osh7 will be performed in a like manner described for Psk1 phosphosite mapping and samples will be submitted to LC/MS/MS.

Osh7 is a member of the Osh family of proteins, which are homologous to the mammalian oxysterol-binding proteins. The Osh family of proteins are needed to maintain proper levels of ergosterol, the primary sterol in yeast, with an observed overproduction of ergosterol when the Osh family of genes are knocked out (Beh et al., 2001). The Osh family of proteins also appears to be essential for polarized cell growth. When the Osh family of genes are knocked out, there is a growth defect that results from the mislocalization of key proteins that regulate polarized cell growth including Cdc42, Rho1, and Sec4 (Kozminski et al., 2006). It

appears that each protein in the Osh family has some specificity to the type of lipid they transport, with Osh7 having a specificity for phosphatidylserine. A function of Osh7 that has been discovered is that it transfers phosphatidylserine from the endoplasmic reticulum to the cell membrane (Maeda et al., 2013). Thus, these results may help explain the altered lipid metabolism seen in *SNF1*-deficient yeast as well as AMPK activated mammalian cells (Hardie, 2007).

Conclusion

The in vitro data suggest that Osh7 is a novel substrate of Snf1. This may explain the mechanism by which Snf1 inhibits lipid synthesis. Due to the role of Osh7 as a phosphatidylserine transporter and the necessity of the Osh genes for polarized cell growth, it is likely that Snf1 phosphorylation of Osh7 inhibits its activity in some way. There are several Osh7 homologs in humans and there is not very much known about Osh7 or the other Osh proteins. These results open molecular pathways for future study that are likely to have broad impacts in the field of metabolic regulation and lipid metabolism.

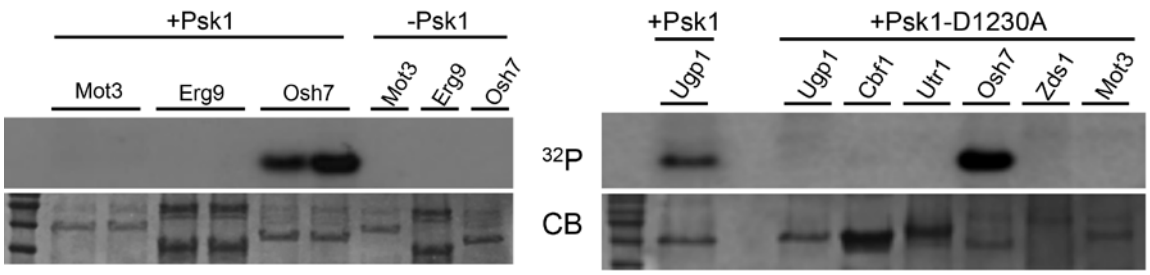

Figure 4.1: Osh7 is phosphorylated by both WT and kinase dead Psk1 (D1230A) preps (DeMille et al., 2014). His-tagged WT and kinase dead (D1230A) Psk1, and Osh7 were purified and incubated with ^{32}P-ATP as described by Demille et al. (DeMille et al., 2014). Samples were run on 8% SDS-PAGE gels, stained with commassie blue, dried and incubated with film overnight prior to developing.

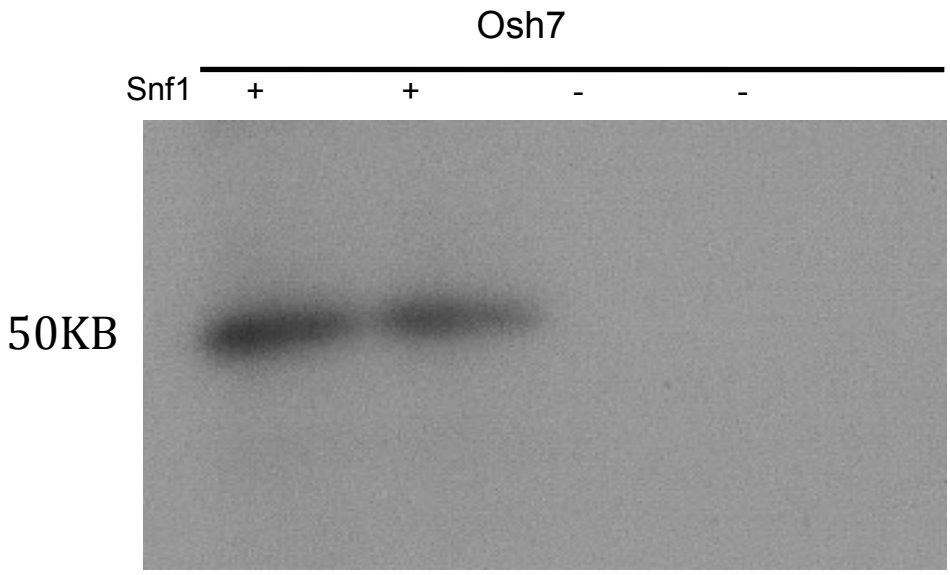

Figure 4.2: Snf1 phosphorylates Osh7 in vitro. HIS-tagged Osh7 was purified using Ni-NTA beads (Qiagen), and incubated with α-Myc beads conjugated with Snf1-myc protein in kinase buffer and ^{32}P-ATP. Samples were run on 8% SDS-PAGE gels, stained with commassie blue, dried and incubated with film overnight prior to developing.

CHAPTER 5: Conclusion

The in vivo and in vitro data collected from these experiments support the hypothesis that Snf1 directly phosphorylates and activates PAS kinase. First, in vitro kinase assays suggest that Snf1 is able to directly phosphorylate Psk1 and Psk1 displays a mobility shift when purified from cells containing Snf1. Co-purification results and Y2H data also confirmed that the Snf1 complex and Psk1 directly interact in the cell. These results provide a mechanism by which cells can link cellular energy status to cellular metabolism because Snf1 senses low cellular energy and PAS kinase regulates cellular glucose homeostasis.

As discussed in the introduction, Snf1 is the major energy status sensing enzyme in the cell. Snf1 activity is directly affected by the ATP levels of the cell and helps regulate cellular activity to conserve and create energy. Snf1 is necessary and sufficient to activate PAS kinase in response to nutrients (Grose et al., 2007), and explains how PAS kinase can be regulated based on the changing metabolic status of the cell. It also provides a dynamic method for the cell to quickly regulate the allocation of nutrients. Understanding the molecular mechanisms of PAS kinase activation will also help us to better predict and manipulate the activity of PAS kinase. In our current model for PAS kinase regulation and function, this phosphorylation would inhibit PAS domain binding, and stabilize the active form of PAS kinase (Figure 5.1). When new substrates of PAS kinase are identified, it will be likely PAS kinase will change their activity to allow for the allocation of nutrients to be shifted away from energy consumption pathways including storage. One example of this is how PAS kinase shifts Ugp1 activity away from glycogen production (Smith and Rutter, 2007).

So far we have not been able to identify the critical phosphorylation site of Psk1 activation. The difficulty in mapping critical phosphorylation sites can be explained by the large

size of the protein and the multiple phosphorylation events. However, the ability of Snf1 to phosphorylate the kinase domain truncation Psk-ΔN931 would suggest that this site is within the kinase domain region of the protein. It is expected that the phosphosite mapping of Psk1 by in vitro kinase assays will allow for a more specific Snf1 phosphorylation site.

The results of these experiments contribute to our understanding of basic cellular processes and the regulation of an important metabolic enzyme PAS kinase. We now understand better the process that regulates PAS kinase activity under nutrient stress conditions. It would be interesting to learn if this is a conserved pathway in mammalian cells as well.

In addition to identifying an interaction between Snf1 and Psk1, we have also identified a possible novel substrate of Snf1, Osh7. It is not known yet what effect Snf1 phosphorylation has on the protein, but due to the global effects of Snf1 on the cell, it is likely that Snf1 phosphorylation regulates its ability to transfer phosphatidylserine to the cell membrane and thus regulating polarized cell growth.

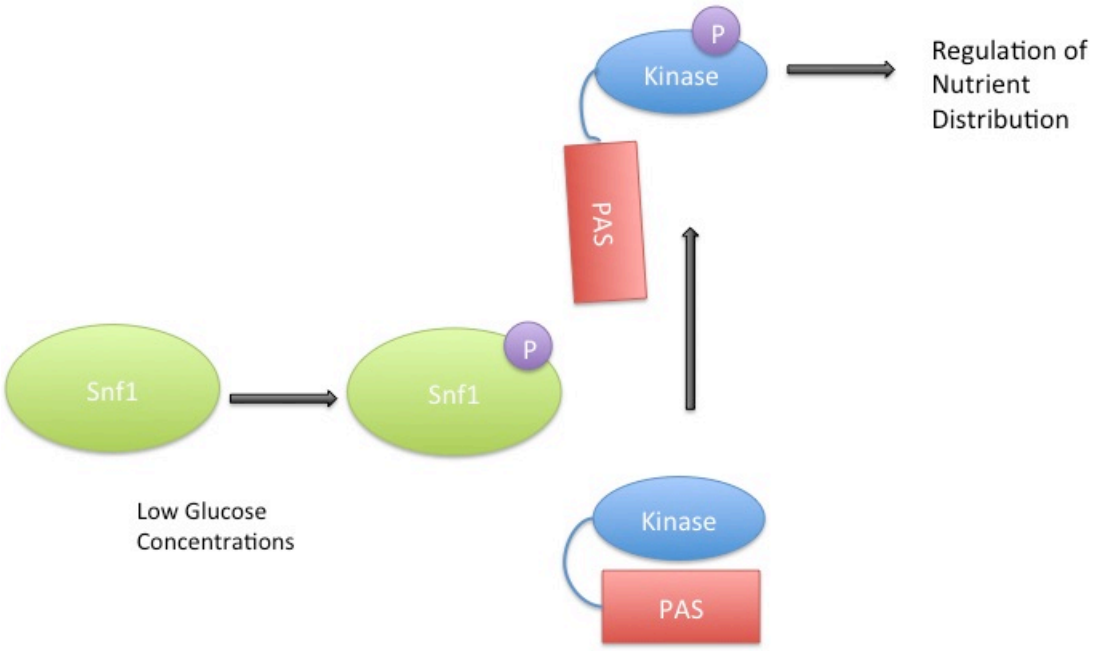

Figure 5.1: Model for activation of PAS kinase under nutrient stress. Snf1 is activated when glucose concentrations are low, which in turn phosphorylates and activates PAS kinase by releasing inhibition by the PAS domain. PAS kinase then regulates the distribution of nutrients (glucose).

Table 5.1: Yeast Strains Used

Strain	**Background**	**Genotype**	**Abbreviations**	**a/α**	**Reference or Source**
JGY1	W303	*ade2-1 can1-100 his3-11, 15 leu2-3, 112 trp1-1 ura3-1*	WT	a	David Stillman University of Utah
JGY4	W303	*psk1*::*his3 psk2*::*kan*-MX4 *ade2-1 can1-100 his3-11,15 leu2-3, 112 trp1-1 ura3-1*	*psk1psk2*	a	Grose et al. (2007)
JGY91	W303	*snf1*::hphMX4 *ade2-1 can1-100 his3-11,15 leu2-3, 112 trp1-1 ura3-1*	*snf1*	a	Grose et al. (2007)
JGY95	W303	*reg1*::hphMX4 *ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3-1*	*reg1*	a	Grose et al. (2007)
Y2H Gold JGY 1031		*LYS2*::*GAL1*UAS-*GAL1*TATA-His3, *GAL2*UAS-Gal2TATA-Ade2 *URA3*::*MEL1*UAS-*MEL1*TATA, *AUR1*-C*MEL1*, *ura3-52 his3-200 ade2-101 trp1- 901 leu2-3, 112 gal4del gal80del met-*	Y2H Gold	a	Clontech

Table 5.2: Plasmids

Plasmid	Gene	Description	Backbone	Yeast Origin	Selection	Reference or Source
pJG421	EV	pGAD-C1 empty Y2H prey vector	YEp-GAD	2u	LEU	James *et al.,* (1998)
pJG422	EV	pGAD-C2 empty Y2H prey vector	YEp-GAD	2u	LEU	James *et al.,* (1998)
pJG424	EV	pGBD-C1 empty Y2H bait vector	YEp-GBD	2u	TRP	James *et al.,* (1998)
pJG425	EV	pGBD-C2 empty Y2H bait vector	YEp-GBD	2u	TRP	James *et al.,* (1998)
pJG441	*PSK1*	Full-length Psk1 in pJG425	YEp-GBD	2u	TRP	DeMille *et al.,* (2104)
pJG442	*PSK1*	Full-length Psk1 in pJG422	YEp-GAD	2u	LEU	Desi DeMille, This Study
pJG598	*PSK1*	ΔN692Psk1 in pJG425	YEp-GBD	2u	TRP	DeMille *et al.,* (2014)
pJG709	*PSK1*	ΔN692Psk1 in pJG422	YEp-GAD	2u	LEU	Desi DeMille, This Study
pJG858	*PSK1*	pGAL1-10, Psk1-HIS/HA	pRS426	2u	URA	DeMille *et al.,* (2014)
pJG960	*PSK1*	ΔN692Psk1-HIS/HA in pJG858	pRS426	2u	URA	DeMille *et al.,* (2014)
pJG1000	*PSK1*	ΔN931PSK1-HIS/HA in pJG858	pRS426	2u	URA	DeMille *et al.,* (2014)
pJG1046	*GAL83*	pGAL1-10, Gal83-HIS/HA	pRS426	2u	URA	This Study
pJG1047	*SIP2*	pGAL1-10, Sip2-HIS/HA	pRS426	2u	URA	This Study
pJG1170	*PSK1*	pGAL1-10, Psk1D1230A-HIS/HA	pRS426	2u	URA	DeMille *et al.,* (2014)
pJG1193	*SNF1*	Snf1-8xmyc	pRS313	2u	URA	Mark Johnston University of Colorado Denver
pJG1203	*ROD1*	Rod1-HIS in pJG998	pET15b		AMP	Brady Evans, This Study
pJG1236	*GAL83*	Gal83 in pJG424	YEp-GBD	2u	TRP	Brady Evans, This Study
pJG1237	*GAL83*	Gal83 in pJG421	YEp-GAD	2u	LEU	Brady Evans, This Study
pJG1238	*SIP2*	Sip2 in pJG424	YEp-GBD	2u	TRP	Brady Evans, This Study
pJG1239	*SIP2*	Sip2 in pJG421	YEp-GAD	2u	LEU	Brady Evans, This Study
pJG1240	*SNF1*	Snf1 in pJG424	YEp-GBD	2u	TRP	Brady Evans, This Study
pJG1241	*SNF1*	Snf1 in pJG421	YEp-GAD	2u	LEU	Brady Evans, This Study
pJG1258	*GAL83*	aa1-342Gal83 in pJG424	YEp-GBD	2u	TRP	Brady Evans, This Study
pJG1259	*GAL83*	aa1-342Gal83 in pJG421	YEp-GAD	2u	LEU	Brady Evans, This Study
pJG1260	*GAL83*	aa141-417Gal83 in pJG424	YEp-GBD	2u	TRP	Brady Evans, This Study

pJG1261	*GAL83*	aa141-417Gal83 in pJG421	YEp-GAD	2u	LEU	Brady Evans, This Study
pJG1262	*GAL83*	aa1-140Gal83 in pJG424	YEp-GBD	2u	TRP	Brady Evans, This Study
pJG1263	*GAL83*	aa1-140Gal83 in pJG421	YEp-GAD	2u	LEU	Brady Evans, This Study
pJG1264	*GAL83*	aa141-342Gal83 in pJG424	YEp-GBD	2u	TRP	Brady Evans, This Study
pJG1265	*GAL83*	aa141-342Gal83 in pJG425	YEp-GAD	2u	LEU	Brady Evans, This Study

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