AN EVOLUTIONARY PROTEOMICS APPROACH FOR THE IDENTIFICATION OF SUBSTRATES OF THE CAMP-DEPENDENT PROTEIN KINASE IN *SACCHAROMYCES CEREVISIAE*

DISSERTATION

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ABSTRACT

Eukaryotic cells utilize a network of signal transduction pathways to sense their environment and control their growth and proliferation. Protein kinases are a large group of enzymes that coordinate responses to extracellular and intracellular stimuli via phosphorylation of specific downstream targets. In *S. cerevisiae*, growth is controlled, in part, by the Ras signaling pathway via the cAMP-dependent protein kinase, PKA. PKA is a serine/threonine-specific protein kinase that has been shown to regulate any aspects of cell growth and metabolism in this budding yeast and other eukaryotes. Unfortunately, finding protein kinase substrates by conventional methods is a difficult and time-consuming task. As a result, few targets of any given protein kinase are known. To simplify this task, we developed an evolutionary proteomics strategy for the identification of PKA substrates in *S. cerevisiae* and related yeast species.

This evolutionary proteomics approach is sequenced-based and takes advantage of the fact that most PKA substrates contain the consensus sequence, R-R-x-S/T-B. In this consensus, "x" refers to any amino acid, "B" to hydrophobic residues and "S" or "T" to the site of phosphorylation. The general approach consists of two basic steps. In the first, we identified all of the proteins in the *S. cerevisiae* proteome that contain this PKA target consensus sequence. In the second, we asked whether these potential target sites are conserved in the orthologous proteins present in other budding yeast

species. For this latter step, we used the recently released genome sequences of six different yeast, including five *Saccharomyces* species and *Candida albicans*. The underlying premise of this approach is that PKA sites important for general aspects of cell biology are more likely to be conserved across these evolutionary distances. We are presently testing this basic premise with a small number of proteins predicted to be physiologically relevant PKA substrates. In this thesis, I will discuss my recent work with one of these potential targets, Atg1p.

Atg1p is a key regulator of autophagy, a membrane trafficking pathway that is responsible for much of the protein and organelle turnover occurring during periods of nutrient depravation. This catabolic pathway is highly conserved and Atg1p homologs exist in essentially all eukaryotic organisms. We have found that the *S. cerevisiae* Atg1p contains two PKA consensus sites that have been conserved in all budding yeast and *C. albicans*. My work has shown that Ras/PKA signaling activity does indeed control autophagy *in vivo* and that Atg1p is phosphorylated at the two predicted sites by PKA. PKA phosphorylation of Atg1p does not effect its protein kinase activity, but appears instead negatively regulate the association of Atg1p with the pre-autophagosomal structure (PAS). We hypothesize that this regulation might prevent Atg1p from reaching specific substrates that are important for the autophagy process.

Dedicated to my family

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FIELDS OF STUDY

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CHAPTER 1

INTRODUCTION

1.1. Ras/PKA signaling in *Saccharomyces cerevisiae*

Nutrient availability is a major factor controlling growth and proliferation of all living cells. Cells that are deprived of essential nutrients arrest in the G_1 phase of the mitotic cell cycle and then enter a specific non-proliferating resting state, known as stationary phase in yeast and G_0 in mammals [1]. This growth arrest is accompanied by specific metabolic and morphological changes, such as the accumulation of proteases, the formation of uniquely-folded chromosomes, the accumulation of storage carbohydrates (glycogen, trehalose), an increase in thermo-tolerance, a restructuring of the cell wall and a significant decrease in the rates of RNA pol II transcription and protein translation [1]. Once cells have entered into stationary phase, they can remain viable during prolonged periods of starvation. In addition to entry into stationary phase, yeast cells can also undergo filamentous growth upon nutrient limitation. During filamentous growth, yeast cells do not separate after nuclear division and remain physically attached to form chains of elongated cells, called pseudohyphae. It has been

hypothesized that pseudohypal differentiation is an adaptive mechanism that allow fungi to search for a nutrient-rich environment [1,2]. The entry into stationary phase and filamentous growth are both regulated by a complex network of signal transduction pathways that allow these cells to appropriately adjust their metabolic activities in response to changes in the environment.

The Ras/PKA signaling pathway plays a key role in nutrient-sensing and the regulation of a diverse set of biological processes including growth, stress resistance, sporulation, pseudohyphal growth, and entry into stationary phase. An overview of this signaling pathway in *S. cerevisiae* is shown in Figure 1.1. This budding yeast contains two functionally redundant Ras proteins, Ras1p and Ras2p that respond to a number of stimuli, including glucose availability [2,3]. These Ras proteins oscillate between inactive GDP-bound form and active GTP-bound form. The intrinsic GTPase activity of Ras1p and Ras2p is stimulated by the GTPase-activating proteins (GAPs) encoded by the *IRA1* and *IRA2* genes. In addition, the *CDC25* gene encodes a guanine nucleotide exchange factor that activates Ras by catalyzing the exchange of the GDP present on inactive Ras proteins for GTP. The GTP-bound Ras then binds to and activates adenylyl cyclase, a membrane-associated protein that catalyzes the synthesis of cAMP from ATP. The increased levels of cAMP activate the cAMP-dependent protein kinase (PKA) by binding to its regulatory subunit, encoded by the *BCY1* gene. This binding inactivates Bcy1p by inducing a conformational change that results in the release of free catalytically active PKA. Active PKA phosphorylates multiple substrates, and thus, brings about important metabolic changes that drive cell growth and proliferation [1, 2].

Hydrolysis of cAMP by the cAMP phosphodiesterases, Pde1p and Pde2p, restores PKA to its inactive state.

The proper regulation of the Ras/PKA pathway is essential for a normal response to nutrient availability. Cells with constitutively elevated levels of Ras/PKA activity are unable to cease cell growth and adopt stationary phase characteristics as nutrients are depleted. Constitutively elevated levels of Ras/PKA activity result from either a mutation that inactivates the intrinsic GTPase activity of Ras2p (*RAS2*^{val19}), or a deletion of the gene encoding Bcy1p, the regulatory subunit of PKA [3-5]. Conversely, mutants with lowered levels of Ras/PKA signaling adopt stationary phase characteristics even in a nutrient-rich environment [3, 6-8]. These results suggest that this signaling pathway might be controlling the transition between stationary phase and the mitotic cycle. However, the manner in which this regulation is achieved is poorly understood since only a few of the PKA substrates important for these processes have been identified.

PKA is one of the simplest and best-characterized protein kinases in eukaryotic cells. In *Saccharomyces cerevisiae,* there are three catalytic subunits of PKA, encoded by the *TPK1*, *TPK2*, and *TPK3* genes. Strains lacking all three PKA catalytic subunits are inviable, but strains containing any one of the three *TPK* genes are viable [6]. Despite this functional redundancy, phenotypic analyses of individual *tpk* mutants indicate that slight functional differences do exist. For example, Tpk2p is essential for pseudohyphal growth, whereas Tpk3p inhibits this process; and Tpk1p has apparently no effect on the development of phesudohyphae [9-13]. Therefore, Tpk proteins appear to mediate both shared and distinct processes important for cellular proliferation.

A variety of studies have led to the identification of the specific sequence motif, that is identical to the general consensus recognition site of all known PKA substrates and its physiological inhibitors [14] (Figure 1.2). Previous work has identified a consensus site of R_{-3}-R_{-2}-x_{-1}-S/T-B_{+1} for this enzyme, where "x" refers to any amino acid and "B" to a residue with a hydrophobic side chain [15, 16]. PKA binds to a subset of the proteins that contain this consensus site, and phosphorylates the serine or threonine residue of this sequence [17]. Interestingly, the regulatory subunit of PKA, Bcy1p, also contains this consensus site. Moreover, previous work has shown that the phosphorylation of Bcy1p by PKA strengthens the interaction between the regulatory and catalytic subunits during PKA holoenzyme formation. However, not all proteins that contain this motif are phosphorylated by PKA. Thus, in spite of its importance, this consensus site does not act as an absolute predictor of whether a protein is, or is not, a substrate of PKA.

The cAMP signaling cascade is one of the most common and versatile signaling pathways in eukaryotic cells. In higher eukaryotes, cAMP is produced in response to extracellular stimuli such as hormones and growth factors and is involved in regulation of cellular functions in almost all tissues in mammals. Therefore, further studies to identify new components and targets of this pathway can take advantage of similarities in the cAMP signaling pathway between *S.cerevisiae* and higher eukaryotes. The information obtained from studies in model organisms such as *S.cerevisiae*, could be relayed to the higher organisms.

1.2 Targets of the PKA

A complete understanding of the biology of the cAMP-dependent protein kinase (PKA) requires the identification of the substrates of this enzyme. Unfortunately, this identification process tends to be a difficult and labor-intensive task [18]. As a result, relatively few targets of the *S. cerevisiae* PKA are known (Table 1.1). Enzymes involved in carbohydrate and lipid metabolism were some of the first discovered targets for PKA. In cells growing on rich medium, high PKA activity results in phosphorylation and subsequent up-regulation of enzymes involved in glycolysis, such as fructose-1,6-bisphosphate (Fbp1p) [19] and phosphofructokinase 2 (Pfk26p) [20]. At the same time, PKA dependent phosphorylation of enzymes involved in gluconeogenesis inhibits their activity [21-23]. The gluconeogenesis enzymes include glycogen synthase (Gsy2p) [24], glycogen phosphorylase (Gph1p), trehalose phosphate synthase (Tps1p), and trehalase (Ath1p and Nrh1p) [25, 26] [27-29].

In addition to the post-translational control of enzymes, PKA activity is essential for the repression of transcription of genes responsible for the general stress response. Such stresses include temperature shift, osmotic shock, nutrient starvation, and DNA damage [30, 31]. These stress-response genes contain a specific DNA sequence in their promoters, called the STRE (STress Response Element). The Zn^{2+}-finger transcription factors, Msn2p and Msn4p, bind to this STRE and mediate gene activation in response to environmental stresses [32-35]. Previous work has shown that the Ras signaling pathway negatively regulates expression of these stress-response genes through the direct phosphorylation of Msn2p. On the other hand, the expression of several

stationary-phase specific genes, such as *SSA3*, is Msn2p/Msn4p-independent, but still negatively regulated by cAMP. This latter control appears to be mediated by a sequence element in the promoters of these genes known as the post-diauxic shift (PDS) promoter element [36]. This element is bound by a Zn^{2+}-finger transcription factor, Gis1p, that is activated by the protein kinase Rim15p [37]. Recent studies have identified Rim15p as a substrate of PKA and have shown that this phosphorylation is required for proper stationary phase entry [38].

The repression of Msn2p/Msn4p and Gis1p is not the only way in which PKA inhibits the expression of stress-related genes. There are large number of cAMP-repressed genes that are induced when glucose becomes exhausted, during a period knows as the diauxic transition. However, neither Msn2p/Msn4p nor Gis1p regulate the expression of these genes [39]. These observations suggest that there are other PKA-regulated transcription factors responsible for the induction of the diauxic shift. Recent data indicate that PKA directly targets proteins within the RNA polymerase II holoenzyme, and that this phosphorylation results in the transcriptional repression of specific stress-related genes [40, 41]. In particular, Ras/PKA activity positively regulates the Srb8-11p complex in the RNA polymerase II holoenzyme. This Srb8-11p complex has been shown to negatively regulate RNA polymerase II transcription of a specific set of stress-induced genes [42]. PKA directly phosphorylates one of the components of this Srb complex, Srb9p, and thereby regulates the transcription of a set of genes involved in the diauxic shift and stationary phase entry [43].

In addition to transcriptional regulation and post-translational modification of its targets, PKA modulates its own activity by controlling the subcellular localization of its regulatory subunit, Bcy1p. Upon carbon source deprivation, Bcy1p is found to be phosphorylated at multiple serine residues within its N-terminal 124 residues. This region is both necessary and sufficient for the proper nutrient-regulated localization of Bcy1p [44, 45]. This Bcy1p phosphorylation is dependent upon a functional *YAK1* gene [45, 46]. Phosphorylation of the serine residues within this N-terminal domain of Bcy1p increases its affinity for Zds1p, a putative homolog of a mammalian AKAP (A Kinase Anchoring Protein) and results in the retention of Bcy1p in the cytoplasm. Interestingly, expression of *YAK1* is negatively regulated by PKA. This observation suggests the existence of an autoregulatory loop in which PKA controls the phosphorylation and subcellular localization of its own regulatory subunit. This loop appears to involve Msn2p, as PKA directly phosphorylates and inhibits the nuclear localization of Msn2p [47-49]. Thus, as nutrients are depleted and PKA activity decreases, Msn2p becomes dephosphorylated, enters the nucleus, and activates the expression of *YAK1*.

Another important target of the *S. cerevisiae* PKA is the cAMP phosphodiesterase, Pde1p [50]. Pde1p contains a consensus PKA phosphorylation site and is phosphorylated by PKA *in vivo*. This phosphorylation of Pde1p activates its phosphodiesterase activity, which is important for the down-regulation of cAMP signaling. Accurate control of the intracellular concentration of cAMP is required for the proper response to sudden changes in the nutrient supply.

Yeast PKA activity is required in numerous cellular processes, including growth, carbon storage, stress response and differentiation. However, our knowledge of the downstream targets of PKA is rather limited. The identification of additional PKA substrates will improve our understanding of the role played by the Ras/PKA signaling pathway in the control of yeast growth and proliferation.

1.3 TOR signaling pathway in *S.cerevisiae*

In addition to Ras/PKA signaling, TOR (Target Of Rapamycin) kinases also regulate the growth of proliferating cells in response to nutrients. In the budding yeast *S. cerevisiae*, there are two TOR kinases, Tor1p and Tor2p. TORs were initially identified about 10 years ago through a genetic screen for rapamycin-resistant mutations in yeast. Rapamycin is a potent immunosuppressive drug that inhibits the proliferation of mammalian T-lymphocytes [51-53]. Rapamycin forms an active drug-protein toxin with the prolylisomerase, FKBP12. This drug-protein complex binds to Tor proteins and inhibits their activity [54]. The Tor proteins have been highly conserved through evolution and are found in all eukaryotic cells. A signature feature of these proteins is a highly-conserved C-terminal domain that is homologous to the catalytic domain of phosphatidylinositol 3-kinase (PI3K) and phosphatidylinositol 4-kinases (PI4K). Thus, the TOR proteins have been termed phosphatidylinositol kinase-related kinases [55]. However, none of the TORs has been found to posses lipid kinase activity. Instead, Tor

proteins appear to be the Ser/Thr- specific protein kinases [56-58]. A variety of studies has indicated that the integrity of this kinase domain is essential for Tor protein function.

The function of TOR appears to be conserved from yeast to mammals. In yeast, TOR signaling coordinates the activity of various metabolic pathways with nutrient availability. In cell growing on rich media, the Tor proteins positively regulate processes important for cell growth, such as the polarization of the actin cytoskeleton, translation initiation, transcription of rRNA, tRNA and ribosomal protein genes, amino acid transport, and the transcription of genes involved in glycolysis [35, 59-63]. In addition to this positive role, Tor kinases also inhibit the transcription of specific stress response genes and genes activated upon nitrogen limitation [64-66]. When nutrients are limiting, Tor activity decreases and this induces a growth arrest that is reminiscent of stationary phase. This growth arrest is accompanied by changes in transcription efficiency, the suppression of protein synthesis, and regeneration of amino acids and other small metabolites via autophagy [67] (Figure 1.3).

Significant progress towards understanding the downstream functions of the Tor proteins has been made in the past decade. Several research groups have shown that Tor signaling controls at least some of its downstream effectors via regulation of a type 2A-related phosphotase, Sit4p. Under nutrient-rich conditions, Tor activity promotes the association of Sit4p with Tap42p, and therefore maintains Sit4p in an inactive state. When nutrients become limiting (or upon rapamycin treatment), Sit4p is released from Tap42p and activated. Active Sit4p dephosphorylates and activates several targets such as the GATA-type transcription factor, Gln3p, and the Ser/Thr-specific protein kinases,

Npr1p and Tip41p [64, 68, 69]. However, it is not yet known if the Tor proteins directly regulate Tap42p or how Tap42p affects Sit4p activity.

In addition to the direct regulation of protein activity via phosphorylation, Tor signaling also represses starvation-specific transcription by sequestering several nutrient-responsive transcription factors in the cytoplasm. For example, Tor activity prevents the transcription of genes normally induced upon nitrogen limitation by promoting the association of Gln3p with its cytoplasmic regulator Ure2p. The Gln3p-Ure2p interaction requires the Tor-dependent phosphorylation of ether Gln3p and/or Ure2p [61, 64]. Tor activity is also responsible for sequestrating of several other transcription factors, such as Msn2p and Msn4p, and the basic helix-loop-helix/leucine zipper (bHLH/Zip) factors, Rgt1p and Rgt3p, in the cytoplasm [65, 66]. The Gln3p, Rgt1p and Rgt3p transcription factors are activated specifically in response to nitrogen starvation, whereas Msn2p and Msn4p are activated in response to other types of nutrient and environmental stresses. Therefore, Tor signaling appears to discriminate between different nutrient conditions and this allows the cell to respond appropriately to a given condition. However, the precise mechanisms by which Tor senses and discriminates between these nutrient and environmental stresses are still unknown.

Both the Tor and Ras/PKA signaling pathways regulate cell growth and proliferation in response to nutrient availability. Inactivation of either of these pathways results in a growth arrest similar to stationary phase [8]. Thus, it is possible that the two signaling pathways will form an interacting network. This hypothesis is supported by the fact that both the Tor and Ras/PKA signaling pathways regulate the expression of

stationary phase markers through the negative regulation of the activities of the Msn2p and Msn4p transcription factors and the Rim15p [37, 38]. Unfortunately, the precise nature of any interplay between these two signaling pathways remains unknown.

1.4. Autophagy

Cell homeostasis is dependent upon the balance between the biosynthesis and degradation of macromolecules. Autophagy is the major cellular pathway for macromolecular and organelle degradation in eukaryotic cells. Autophagy is an evolutionarily conserved process that occurs in all eukaryotic cells, from yeast to humans. In unicellular eukaryotic organisms, such as fungi, autophagy allows cells to survive prolonged periods of starvation by recycling portions of the cytoplasm and organelles. Higher eukaryotes utilize autophagy for many physiological processes, including the survival during nutrient starvation, the control of cell proliferation and for normal aging. In addition, mutations that disrupt the autophagy process result in many human diseases, including cancer and cardiomyopathies, and may contribute to specific muscular and neurodegenerative disorders, such as Hungtinton's, and Alzheimer's diseases) [70, 71].

Autophagy is a dynamic process that is inhibited under nutrient-rich conditions and induced upon starvation. Autophagy consists of a multi step pathways that involves the sequestration of portions of the cytoplasm (including organelles) within double-membrane vesicles, known as autophagic vacuoles in mammalian cells or

autophagosomes in yeast. Autophagosomes deliver their cargo to the vacuole (or lysosome in mammalian cells), where the sequestered cargo is degraded by specific hydrolyses [70, 72-74] (Figure 1.4). Genetic screens have been used in yeast to identify over 20 novel *ATG* (AuTophaGy-related) genes that are involved in the autophagy process [75, 76]. Analysis of *atg* mutants has shown that many regulatory components of autophagy are also required for biosynthetic pathway, known as the cytoplasm-to vacuole-targeting (Cvt) pathway. The Cvt pathway operates under nutrient-rich conditions and has been shown to be important for the vacuolar delivery of at least two hydrolyses, aminopeptidase I (Ape1p) and α-mannosidase (Ams1p) [77-79]. Ape1p is synthesized as a precursor in the cytosol and rapidly oligomerizes into dodecatomers, which further assemble into a large complex, termed the Cvt complex. The Cvt complex is enwrapped in a double membrane vesicle, which is smaller (140-160nm in diameter) than an autophagosome (300-900nm in diameter) and excludes other cytosol components. After fusion of the Cvt vesicle with the vacuole, the inner vesicle degrades in the lumen, allowing release and maturation of precursor Ape1p.

Both autophagy and Cvt transport involve the sequestration of cytoplasmic material within a double-membrane vesicle, fusion of the autophagosome/ Cvt vesicle with the vacuole, and degradation of the cargo in the vacuole. In *S. cerevisiae*, many of the components of the autophagic machinery are also used for the Cvt transport. The major difference between the pathways is at the level of cargo selection. Autophagy is responsible for the nonselective delivery of cytoplasmic material to the vacuole during starvation, whereas Cvt specifically delivers particular hydrolyses to the vacuole.

To ensure the specificity of the cargo, the Cvt pathway utilizes the Atg11p and Atg19 proteins. These proteins are not involved in autophagy, but play a critical role in the recognition and packaging of Ape1p [79-82].

One of the proteins that is thought to regulate the switch between Ctv transport and autophagy is the serine/threonine-specific protein kinase, Atg1p [83]. Deletion of the *ATG1* gene completely blocks both the Cvt pathway and autophagy, indicating that Atg1p activity is essential for both processes [79, 83]. However, the absolute level of Atg1p activity appears to be a major determinant of whether the cell will carry out Cvt transport or autophagy. In nutrient-rich media, Atg1p kinase activity is low and Cvt transport predominates. Following either nitrogen starvation or rapamycin treatment, Atg1p activity increases and autophagy is induced. This change in Atg1p activity appears to be controlled by the phosphorylation state of Atg13p, a second important regulator of autophagy [77, 84, 85]. In rich media, Atg13p is hyperphosphorylated and unable to interact with Atg1p. Upon nutrient depravation (or rapamycin treatment), Atg13p is rapidly dephosphorylated and subsequently found in a complex with Atg1p [81, 82, 85]. Atg1p and Atg13p interact with multiple proteins, including Atg11p, Atg17p, Atg20p, Atg24p, and Vac8p. These proteins play an important role in autophagy and/or the Cvt pathway [81, 85, 86]. Unfortunately, the target, or targets, of Atg1p have not yet been identified and thus we do not yet know how this kinase regulates the switch between autophagosome and Cvt vesicle biogenesis.

Autophagosomes and Cvt vesicles do not form randomly in the cytoplasm. Instead, they initiate at a specific site, known as the pre-autophagosomal structure (PAS) [87, 88]. Unlike vesicle formation through the secretory pathways, autophagic vesicles do not bud off from pre-existing organelles, but instead appear to form *de novo*. However, to date little is known about the source of the membrane used to form the autophagosome. One key component for the formation of these transport intermediates the phosphatidylinositol 3-kinase, Vps34p. Kinase Vps34p is part of a complex, the phosphatidylinositol 3-kinase complex I (PtdIns 3-kinase complex I),that found at the PAS and contains the serine/threonine protein kinase Vps15p, Atg6p, and Atg14p [89].

During autophagosome formation PtdIns 3-kinase complex I is associated with several other proteins such as the transmembrane protein Atg9p, peripheral membrane proteins, Atg2p and Atg23p, and PdsIns(3)P biding protein Atg18p. The Atg9p is essential for Cvt vesicles and autophagosome formation, whereas Atg23p is required for the Cvt pathway and efficient autophagy [90]. Studies using fluorescence microscopy techniques demonstrated that Atg9p and Atg23p are distributed in several subcellular punctate structures, only one of which localizes to the PAS and none of those structures were found on complete autophagosome/Cvt vesicles [90-92]. These results led to the model that Apg9p and Atg23p are recruited to the PAS during vesicle formation and then retrieved from the autophagosome prior its completion into yet undefined punctate structures. Previous work have demonstrated that Atg1p and Atg13p are required for this process [91]. This complex alone is sufficient to organize the proper trafficking of Atg23p. To date, its unknown if Atg23p redistribution requires additional proteins, but it

has been demonstrated that Atg23p redistribution absolutely requires Atg1p kinase activity. As an integral membrane protein, Atg9p cannot simply dissociate from the lipid bilayer. The retrieval of Atg9p requires the generation of phosphatidylinositol-3-phosphate (PtdIns(3)P) by the PtdIns 3-kinase complex I [87]. The Atg1p-Atg13p complex recruits Atg18p and Atg2p to the PAS and allows Atg9p to leave the PAS (Figure 1.5). Its remains to be established how the Atg1p-Atg13p complex regulates this event, but it has been shown that retrieval of Atg9p does not require the protein kinase activity of Atg1p. [91].

Thus, Atg1p and Atg13p are essential parts of Atg machinery. These proteins interact with several components of the PAS to coordinate correct double-membrane assembly. It is possible, that by inducing the retrograde transport of the Atg9p and Atg23p at a specific time, the Atg1p-Atg13p complex will determine the size of double-membrane vesicles (Cvt vesicles or autophagosome), and therefore coordinate a switch between Cvt transport and autophagy.

In recent years, great advances have been made in the identification of the autophagic machinery and the functions of these components. Interestingly, many of the yeast autophagy genes have candidate orthologs in higher eukaryotes [93]. Furthermore, inactivation of several of these orthologs has illustrated the importance of autophagy to the normal development of plants, nematodes, flies, and mammals. The control of autophagy is dependent upon a variety of signaling pathways, including the Tor [81, 85] and Ras/PKA [67] signaling pathways, specific tyrosine kinase receptors [94], protein kinase A in mammals [95], casein kinase II [96], MAP kinases [97, 98] and intracellular

calcium [99]. However, the mechanism by which many of these signaling pathways contribute to the control of autophagy are still largely unknown and require identification of the relevant downstream targets.

1.5 Significance

One of the simplest and best-characterized protein kinases in the budding yeast *Saccharomyces cerevisiae* is cAMP-dependent serine-threonine protein kinase (PKA). PKA is highly conserved from yeast to humans. Although the regulation mechanism is not identical in all cells, PKA is invariably involved in growth control and development. Therefore, identification of PKA targets in yeast may provide significant insight into the mechanisms that regulate cell growth in humans.

Recent technological advances have resulted in the accumulation of large amounts of genomic DNA sequences for a variety of organisms. How to use these data to increase our understanding of biology represents one of the major challenges we face today. Here, we describe a bioinformatics approach that uses this sequence information to identify the biologically-relevant occurrences of a protein motif of interest. In this approach, the evolutionary conservation of a particular sequence element is assessed within a group of related organisms. The underlying premise is that a higher degree of conservation would identify the sequence elements that are functional *in vivo* [100, 101]. This approach was used here to identify 45 candidate substrates of the cAMP-dependent

protein kinase (PKA) in the yeast, *Saccharomyces cerevisiae*. Remarkably, phosphorylation analyses indicated that essentially all of these candidates are phosphorylated by this kinase *in vitro*. Moreover, as proof of principle, we show that one of these targets, the autophagy-related protein kinase, Atg1p, is regulated by direct PKA phosphorylation *in vivo*. These data therefore demonstrate the general potential this strategy has for determining the physiological relevance of any short sequence motif found in any type of protein.

This body of work not only suggests novel targets for Ras/PKA signaling pathway, but also provides new insights into the manner in which signal transduction pathways may coordinate the transition between biosynthetic and degradative processes in the cell, such as Cvt transport and autophagy. We have demonstrated that a key regulator of autophagy and Cvt pathway, Atg1p is a direct target of PKA in regulation of both of these pathways. In addition, research presented here suggests a connection between Ras/PKA signaling and Tor function

Protein:	Function:	PKA consensus site:	References:
Abf2p	Mitochondrial histone	$KRP_{21}T_{22}SA$	[102]
Bcy1p	PKA regulatory subunit	$RRT_{143}S_{144}V$	[46]
Cdc19p	pyruvate kinase	$RRT_{21}S_{22}I$	[103]
Cdc20p	Cell-cycle regulated activator of anaphase-promoting complex/cyclosome (APC/C)	$RRDS_{88}S_{89}F$ $RRPS_{602}S_{603}T_{604}$	[104]
Cdc25p	Guanine nucleotide exchange factor	$RRSS_{151}L$ $RRGS_{174}S$	[105, 106]
Cki1p	Choline kinase	$RRHS_{30}L$ $RRAS_{85}A$	[107]
Fbp1p	Fructose-1,6-bis-phosphatase (FBPase)	$RRDS_{11}T$	[19]
Msn2p	Transcription factor	$RRFS_{288}D$	[49]
Pde1p	Low affinity 3',5'-Cyclic-nucleotide phosphodiesterase	$RRES_{252}E$	[50]
Pfk26p	Phosphofructokinase 2	$RRPT_{157}T_{158}I$ $RRYS_{644}V$	[20]
Rim15p	Protein kinase	$RRGS_{709}P$ $RRKS_{1094}L$ $RRSS_{1416}K$ $RRTS_{1463}S$ $RRRS_{1616}G$	[38]
Rst2p	Transcription factor	$RRS_{139}T_{140}T_{141}A$ $RRAT_{253}I$	[108]
Sko1p	Transcriptional repressor	$RRMS_{380}S$	[109]
Sok2p	Transcription factor	$KKCT_{598}M$	[110]
Spt5p	Transcription elongation factor	$RRPT_{441}F$	[111]
Ssn2p	Srb complex of the RNA pol II holoenzyme	$RRKS_{608}V$ $RRLS_{1236}G$	[43]
Tlg2p	t-SNARE	$RRLS_{90}E$	[112]
Yak1p	Ser/Thr protein kinase	$RRQS_{206}L$ $RRLS_{240}A$	[113]

Table 1.1: Known targets of PKA.

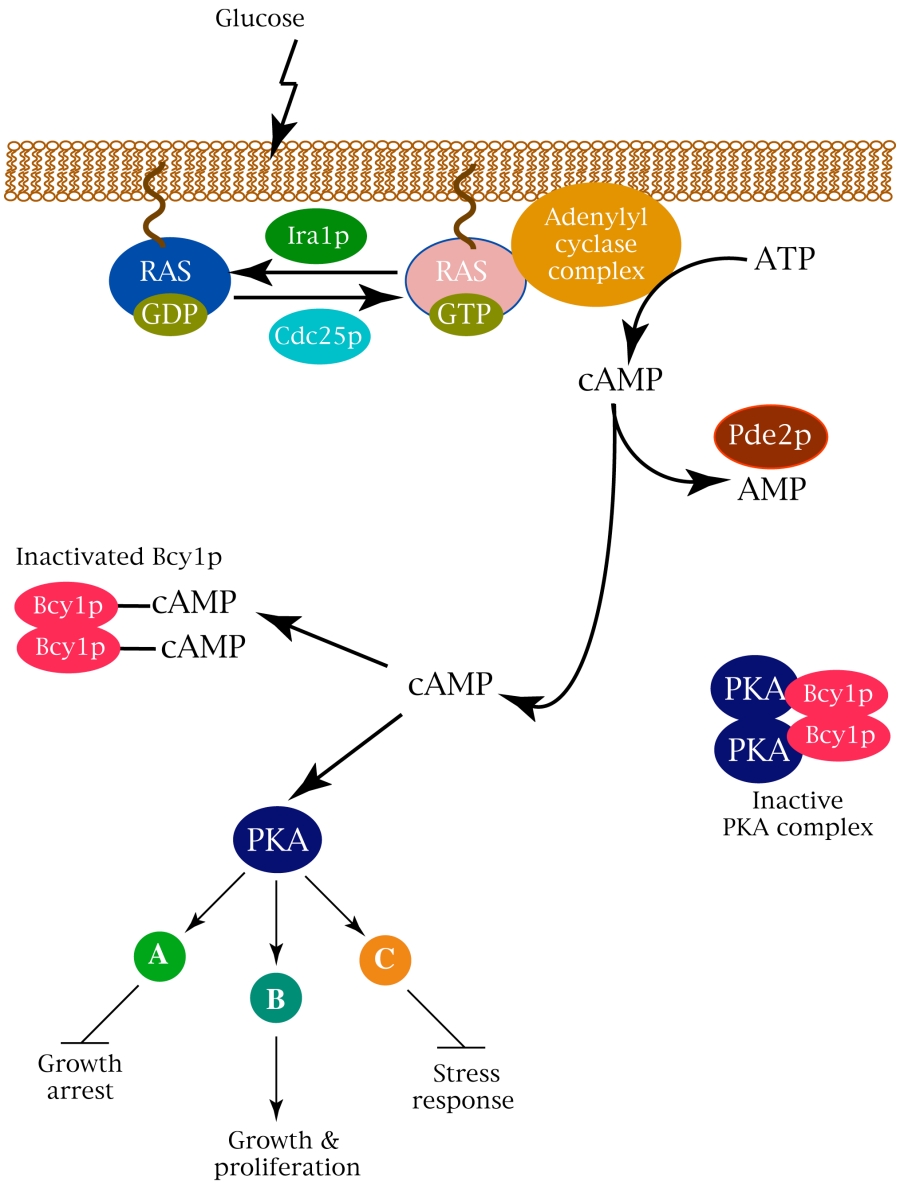

Figure 1.1: The cAMP-dependent Ras/PKA signaling in *S. cerevisiae*.

Figure 1.2: The minimal consensus site of cAMP-dependent protein kinase (PKA).

All known PKA substrates share the minimal consensus site of cAMP-dependent protein kinase is R_{-3}-R_{-2}-x_{-1}-S/T-B_{+1}, where "x" refers to any amino acid, "B" to a residue with a hydrophobic side chain and the S/T to the serine or threonine residue that is the site of phosphate addition. The region required for high affinity is highlighted in blue and phosphorylated residues (Serine) in red [114]. Red diamonds indicates minimum consensus site highlighted in purple, with the essential residues.

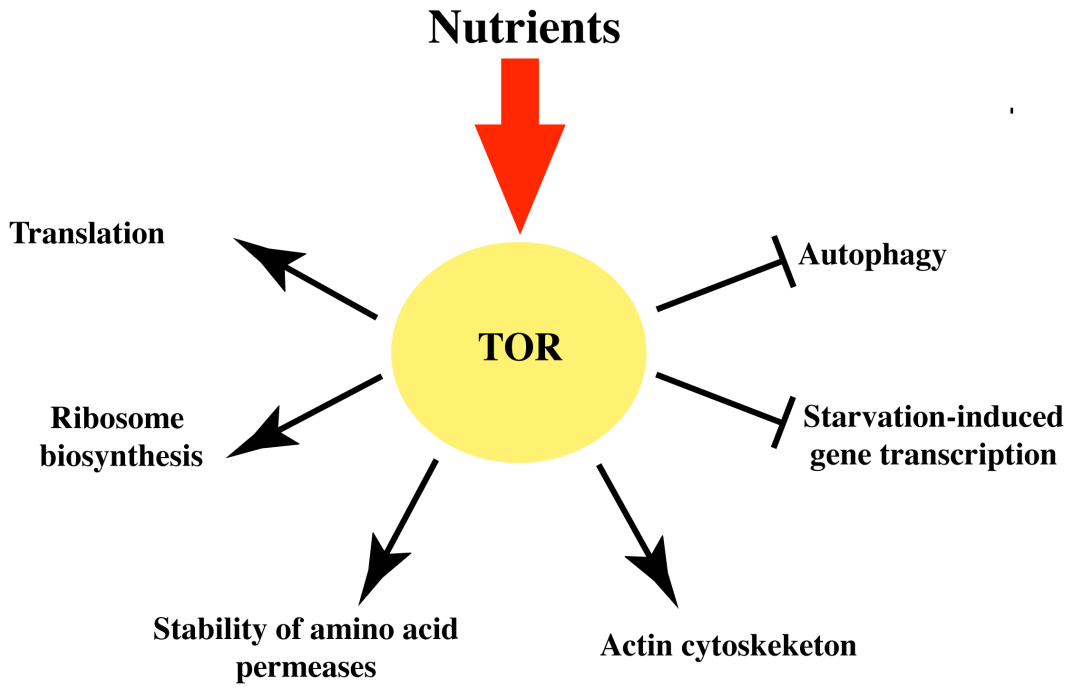

Figure 1.3: TOR signal transduction pathways in yeast.

CHAPTER 2

AN EVOLUTIONARY PROTEOMICS APPROACH FOR THE IDENTIFICATION OF SUBSTRATES OF THE CAMP-DEPENDENT PROTEIN KINASE (PKA)

2.1 Introduction

Protein kinases play an important role in essentially all aspects of eukaryotic biology, from specifying cell fates in the early embryo to controlling cell proliferation in the adult. These enzymes modify protein function by transferring the terminal phosphate from ATP to the hydroxyl group of particular serine, threonine or tyrosine residues in a defined set of targets. A complete understanding of the biology of any protein kinase therefore requires the identification of the substrates of this enzyme. Unfortunately, this identification process is often a difficult and labor-intensive task [18]. With this in mind, we set out to test whether this process could be simplified with a bioinformatics approach that identifies proteins with consensus phosphorylation sites that have been conserved through evolution. The underlying premise is that a high

degree of sequence conservation would identify those proteins that are most likely to be substrates of this protein kinase *in vivo*. This comparative analysis between related species has been wildly utilized to identify important features of both entire genomes and individual genes [100, 101, 184, 186]. The *Saccharomyces* species used for this analysis represent the three major groups of this genus and the pathogenic yeast, *Candida albicans* (Figure 2.1) [184, 185]. The *Saccharomyces* species used in this study were *sensu stricto* species, *S. mikatae*, *S. kudriavzevii*, and *S. bayanus*, the *sensu lato* species, *S. castellii* and the petite-negative species, *S. kluyveri* [184]. The recent work has indicated that PKA activity is regulated by the Ras proteins in yeast that is most distantly related to *S. cerevisiae*, in *C. albicans* [124, 125]. These observations therefore suggest that a Ras/PKA signaling pathway is functional in all of the yeasts being used in this study.

This chapter describes the initial application of the evolutionary proteomic approach to the consensus target site that is recognized by the cAMP-dependent protein kinase, PKA in budding yeast *Saccharomyces cerevisiae*. This kinase is an important regulator of cell growth and proliferation in *S. cerevisiae*. Although several PKA substrates have been described (see Table 1.1), the biological activities of these proteins are not sufficient to explain the global effect that PKA activity has on the growth of this organism. Therefore, identification of additional targets is necessary for a complete understanding of the biological role of PKA in this budding yeast.

2.2 Materials and Methods

2.2.1 Growth media: Standard *E.coli* growth conditions and media were used throughout this study [116]. Yeast YPAD and SC growth media were as described [117, 118]. SC-glucose medium refers to a synthetic complete medium containing 0.67% yeast nitrogen base (DIFCO), 2% glucose and those growth supplements required for cell proliferation.

2.2.2 Protein sequence comparisons: The pattern match program, PatMatch, at the SGD website (www.yeastgenome.org) was used to identify the consensus PKA sites in the *S. cerevisiae* proteome. The proteins containing these PKA sites were then aligned with their orthologs from the budding yeast species used in this analysis with the Blastp and Dialign alignment programs. The final sequence alignments were also examined by eye to ensure that no conserved PKA site had been missed. The protein sequences for the five *Saccharomyces* species used in this analysis were obtained from the web site for the Genome Sequencing Center at Washington University (genome.wustl.edu). The *C. albicans* sequences were obtained from the CandidaDB website (www.pasteur.fr/ Galar_Fungail/CandidaDB) developed by the Galar Fungail European Consortium.

2.2.3 Analysis of protein phosphorylation: In general, the *in vitro* phosphorylation assays were performed with glutathionine S-transferase (GST) fusion proteins that were under the control of the *GAL1* promoter in the yeast strain, Y258 (*MATa his4-580 ura3-52 leu2-3,112 pep4-3*) [119]. The strains were grown to mid log phase in SC medium,

containing 2% raffinose and than transferred to medium containing 2% raffinose and 5%galactose for 4 hrs at 30^{O}C. The GST fusion proteins were then isolated on glutathione-agarose beads (Pierce) and incubated with 1 μCi [γ-^{32}P] ATP (Perkin Elmer) and either 0 or 5 U of bovine PKA catalytic subunit (Sigma) as described [43]. A Western immunoblot was performed with a α-GST antibody (Cell Signaling) to quantify the relative amount of GST fusion protein present for each candidate.

2.3 Results

2.3.1 The evolutionary proteomics strategy.

The bioinformatics approach used here consisted of two major steps. In the first, a pattern match program was used to identify the *S. cerevisiae* proteins that contain a consensus PKA phosphorylation site. Previous work has identified a consensus site of R_{-3}-R_{-2}-x_{-1}-S/T-B_{+1} for this enzyme, where "x" refers to any amino acid and "B" to a residue with a hydrophobic side chain [120-122]. A search of the *S. cerevisiae* proteome found 553 occurrences of this consensus sequence in 491 proteins (Table 2.1). Fifty-one proteins were found to have multiple sites, with five being the most sites present in any one protein.

For the second stage of this analysis, we asked whether these potential PKA sites were conserved in the orthologous proteins present in five different *Saccharomyces* species and the pathogenic yeast, *Candida albicans* (Figure 2.1) [123]. Recent work has indicated that in *C. albicans*, as in *S. cerevisiae*, PKA activity is regulated by the Ras proteins [124, 125]. Thus, a Ras/PKA signaling pathway is likely functional in all of the yeasts being used in this study.

This analysis found that the number of potential PKA sites dropped as the evolutionary distance from *S. cerevisiae* increased (Figure 2.1). Only 92 of the original 553 sites (~17%) present in *S. cerevisiae* were conserved in the other *Saccharomyces* species and *C. albicans*. The 85 proteins that contain these conserved sites are involved in a wide variety of processes important for cell growth (Figure 2.2; Table 2.2). This

observation is consistent with the highly pleiotropic phenotypes associated with mutations affecting the Ras/PKA pathway [23, 115]. However, since many of these potential substrates are highly conserved proteins, it is not clear whether the observed conservation of their PKA sites is significant in so far as this comparative analysis is concerned. As a result, we limited the subsequent analysis to those proteins that possessed a conserved site in a region exhibiting less than 50% identity between the *S. cerevisiae* and *C. albicans* proteins. This constraint reduced the number of potential PKA substrates to 45 proteins, or less than 1% of the total proteome (Table 2.3).

2.3.2 Predicted PKA targets are phosphorylated by PKA *in vitro*.

The most important question is whether these identified candidates are indeed substrates for PKA. Therefore, we tested whether proteins in this candidate group were more likely than other *S. cerevisiae* proteins to be phosphorylated by PKA in an *in vitro* assay. Representative proteins from four different groups were examined: proteins lacking a PKA consensus site; proteins with sites only in *S. cerevisiae*; proteins with sites conserved amongst the *sensu stricto Saccharomyces* species (*Saccharomyces cerevisiae*, *Saccharomyces mikatae*, *Saccharomyces kudriavzevii* and *Saccharomyces bayanus*); and proteins with sites conserved out to *C. albicans*. Remarkably, we found that 19 of the 20 candidates tested from this latter group were phosphorylated by PKA (Figure 2.3). In contrast, only 20-30% of the proteins containing less conserved sites were labeled in this assay (Figure 2.4 and Figure 2.6). Finally, none of the proteins that lacked a consensus site were efficiently phosphorylated by PKA; one of these proteins,

Cox6p, was weakly phosphorylated in this assay (Figure 2.6). Thus, the presence of a highly conserved consensus site was a very strong predictor of PKA phosphorylation (Figure 2.7). This striking correlation between the conservation of the PKA site and its tendency to be phosphorylated suggested that the candidates with the most highly conserved sites might be targets of PKA. Consistent with this prediction, five of the these candidate proteins, Bcy1p, Cki1p, Msn2p, Rim15p, and Yak1p, have been previously shown to be substrates of this kinase (Table 2.3) [38, 46, 49,107, 113].

2.4 Discussion

Previous data have indicated that PKA both drives cellular proliferation and inhibits the stress response. However, the manner in which this is achieved is poorly understood since only a few of the PKA substrates important for these processes have been identified. In the present study, we develop an evolutionary proteomics strategy to identify new targets of PKA. This approach led to identification of 45 potential PKA targets. These targets play an important role in variety of different cellular processes such us protein and lipid biosynthesis, RNA pol II and pol III transcription, chromatin assembly, bud site selection, protein targeting, cell size regulation, cell wall biosynthesis, and sporulation (Table 2.3).

The striking correlation between the conservation of the site and its tendency to be phosphorylated suggests that these candidates might be substrates of PKA *in vivo*. Consistent with this prediction, five of these candidate proteins have a PKA consensus site that is conserved amongst all *Saccharomyces* species and *C.albicans*, and have been previously shown to be targets of this kinase *in vivo* (see Tables 1.1 and 2.3). These proteins include Bcy1p [126], Yak1p [113], Msn2p [48], Cki1p [127], and Rim15p [38]. Yak1p, Msn2p, and Rim15p are important for the PKA-mediated transcriptional regulation of genes involved in the response to a variety of environmental stresses and stationary phase entry. It has been shown that PKA directly phosphorylates these targets, and that this phosphorylation has a negative role in the regulation of their activity. The evolutionary proteomics approach described here identified an additional

PKA targets that are important for the expression of stress-specific proteins, namely the ATF/CREB family activator proteins, Aca1p and Cst6p. Eukaryotic organisms from yeast to human contain multiple CREB family proteins that activate or repress the expression of specific genes through a conserved promoter element, known as the cAMP responsive element (CRE) [128]. In multicellular organisms, CREB proteins (CRE-binding) stimulate transcription in response to cyclic AMP and calcium, and this transcriptional induction by cAMP requires the activation of PKA [129, 130]. CREB proteins control diverse biological functions such as memory [131], opiate tolerance [132], spermatogenesis [133], circadian rhythms [134], and skeletal and neural development [135]. In addition, CREB activator proteins in fission yeast and mammalian cells play an important role in mediating the response to activation of a PKA and to a wide variety of environmental stresses [129, 130, 136]. Phenotypic analysis of the *S.cerevisiae* CREB proteins, Aca1p and Cst6p, indicates that these proteins are important for growth on nonoptimal carbon sources as well as the resistance to a variety of stresses [137]. These data led to the hypothesis, that PKA can directly regulate the activity of Aca1p and Cst6p via phosphorylation. Our data are consistent with this hypothesis as we found that PKA phosphorylates Cst6p *in vitro* (see Figure 2.3). Additional experiments are needed to elucidate the effect of PKA phosphorylation on this transcriptional regulation. Unfortunately, the precise connection between Ras/PKA signaling activity and the other targets identified by our evolutionary proteomics approach has not yet been made.

Functional sequences, DNA or protein, usually are highly conserved in evolution. Therefore, functionally important element would remain conserved in diverse organisms, and redundant sequences would disappear in closely related organisms. The *sensu stricto* species (*S. baynus*, *S. mikatae*, and *S. kudriavzevii*) are physiologically similar to *S. cerevisiae*, are capable of forming stable diploids with each other, and have a very similar karyotype. Most of the proteins of this group are more than 80% identical to their *S. cerevisiae* homologs. A phosphorylation analysis of randomly selected proteins with PKA sites present only in *S. cerevisiae* or with PKA sites conserved amongst *sensu stricto Saccharomyces* species demonstrated that only 20-30% of the targets are phosphorylated by PKA (see Figure 2.4 and 2.5). These results suggest that most of PKA sites in this group of proteins are not likely to be functionally relevant.

The *sensu lato* and *petite-negative Saccharomyces* species are quite diverged from *S. cerevisiae*. They have significantly different physiological characteristics and have a smaller number of chromosomes [138]. Only about 30% of the proteins of *sensu lato* species share more than 50% identity to their *S. cerevisiae* homologs [123]. Our data indicate that only 221 of the original 553 sites present in *S. cerevisiae* (~39.8%) were conserved in the *sensu lato* and *petite-negative Saccharomyces* species. If proteins exhibiting more than 50% identity between the *S. secevisiae* and *S. kluyveri* orthologs are excluded, this comparative analysis would identify an additional 76 proteins (less than 2% of total proteome) as a potential PKA substrates (see Table 2.2). Four previously identified targets of PKA, Cdc25p [105, 106], Pfk26p [20], Spt5p [111], and Ssn2p [43], were identified in this group of potential PKA targets (see Tables 1.1 and

Table 2.2). Although, the *C. albicans* orthologs of these proteins do not share the conserved PKA consensus site, future characterization of these proteins with PKA sites conserved to the *sensu lato* and *petite-negative Saccharomyces* species will likely shed important insights on the PKA regulation of *S. cerevisiae* biology.

Sequence Name	Site	*cerevisiae*	*bayanus*	*kudriavzevi*	*mikatae*	*castellii*	*kluveri*	*albicans*
YAL038w/CDC19	19	RRTSI	RRTSI	-----	RRTSI	RRTSI	RRTSI	RRSSI
YBL072c/RPS8A	194	RRLTA	RRLTA	RRLTA	RRLTA	RRLTA	RRLTA	RRLTA
YBL029w	350	RRGTY	RRGTY	RRGTY	RRGTY	RRATY	RRATY	RRGSY
YBL011w/SCT1	110	RRVSF	RRVSF	RRVSF	RRVSF	RRVSF	-----	RRISF
YBL008w/HIR1	236	RRPSW	RRPSW	RRPSW	RRPSW	RRLSW	RRLSW	RRMSW
YBR028c	70	RRSSA	RRSSA	RRSSA	RRSSI	RRSSI	RRSSL	RRKSS
YBR140c/IRA1	1750	RRNSC	RRNSC	-----	RRNSC	RRNTC	-----	RRNST
YBR220c	85	RRRSW	RRRSW	RRRSW	RRRSW	RRRSW	RRRSW	RRRSW
YBR229c/ROT2	816	RRSSM	-----	RRSSK	RRSSK	RRSSK	-----	RRSSK
YCR011c/ADP1	536	RRVSI	RRVSI	RRVSI	RRVSI	RRVSI	RRVSI	RRVSI
YDL224c/WHI4	560	RRLSF	RRLSF	RRLSF	RRLSF	RRLSF	RRLSF	RRLSF
YDR005c/MAF1	98	RRISF	RRISF	RRISF	RRISF	RRMSV	RRISV	RRASS
YDR099w/BMH2	57	RRASW	RRASW	RRASW	RRASW	RRASW	RRASW	RRASW
YDR169c/STB3	283	RRSSI	RRSSI	RRSSI	RRSSI	RRMSR	RRSSS	RRKSS

Continued

Table 2.1: Eighty-five *S. cerevisiae* proteins contain PKA sites that are conserved in the orthologs present. The "-----" indicates that PKA site is absent in the conserved ortholog protein.

Table 2.1 continued

Sequence Name	Site	*cerevisiae*	*bayanus*	*kudriavzevi*	*mikatae*	*castellii*	*kluveri*	*albicans*
YDR172w/SUP35	338	RRYTI	RRYTI	RRYTI	-----	RRYTL	-----	RRYTI
YDR283c/GCN2	574	RRRSF	RRRSF	-----	RRRSF	-----	-----	RRLSN
YDR293c/SSD1	161	RRHSL	RRHSL	RRHSL	RRHSL	RRHSL	RRHSL	RRHSL
YDR293c/SSD1	500	RRSSL	RRSSL	RRSSL	RRSSL	RRNSL	RRGSL	RRGSL
YDR385w/EFT2	710	RRATY	RRATY	RRATY	RRATY	RRATY	RRATY	RRATY
YDR463w/STP1	269	RRDTY	RRDTY	RRDTY	RRDTY	RRDTY	RRDTY	RRDTY
YDR505c/PSP1	235	RRSSY	RRSSY	RRSSY	RRSSY	RRSSY	-----	RRPSY
YER045c/ACA1	102	RRISI	RRISI	RRISI	RRISI	RRISI	-----	RRISI
YER049w	166	RRISF	RRISF	RRISF	RRISF	RRISF	RRVSF	RRVSF
YER099c/PRS2	87	RRITA	-----	RRITA	RRITA	RRITA	-----	RRITA
YER102w/RPS8B	194	RRLTA	RRLTA	RRLTA	RRLTA	RRLTA	RRLTA	RRLTA
YER125w/RSP5	758	RRFTI	RRFTI	RRFTI	RRFTI	RRFTI	-----	RRFTI
YER177w/BMH1	57	RRASW	RRASW	RRASW	RRASW	RRASW	RRASW	RRASW
YFL045c/SEC53	122	RRGTF	RRGTF	-----	RRGTF	RRGTF	RRGTF	RRGTF
YFL033c/RIM15	1618	RRRSG	RRRSG	RRRSG	RRRSG	RRESG	RRRSG	RRESS

Continued

Table 2.1 continued

Sequence Name	Site	*cerevisiae*	*bayanus*	*kudriavzevi*	*mikatae*	*castellii*	*kluveri*	*albicans*
YFR021w/ATG18	285	RRGTY	RRGTY	RRGTY	RRGTY	RRGTY	RRGTY	RRGTY
YGL197w/MDS3	754	RRSSL	RRSSL	RRSSL	RRSSL	RRSSF	-----	RRQSF
YGL180w/ATG1	512	RRLSI	RRLSI	RRLSI	RRLSI	RRLSI	RRISL	RRISL
YGL093w/SPC105	74	RRVSF	RRVSF	RRVSF	RRVSF	RRVSF	RRVSF	RRVSF
YGL003c/CDH1	210	RRLSA	-----	-----	-----	RRTSV	-----	RRKTN
YGR032w/GSC2	836	RRISF	RRISF	RRISF	RRISF	RRISF	RRITF	RRISF
YGR032w/GSC2	1375	RRYTL	RRYTL	RRYTL	RRYTL	RRYTL	RRISL	RRYTL
YGR113w/DAM1	28	RRSSM	RRSSI	RRSSM	RRSSM	RRSSL	RRSSL	RRHSH
YGR113w/DAM1	254	RRKSI	RRKSI	RRKSI	RRKSI	RRKSI	RRKSI	RRVSR
YGR121c/MEP1	45	RRKSA	RRKSA	RRKSA	RRKSA	RRKSA	RRKSA	RRKSA
YHL011c/PRS3	86	RRITA	RRITA	RRITA	RRITA	RRITA	-----	RRITV
YHR006w/STP2	255	RRDTY	RRDTY	RRDTY	RRDTY	RRDTY	RRDTY	RRDTY
YHR032w	12	RRSSI	RRSSI	RRSSI	RRSSI	RKSSV	-----	RRASI
YHR117w/TOM71	200	RRASA	RRASA	RRASA	RRASA	RRASA	RRASA	RRATS
YHR165c/PRP8	1604	RRFTL	RRFTL	-----	RRFTL	-----	RRFTL	RRFTL

Continued

Table 2.1 continued

Sequence Name	Site	*cerevisiae*	*bayanus*	*kudriavzevi*	*mikatae*	*castellii*	*kluveri*	*albicans*
YIL047c/SYG1	857	RRSSV	RRSSV	RRSSV	RRSSV	RRTTF	-----	RRKST
YIL036w/CST6	263	RRISI	RRISI	RRISI	RRISI	RRISI	RRISI	RRISI
YIL033c/BCY1	142	RRTSV	RRTSV	RRTSV	RRTSV	RRTSV	RRTSV	RRTSV
YJL165c/HAL5	214	RRNSL	RRNSL	RRNSL	RRNSL	-----	-----	RRITG
YJL141c/YAK1	292	RRASL	RRASL	RRASL	RRASL	RRASL	RRASL	RRCSI
YJL057c/IKS1	93	RRASA	RRASG	RRASS	RRASS	-----	-----	RRSSS
YJL046w	229	RRFSG	RRFSG	RRFSG	RRFSG	RRLSG	RRRSG	RRRSG
YJR001w/AVT1	47	RRQSI	RRQSI	RRQSI	RRQSI	RRQSI	RRQSI	RRPSH
YJR066w/TOR1	2253	RRTTY	RRTTY	RRTTY	RRTTY	RRTTY	RRTTY	RRTTY
YJR092w/BUD4	592	RRNTL	RRNTL	RRNTL	RRNTL	RRNTI	RRNTL	RRLSA
YKL203c/TOR2	2256	RRTTY	RRTTY	RRTTY	RRTTY	RRTTY	RRTTY	RRTTY
YKL180w/RPL17A	126	RRRTY	-----	RRFSA	RRRTY	-----	RRRTF	RRRTY
YKL072w/STB6	511	RRNSI	RRNSI	RRNSI	RRNSI	RRKSI	RRTSI	RRNSF
YKL064w/MNR2	162	RRKSV	RRKSV	RRKSV	RRKSV	-----	-----	RRSST
YKL064w/MNR2	618	RRSSA	RRSSA	RRSSA	RRSSA	RRSSG	RRHSS	RRLSS

Continued

Table 2.1 continued

Sequence Name	Site	*cerevisiae*	*bayanus*	*kudriavzevi*	*mikatae*	*castellii*	*kluveri*	*albicans*
YKL058W/TOA2	11	RRSTI	RRSTI	RRSTI	RRSTI	RRSTI	RRSTI	RRSTI
YLL029W	53	RRASL	RRASL	RRASL	RRASL	RRAST	-----	RRNST
YLL018C/DPS1	543	RRASL	RRASL	RRASL	RRASL	RRASL	RRASL	RRASL
YLR029C/RPL15A	187	RRKTW	RRKTW	RRKTW	RRKTW	-----	RRKTW	RRHTW
YLR069C/MEF1	312	RRSTI	RRATI	-----	RRATI	RRATI	RRATI	RRSTI
YLR133W/CKI1	27	RRHSL	RRPSL	RRHSL	RRHSL	RRPSL	RRPSL	RRPSL
YLR177W	176	RRSSY	RRSSY	RRSSY	RRSSY	RRSSY	RRSSY	RRPSQ
YLR223C/IFH1	1038	RRQSM	RRQSM	RRQSM	RRQSM	RRQSI	RRQSI	RRASI
YLR342W/FKS1	817	RRISF	RRISF	RRISF	RRISF	RRISF	RRITF	RRISF
YLR342W/FKS1	1356	RRYTL	RRYTL	RRYTL	RRYTL	RRYTL	-----	RRYTL
YLR359W/ADE13	334	RRISL	RRISL	RRISL	RRISL	RRISL	RRISL	RRISL
YLR403W/SFP1	102	RRESI	RRESI	RRESI	RRESI	RRDSI	RRESI	RRDSV
YLR419W	663	RRISA	RRISA	RRISA	RRISA	RRISA	RRISA	RRLSA
YMR037C/MSN2	579	RRKSA	RRKSA	RRKSA	RRKSA	RRKSS	-----	RRKSS
YMR053C/STB2	591	RRNSF	RRNSF	RRNSF	RRNSF	RRNSL	RRTSI	RRNSF

Continued

Table 2.1 continued

Sequence Name	Site	*cerevisiae*	*bayanus*	*kudriavzevi*	*mikatae*	*castellii*	*kluveri*	*albicans*
YMR121c/RPL15B	187	RRKTW	RRKTW	RRKTW	-----	-----	RRKTW	RRHTW
YMR196w	978	RRKSA	RRKSA	-----	RRKSA	RRKSA	RRKSA	RRKSG
YMR306w/FKS3	703	RRISF	RRISF	RRISF	RRISF	RRISF	RRITF	RRISF
YNL197c/WHI3	565	RRLSF	RRLSF	RRLSF	RRLSF	RRLSF	RRLSF	RRLSF
YNL154c/YCK2	165	RRFSV	RRFSV	RRFSV	RRFSV	RRFSV	RKFSV	RRFSV
YNL099c/OCA1	198	RRFTG	RRFTG	RRFTG	RRFTG	RRFTG	RRFTG	RRFTG
YOL081w/IRA2	1742	RRNSC	RRNSC	RRNSC	RRNSC	RRNSP	-----	RRNST
YOL075c	192	RRLSI	RRVSI	RRLSI	RRLSI	RRLSV	RRVSI	RRVSL
YOL075c	845	RRVTM	RRVSI	RRVSI	RRVSI	RRVTM	RRVSL	RRVSI
YOR092w/ECM3	309	RRNSI	RRNSI	RRNSV	RRNSI	-----	RRGSI	RRTSN
YOR133w/EFT1	710	RRATY	RRATY	RRATY	RRATY	RRATY	RRATY	RRVTY
YOR196c/LIP5	295	RRATY	RRATY	-----	RRATY	-----	RRATY	RRATY
YOR209c/NPT1	181	RRRSL	RRRSF	-----	RRRSL	RRRSF	-----	RRRSY
YOR370C/MRS6	6	RRPSM	RRPSM	RRPSM	RRPSM	RRPSM	RRPSM	RRKSM
YOR370C/MRS6	13	RRPSF	RRPSF	RRPSF	RRPSF	RRPSI	RRPSF	RRPST

Continued

Table 2.1 continued

Sequence Name	**Site**	***cerevisiae***	***bayanus***	***kudriavzevi***	***mikatae***	***castellii***	***kluveri***	***albicans***
YPL260W	366	RRWTL	RRWTL	RRWTL	RRWTL	RRWTL	-----	RRWTL
YPL217C/BMS1	113	RRLTF	RRLTF	RRLTF	RRLTF	RRLTM	RRLTF	RRLTF
YPR138C/MEP3	44	RRKSA	RRKSA	RRKSA	RRKSA	RRKSA	RRKSA	RRKSA
YPR185W/APG13	578	RRNSL	RRNSL	RRNSL	RRNSL	RRNSL	RRNSL	RRSSN

Sequence Name	Site	*cerevisiae*	*bayanus*	*kudriavzevi*	*mikatae*	*castellii*	*kluveri*
YBL054w	277	RRNSF	RRNSF	RRNSF	RRNSF	RRSSF	RRGSM
YBL054w	315	RRSSF	RRSSF	RRSSV	RRSSF	RRSSF	RRSSM
YBL054w	330	RRASL	RRPSL	RRASL	RRASM	RRGSM	RRESF
YBL023c/MCM2	794	RRESI	RRESI	-----	RRESI	RRESI	RRESI
YBR102c/EXO84	73	RRLSI	RRLSI	RRLSI	RRLSI	RRLSI	RRLSV
YBR198c/TAF90	758	RRRTV	RRRTV	RRRTV	RRRTV	RRRTI	RRRTI
YCR077c/PAT1	454	RRSSY	RRSSY	RRSSY	RRSSY	RRSSY	RRSSY
YDL231c/BRE4	745	RRYSG	RRYSG	RRYSG	RRYSG	RRYSG	RRNSG
YDL231c/BRE4	988	RRDSV	RRDSV	RRDSV	RRVSF	RRDSV	RRDSV
YDL102w/CDC2	839	RRDSC	RRDSC	-----	RRDSC	RRDSC	RRDSC
YDL051w/LHP1	16	RRNSF	RRNSF	RRNSF	RRNSF	RRNSF	RRGSY
YDR122w/KIN1	796	RRESL	-----	RRESL	RRESL	RRESL	RRESL
YDR207c/UME6	642	RRLSA	RRLSA	-----	RRLSA	RRLSA	RRLSA
YDR216w/ADR1	227	RRASF	RRASF	-----	RRASF	RRASF	RRGSF

Continued

Table 2.2: Potential targets with PKA sited conserved up to *S. castellii* and *S. kluyverii* (but not to *Candida albicans)*. Genes encoding the four proteins previously shown to be substrates of PKA are underlined. The "-----" indicates that PKA site is absent in the conserved ortholog protein.

Table 2.2 continued

Sequence Name	Site	*cerevisiae*	*bayanus*	*kudriavzevi*	*mikatae*	*castellii*	*kluveri*
YDR309c/GIC2	342	RRNSI	RRNSI	RRNSV	RRNSI	RRSSQ	RRKSD
YDR338c	28	RRISL	RRISL	-----	RRISL	RRISL	RRISL
YDR443c/SSN2	605	RRKSV	RRRSV	RRKSV	RRKSV	RRKSV	RRRSV
YDR443c/SSN2	1233	RRLSG	RRLSG	RRLSG	RRLSG	RRLSG	RRLSS
YER065c/ICL1	50	RRGTF	RRGTF	RRGTF	RRGTF	RRGTF	RRGTF
YER088c/DOT6	279	RRSSF	RRSSF	RRSSL	RRSSF	RRSSF	RRGSM
YER088c/DOT6	365	RRESV	RRESV	RRESV	RRESV	RRESI	RRGSI
YER129w/PAK1	1071	RRRSI	RRRSI	RRRSI	RRRSI	RRGSI	RRGSL
YER164w/CHD1	750	RRISI	RRISI	RRISI	RRISI	RRISI	RRISI
YFL050c/ALR2	611	RRKTM	RRKTM	RRKTM	RRKTM	RRKTM	RRKTM
YFL033c/RIM15	1091	RRKSL	RRKSL	RRKSL	RRKSL	RRRSP	RRHST
YGL195w/GCN1	1384	RRESV	RRESV	RRESV	-----	RRESV	RRESA
YGL180w/ATG1	505	RRPSL	RRPSL	RRPSL	RRPSL	RRPSV	RRPSL
YGL098w/USE1	85	RRYSI	-----	RRYSI	RRYSI	RRYSV	RRYSI
YGL014w/PUF4	202	RRQTF	RRQTF	RRQTF	RRQTF	RRQTF	RRQTF

Continued

Table 2.2 continued

Sequence Name	Site	*cerevisiae*	*bayanus*	*kudriavzevi*	*mikatae*	*castellii*	*kluveri*
YGR079w	350	RRKSG	RRKSG	RRKSG	RRKSG	RRESG	RRKSG
YGR083c/GCD2	118	RRATI	RRATI	RRATI	RRATI	RRASI	RRASL
YGR156w/PTI1	5	RRRTG	RRRTG	RRRTG	-----	RRRTA	RRNTR
YGR237c	346	RRYTW	RRYTW	RRYTW	RRYTW	RRFTW	RRHTW
YHL024w/RIM4	522	RRKSM	RRKSM	RRKSM	RRKSM	RRNSF	RRGSF
YHR013c/ARD1	6	RRATI	RRATI	RRATI	RRATI	RRATI	RRATI
YHR048w	138	RRITF	RRTTF	RRITF	RRITF	RRPTF	RRKTF
YHR102w/KIC1	175	RRQTM	RRQTM	RRQTM	RRQTM	RRQTM	RRQTM
YIL107c/PFK26	641	RRYSV	RRYSV	RRYSV	RRYSV	RRYSV	RRYSI
YIL046w/MET30	336	RRLSG	RRLSG	RRLSG	RRLSG	RRLSG	RRLTG
YIR010w/DSN1	247	RRLSM	-----	RRLSM	RRLSM	RRLSI	RRLSM
YJL141c/YAK1	237	RRLSA	-----	RRLSA	RRLSA	RRLSA	RRLSA
YJL084c	835	RRESV	-----	RRESV	-----	RRKSI	RRNSA
YJL070c	6	RRPSL	RRPSL	RRPSL	RRPSL	RRPSL	RRPSI
YJR001c/AVT1	112	RRLSV	RRLSV	RRLSV	RRLSV	RRLSV	RRLSV

Continued

Table 2.2 continued

Sequence Name	Site	*cerevisiae*	*bayanus*	*kudriavzevi*	*mikatae*	*castellii*	*kluveri*
YJR066c/TOR1	854	RRQTV	-----	RRGTV	RRQTV	RRETV	RRETG
YKL203c/TOR2	863	RRGTV	RRGTV	RRGTV	RRGTV	RRETV	RRETG
YKL203c/TOR2	1245	RRLSI	RRLSI	-----	RRLSI	RRLSI	RRLSI
YKL078w/DHR2	552	RRLSL	RRLSL	RRLSL	RRLSL	RRFTL	RRLSL
YKR031c/SPO14	283	RRPSI	RRPSL	RRPSI	RRPSL	RRTSL	RRGSA
YKR037c/SPC34	196	RRKTI	-----	RRKTI	RRKTI	RRKTM	RRKTM
YKR075c	84	RRWSI	RRWSI	RRWSI	RRWSI	RRWSI	RRWTI
YKR089c	874	RRHSI	RRHSV	RRHSV	RRHSV	RRNSA	RRTST
YLL021w/SPA2	1399	RRMTI	RRMTI	RRMTI	RRMTL	RRMTV	RRMTI
YLL015w/BPT1	928	RRASL	RRASL	RRASL	-----	RRASD	RRASN
YLL013c/PUF3	53	RRSSF	RRSSF	RRSSF	RRSSF	RRSSF	RRPSI
YLR096w/KIN2	823	RRESL	-----	RRESL	RRESL	RRESL	RRESL
YLR310c/CDC25	148	RRSSL	RRSSL	RRSSL	RRSSL	RRGSA	RRNSF
YML010w/SPT5	438	RRPTF	RRPTF	RRPTF	RRPTF	RRPTF	RRATY
YMR037c/MSN2	630	RRSSV	RRSSV	RRSSY	RRSSV	RRASV	RRSSR

Continued

Table 2.2 continued

Sequence Name	Site	*cerevisiae*	*bayanus*	*kudriavzevi*	*mikatae*	*castellii*	*kluveri*
YMR216c/SKY1	380	RRHTI	RRHTI	RRHTI	RRHTI	RRHTI	RRHTI
YMR221c	266	RRKSV	RRKSV	RRKSV	RRKSV	RRKSV	RRKSV
YMR292w/GOT1	64	RRGSL	RRGSL	RRGSL	RRGSL	RRGSI	RRGSL
YNL221c/POP1	97	RRRTA	RRRTA	RRRTA	RRRTA	RRRTA	RRTTA
YNL121c/TOM70	171	RRASA	RRASA	RRASA	RRASA	RRASA	RRASA
YNL076w/MKS1	515	RRQSM	RRESM	RRESM	RRESM	RRESM	RRESM
YNL054w/VAC7	686	RRYSG	RRYSG	RRYSG	RRYSG	RRYSG	RRYSG
YNR039c/ZRG17	110	RRQSL	RRQSL	RRQSL	RRQSL	RRHSL	RRHSF
YOL130w/ALR1	612	RRKTM	RRKTM	RRKTM	RRKTM	RRKTM	RRKTM
YOR062c	84	RRWSI	RRWSI	RRWSV	RRWSI	RRWSI	RRWTI
YOR073w	193	RRQSM	RRQSM	RRQSM	RRQSM	RRQSM	RRESL
YOR113w/AZF1	300	RRNSF	RRNSS	RRNSS	RRNSS	RRNSS	RRDSS
YOR233w/KIN4	83	RRDTI	RRDTI	RRDSI	RRDTI	RRDTI	RRDTV
YOR291w	939	RRMSV	RRMSV	RRMSV	RRMSV	RRMSV	RRMSV
YOR307c/SLY41	11	RRNSV	RRNSV	RRNSV	RRNSV	RRNSV	RRASI

Continued

Table 2.2 continued

Sequence Name	**Site**	***cerevisiae***	***bayanus***	***kudriavzevi***	***mikatae***	***castellii***	***kluveri***
YOR352w	128	RRLSM	RRLSM	RRLSM	RRLSM	RRLSM	RRLSM
YPL011c/TAF47	343	RRTSL	RRTSL	RRTSL	RRTSL	RRESI	RRESF
YPR032w/SRO7	599	RRFSL	RRFSL	RRFSL	RRFSL	RRFSL	RRFSL
YPR131c/NAT3	88	RRISL	RRISL	RRISL	RRISL	RRISL	RRISL
YPR160w/GPH1	28	RRLTG	RRLTG	RRLTG	RRLTG	RRLTG	RRLTG
YPR187w/RPO26	65	RRKTL	RRKTL	RRKTL	RRKTL	RRKTL	RRRTL

Biological process:	**Gene name:**
Transport (8)	*ATG1, ATG13, ATG18, AVT1, MNR2, MRS6, TOM71, YOL075c*
Signal transduction (6)	*BCY1, IRA1, IRA2, RIM15, SYG1, YAK1*
RNA pol II transcription (6)	*ACA1, CST6, MSN2, STP1, STP2, TOA2*
Cell size regulation (3)	*SFP1, WHI3, WHI4*
Chromatin assembly (3)	*STB2, STB3, STB6*
Cell wall synthesis (2)	*SSD1, ECM3*
Spindle assembly (2)	*DAM1, SPC105*
Bud site selection (1)	*BUD4*
Lipid biosynthesis (1)	*CKI1*
Protein biosynthesis (1)	*IFH1*
RNA pol III transcription (1)	*MAF1*
Sporulation (1)	*MDS3*
Unknown (10)	*CDS1, IKS1, PSP1, YBL029w, YHR032w, YJL046w, YLL029w, YLR177w, YLR419w, YPL260w*

Table 2.3: The forty-five candidate PKA substrates identified by the evolutionary proteomics approach. Genes encoding the five proteins previously shown to be substrates of PKA are underlined.

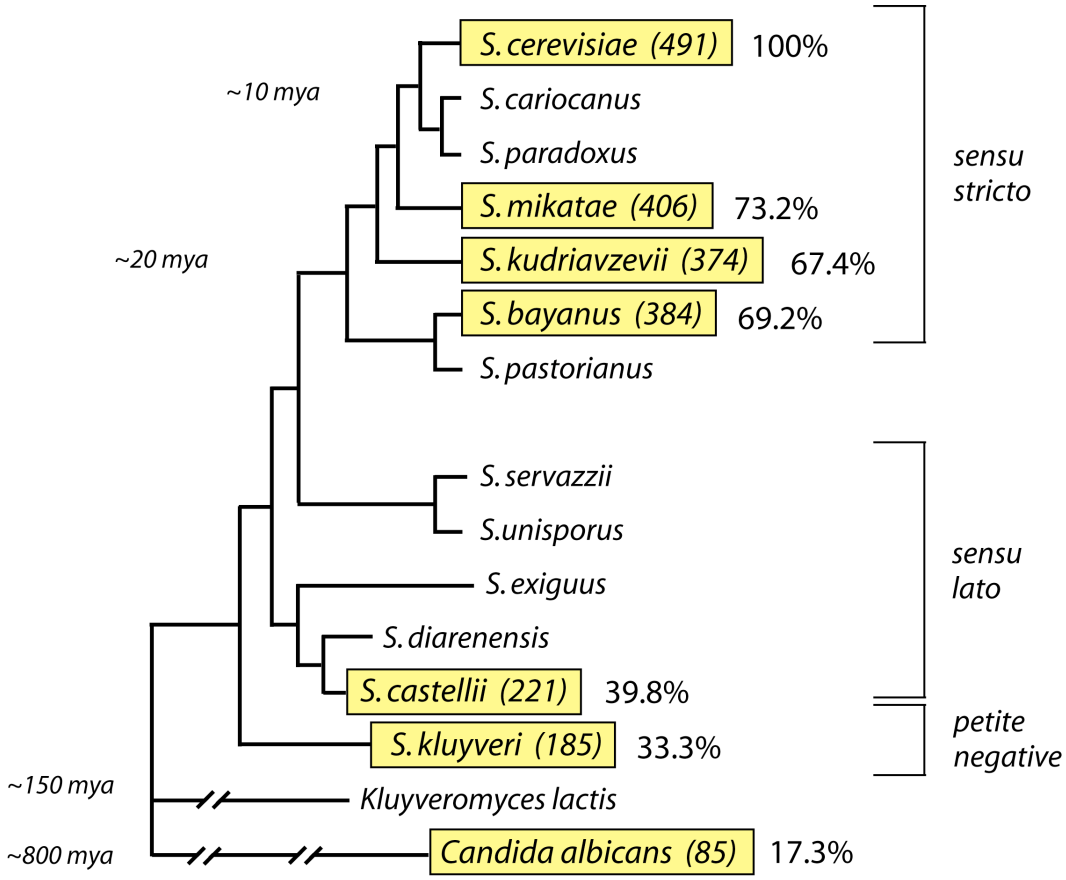

Figure 2.1: A phylogenic tree showing the relative evolutionary distances between the budding yeast species.

The relative number of *S. cerevisiae* candidates that have PKA sites conserved in the orthologous proteins present in each of the indicated budding yeast species is shown.

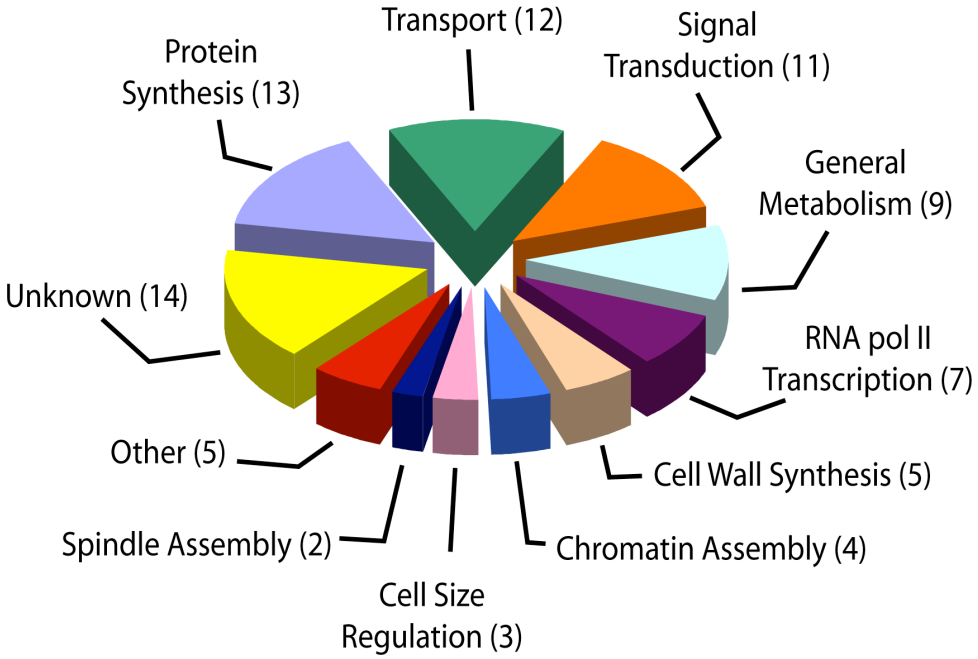

Figure 2.2: An evolutionary proteomics approach identified 85 potential substrates of PKA in *S. cerevisiae*.

This graph shows the number of candidates (in parentheses) that participate in the indicated cellular processes.

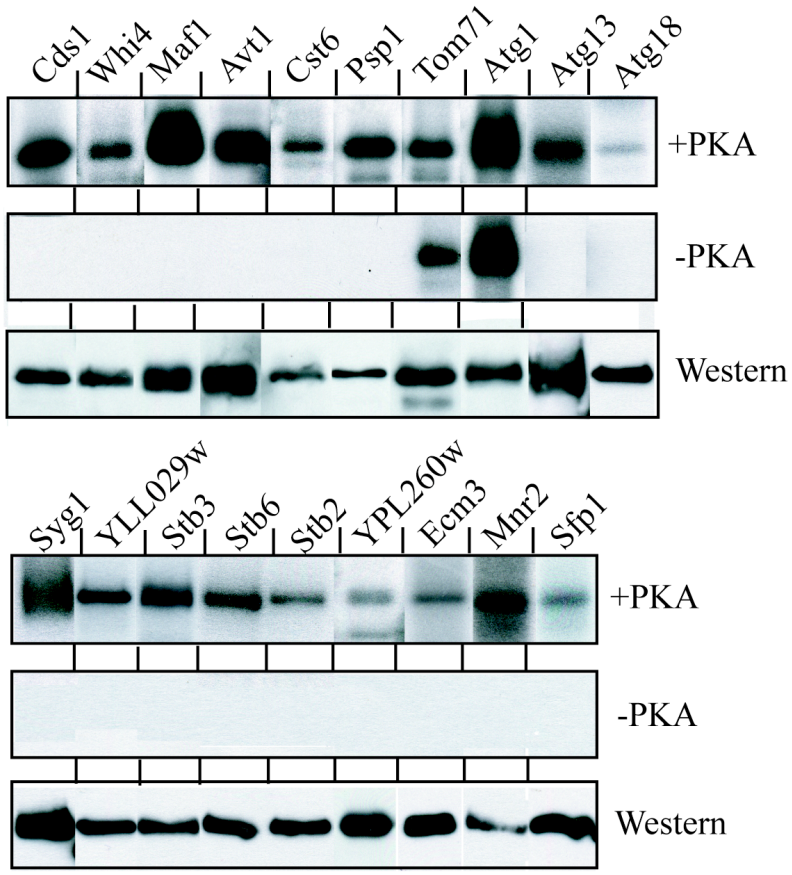

Figure 2.3: A phosphorylation analysis of the representative candidate substrates that had PKA sites conserved out to pathogenic yeast, *Candida albicans*.

The amount of label incorporated into a full-length GST fusion from [γ-^{32}P] ATP was assesses in an *in vitro* assay performed in the presence or absence of bovine PKA (see Materials and Methods).

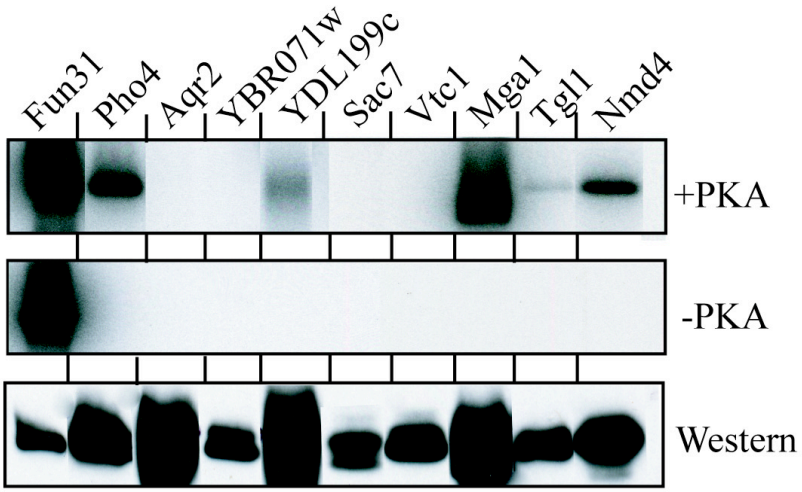

Figure 2.4: A phosphorylation analysis of proteins with PKA sites conserved amongs the *sensu stricto Saccharomyces* species (*S. mikatae, S. kudriavzevii,* and *S. bayanus*).

The amount of label incorporated into a full-length GST fusion from [γ-^{32}P] ATP was assesses in an *in vitro* assay performed in the presence or absence of bovine PKA (see Materials and Methods).

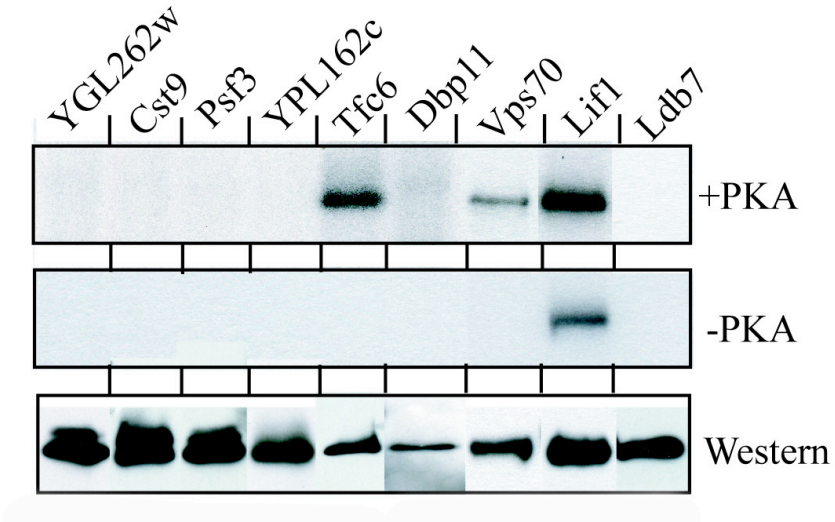

Figure 2.5: Proteins with PKA sites only in *S. cerevisiae*.

The amount of label incorporated into a full-length GST fusion from [γ-^{32}P] ATP was assesses in an *in vitro* assay performed in the presence or absence of bovine PKA (see Materials and Methods).

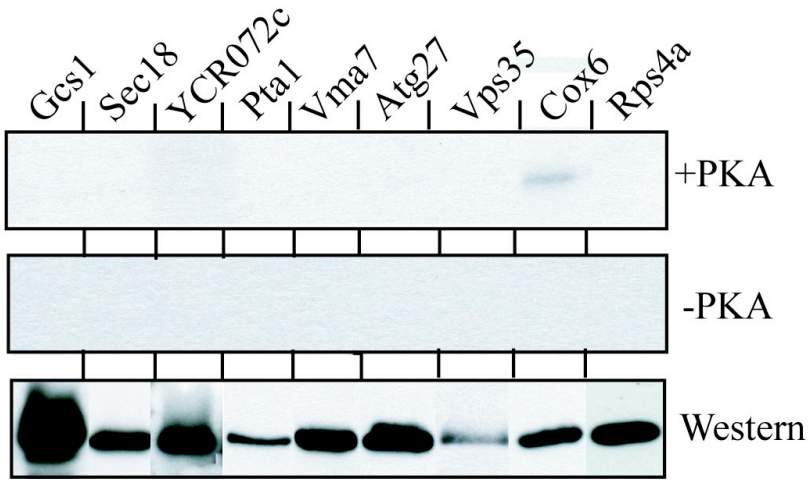

Figure 2.6: A phosphorylation analysis of representative proteins that did not contain PKA sites.

The amount of label incorporated into a full-length GST fusion from [γ-^{32}P] ATP was assesses in an *in vitro* assay performed in the presence or absence of bovine PKA (see Materials and Methods).

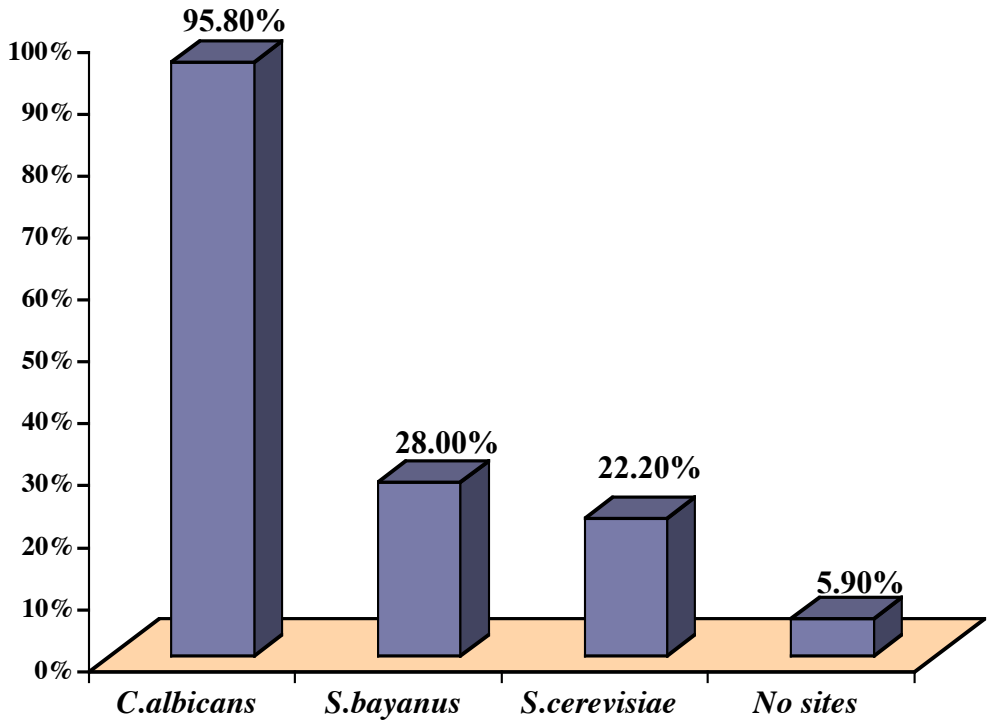

Figure 2.7: A summary graph indicating the frequency of PKA phosphorylation for each of the above groups of proteins.

CHAPTER 3

THE RAS/PKA SIGNALING PATHWAY REGULATES AN EARLY STEP OF THE AUTOPHAGY PROCESS IN *SACCHAROMYCES CEREVISIAE*

3.1 Introduction

The Ras/PKA signaling pathway plays an important role in regulating cell growth and proliferation as well as the entry into the resting state and the subsequent survival of stationary phase cells. The survival of these resting cells is also dependent upon autophagy, a membrane trafficking pathway that is induced upon nutrient deprivation. Autophagy is responsible for targeting bulk protein and other cytoplasmic constituents to the vacuolar compartment for their ultimate degradation. Previous work has implicated the Tor signal transduction pathway in the control of both autophagy and stationary phase entry in *S. cerevisiae*. The inactivation of this signaling pathway results in the induction of autophagy and in a growth arrest that resembles that associated with stationary phase cells [85, 139]. The Tor proteins are conserved serine/threonine-specific protein kinases that appear to coordinate the overall growth rate with nutrient availability in eukaryotic cells [140, 141]. Consistent with this proposition, a wide

variety of Tor pathway targets important for the control of cell growth have been identified in both yeast and other eukaryotic cells [140-143]. We are interested in characterizing the role of the Ras/PKA signaling pathway in the regulation of autophagy. Although the nature of this regulation has not been examined in detail, a recent study has found that increased levels of Ras/PKA signaling activity result in a decrease in the autophagic activity induced by the drug, rapamycin [144]. Rapamycin is a potent inhibitor of Tor activity *in vivo* and is often used to study the consequences of Tor pathway inactivation [54, 57]. Interestingly, three proteins important for autophagy, Atg1p, Atg13p and Atg18p, were all identified as potential substrates for PKA. Thus, these data were consistent with the Ras/PKA pathway having some regulatory role in the autophagy process.

In this study, we examined this regulation in detail and found that Ras/PKA signaling activity controls a relatively early step in the autophagy pathway. This Ras sensitive step likely precedes the formation of the autophagosome, as these transport intermediates were not formed in cells with elevated levels of Ras/PKA signaling activity. This signaling pathway also inhibited a vacuolar trafficking pathway that is related to autophagy but functions during the log phase of growth. This cytoplasm to vacuole targeting (Cvt) pathway shares many mechanistic features with autophagy and most of the proteins required for autophagy are also required for Cvt transport [77-79, 145]. In all, these data indicate that Ras/PKA activity is not regulating the switch between autophagy and the Cvt pathway *in vivo* and is instead controlling an activity that is required during the early stages of both of these membrane trafficking pathways.

3.2. Materials and Methods

3.2.1 Growth media: Standard *Escherichia coli* growth conditions and media were used throughout this study [116]. The yeast rich growth medium, YPAD, consists of 1% yeast extract, 2% Bacto-peptone, 50mg/l adenine-HCl, and 2% glucose. The yeast YM minimal growth medium consists of 0.67% yeast nitrogen base lacking amino acids, 2% glucose, and all of the growth supplements required for cell proliferation [117, 118]. The nitrogen starvation medium, SD-N, consists of 0.17% yeast nitrogen base lacking amino acids and ammonium sulfate and 2% glucose. Growth media reagents were from DIFCO.

3.2.2 Plasmid constructions: The construction of the *MET3-RAS2*^{val19} integrating plasmid, pPHY446, was described previously [40]. The integration of this allele was directed to the *RAS2* locus by digesting this plasmid with Xmn I before transformation of yeast cells. For the high copy number *TPK1* plasmid, pPHY2056, a 1.7 kb PCR fragment containing the *TPK1* gene was cloned into the pRS426 plasmid [117, 146]. A dominant negative allele of *RAS2*, known as *RAS2*^{ala22}, was generated by a site-directed mutagenesis of a *RAS2* plasmid, pJW82 that was kindly provided by Dr. Jasper Rine. The pJW82 plasmid has *RAS2* placed under the control of the inducible promoter from the *GAL1* gene. The site-directed mutagenesis was performed with the Transformer mutagenesis kit (Clontech) and resulted in the substitution of an alanine residue for the

glycine normally found at position 22 of Ras2p. The resulting plasmid was called pPHY2128. The effects of this dominant negative allele have been described previously [147, 148].

For the Ape1p over-expression experiments, the *APE1* coding sequences were placed under the control of the inducible promoter from the yeast *CUP1* gene; *CUP1* encodes a copper-binding metallothionein [149, 150]. Expression from the *CUP1* promoter was induced by the addition of 100 μM $CuSO_4$ to the growth medium.

3.2.3 Yeast strain construction and growth conditions: The strains used in this study are listed in Table 3.1. Unless otherwise noted, strains were from our lab collections or were derived during the course of this work. Standard yeast genetic methods were used for the construction of all strains [118]. Strains carrying the *MET3-RAS2*^{val19} allele were grown in media containing 500 mM methionine to keep the *MET3* promoter in its repressed state. *RAS2*^{val19} expression was induced by transferring these cells to media that lack methionine [40, 151]. For the experiments with the dominant negative allele, *GAL1-RAS2*^{ala22}, a wild-type strain, TN125, was transformed with either pPHY2128 or a control vector, pRS416. These strains were grown to mid-log phase in YM-glucose minimal medium and were transferred to YM medium containing 5% galactose and 2% raffinose. Culture samples were collected at the indicated time intervals and alkaline phosphatase assays were performed to assess autophagy activity, as described below.

3.2.4 Alkaline phosphatase-based autophagy assays: Autophagy levels were measured with an alkaline phosphatase-based assay that has been described previously [152]. In general, transferring cells to a medium that lacks a nitrogen source, SD-N, induced autophagy. Alkaline phosphatase levels were typically determined for cells that had been incubated in SD-N medium for 0 and 15 hrs at 30^{O}C. The increase in alkaline phosphatase activity that was observed following the period of starvation was a direct measure of the autophagy activity present in those cells. For the assays, the cells were re-suspended in 200 µl of the assay buffer (250 mM Tris-SO_4, pH 9.4 - 10 mM $MgSO_4$ – 10 µM $ZnSO_4$) and disrupted by vortexing with glass beads. The cell lysates were clarified by centrifugation at 10,000 x g for 5 min and 50 µl of the resulting protein extract was added to a tube containing 500 µl of assay buffer and 50 µl of a 1 mg/ml solution of p-nitrophenylphosphate (pNPP). This reaction mix was incubated for 30 min at 35^{O}C and the reaction was then stopped by the addition of 500 ml of 2 M glycine-NaOH, pH 11. The absorbance at 405nm of the resulting solution was then measured. One unit of activity is defined as the release of 1 mmol of p-nitrophenyl/min/mg of protein. The protein concentrations in the cell extracts were determined with a bicinchoninic acid (BCA) protein assay kit (Sigma). For the rapamycin experiments, autophagy assays were performed on cells that had been treated with 0.2 µg/ml rapamycin for 0, 1 or 3 h at 30^{O}C.

3.2.5 Western immunoblotting and immunoprecipitations: For the Western immunoblots, protein extracts were prepared by a glass-beading method described previously [153, 154]. The resulting protein extracts were separated on SDS-polyacrylamide gels and transferred to nitrocellulose membranes (Hybond ECL, Amersham Biosciences, Inc.) at 4^{O}C. The membranes were then probed with either anti-Ape1p [155], anti-Pgk1p or anti-Apg13p antisera and the immunoreactive proteins were detected with anti-rabbit IgG (Amersham Biosciences, Inc.) used at a dilution of 1:3000. The Supersignal chemiluminescent substrate (Pierce) was subsequently used to illuminate the reactive bands. The carboxypeptidase Y (CPY) immunoprecipitation experiments were performed as described [153, 156].

3.2.6 Electron microscopy: The *pep4*Δ (TVY1) and *vam3*^{ts} (TDY2) strains carrying either a control vector or an integrated version of the *MET3-RAS2*^{val19} construct were grown overnight in YM-glucose medium containing 500 μM methionine. The cells were washed twice with water and transferred to an YM-glucose medium lacking methionine for 4 h to induce *RAS2*^{val19} expression. The cells were then washed twice with water and transferred to SD-N for 3 h. All incubations for the *pep4*Δ strains were performed at 30^{O}C. For the *vam3*^{ts} strains, the incubations in YM-glucose media were performed at 26^{O}C, whereas the incubations in the SD-N medium were done at 37^{O}C to inactivate the temperature-sensitive Vam3p present. Cells were fixed with permanganate, dehydrated with acetone, and embedded with Spurr's resin as described

[157]. Seventy nm sections were mounted on nickel grids, and stained with uranyl acetate and lead citrate to be finally imaged with a Philips CM-100 transmission electron microscope.

3.3 Results

3.3.1 Elevated levels of Ras/PKA signaling activity inhibited autophagy.

We were interested in examining the role of the Ras/PKA pathway in the regulation of autophagy. For our initial experiments, autophagy activity was measured with an assay that analyzes the vacuolar delivery of a cytoplasmic variant of the Pho8p alkaline phosphatase following a shift to conditions of nitrogen starvation [152]. The wild-type Pho8p is synthesized as an inactive zymogen that is delivered to the vacuolar compartment via the traditional secretory pathway [158, 159]. Upon arrival in the vacuole lumen, this zymogen is activated by a proteolytic cleavage that removes a C-terminal propeptide [158]. The cells used for the autophagy assay express a Pho8p variant, Pho8pΔ60p, that lacks a transmembrane domain that functions as an internal signal sequence and normally directs this protein into the secretory pathway [152, 158]. As a result, Pho8pΔ60p is found in the cytosol in its inactive, precursor form. Following the induction of autophagy, this protein is packaged into autophagosomes and delivered to the vacuole where it can be activated [74, 158]. Previous work has indicated that the levels of alkaline phosphatase activity present are proportional to the amount of autophagy occurring in these cells [74, 158].

The Pho8pΔ60p assay was used to examine the effect that elevated levels of Ras/PKA signaling activity would have on the autophagy process. To increase Ras/PKA activity, a dominant hyperactive allele of *RAS2*, known as $RAS2^{val19}$, was introduced into the assay strain. The presence of this allele results in constitutively

elevated levels of Ras/PKA signaling activity [2, 7]. For most of the experiments in this study, we used an inducible version of this allele where *RAS2*^{val19} was placed under the control of the promoter from the yeast *MET3* gene [40, 160, 161]. This promoter is repressed when cells are grown in media containing methionine and induced when methionine is absent [40, 151]. For the autophagy assays, alkaline phosphatase activity was assessed after 0 and 15 h of nitrogen starvation. The level of autophagy was indicated by the difference in enzymatic activity found at these two time-points [152].

We found that the presence of *RAS2*^{val19} resulted in an essentially complete block in autophagy (Figure 3.1A). The defect observed in the *RAS2*^{val19} cells was comparable to that seen in mutants lacking proteins essential for autophagy, like *atg1*Δ and *atg13*Δ (Figure 3.1A) [83, 84]. Since the yeast Ras proteins have multiple effectors [2, 115, 162, 163], we tested whether this inhibition of autophagy was due to the cAMP/PKA effector pathway. For this analysis, a high copy plasmid containing the *TPK1* gene was introduced into the assay strain. *TPK1* encodes one of the three PKA catalytic subunits in *S. cerevisiae* and its over-expression results in high levels of PKA activity [5]. We found that the presence of this *TPK1* plasmid also resulted in a severe inhibition of autophagy (Figure 3.1B). Thus, constitutively elevated levels of Ras/PKA activity prevented the induction of autophagy that normally occurs upon nitrogen starvation.

We also tested whether the presence of the *RAS2*^{val19} allele would inhibit the autophagy induced by rapamycin, a drug that inhibits Tor activity *in vivo* [54, 142]. For these experiments, cells were treated with rapamycin for either 0, 1 or 3 h and the levels of autophagy activity were assessed with the alkaline phosphatase-based assay system. In wild-type cells, treatment with rapamycin resulted in a significant increase in autophagy activity at both time-points (Figure 3.2). In contrast, no increase in autophagy activity was observed in the *RAS2*^{val19} mutant cells (Figure 3.2). This block to

autophagy was essentially as complete as that observed in the *atg1*Δ and *atg13*Δ mutants. Thus, the Ras/PKA signaling pathway inhibited the autophagy activity induced by either nitrogen starvation or the rapamycin-mediated inactivation of the Tor pathway.

3.3.2 Inactivation of the Ras/PKA pathway led to an induction of autophagic activity.

The above data indicated that the Ras/PKA signaling pathway negatively regulates autophagy. The experiments in this section tested whether the inactivation of this pathway would be sufficient to induce autophagy even under growth conditions that normally inhibit this process. For these experiments, we used a dominant negative allele of *RAS2*, known as $RAS2^{ala22}$ [148, 149, 164]. This allele encodes an altered Ras2p that binds tightly to Cdc25p, the guanine nucleotide release factor that promotes the formation of the active Ras2p-GTP complex [148, 149]. The overexpression of this altered protein prevents Cdc25p from activating the normal Ras2p (and Ras1p) and results in a loss of Ras/PKA signaling activity and in a stationary phase-like growth arrest [148]. We used a construct where the $RAS2^{ala22}$ coding sequences were placed under the control of the promoter from the *GAL1* gene. This construct provides high levels of expression only in media that contain galactose as the primary carbon source [165]. The basic experiment therefore involved measuring autophagy levels during growth in galactose media. As expected, we found that cells containing the *GAL1-*$RAS2^{ala22}$ construct exhibited a growth defect in galactose media (Figure 3.3A). For these experiments, cells were grown to mid-log phase in YM-glucose medium and then transferred to an YM medium that contained 5% galactose. Transfer to this galactose medium resulted in a slower growth rate, and ultimate growth arrest, of those cells containing the *GAL1-*$RAS2^{ala22}$ hybrid gene. An essentially complete arrest was observed

after 36 to 40 hrs of growth in the galactose medium (Figure 3.3A). Consistent with previous work [148], we found that the majority of cells in the growth-arrested cultures were unbudded and thus were likely in the G_1/G_0 phase of the cell cycle (data not shown). Shortly after this arrest, an induction in autophagy activity was observed specifically in those cells that were expressing the dominant negative Ras2p (Figure 3.3B). The autophagy levels present were comparable to those observed in wild-type cells after nitrogen starvation. In contrast, no significant increase in autophagy activity was observed at these times in the wild-type control cells (Figure 3.3B). Therefore, these data are consistent with the proposition that the inactivation of the Ras/PKA pathway is sufficient to induce autophagy activity in *S. cerevisiae*.

3.3.3 Elevated levels of Ras/PKA signaling activity inhibited the formation of both autophagosomes and autophagic bodies.

The above work indicated that Ras/PKA signaling activity regulated autophagy but did not identify the step that might be affected. We therefore turned to electron microscopy (EM) in an attempt to gain more insight into the nature of this Ras-mediated block to autophagy. The primary advantage of this EM analysis is that it can provide qualitative information that can supplement the quantitative data provided by the alkaline phosphatase-based assays [82, 166]. For these experiments, the *MET3-RAS2*^{val19} allele was introduced into the *pep4*Δ and *vam3*^{ts} mutant strains. *PEP4* encodes a vacuolar protease that is required for the degradation of autophagic bodies [167, 168]. *VAM3,* on the other hand, encodes a t-SNARE protein that is required for the fusion of the autophagosome with the vacuolar membrane [169]. Therefore, following the induction of autophagy, *pep4*Δ mutants accumulate autophagic bodies within the

vacuole, and *vam3* mutants accumulate autophagosomes in the cytoplasm [67, 169] (Fig. 3). The basic experimental approach was to test whether elevated levels of Ras/PKA signaling activity would have any effect on the accumulation of these autophagy pathway intermediates. To induce *RAS2*^{val19} expression, the cells carring the *MET3-RAS2*^{val19} allele were grown in YM medium lacking methionine for 4 h and then transferred to SD-N starvation medium for 3 h. The *vam3*^{ts} allele encodes a temperature-sensitive protein that is functional at 26^{O}C but not at 37^{O}C [169]. Therefore, for the *vam3*^{ts} experiments, the strains were grown at 26^{O}C in the YM media and incubated at 37^{O}C in the SD-N starvation medium. For the *pep4*Δ strains, all incubations were performed at 30^{O}C. After starvation, the *pep4*Δ and *vam3*^{ts} cells were processed for the EM analysis as described in the Materials and Methods. We found that the presence of the *RAS2*^{val19} allele blocked the formation of both the autophagic bodies in the *pep4*Δ mutant and the autophagosomes in the *vam3*^{ts} strain (Figure 3.4). No obvious autophagy-related structures were identifiable in either mutant strain when the *RAS2*^{val19} allele was being expressed. In contrast, both of these intermediate structures were readily observed in the relevant control strains (Figure 3.4). These studies therefore indicate that the Ras/PKA pathway must be inhibiting an early step in the autophagy pathway, a step that precedes (or is required for) the formation of the autophagosome.

3.3.4 The presence of the *RAS2*^{Val19} allele inhibited the Cvt- and starvation-dependent processing of the vacuolar aminopeptidase Ape1p.

The Cvt pathway is responsible for delivering a subset of vacuolar proteins, including the aminopeptidase Ape1p [170, 171]. Cvt transport occurs constitutively in growing cells and represents an alternative delivery route to the traditional secretory pathway [155, 170]. Cvt transport and autophagy appear to be mechanistically similar as most of the proteins required for one process are also required for the other [77, 79]. In addition, the same type of double membrane vesicle is observed during both processes, the autophagosome during autophagy and the Cvt vesicle during Cvt transport [172, 173]. Therefore, we were interested in testing whether the Ras/PKA pathway also regulated Cvt transport. Ape1p is the best-characterized cargo of the Cvt pathway [172]. It is synthesized as an inactive precursor (prApe1p) that is activated by a proteolytic cleavage that occurs in the vacuole [155]. This processing of prApe1p serves as a marker for vacuolar delivery and is the standard assay used to measure Cvt transport activity. Therefore, to assess the effect of Ras/PKA activity on this pathway, we analyzed Ape1p processing in cells that expressed the *RAS2*^{val19} allele. For these experiments, we prepared protein extracts from log phase cultures of wild-type and *RAS2*^{val19} cells and performed an immunoblot with an antibody specific for Ape1p to monitor prApe1p processing. Surprisingly, we found that Ape1p was not present in the extracts from the *RAS2*^{val19} cells (Figure 3.5A). A whole genome expression analysis indicated that this absence of Ape1p was likely due, at least in part, to a defect in RNA polymerase II transcription (our unpublished data). These microarray experiments indicated that *APE1* mRNA levels were down 3- to 4-fold in *RAS2*^{Val19} cells relative to an isogenic wild-type control strain. Interestingly, the mRNA level of a gene encoding a second protein carried by the Cvt pathway, *AMS1*, was also down ~2-fold in the

RAS2^{val19} cells. *AMS1* encodes the vacuolar hydrolase, α-mannosidase I [174]. Therefore, Ras/PKA signaling activity was apparently inhibiting the expression of the only two known cargo proteins for the Cvt pathway.

To analyze Cvt activity, the *APE1* gene was placed under the control of the promoter from the *CUP1* gene (see Materials and Methods). *CUP1* encodes a copper-binding metallothionein and expression from the *CUP1* promoter is induced by the presence of copper in the growth medium [149, 150]. Cells containing this *CUP1-APE1* construct exhibited Ape1p levels in YM-glucose media that were just slightly higher than those normally provided by the wild-type *APE1* promoter (data not shown; see Figure 3.5B). More importantly, the expression levels from this promoter were not significantly influenced by Ras/PKA signaling activity (Figure 3.5B). Thus, this construct was used to assess Cvt activity in wild-type and *RAS2*^{val19} strains by analyzing Ape1p processing as described above.

We found that the presence of *RAS2*^{val19} resulted in an almost complete block in prApe1p maturation; essentially all of the Ape1p found in the *RAS2*^{val19} strain was in its precursor form (Figure 3.5B). In contrast, ~50% of the Ape1p detected in the wild-type strain was migrating as the mature vacuolar form (Figure 3.5B). The incomplete processing observed in this latter strain was likely due to the saturation of the Cvt targeting system brought on by the slightly higher expression provided by the *CUP1* promoter elements (see below). It is important to point out that the presence of *RAS2*^{val19} did not inhibit the vacuolar delivery and subsequent processing of CPY (Figure 3.5C). CPY is a vacuolar hydrolase that is synthesized as an inactive zymogen and delivered via the traditional secretory pathway [159, 168]. The processing of both CPY and Ape1p to their mature forms occurs in the vacuole and is dependent upon proteinase A activity; proteinase A is the product of the *PEP4* gene [155, 175, 176]. Thus, these data

suggest that the vacuole in *RAS2*^{val19} cells is competent for prApe1p processing and that the prApe1p maturation defects observed here were likely due to a block in the delivery of this precursor to the vacuolar compartment. In all, these data indicated that, in addition to autophagy, the Ras/PKA signaling pathway is also negatively regulating the Cvt targeting pathway in *S. cerevisiae*.

Previous work has shown that the over-expression of Ape1p results in the saturation of the Cvt system and in the accumulation of prApe1p in the cytosol [77, 155]. However, upon transfer to a starvation medium, this prApe1p is efficiently processed to its mature form suggesting that this precursor can be delivered to the vacuole via the autophagy pathway [77]. The availability of the *CUP1-APE1* construct thus provided an additional method for evaluating the effect of Ras/PKA signaling activity on autophagy. For cells containing the *CUP1-APE1* construct, the presence of copper in the growth medium resulted in Ape1p levels that were ~40-fold higher than those seen in wild-type cells (data not shown). This level of over-production saturated the Cvt system and resulted in >95% of the Ape1p being present in its precursor form in growing cells (Figure 3.6). However, this prApe1p was efficiently processed upon transfer to the SD-N starvation medium; complete processing typically required ~4 h of incubation at 30^{O}C (Figure 3.6). Consistent with previous work suggesting that this processing was mediated by the autophagy pathway, we found that the maturation of the over-expressed prApe1p required the activities of the *ATG1* and *ATG13* genes (Figure 3.6). The presence of the *RAS2*^{val19} allele also resulted in an almost complete block to

this starvation-induced processing of prApe1p (Figure 3.6). Thus, elevated levels of Ras/PKA signaling activity inhibited the vacuolar delivery of Ape1p by both the Cvt and autophagy transport pathways.

3.3.5 The presence of *RAS2^{val19}* did not block the rapamycin-induced dephosphorylation of Atg13p.

Previous work has shown that Atg13p is highly phosphorylated in log phase cells and that most of the added phosphate is removed following either nitrogen starvation or treatment with rapamycin [81, 85]. Interestingly, the hyperphosphorylated forms of Atg13p migrate more slowly in SDS-polyacylamide gels and can be easily distinguished from the more rapidly migrating, hypophosphorylated species found in starved or rapamycin-treated cells [81, 85] (see Figure 3.7). Although this phosphorylation of Atg13p is clearly dependent upon Tor activity, it is not yet known whether the Tor proteins directly phosphorylate Atg13p or regulate the activity of a second protein kinase. Therefore, we tested whether PKA might be responsible for this hyperphosphorylation of Atg13p. Specifically, we asked whether the presence of constitutively elevated levels of Ras/PKA signaling activity would prevent the dephosphorylation of Atg13p that normally occurs upon rapamycin treatment. For this experiment, protein extracts were prepared from wild-type and *RAS2^{val19}* cells that had been treated with rapamycin for 0 or 60 min. The extent of Atg13p phosphorylation was then assessed by examining the relative mobility of this protein on SDS-polyacrylamide gels. We found that the presence of the *RAS2^{val19}* allele had no significant effect on the mobility of the Atg13p present in either log phase or rapamycin treated cells (Figure 3.7). Thus, PKA does not appear to be responsible for the hyperphosphorylated forms of Atg13p that are observed in log phase cells.

3.4 Discussion

This study examined the effects that the Ras/PKA signaling pathway had on autophagy in the budding yeast, *S. cerevisiae*. All of the work presented here is consistent with the Ras/PKA pathway having a negative role in the control of this transport process. For example, elevated levels of Ras/PKA signaling activity, like those associated with the hyperactive $RAS2^{val19}$ allele, were found to result in an essentially complete block to the autophagy process. The autophagy levels present in RAS^{val19}cells were as low as those found in mutants, like *atg1*Δ, that were previously characterized as having a nonfunctional autophagy pathway. In addition, the mutational inactivation of the Ras/PKA pathway led to an induction of autophagy activity even in relatively rich growth media that normally inhibit this transport process. The autophagy levels observed in these latter studies were comparable to those found in nitrogen-starved cells. Thus, the presence of Ras/PKA signaling activity appears to be both necessary and sufficient for the inhibition of the autophagy process. Signaling through this pathway is required to keep the autophagy pathway inactive in growing cells and an absence of Ras/PKA activity is apparently necessary for the induction of autophagy that is normally associated with nutrient deprivation. This type of a regulatory role would be consistent with a recently proposed model suggesting that the Ras/PKA pathway is a key component of a growth checkpoint mechanism that functions in *S. cerevisiae* cells to ensure that the overall metabolic rate is balanced with the available nutrient supply [115].

The precise step of the autophagy pathway affected by Ras/PKA signaling activity is not yet known, but the EM analysis conducted here suggests that this signaling pathway is targeting a relatively early event in this transport process. In particular, we found that the presence of the *RAS2*^{val19} allele blocked the accumulation of autophagosomes that normally occurs in the cytosol of *vam3*^{ts} mutants [169]. Thus, the Ras/PKA pathway appears to be regulating an event that is required either before, or during, the formation of the autophagosome (Figure 3.8). Possible targets of this regulation would include components of the PAS, the structure that is the site of autophagosome formation in *S. cerevisiae* cells [87, 88], or the membrane source for the sequestering vesicles. Finally, it is important to point out that these results do not preclude the possibility that a later step of the autophagy pathway might also be controlled by this signaling pathway. Further work will be necessary to identify this early-acting target and any other steps of the autophagy process that might be regulated by Ras/PKA signaling activity.

Further insight into the role of Ras/PKA signaling activity was provided by our analysis of Cvt transport. The Cvt pathway is mechanistically similar to autophagy but generally operates under a different set of nutritional conditions [77, 78, 145]. Whereas autophagy is induced by nutrient deprivation, Cvt transport is responsible for the delivery of proteins to the vacuole in growing cells. Therefore, these transport pathways could represent two mutually exclusive options and Ras/PKA activity could have been acting as a switch to promote transport through the Cvt route. However, our data clearly

argue against such a role for this signaling pathway as elevated Ras/PKA activity also inhibited Cvt transport. Instead, these observations suggest that the Ras/PKA pathway is regulating a process that is required during the early stages of both Cvt transport and autophagy. Finally, it is interesting to note that elevated levels of Ras/PKA activity not only inhibited Cvt transport, but also resulted in a decrease in the expression of the only two known Cvt cargo proteins, Ape1p and Ams1p. Thus, the Ras/PKA pathway appears to be able to down-regulate traffic through the Cvt pathway at multiple levels.

The data in this chapter indicate that autophagy in *S. cerevisiae* is regulated by the activities of both the Ras/PKA and Tor signaling pathways. An important question that remains concerns the manner in which this control is coordinated. Our data, and that from a recent study, suggest that the Ras proteins are functioning either (i) independently, or (ii) downstream of the Tor signaling pathway with respect to autophagy [144] (Figure 3.9). This assertion follows from the observation that the constitutively-activated $RAS2^{val19}$ allele blocks the induction of autophagy normally associated with the inactivation of the Tor pathway. These data effectively rule out the possibility that the Ras proteins are functioning upstream of the Tor pathway. These same two possibilities have been suggested by studies examining other processes that are controlled by these signaling pathways [63, 115]. In addition, a recent study of the Rim15p kinase has suggested that the Ras and Tor pathways function independently to control aspects of Rim15p function [177]. Rim15p is a protein kinase that is required for the acquisition of stationary phase properties [37, 38]. In contrast, a second study

has suggested that the Ras proteins function downstream of the Tor signaling pathway [144]. This study examined the effects of these pathways on a variety of processes known to be controlled by Tor signaling activity, including protein translation and RNA polymerase I and III transcription. However, the authors of this latter study acknowledge that their data could also be explained by a model proposing that the Ras and Tor pathways act independently on the targets under study [144]. Therefore, the precise relationship of these two pathways to one another is not yet clear. A definitive order could be imposed if we had a constitutively active allele of one of the *TOR* genes; this allele would be essentially the *TOR* equivalent of the $RAS2^{val19}$ allele. This type of an allele would allow us to test whether the activation of the Tor pathway would be able to suppress phenotypes associated with a loss of Ras/PKA signaling activity. In lieu of such a reagent, additional work would appear to be required before we can definitely assign a relative order to these signaling pathways.

In summary, the data presented here suggest that the Ras/PKA pathway regulates an early step of both the autophagy and Cvt transport processes. Clearly, one of the main priorities of our future work will be the identification of the PKA substrate that is responsible for the regulatory effects observed in this study.

Strain	Genotype	Alias	Reference
TN125	*MAT a ade2 his3 leu2 lys2 trp1 ura3 pho8::pho8Δ60*	PHY2801	[85]
TDY2	*MAT a his3-Δ200 leu2-3,112 lys2-80 trp1-Δ901 ura3-52 suc2-Δ9 vam3Δ::LEU2 p(vam3^{ts})*	PHY3671	[169]
YYK126	*MAT a ade2 his3 leu2 lys2 trp1 ura3 pho8::pho8Δ60 atg1Δ::LEU2*	PHY 2802	
YYK130	*MAT a ade2 his3 leu2 lys2 trp1 ura3 pho8::pho8Δ60 atg13Δ::TRP1*	PHY 2803	
PHY3513	TN125, *RAS2::HIS3-RAS2^{Val19}*		[178]
PHY3992	TVY1, *RAS2::HIS3-RAS2^{Val19}*		[178]
PHY3993	TVY2, *RAS2::HIS3-RAS2^{Val19}*		[178]
TVY1	*MAT* a *his3-Δ200 leu2-3,112 lys2-80 trp1-Δ901 ura3-52 suc2Δ pep4Δ::LEU2*	PHY3670	[179]

Table 3.1 Yeast strains used in this study

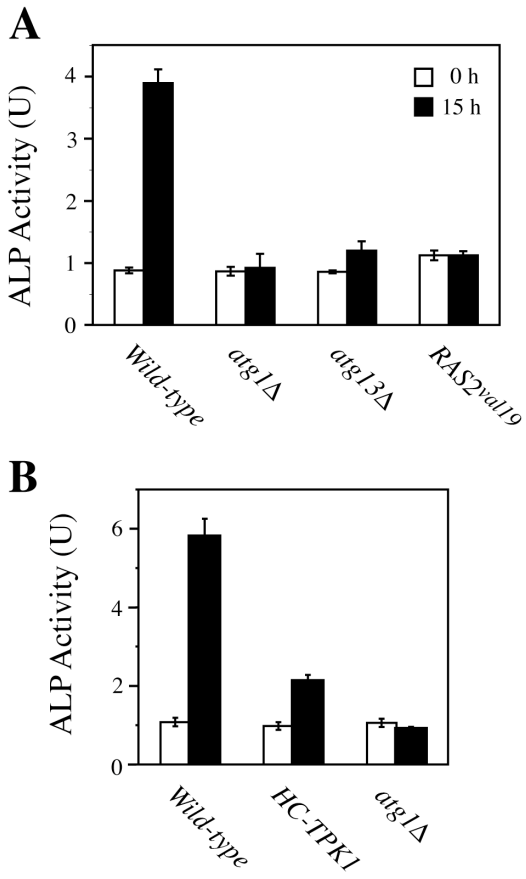

Figure 3.1: Elevated levels of Ras/PKA signaling activity inhibited autophagy.

A, *RAS2*^{val19} mutants exhibited very low levels of autophagy following nitrogen starvation. The indicated strains were grown to mid-log phase at 30 °C and transferred to the nitrogen starvation medium, SD-N, for 0 or 15 h. Autophagy levels were measured with the alkaline phosphatase-based assay as described under "Materials and Methods." The levels of autophagy induction are indicated by the relative increase in alkaline phosphatase activity induced by the nitrogen starvation.

B, Increased levels of PKA activity inhibited autophagy. A high level of PKA activity was achieved by introducing the high-copy plasmid, containing *TPK1* gene. Alkaline phosphatase assays were performed as described above in A.

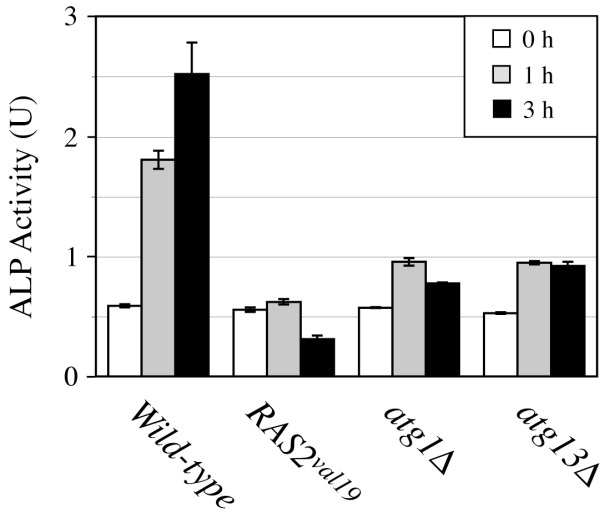

Figure 3.2: Elevated levels of Ras/PKA signaling activity inhibited the rapamycin-mediated induction of autophagy.

The indicated strains were grown to mid-log phase in YM-glucose medium and then treated with 0.2 μg/ml rapamycin for 0, 1, or 3 h. Autophagy levels were then assessed with the alkaline phosphatase-based assay system. The strains analyzed were those used in Figure 3.1 A.

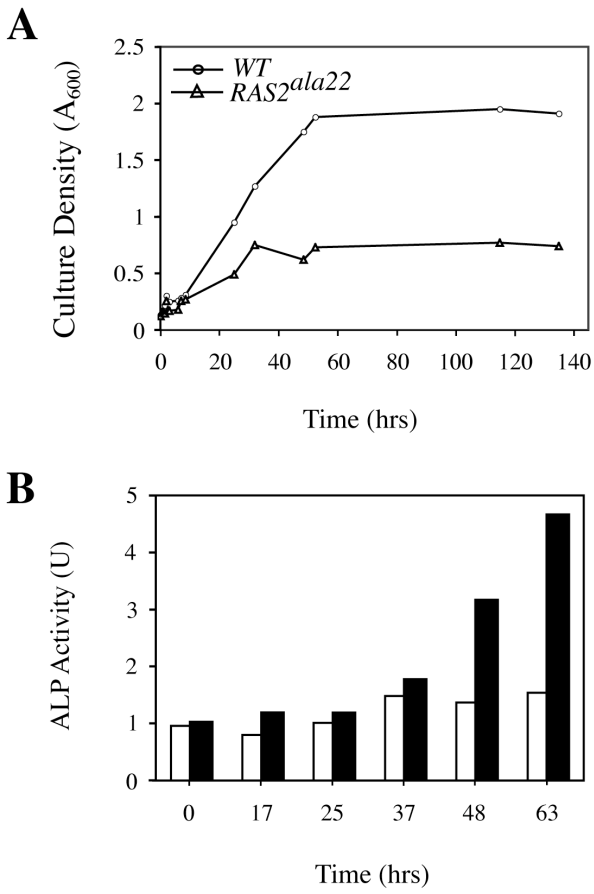

Figure 3.3: Inactivation of the Ras/PKA signaling pathway was sufficient to induce an autophagy response.

A, Expression of the dominant negative *RAS2*^{ala22} allele resulted in a growth arrest after transfer to the galactose medium. Wild-type cells (TN125) carrying either a control vector, pRS416 (circles), or the *GAL1-RAS2*^{ala22} plasmid, pPHY2128 (triangles), were grown to mid-log phase in YM-glucose medium and transferred to an YM medium that contained 5% galactose and 2% raffinose. The culture density was assessed by spectrophotometric measurement of the OD_{600} at the indicated times after the transfer to galactose-containing growth medium.
B, Inactivation of the Ras/PKA pathway resulted in an induction of autophagy. Autophagy levels were assessed with the alkaline phosphatase-based assay as described under "Materials and Methods."

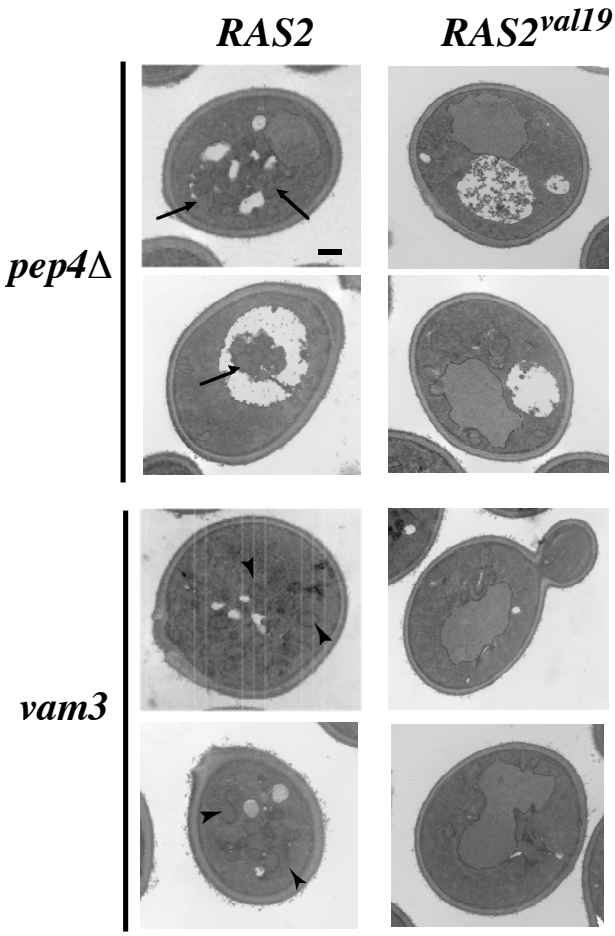

Figure 3.4: Elevated levels of Ras/PKA signaling activity blocked the formation of autophagy pathway intermediates.

The cells were collected and processed for electron microscopy as described in the "Materials and Methods." The arrows in the figure indicate the autophagic bodies accumulating in the vacuoles of the *pep4Δ* strain, and the arrowheads indicate the autophagosomes present in the cytoplasm of the *vam3*^{ts} strain. Note that both of these structures are absent in cells expressing the *RAS2*^{val19} allele. The bar indicates 1 μm.

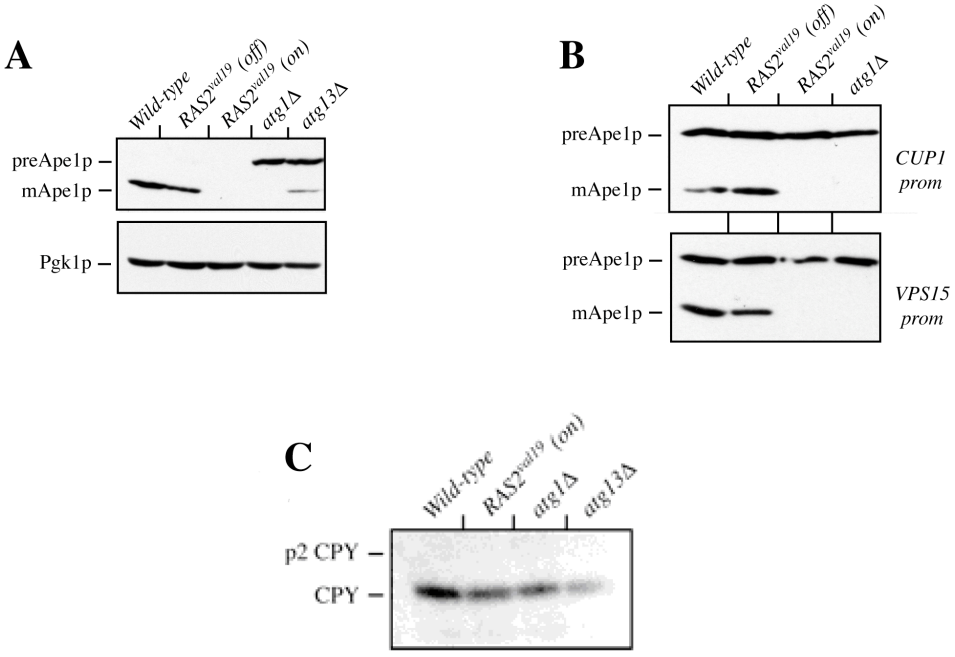

Figure 3.5: Elevated levels of Ras/PKA signaling activity inhibited the expression and processing of the Cvt cargo protein, Ape1p.

A, levels of Ape1p were greatly diminished in *RAS2*^{val19} mutants. Protein extracts were prepared from mid-log phase cultures of the indicated strains. The levels of Ape1p present in these extracts were assessed by a Western immunoblot with a polyclonal antiserum specific for this protein. The positions of the mature (Ape1p) and precursor (prApe1p) forms of Ape1p are shown. The relative levels of a cytoplasmic protein, Pgk1p, were assessed to ensure that similar amounts of protein were present in each sample.
B, elevated levels of Ras/PKA signaling activity inhibited the processing of Ape1p. Strains with the indicated genotypes were transformed with a plasmid containing the *CUP1-APE1* construct. These strains were grown to mid-log phase in YM-glucose medium and the relative levels of the two forms of Ape1p were assessed by Western immunoblotting. The strains analyzed were as in A.
C, elevated levels of Ras/PKA signaling activity did not affect the processing of the vacuolar hydrolase, CPY. The processing of CPY was assessed as described under "Materials and Methods".

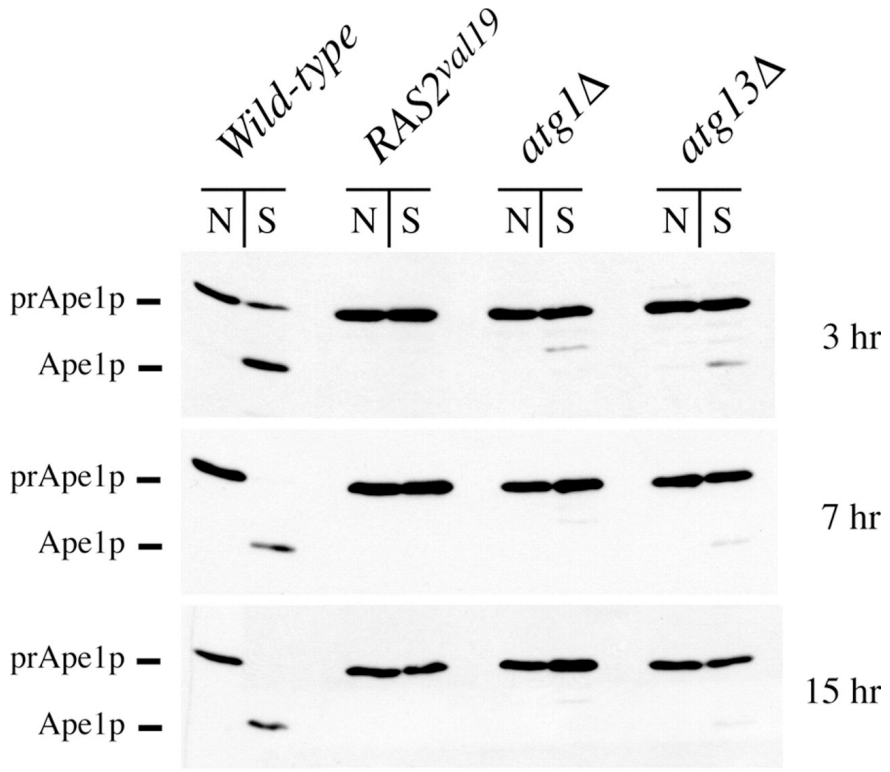

Figure 3.6: The starvation-induced processing of Ape1p was inhibited by Ras/PKA signaling activity.

The *CUP1-APE1* construct was introduced into strains with the indicated genotypes. The strains were grown to mid-log phase at 30 °C in an YM-glucose medium containing 100 μM CuSO4 to induce high-level expression from the *CUP1* promoter. The cultures were then transferred to the nitrogen starvation medium, SD-N, and incubated at 30 °C for 3, 7, or 15 h. Protein extracts were prepared from these cultures, and the relative levels of Ape1p processing were assessed with a Western immunoblot using a polyclonal antiserum specific for Ape1p. The relative processing at each starvation point (S) was compared with that observed in the mid-log phase, or nonstarved, control culture (N). The positions of the mature and precursor forms of Ape1p are shown.

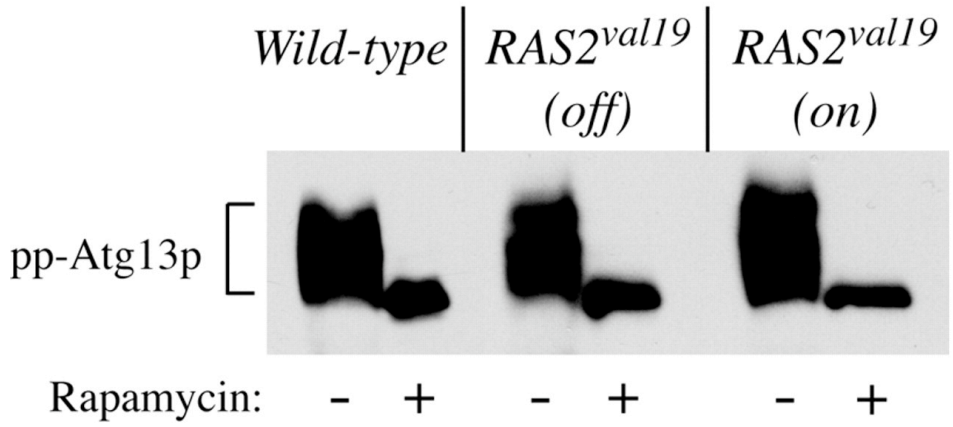

Figure 3.7: The rapamycin-induced dephosphorylation of Atg13p was not inhibited by the presence of the *RAS2*^{val19} allele.

Wild-type and *RAS2*^{val19} cells carrying a high-copy *ATG13* plasmid were grown to mid-log phase and then treated with 0.2 μg/ml rapamycin for 0 or 60 min. Protein extracts were prepared and the relative mobility of Atg13p in an SDS-polyacrylamide gel was assessed by Western immunoblotting with a polyclonal antiserum specific for Atg13p. The positions of the hyperphosphorylated forms of Atg13p found in log phase cells are indicated (pp-Atg13p).

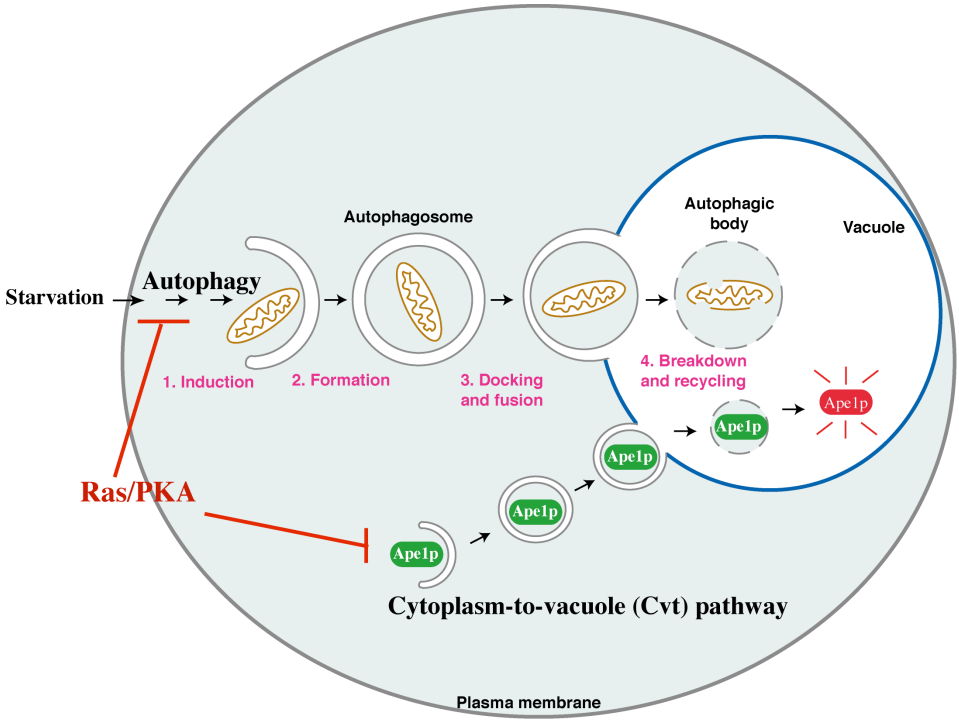

Figure 3.8: The Ras/PKA signaling pathway regulates an early event in autophagy, an event
that precedes the formation of the autophagosome.

The schematic shows a number of the steps involved in the autophagy and Cvt pathways and the relative position of the event most likely to be controlled by Ras/PKA signaling activity.

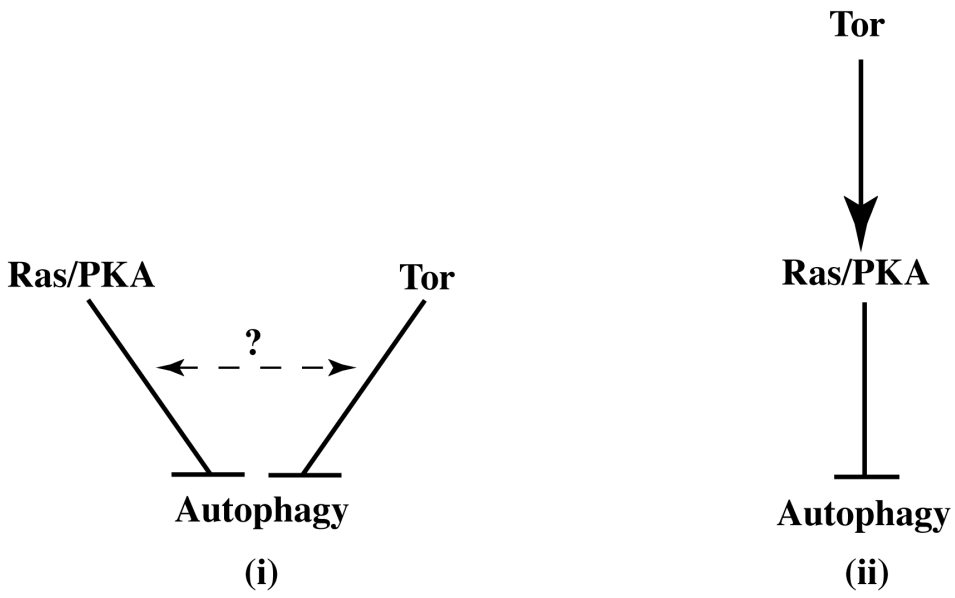

Figure 3.9: Schematic depicting the relative order of action of the Ras/PKA and Tor signaling pathways on the autophagy process.

The data presented here, and in other recent studies, are consistent with the Ras/PKA pathway acting either (i) independently or (ii) downstream of the Tor signaling pathway. See the text for further details.

CHAPTER 4

THE RAS/PKA SIGNALING PATHWAY REGULATES AN EARLY STEP OF THE AUTOPHAGY PROCESS IN *SACCHAROMYCES CEREVISIAE* VIA PHOSPHORYLATION OF ATG1P

4.1 Introduction.

Autophagy is a highly conserved, membrane trafficking pathway responsible for much of the protein and membrane turnover in eukaryotic cells [74, 180]. In *S. cerevisiae*, autophagy is regulated by both the TOR and Ras/PKA signaling pathways (see Chapter 3) [85, 178]. In nutrient-rich conditions, Atg13p is phosphorylated in a Tor-dependent manner and this phosphorylation has been shown prevent its association with Atg1p. Starvation or rapamycin treatment represses Tor activity, and results in the rapid dephosphorylation of Apg13p. This dephosphorylation of Atg13p results in the formation of a more stable Atg1p-Atg13p complex and in the induction of autophagicactivity [81, 82, 85]. Our previous data suggest that the Ras/PKA pathway also regulates an early step of the autophagy processes (see Chapter 3). However, the PKA substrates that are responsible for these regulatory effects are unknown. We have

used an evolutionary proteomic approach to identify a number of potential substrates for PKA in *S. cerevisiae*. Interestingly, this approach identified three proteins important for autophagy, Atg1p, Atg13p, and Atg18p (Figure 2.3 and 4.1), as potential PKA substrates. These proteins contain conserved PKA consensus sites and we have shown that they can be phosphorylated by PKA *in vitro*. As a proof of principle for this evolutionary proteomics approach, we tested whether one candidate in particular was regulated by PKA phosphorylation *in vivo*. For this analysis, we chose to examine the autophagy-related protein kinase, Atg1p.

Atg1p is a serine/threonine-specific protein kinase that is a key regulator of the initial induction stage of autophagy [74, 83]. There are Atg1p homologues present in all eukaryotic genomes examined, suggesting that the function of this protein is conserved. The precise mechanism by which Atg1p participates in the induction of autophagosome formation is unknown. However, it is known that *atg1Δ* mutants accumulate a precursor form of aminopeptidase I, Ape1p, implying a role for Atg1p in early events of autophagosome/Cvt vesicle formation [79]. Previous work has shown that Atg1p catalyzes an autophosphorylation and that relative Apg1p kinase activity is low in dividing cells [83]. Upon the induction of autophagy, an increase is observed in both the association of Atg1p with Atg13p and in Atg1p protein kinase activity [85]. In these latter studies, Atg1p protein kinase activity was assessed in an *in vitro* assay with myelin basic protein (MBP) as the substrate. The work presented here shows that the autophagy-related kinase, Atg1p, is phosphorylated and regulated by PKA phosphorylation *in vivo*.

4.2. Materials and Methods

4.2.1 Growth media: Standard *Escherichia coli* growth conditions and media were used throughout this study [116]. The yeast rich growth medium, YPAD, consists of 1% yeast extract, 2% Bacto-peptone, 50mg/ml adenine-HCl, and 2% glucose. The yeast YM minimal growth medium consists of 0.67% yeast nitrogen base lacking amino acids, 2% glucose, and all of the growth supplements required for cell proliferation [117, 118]. The nitrogen starvation medium, SD-N, consists of 0.17% yeast nitrogen base lacking amino acids and ammonium sulfate and 2% glucose. Growth media reagents were from DIFCO.

4.2.2 Plasmid constructions: The plasmids used in this study are listed in Table 4.1. Unless otherwise noted, plasmids were from our lab collections or were derived during the course of this work.

The site-directed mutagenesis of pPHY1115 and pPHY1249 generated the altered PKA sites of Atg1p. The site-directed mutagenesis was performed as described previously [181, 182]. The $S_{508}A$, $S_{515}A$, and $S_{508}A$ $S_{515}A$ versions of Atg1p encoded by pPHY1115 were generated to produce pPHY1191, pPHY1196, and pPHY1240, respectively. The $S_{508}A$, $S_{515}A$, and $S_{508}A$ $S_{515}A$ versions of Atg1p encoded by pPHY1249 were generated to produce pPHY1251, pPHY1253, and pPHY1255, respectively. The $K_{54}A$ substitution in Atg1p encoded by pPHY 1115 was generated to produce the kinase inactive version of Atg1p (pPHY 1886). The $K_{54}A$ $S_{508}A$, $K_{54}A$

$S_{515}A$, and $K_{54}A$ $S_{508}A$ $S_{515}A$ versions of Atg1p encoded by pPHY1115 were generated to produce pPHY1888, pPHY1892, and pPHY1893, respectively.

The Protein A-Atg1p fusion proteins were constructed with a vector, pPHY1044, that contains two repeats of the immunoglobulin binding region of Protein A from *Staphylococcus aureus* [153]. PCR fragments encoding Atg1p amino acid residues 345 - 559 or 500 - 523 were subcloned into pPHY1044 to produce pPHY1573 and pPHY1706, respectively. The templates for the PCR reactions were either the wild-type allele (pPHY1249) or mutant alleles that encoded alterations of the serine residues in the two potential PKA sites.

4.2.3 Yeast strain construction and growth conditions: The strains used in this study are listed in Table 4.2. Unless otherwise noted, strains were from our lab collections or were derived during the course of this work. Standard yeast genetic methods were used for the construction of all strains [118].

4.2.4 Alkaline phosphatase-based autophagy assays: Protein extract preparation and alkaline phosphatase based assay were carried out as described in Materials and Methods section 3.2.4.

4.2.5 Western immunoblotting and immunoprecipitations: For the Western immunoblots, protein extracts were prepared by a glass-beading method described previously [153, 154]. The resulting protein extracts were separated on SDS-

polyacrylamide gels and transferred to nitrocellulose membranes (Hybond ECL, Amersham Biosciences, Inc.) at 4^{O}C. The membranes were then probed with either anti-Ape1p [155]; anti-GFP (Cell Signaling Technology, Inc.) or anti-*c-myc* (Cell Signaling Technology, Inc.) antisera and the immunoreactive proteins were detected with anti-rabbit or anti-mouse IgG (Amersham Biosciences, Inc.) used at a dilution of 1:3000. The Supersignal chemiluminescent substrate (Pierce) was subsequently used to illuminate the reactive bands.

The plasmids encoding the protein A-Atg1p fusion proteins or protein A alone were introduced into the yeast strains PHY2300 or PHY1942 (Table 4.2). The immunoprecipitations assays were performed as described previously [153]. Briefly, the resulting protein extracts were clarified, added to 70 μl of a 50% solution of IgG-Sepharose beads (Amersham Biosciences, Inc.), and incubated for 2h at 4°C. The IgG-Sepharose beads were washed six times with 1 ml of ice-cold TBS buffer containing protease inhibitors. The beads were used in protein kinase A phosphorylation assays, or resuspended in 50 μl of IP sample buffer (125mM Tris-HCl, pH 6.8, 20% glycerol, 10% β-mercaptoethanol, and 6% SDS), and then heated to 100°C for 5 min to release the bound proteins. The latter proteins were separated in 12% SDS- PAGE gels, and the relative amount of protein A-Atg1p was assessed by Western immunoblotting with anti-rabbit IgG (Amersham Biosciences, Inc.). The Supersignal chemiluminescent substrate (Pierce) was subsequently used to illuminate the reactive bands.

For the immunoprecipitation of the *myc*-tagged Atg1 proteins, 1µl of the anti-myc antisera (Roche Applied Science) was added to the clarified protein extract and incubated for 2h at 4°C. Antibody-protein complex was precipitated by adding 70µl of Protein A-sepharose (Amersham Biosciences, Inc.) instead of the IgG-Sepharose beads, and the samples were processed as described above. The bound proteins were separated in 7.5% SDS-PAGE gels, and the relative amount of Atg1p was assessed by Western immunoblotting with an anti-*myc* antibody (Cell Signaling Technology, Inc.). The reactive bands were detected as described above.

4.2.6. Analysis of protein phosphorylation: The HA-tagged Atg1p and PrA-Atg1 fusion proteins were expressed in the protease-deficient strain, PHY1942. The PrA-Atg1p fusion proteins bound to IgG-sepharose, or the HA-tagged Atg1p isolated on anti-HA affinity matrix, were used as the substrates for these assays. In general, 30 µl of the bound protein substrates purified on these beads were washed once with 1 ml of ice-cold PKA phosphorylation buffer (50 mM potassium phosphate, pH 7.15, 5 mM NaF, 10 mM $MgCl_2$, 4.5 mM dithiothreitol, protease inhibitors and phosphatase inhibitors). For ^{32}P incorporation, the beads were incubated with 38µl of the phosphorylation buffer, 10 µCi of the γ-[^{32}P]-ATP, and 10 units of bovine PKA catalytic subunit (Sigma). The reaction mixtures were incubated at 25°C for 30 min, and then washed six times with 1 ml of PBS buffer (137 mM NaCl, 2.5 mM KCl, 10.4 mM Na_2HPO_4, 1.7 mM NaH_2PO_4, 1 mM dithiothreitol, protease inhibitors, and phosphatase inhibitors). The resulting proteins were eluted in 50 µl of IP sample

buffer, and separated in 12% or 7.5% SDS-PAGE gels. After electrophoresis, the gels were fixed in a solution of 50% methanol, 10% acetic acid, and 10% trichloroacetic acid for 20 min, rinsed with distilled water, dried, and then exposed to x-ray film. For the *in vivo* phosphorylation experiments, yeast cells were labeled with [^{32}P] inorganic orthophosphate and the labeled PrA-Atg1 was precipitated as described [43, 156].

4.2.7. Fluorescence microscopy: The CFP-Cvt9 and YFP-Atg1 fusions were under the control of the inducible promoter from the yeast *CUP1* gene [88]. Expression of these fusion proteins was induced by the addition of 100 μM $CuSO_4$ for 1 hr at 30^{O}C. For the starvation experiments, the cells were transferred to the nitrogen starvation medium, SD-N, containing 100 μM $CuSO_4$ for 1 hr at 30^{O}C. The GFP-Atg23 fusion protein has been described previously [91]. The samples were imaged with an Axioplan 2 Imaging E Mot microscope (Carl Zeiss, Inc.) equipped with a 100x Plan Neofluar objective (1031-172), filter sets 31044v2 (CFP), 41017 (Endow GFP), 41028 (YFP) (Chroma Technology Corp.) and a model C4742-95-12ERG CCD camera (Hamamatsu Photonics K.K). Image processing and contrast enhancement were performed with OpenLab 3 (Improvision Inc.) and Photoshop (Adobe Systems Inc.) software.

4.3. Results

4.3.1. The autophagy-related protein kinase, Atg1p, is a sustrate of PKA in vitro.

Atg1p has two conserved sequences, R-R-P-S_{508}-L and R-R-L-S_{515}-I, that conform to the PKA consensus site. We found that the full-length Atg1p was phosphorylated *in vitro* by PKA and that this phosphorylation required the presence of the serine residues in these consensus sites (Figure 4.2). The analysis of this phosphorylation was somewhat complicated by the fact that Atg1p is itself a protein kinase that can catalyze an autophosphorylation reaction [83, 85]. This autophosphorylation retards the mobility of this protein in SDS-PAGE gels, causing the wild-type Atg1p to run as a doublet (Figure 4.2; see below). Interestingly, label incorporation into the faster migrating form of Atg1 was completely dependent upon both PKA activity and the two consensus PKA sites (Figure 4.2). At the same time, the phosphorylation levels of the slower migrating band in the wild-type protein or proteins with altered PKA sites were comparable (Figure 4.2). Thus, these data are the first indication that the PKA phosphorylation of Atg1p did not regulate Atg1p kinase activity.

We also tested whether these two PKA consensus sites could be phosphorylated directly *in vitro*. We generated protein A fusions that encompass both of these PKA consensus sites from Atg1p. In our *in vitro* phosphorylation assay, the ability of bovine PKA to phosphorylate a Protein A (PrA)-Atg1p fusion protein was also dependent upon these same serine residues (Figure 4.3).

4.3.2. Atg1p was phosphorylated by PKA *in vivo*.

A phosphorylation analysis in wild-type and *pkaΔ* strains was performed to test whether Atg1p was phosphorylated by PKA *in vivo*. Indeed, PrA-Atg1p was heavily phosphorylated *in vivo* (Figure 4.4). However, we were unable to determine that this phosphorylation was dependent on the two aforementioned consensus PKA sites. The phosphorylation level of wild-type Atg1p was higher than that of Atg1p lacking the PKA target sites. Nevertheless, the altered Atg1p was still quite heavily phosphorylated. In addition, phosphorylation of Atg1p was detectable in both wild-type and *pkaΔ* strains. Perhaps this is because Atg1p was heavily phosphorylated on alternate serine and threonine residues by other kinases (Figure 4.4, A). To address this problem, we generated smaller PrA-Atg1p fusions. These fusion proteins encompass the Protein A immunoglobulin binding region fused in frame to residues 500 to 523 of Atg1p. We found that the *in vivo* phosphorylation of this PrA-Atg1 fusion required the presence of both the two PKA consensus sites and PKA (Figure 4.4, B). Thus, these data strongly indicate that Atg1 is likely a *bona fide* substrate of PKA in *S. cerevisiae* cells.

4.3.3. PKA phosphorylation does not influence Atg1p protein kinase activity.

Previous studies have indicated that Atg1p kinase activity is required for both autophagy and the Cvt [83, 85]. Indeed, these data suggested that a basal level of Atg1p kinase activity was essential for Cvt transport and that an elevated level of activity was required for autophagy [85]. Therefore, in nutrient-rich conditions, where PKA levels are relatively high, PKA might phosphorylate Atg1p and thereby inhibit its kinase activity. To test the effects of PKA phosphorylation on Atg1p function in the autophagy process, we measured the kinase activity of wild-type Atg1p or an Atg1p that lacks both PKA sites with an *in vitro* phosphorylation analysis. This *in vitro* protein kinase assay was performed in the presence of γ–[^{32}P]-ATP and employed the myelin basic protein (MBP) as a substrate for Atg1p. We found that the phosphorylation levels of MBP were comparable, perhaps because the PKA phosphorylation of Atg1p does not influence its protein kinase activity (Figure 4.5).

4.3.4. PKA phosphorylation regulates the subcellular localization of Atg1p.

Since PKA phosphorylation did not appear to influence Atg1p protein kinase activity (Figure 4.6), we examined the effect this modification had upon the subcellular localization of Atg1p. Previous work has shown that Atg1p is predominantly cytoplasmic in dividing cells and is recruited to a specialized site, known as the pre-autophagosomal structure (PAS), upon the induction of autophagy (Figure 4.6) [88,

91]. Most of the Atg proteins that have been identified are associated with this structure. For this experiment, we used a CFP-Cvt9p fusion protein as a marker for the PAS [91]. Cvt9p is essential for Cvt pathway activity and has been shown to physically interact with Atg1p. Furthermore, the localization of Cvt9p does not change significantly upon the change of the nutritional status of the cell. We found that YFP-Atg1p that lacked both PKA sites, YFP-Atg1-AAp, was constitutively localized to the PAS (Figure 4.6). Moreover, the introduction of a constitutively-active allele of *RAS2*, known as $RAS2^{val19}$, blocked YFP-Atg1p localization to the PAS in a manner that was dependent upon the two PKA sites in Atg1p (Figure 4.6). PKA phosphorylation therefore appears to negatively regulate the association of Atg1p with the PAS and thus may prevent Atg1p from reaching its normal substrates in the cell.

4.3.5. Redistribution of Atg23-GFP requires the Atg1p kinases activity at the PAS.

Atg23p is essential for the maturation of aminopeptidase I (Ape1p) via the Cvt pathway and it also plays an important role in efficient autophagosome formation during autophagy. Atg23p is a peripheral membrane protein that is found associated with subcellular punctate structures, only one of which co-localizes with the PAS [90, 92]. A recent study has investigated the relationship between these two Atg23p populations [91]. These data indicate that Atg23p cycles between the PAS and the dispersed punctate structures. In addition, this recycling of Atg23p from the PAS requires Atg1p kinase activity [91]. Therefore, the proper redistribution of Atg23p

requires that Atg1p be active and at the PAS. However, our data indicate that PKA phosphorylation negatively regulates the association of Atg1p with the PAS (Figure 4.6), and therefore suggests that Atg23p recycling would be defective in a *RAS2*^{val19} mutant. Consistent with this prediction, we found that the presence of the *RAS2*^{val19} allele had a similar effect on the localization of Atg23p as did mutations that disrupted Atg1p protein kinase activity (Figure 4.7) [91]. Both of these alterations result in the redistribution of Atg23p to the PAS and therefore suggest that PKA phosphorylation might influence Atg1p activity *in vivo*.

4.3.6. The elimination of the two PKA phosphorylation sites within Atg1p did not reverse the *RAS2*^{val19} repression of autophagy.

Our previous data showed that elevated levels of Ras/PKA signaling activity leads to complete inhibition of both autophagy and the Cvt pathway (Chapter 3; [178]). One way in which Ras/PKA signaling could regulate autophagy is by direct phosphorylation of Atg1p by PKA. The PKA-dependent phosphorylation of Atg1p blocks Atg1p localization to the PAS and therefore inhibits an early step of the autophagy and Cvt transport processes, autophagosome or Cvt-vesicle formation. Thus, the elimination of the target PKA sites might render Atg1p insensitive to the effects of Ras/PKA signaling and lead to constitutively high levels of autophagy. To test this possibility, a dominant hyperactive allele of *RAS2*, *RAS2*^{val19}, was introduced into a yeast strain that carries a plasmid encoding either wild-type Atg1p or an Atg1p

that lacks its two PKA phosphorylation sites. Autophagy activity was then measured with an assay that analyzes the vacuolar delivery of a cytoplasmic variant of the Pho8p alkaline phosphatase following a shift to conditions of nitrogen starvation (see Materials and Methods). To assess Cvt transport activity we analyzed Ape1p processing under nutrient-rich conditions in the same strain backgrounds. We found that the removal of these two PKA sites did not suppress the *RAS2*^{val19} inhibition of either autophagy (Figure 4.8) or the Cvt pathway (data not shown). One possible explanation for these observations is that Atg1p is not the only target of PKA relevant to the regulation of autophagy and the Cvt pathway. To examine this possibility, we are currently testing whether the loss of the PKA phosphorylation sites in Atg1p, Atg13p, and Atg18p will render autophagy and Cvt transport insensitive to the Ras/PKA pathway.

4.4. Discussion:

Previous studies have suggested that the Ras/PKA signaling pathway regulates autophagy and Cvt trafficking in the budding yeast, *S. cerevisiae* (Chapter 3; [178]. *In vivo* analyses of the Ras/ PKA signaling suggests that this pathway is targeting a relatively early event in both of these transport processes, an event that is needed for double-membrane vesicle biogenesis. However, the manner in which this regulation is achieved is still poorly understood. A major step forward to better understanding the Ras/PKA effect on autophagy was made through our evolutionary proteomics approach. This bioinformatics approach identified three potential targets of PKA that are important for the regulation of autophagy. These proteins, Atg1p, Atg13p and Atg18p, all contain putative PKA phosphorylation sites and all are phosphorylated by PKA in an *in vitro* kinase assay (see Figure 2.3). These observations therefore suggested that the Ras/PKA pathway might be regulating autophagic activity in yeast cells via the direct phosphorylation of these targets. In particular, the work presented here suggests that the Ras/PKA pathway is controlling the activity of the serine/threonine-specific protein kinase, Atg1p. Our data show that Atg1p is an efficient *in vitro* and *in vivo* substrate for PKA, and that this phosphorylation is dependent on the presence of both of the conserved PKA sites.

Previous work has shown that Atg1p kinase activity is highly elevated in cells grown under starvation conditions, and a kinase-negative *atg1* mutant (*atg1*^{K54A}) is defective in both autophagy and Cvt transport [85]. This work suggests an elevated level of Atg1p kinase activity is required for autophagy and that a basal Atg1p kinase

activity is essential for the Cvt pathway in growing cells. In addition, it has been shown that Tor proteins likely inhibit the Atg1p activation that occur upon the induction of autophagy. Our previous data suggest that the Ras proteins are functioning either independently, or downstream of the Tor signaling pathway with respect to autophagy. The constitutively activated *RAS2*^{val19} allele blocks the induction of autophagy normally associated with the inactivation of the Tor pathway (see Chapter 3, Figure 3.2). Thus, the Ras/PKA pathway could be also targeting the protein kinase activity Atg1p. However, the work presented here indicates that the PKA phosphorylation of Atg1p does not influence its protein kinase activity. Therefore, Ras/PKA signaling activity must influence the autophagy process through a different mechanism. One possible model of action was suggested by our analysis of the subcellular localization of Atg1p.

Previous studies have shown that most of the known Atg components are associated with the pre-autophagosomal structure (PAS), the potential site of Cvt vesicle and autophagosome formation [87, 88]. Atg1p is unique in this regard, as a fluorescence microscopy analysis has demonstrated that the association of Atg1p with the PAS is regulated by the nutritional state of the cell. In nutrient rich media, the cells are growing and Atg1p was found to be largely cytosolic (Figure 4.6) [88]. The YFP-Atg1p fusion was not found to be associated with any visible pre-vacuolar structures under those conditions. In contrast, a strong recruitment to the PAS was seen shortly after the cells were shifted to a nitrogen starvation medium, the same conditions that

lead to the activation of the Atg1p protein kinase activity and to the induction of autophagy (Figure 4.6) [88]. Interestingly, we found that an Atg1p that lacked both PKA sites, Atg1p-AA, was constitutively localized to the PAS (Figure 4.6). Furthermore, introduction of a constitutively-active allele of *RAS2*, *RAS2*^{val19}, blocked subcellular localization of the wild-type Atg1p, and at the same time had no effect on Atg1p-AA localization (Figure 4.6). These data suggest that Ras/PKA signaling may modulate an activity associated with the Atg1p by negatively regulating its subcellular localization. It has been previously shown that Atg1p physically interacts with Cvt9p, a PAS-associated protein required for Cvt transport but not autophagy [85, 86]. In addition, an autophagy-specific component, Atg17p, also has been shown to physically interact with Atg1p [85]. These results suggest that Atg1p, through its interaction with pathway specific components, may dictate the initiation of either Cvt or autophagy transport in response to specific upstream nutritional cues.

To test the physiological significance of the PKA phosphorylation of Atg1p, we examined the localization of a GFP-Atg23p fusion protein in wild-type and *RAS2*^{val19} mutant cells. Atg23p is a peripheral membrane protein that is essential for Cvt trafficking, but not for the autophagy process. Recent data have shown that Atg23p cycle between the PAS and a set of dispersed punctate structures, and that this redistribution is dependent on the activity of the Atg1p/Atg13p complex in nutrient rich conditions [91]. Mutations that compromise Atg1p protein kinase activity were found to result in the concentration of Atg23p at the PAS. We found that the presence of the *RAS2*^{val19} allele had a similar effect on the Atg23p localization (Figure 4.7). This result

suggests that the PKA phosphorylation of Atg1p might regulate the activity of Atg1p to phosphorylate its normal targets by controlling its association with the PAS.

A model proposing that Ras/PKA signaling negatively regulates aspects of Atg1p function can explain only some of the data generated to this point. However, this model does not account for the fact that cells containing the Atg1-AA variant are still sensitive to the *RAS2*^{val19} allele. Thus, even though Atg1p can no longer be phosphorylated by PKA, *RAS2*^{val19} is still able to inhibit both Cvt transport and autophagy. One explanation for these data is that Atg1p is not the only target of PKA involved in the regulation of autophagy. Two likely candidates, Atg13p and Atg18p were also identified by our evolutionary proteomic approach as potential targets of PKA. Therefore, our future efforts will be directed at testing whether the PKA phosphorylation of these latter Atg proteins is important for the normal control of autophagy in yeast cells.

Atg1p

```
S. cerevisiae      ---RRPSL---RRLSI---
S. kudriavzevii    ---RRPSL---RRLSI---
S. castellii       ---RRPSL---RRLSI---
S. kluyveri        ---RRPSL---RRLSI---
C. albicans        ---RRSSS---RRISL---
C. elegans         ---RRTTL---RRSTI---
```

Atg13p

```
S. cerevisiae      ---RRHSS---RRNSL---
S. bayanus         ---RRHSS---RRNSL---
S. kudriavzevii    ---RRHSS---RRNSL---
S. castellii       ---RRHSS---RRNSL---
S. kluyveri        ---RRHSS---RRNSI---
C. albicans        ---RRVSI---RRSSN---
```

Atg18p

```
S. cerevisiae      ---RRGTY---
S. bayanus         ---RRGTY---
S. kudriavzevii    ---RRGTY---
S. castellii       ---RRGTY---
S. kluyveri        ---RRGTY---
C. albicans        ---RRGTY---
C. elegans         ---RRGTV---
```

Figure 4.1: Potential PKA substrates in autophagy pathway.

A sequence alignment of Atg1p, Atg13p and Atg18p sequences from five different *Saccharomyces* species, *C. albicans*, and *C. elegans*. The general PKA consensus site is R_{-3}-R_{-2}-x_{-1}-S/T-B_{+1}, where "R" (highlighted blue) refers to conserved arginine residues, "x" to any amino acid, "B" (highlighted yellow) to a residue with a hydrophobic side chain, and the S/T to the serine or threonine residue that is the site of phosphate addition (highlighted yellow).

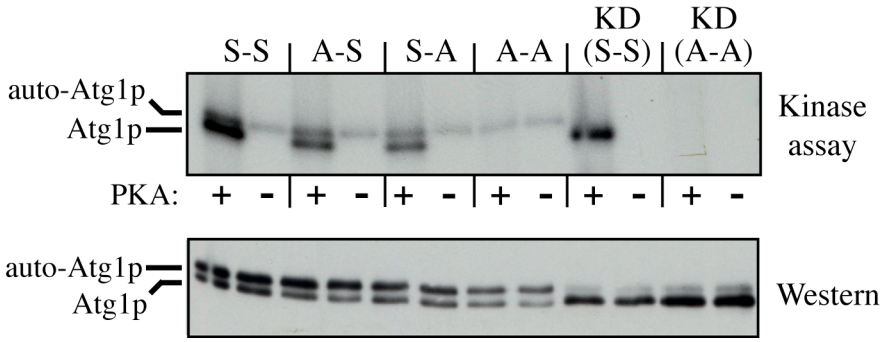

Figure 4.2: The full-length Atg1p is phosphorylated by PKA *in vitro*.

The full-length Atg1p was phosphorylated by bovine PKA *in vitro* at the serine residues within the two consensus PKA sites. KD, kinase-dead version of Atg1p. The residues at positions 508 and 515 with the two PKA sites are indicated. S, serine; A, alanine.

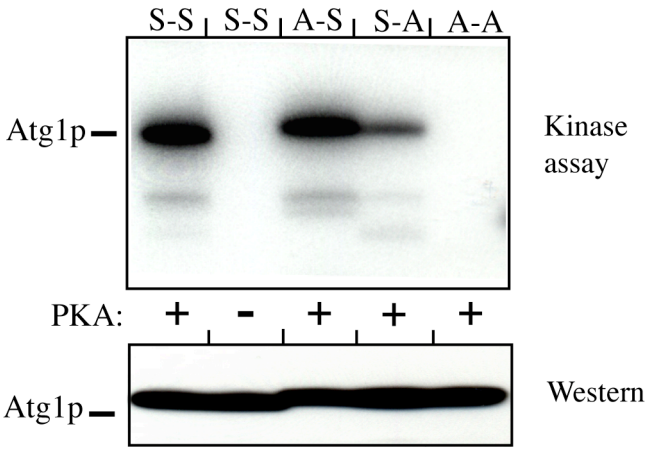

Figure 4.3: The *in vitro* phosphorylation of Atg1p fusion proteins.

The *in vitro* phosphorylation of PrA-Atg1p fusion proteins was dependent upon both bovine PKA activity and the two PKA consensus sites in Atg1p. These fusion proteins contained two repeats of the immunoglobulin binding region of Protein A from *Staphylococcus aureus* fused in frame to residues 345-559 of Atg1p.

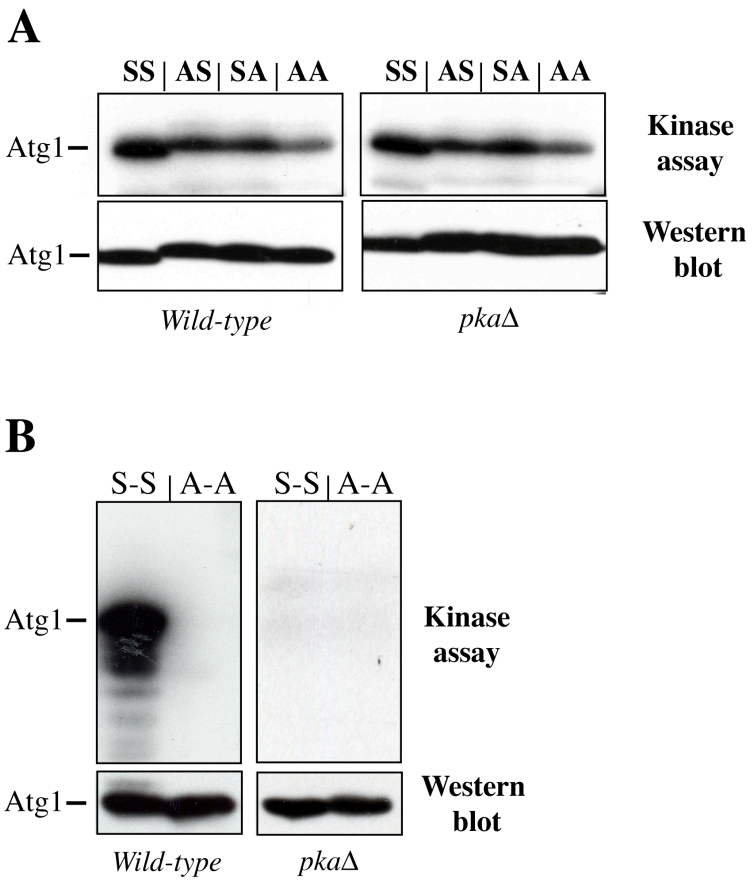

Figure 4.4: The *in vivo* phosphorylation of Atg1p fusion proteins.

For the *in vivo* experiments, yeast cultures were incubated with [^{32}P] orthophosphate and the amount of label incorporated into the PrA-Atg1p fusion proteins was assessed by autoradiography. The residues at positions 508 and 515 with the two PKA sites are indicated: S, serine; A, alanine. (A) The fusion proteins used in this study contained two repeats of the immunoglobulin binding region of Protein A form *S. aureus* fused in frame to residues 345-559 of Atg1p. (B) For this *in vivo* phosphorylation assay, Protein A was fused in frame to residues 500-523 of Atg1p.

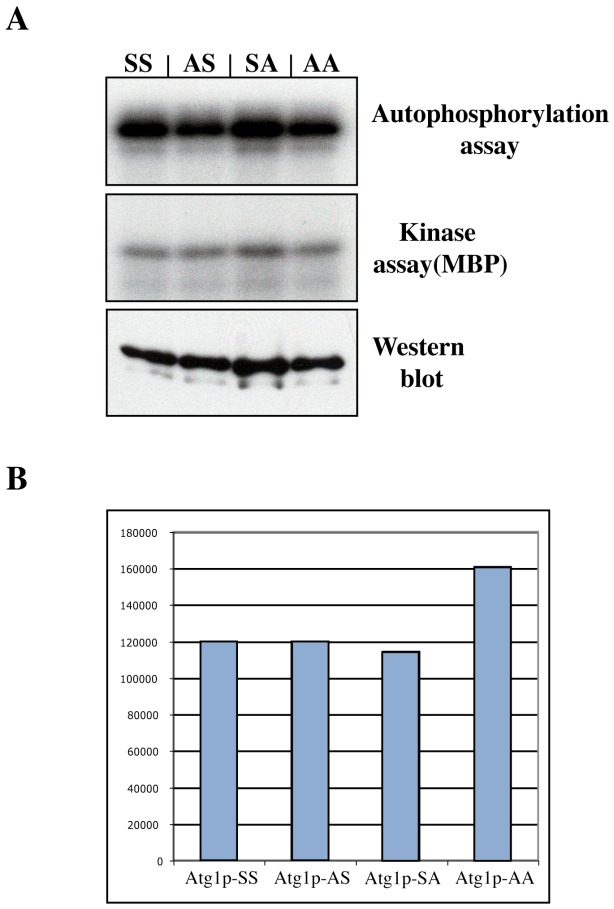

Figure 4.5: PKA phosphorylation of Atg1p does not influence its protein kinase activity.

(A) Alternations of the serine residues within the PKA sites did not influence the kinase activity of Atg1p in *in vitro* kinase assay. The indicated versions of Atg1p were precipitated and an *in vitro* phosphorylation reaction were performed as described in the Materials and Methods. The top panel is the autophosphorylation control and the bottom is the *in vitro* phosphosphorylation assay using the MBP protein as substrate. **(B)** The amount of ^{32}P incorporated into the MBP protein in an *in vitro* kinase assay was quantified by phosphoimage analysis. The Y-axis indicates quantification of the phosphorylation data in (A). The amount of label incorporated into MBP in terms of arbitrary phosphorimaging units.

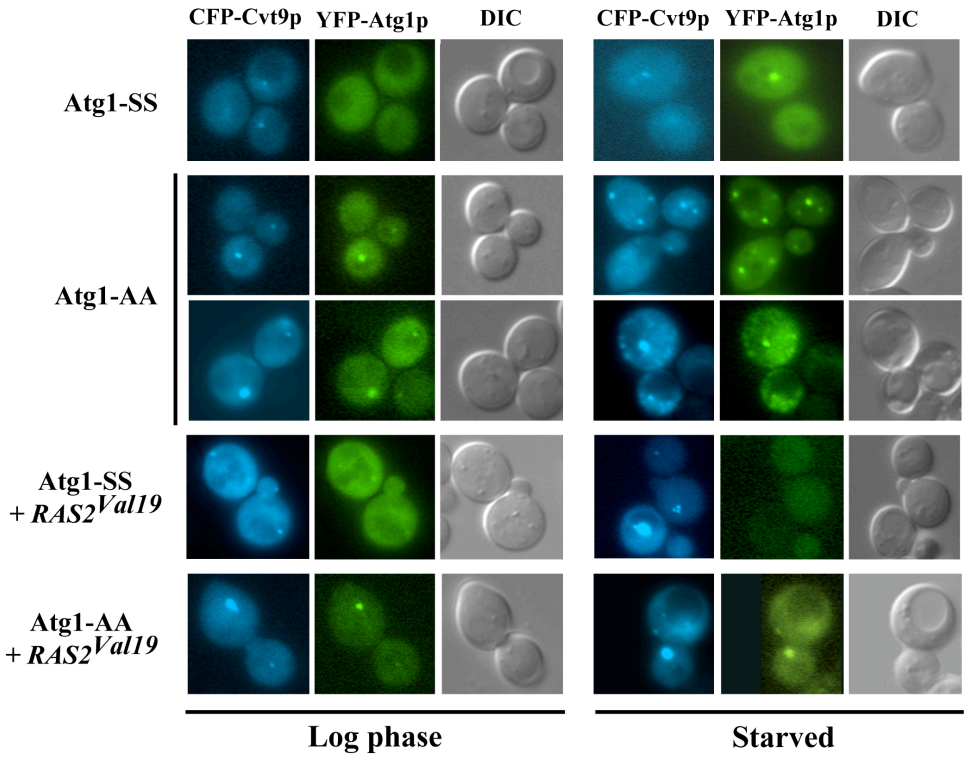

Figure 4.6: PKA phosphorylation regulates the association of Atg1p with the pre-autophagosomal structure (PAS).

A fluorescence microscopy analysis of the subcellular localization of a YFP-Atg1p fusion protein. The CFP-Cvt9p reporter is a marker for the PAS [88].

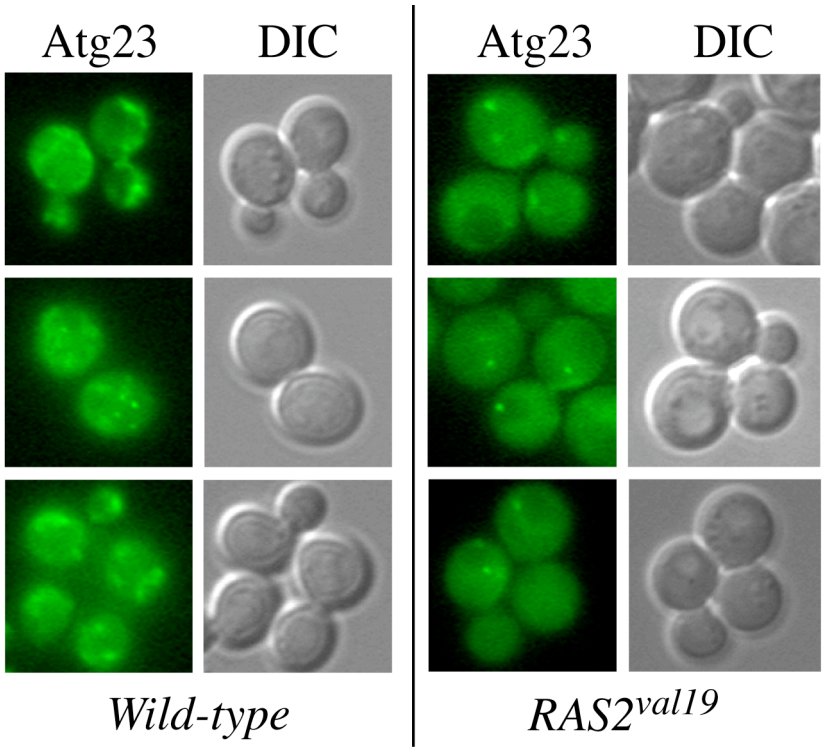

Figure 4.7: The presence of allele *RAS2*^{val19} influences the subcellular localization of Atg23p.

The presence of the *RAS2*^{val19} allele resulted in the redistribution of a GFP-Atg23p fusion protein from a number of punctate structures within the cell to the PAS. The identity of the punctate structures is not yet known.

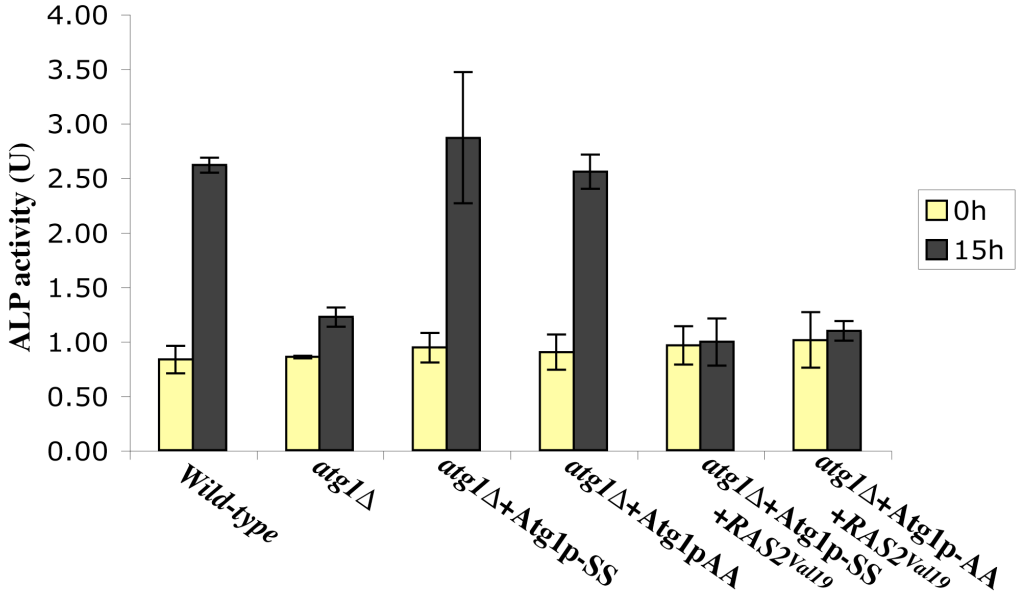

Figure 4.8: The elimination of the two PKA phosphorylation sites within Atg1p did not reverse the *RAS2*^{val19} inhibition of autophagy:

The levels of autophagy induction are indicated by the relative increase in alkaline phosphatase activity that is induced by the nitrogen starvation. Autophagy levels were measured with the alkaline phosphatase-based assay as described in the Materials and Methods.

CHAPTER 5

SUMMARY

This body of work demonstrates how a sequence-based, comparative approach can be used to identify the physiologically-relevant sequence motifs present in eukaryotic proteins. Although this study was focused on identifying substrates for a particular protein kinase, this approach should work for any type of sequence element in any type of a protein. The only prerequisite is that there must be genome sequence information available for an appropriate group of evolutionarily-related organisms. The potential power of such a comparative approach is exemplified by the fact that more potential PKA substrates were identified here than have been found by more traditional means in the past two decades. Although further work is obviously needed to show that these candidates are indeed regulated by PKA phosphorylation *in vivo*, this candidate pool already contains five proteins that have been shown to be substrates of PKA. Moreover, our data indicate that most of those candidates are *in vitro* substrates for PKA. Finally, it is important to point out that the proteins identified here are equally likely to be

substrates of PKA in *C. albicans*. This possibility is interesting in light of recent work indicating that PKA activity is important for virulence in this pathogenic yeast [124, 125].

Interestingly, three of these potential PKA substrates identified by this evolutionary proteomics approach are Atg proteins, Atg1p, Atg13p, and Atg18p. These three proteins are the key regulatory molecules in autophagy process. Autophagy is a membrane trafficking pathway that is induced upon nutrient deprivation and is responsible for targeting bulk protein and other cytoplasmic constituents to the vacuolar compartment for their ultimate degradation. In this study, we characterized the role of the Ras/PKA signaling pathway in the regulation of autophagy and found that Ras/PKA signaling activity controls a relatively early step in this transport pathway. This Ras sensitive step likely precedes the formation of the autophagosome, as these transport intermediates were not formed in cells with elevated levels of Ras/PKA signaling activity. Quantitative assays indicate that these increased levels of Ras/PKA signaling activity result in an essentially complete block to autophagy. This signaling pathway also inhibits a vacuolar trafficking pathway that is related to autophagy but functions during the log phase of growth. This cytoplasm to vacuole targeting (Cvt) pathway shares many mechanistic features with autophagy and most of the proteins required for autophagy are also required for Cvt transport [77-79, 145]. In all, our data indicate that Ras/PKA activity is not regulating the switch between autophagy and the Cvt pathway *in vivo* and is instead controlling an activity that is required during the early stages of both of these membrane trafficking pathways.

In *S. cerevisiae* autophagy is regulated by the activities of both the Ras/PKA and Tor signaling pathways. However, the manner in which this control is coordinated remains unsolved. Our data indicate that the constitutively-activated *RAS2*^{val19} allele blocks the induction of autophagy normally associated with the inactivation of the Tor pathway. These data effectively rule out the possibility that the Ras proteins are functioning upstream of the Tor pathway. Therefore, we propose that the Ras proteins are functioning either (i) independently, or (ii) downstream of the Tor signaling pathway with respect to autophagy [144, 178].

One protein thought to be at the heart of the regulatory switch between Cvt transport and autophagy, is the serine/threonine protein kinase Atg1p. The precise function of this protein in mediating this switch is unclear. Atg1p acts at the stage of double-membrane vesicle biogenesis in both autophagy and Cvt pathways [87]. It has been shown that the Tor kinase is an upstream regulator of the Atg1p activity, but downstream targets of Atg1p have not yet been identified. Moreover, there are two potential phosphorylation sites for PKA in Atg1p that fit the consensus R-R-x-S/T-B. Our data showed that PKA directly phosphorylates both PKA sites in Atg1p *in vitro*. In addition, we found that the *in vivo* phosphorylation of a particular Atg1p fusion protein required the presence of these two PKA consensus sites and PKA activity. Thus, these data suggest that Atg1p is a *bona fide* substrate for PKA in *S. cerevisiae* cells. Disruption of the phosphorylation sites within the PKA target responsible for the *RAS2*^{val19} inhibition of autophagy, should reverse the *RAS2*^{val19} effects on this process. However, the alteration of the PKA sites within Atg1p did not suppress the effects of *RAS2*^{val19} nor

result in the constitutive induction of autophagy. These results suggest that Atg1p is not the only target of PKA relevant to autophagy. We are currently examining this possibility by investigating whether or not PKA phosphorylation might also influence the *in vivo* activities of two other potential targets, Atg13p and Atg18p.

PKA phosphorylation of Atg1p does not influence instead protein kinase activity, but its negatively regulates the association with the pre-autophagosomal structure (PAS). As Atg1p has been shown to interact with several other components of the Atg machinery, this protein kinase might dictate the accurate progression of steps required for vesicle assembly. Thus, PKA phosphorylation might prevent Atg1p form reaching its downstream targets. One of the events that could be catalyzed by Atg1p is the retrieval of certain proteins by retrograde vesicular traffic prior to the completion of the autophagosome and their fusion with the vacuole. The decision of when this retrograde transport route is induced may ultimately determine the size of the double-membrane vesicle transport intermediate and these may be a primary determinant of the choice between the Cvt pathway and autophagy.

The data presented here demonstrate how a systematic comparative approaches can be used to identify functionally relevant sequences in eukaryotic proteins. This approach was used here to identify 45 candidate substrates of the cAMP-dependent protein kinase (PKA) in *Saccharomyces cerevisiae*. Moreover, as proof of principle, we established that one of these targets, the autophagy-related protein kinase, Atg1p, is regulated by PKA phosphorylation *in vivo*. These data therefore demonstrate the general potential this strategy has for determining the physiological relevance of any short sequence motif found in any type of protein.

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