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Mkh1, a Novel MAP Kinase Kinase Kinase in *Schizosaccharomyces pombe*

by

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ABSTRACT

A novel member of the MAPKKK family of protein kinases, *mkh1*$^+$, was isolated and characterized from *Schizosaccharomyces pombe*. *mkh1*$^-$ mutants exhibited defects in cell shape, cytokinesis, response to hyperosmolarity and cell cycle. Deletion of *mkh1*$^+$ did not alter cell growth or division under standard growth conditions. *mkh1*$^-$ cells exhibited a round cell shape, while overexpression of Mkh1 resulted in an elongated cell shape. Cell growth was inhibited by hyperosmotic conditions and resulted in a pseudohyphal phenotype. *mkh1*$^-$ cells required a longer time to reenter the cell cycle after prolonged stationary phase arrest. These phenotypes are very similar to cells lacking Pmk1/Spm1, a recently identified MAP kinase. The genetic evidence suggests that Pmk1/Spm1 acts downstream from Mkh1. There is evidence that Mkh1 and Pck2 act independently to maintain cell morphology and osmoresistance, but act in opposition to regulate pseudohyphal growth.

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LIST OF ABBREVIATIONS

MAP	mitogen-activated protein
MAPK	mitogen-activated protein kinase
DIC	differential contrast microscopy
aa	amino acid(s)
bp	base pair(s)
S. cerevisiae	*Saccharomyces cerevisiae*
S. pombe	*Schizosaccharomyces pombe*
kb	kilobase(s)
PCR	polymerase chain reaction
::	novel junction or insertion

CHAPTER ONE: LITERATURE REVIEW

Introduction:

All cells respond to extracellular and intracellular signals via a diverse number of mechanisms to control gene expression and regulate protein function. The proteins involved in the transduction of signals include ion channels, cell surface and intracellular receptors, GTP-binding proteins, protein kinases and phosphatases. Activation of these proteins ultimately results in regulation of transcription factors or other effector protein function.

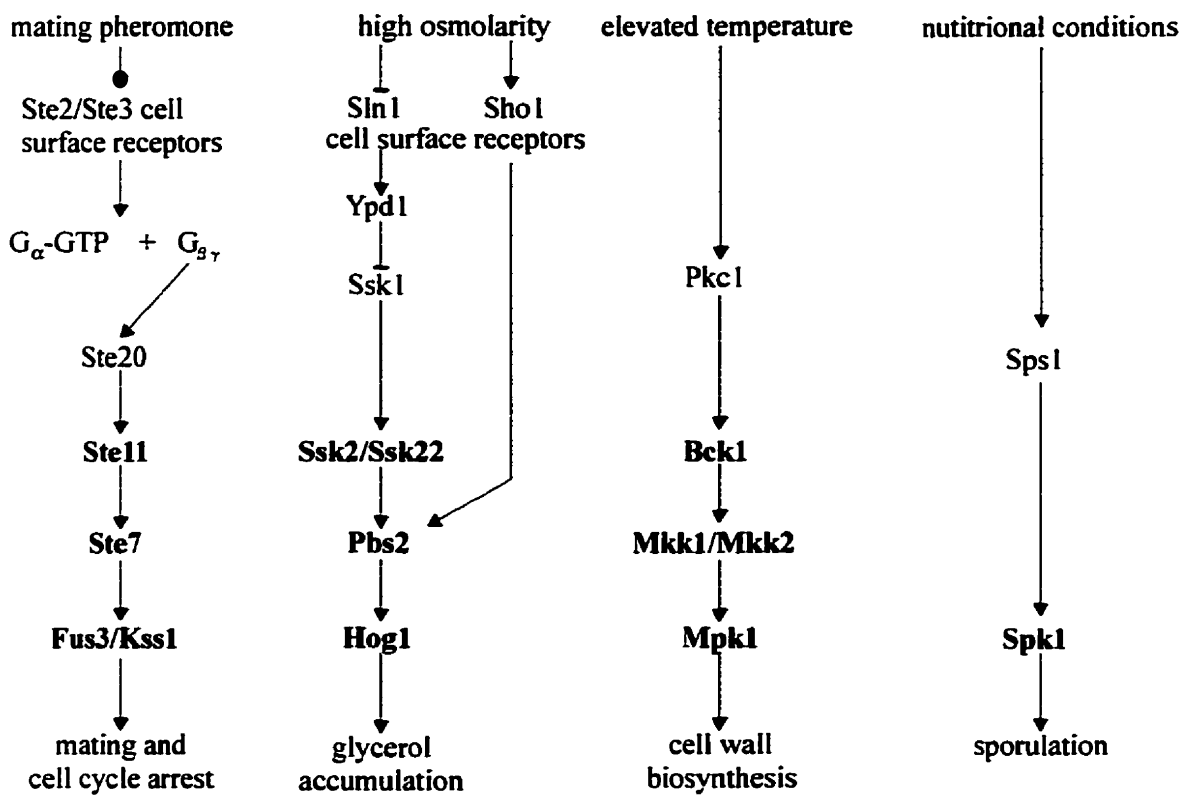
Mitogen-activated protein (MAP) kinase cascades represent a fundamental regulatory mechanisms that have been highly conserved among widely divergent eukaryotic organisms such as budding yeast, fission yeast and mammals. These cascades consist of a trio of sequentially acting protein kinases: MAP kinase kinase kinases (MAPKKKs), which phosphorylate and activate MAP kinase kinases (MAPKKs), which in turn phosphorylate and activate MAP kinases (MAPKs)(reviewed in Herskowitz, 1995; Seger and Krebs, 1995). These cascades are involved in mediating a variety of cellular responses including cell growth, differentiation, and responses to various stresses.

MAP kinase pathways in the budding yeast:

There are six known signalling responses that utilize MAPKs in the budding yeast *Saccharomyces cerevisiae,* four of which are shown in Figure 1.1. The pheromone response pathway is amongst the best understood of all eukaryotic signalling pathways and is often used as the model to which all MAPK cascades are compared. A haploid cell secretes one of two types of mating pheromone, either α factor (from *MAT*α cells) or **a** factor (from *MAT***a** cells). *MAT***a** cells express Ste2, the receptor for α factor. Ste3, the **a** factor receptor, is

Figure 1.1. Representative MAP kinase pathways in budding yeast. Bold text highlights the MAPKKK-MAPKK-MAPK cascade.

Saccharomyces cerevisiae MAPK cascades:



expressed in *MATα* cells. Binding of the pheromone to the receptor results in the dissociation of the G_α subunit from the $G_{\beta\gamma}$ subunits in the heterotrimeric G protein (reviewed in Marsh, et al., 1991). Free βγ subunits serve as positive regulators for downstream signalling (Leberer, et al., 1992a). The βγ subunits activate the Ste20 protein kinase by an unknown mechanism. Ste20, a serine threonine MAPKKK kinase, has been shown genetically to function upstream of the MAPKKK homologue Ste11 (Leberer, et al., 1992b) and has been shown to phosphorylate Ste11 *in vitro* (Wu, et al., 1995). Ste11 phosphorylates and activates the MAPKK homologue Ste7 (Neiman and Herskowitz, 1994). Ste7 then activates a pair of partially redundant MAPKs, Fus3 and Kss1 (Errede, et al., 1993). Fus3 and Kss1 can both activate the transcription factor, Ste12 (Elion, et al., 1993). Fus3, but not Kss1, can also phosphorylate and activate Far1 (Elion, et al., 1993), an inhibitor of cyclin-dependent kinases resulting in G1 arrest (Peter and Herskowitz, 1994). The activation of Ste12 and Far1 causes the cells to undergo a morphological change forming mating projections involved in cell-cell interaction and fusion (reviewed in Marsh, et al., 1991).

Ste5 is a protein that has no obvious enzymatic function, but, it can simultaneously bind Ste11, Ste7 and either Fus3 or Kss1. This has been shown by co-immunoprecipitation, co-sedimentation in glycerol gradients and yeast two hybrid data (Choi, et al., 1994; Marcus, et al., 1994; Printen and Sprague, 1994). The role of Ste5 may be to keep the MAPK complex together and thus minimize signal crosstalk between different MAPK cascades (Levin and Errede, 1995; Herskowitz, 1995). This theory is supported by the observation that a *ste7* gain-of-function variant that can function as the MAPKK in the Bck1 pathway has reduced activity when Ste5 is also expressed (Levin and Errede, 1995), suggesting that Ste5 is

sequestering Ste7. Using yeast two-hybrid and immunoprecipitation, it has been shown that during the pheromone response Gβ subunit associates with Ste5 (Whiteway, et al., 1995). It has already been shown that Ste20 does not appear to interact with Ste5 (Choi, et al., 1994) nor does it phosphorylate Ste5 *in vitro* (Wu, et al., 1995). Perhaps then, the Gβ association to Ste5 acts as a catalyst for the interaction between Ste20 and Ste11.

The HOG pathway, or high osmolarity glycerol response (Figure 1.1), is activated when cells are exposed to high osmolarity. Activation of this pathway results in the elevation of intracellular glycerol concentrations (Brewster, et al., 1993) and a reduction in membrane permeability in an effort to regain iso-osmotic conditions and avoid lysis (reviewed in Levin and Errede, 1995). *S. cerevisiae* can respond to low and high osmolarity with two osmosensors, Sln1 and Sho1 (Maeda et al., 1994; Maeda et al., 1995). The Sln1, Ypd1 and Ssk1 proteins are homologous to prokaryotic phosphorelay signal transduction systems, where Sln1 is the sensor molecule, Ypd1 is the relay molecule and Ssk1 is the response regulator molecule (Posas, et al., 1996). Ssk1 can activate the MAPKKKs Ssk2 and Ssk22. Ssk2 and Ssk22 appear to be functionally redundant and either kinase can activate Pbs2 which in turn activates Hog1. Sho1 can activate Pbs2 independently of Ssk2 and Ssk22 (Maeda et al., 1995). Whether Sho1 activation of Pbs2 is mediated through a different MAPKKK is not yet known. While downstream effectors of Hog1 have not yet been identified, Hog1 activity can induce transcription of *GPD1* (glycerol-3-phosphate dehydrogenase) as well as genes whose promoter region contains stress response element sequences such as *CTT1* (catalase T) and *HSP12* (small heat shock protein)(Schüller et al., 1994; Varela, et al., 1995). Besides required for growth in response to high osmolarity, *hog1*

and *pbs2* mutants also display a failure to complete cytokinesis, resulting in large multinucleated cells with multiple elongated buds (Brewster, et al., 1993).

The cell wall integrity pathway (Figure 1.1) is activated by growth at high temperatures, decreased osmolarity (Davenport, et al., 1995), or exposure to α-factor (reviewed in Levin and Errede, 1995). Protein kinase C (Pkc1) a homologue of the α, β, and γ isoforms of mammalian PKC appears to be involved early in this pathway (reviewed in Herskowitz, 1995). Evidence in support of this comes from studies showing Pkc1 can stimulate Bck1, a MAPKKK, *in vitro* (Levin, et al., 1994) and that dominant gain-of-function *BCK1* alleles can bypass a *pkc1* deletion (Lee, et al., 1992). It is not yet known if Pkc1 directly associates with Bck1. Bck1 can phosphorylate and activate either of the functionally redundant MAPKKs, Mkk1 and Mkk2 (Irie et al., 1993). Either Mkk1 or Mkk2 can then phosphorylate and activate the MAPK Mpk1. *pkc1* null mutants exhibit cell lysis at all temperatures, whereas, *bck1*, *mkk1*, *mkk2*, and *mpk1* mutants only lyse at high temperatures, and display less severe phenotypes (reviewed in Levin and Errede, 1995), suggesting that Pck2 has additional functions. Stimulation of Bck1 results in the transcription of cell integrity genes like *FKS1*, *MNN1*, *CSD2*, *GAS1*, and *KRE6* (Igual, et al., 1996).

Three other responses that utilize MAPK pathways have been identified in *S. cerevisiae* but are not yet well understood. The pseudohyphal response is initiated in diploids by nitrogen starvation (Gimeno, et al., 1992). The invasive growth response is specific to haploids and is activated by nutrient starvation (Roberts and Fink, 1995). Both of these responses require Ste20, Ste11 and Ste7, but not Ste5, Kss1 or Fus3 (Roberts and Fink, 1995;

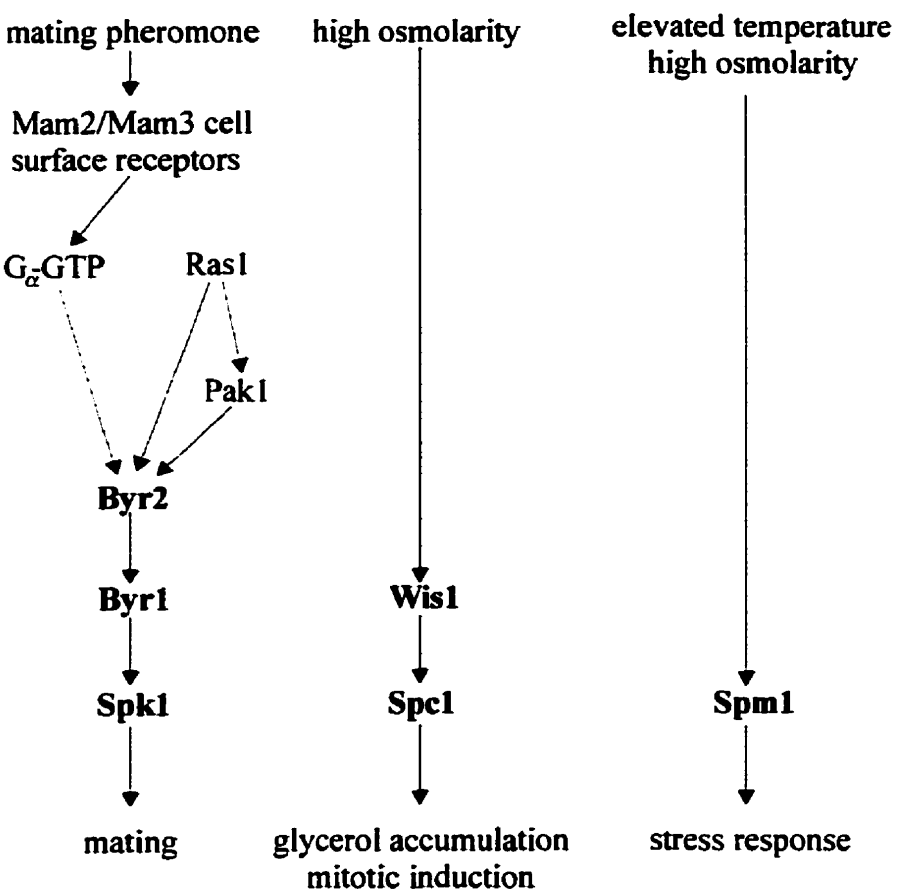
Liu, et al., 1993). The sporulation pathway is activated by nitrogen and nutrient starvation in diploids (Mitchell, 1994). Only two proteins in this pathway have been identified (Figure 1.1). These include a Ste20 homologue, Sps1, which may function upstream of a yet unidentified MAPKKK (Friesen, et al., 1994), and Smk1, a MAPK homologue (Krisak, et al., 1994). Another Ste20 homologue, Cla4, is required for cytokinesis in standard conditions, however, it is unclear what, if any, MAPK pathway is regulated by this gene (Cvrčková, et al., 1995).

MAP kinase pathways in fission yeast:

Previous studies have identified three distinct MAPK pathways in the fission yeast *Schizosaccharomyces pombe* (Figure 1.2). One known MAPK cascade functions within the pheromone response pathway and is highly homologous structurally and functionally to the *S. cerevisiae* pheromone response pathway. There are two mating types in *S. pombe*, P and M. P cells produce P factor which binds the P factor receptor (Mam2) on M cells. Similarly, M cells produce M factor which binds the M factor receptor (Map3) on P cells (reviewed in Marsh, et al., 1991). As in *S. cerevisiae*, receptor activation results in the release of the Gα subunit from the Gβγ subunits. In *S. pombe*, however, it is the Gα subunit which activates the MAPK pathway (Obara, et al., 1991). Another difference between the two yeasts is the role of Ras1 in the *S. pombe* pathway. Ras1 functions upstream of Byr2 and is required for activation of the MAPK cascade when stimulated by mating pheromone; however, Ras1 acts independently of Gα activity to activate Byr2 (Xu, et al., 1994). It has been shown that Ras1 directly binds to Byr2 (Masuda, et al, 1995), however, it is not yet known if the Gα protein also binds directly to Byr2. Pak1 (Shk1), a STE20 homologue, has been shown genetically

Figure 1.2. Representative MAP kinase pathways in fission yeast. Bold text highlights the MAPKKK-MAPKK-MAPK cascade.

Schizosaccharomyces pombe MAPK cascades:



to be downstream of Ras1 and also required for proper functioning of the mating pathway but has not been shown to bind or activate Byr2 (Ottilie, et al., 1995; Marcus, et al., 1995). *pak1*$^+$ can functionally complement a *ste20* null mutant in *S. cerevisiae* (Ottilie, et al., 1995), and *STE20* can complement a *pak1*$^-$ (*shk1*$^-$) mutant in *S. pombe* (Ottilie, et al., 1995; Marcus, et al, 1995). Pak1 activation of Byr2 has not been shown yet, however, the functional similarities to Ste20 suggest that Pak1 activates a MAPKKK involved in mating, which is most likely Byr2. It has also been shown that *spk1*$^+$ can almost entirely complement a *fus3* mutant, and that *byr2*$^+$ can partially complement a *ste11* mutant if *byr1*$^+$ is also expressed (Neiman, et al., 1993). Thus, it appears that the entire MAPK pathway may be functionally conserved between the two species yet the regulation of the pathways is distinct.

Another MAPK pathway in fission yeast involving Wis1 (MAPKK) and Spc1 (Sty1) (MAPK) regulates intracellular osmotic pressure and initiation of mitosis in response to osmotic and nutritional signals (Figure 1.2)(Warbrick and Fantes, 1991; Shiozaki and Russell, 1995a,b; Millar, et al., 1995). This pathway is structurally related to the *S. cerevisiae* Hog1 pathway, however, no results showing a functional homology between the pathways have been reported. Mutations in either Wis1 or Spc1 (Sty1) cause a delay in G2, resulting in cell elongation as well as a loss of viability when maintained at stationary phase, suggesting Wis1 and Spc1 (Sty1) are required for proper response to nutrient limitation (Warbrick and Fantes, 1991; Shiozaki and Russell, 1995b). The MAPKKK that regulates Wis1 has not yet been reported. Spc1/Sty1 activation results in the activation of Atf1 and Atf21 which are ATF2-like transcription factors (Shiozaki and Russell, 1996; Wilkinson, et al., 1996). Atf1 in turn regulates transcription of *gpd1*$^+$, a glycerol 3-phosphate

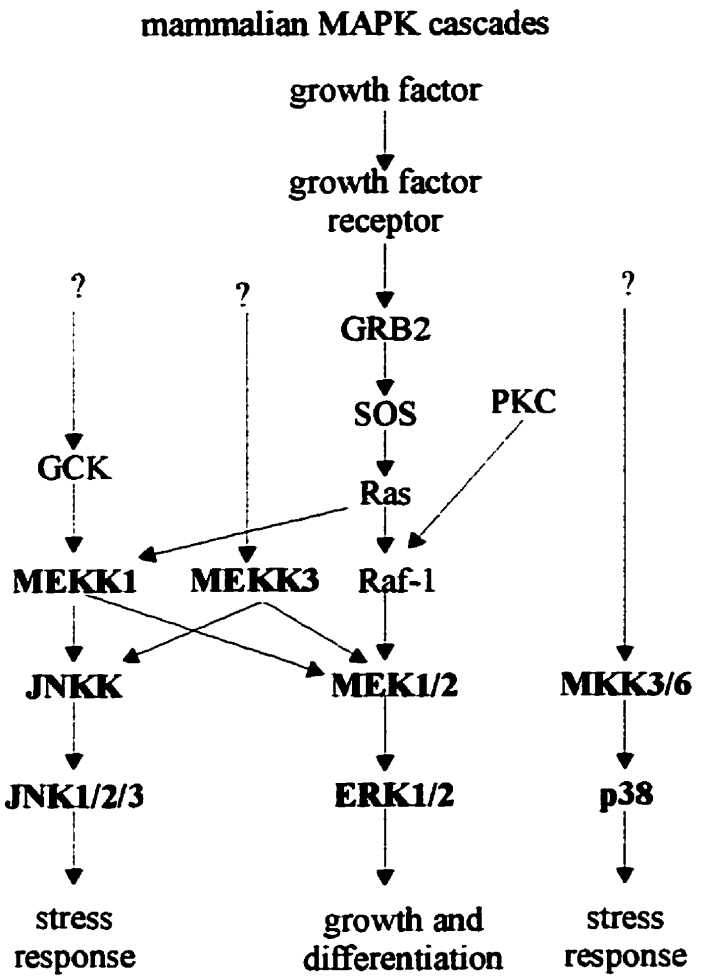
dehydrogenase gene, which is required for glycerol biosynthesis (Wilkinson, et al., 1996). Thus, both Spc1 and the *S. cerevisiae* Hog1 MAPKs regulate levels of glycerol in response to high osmolarity via the same gene.

Very recentl,y a novel MAPK, Pmk1/Spm1, most closely related to *S. cerevisiae* Mpk1, was identified (Toda, et al., 1996; Zaitsevskaya-Carter and Cooper, 1997). Pmk1 (Spm1) is required for normal cell wall integrity, cell shape, cytokinesis, and response to hyperosmotic stress. *pmk1*$^-$ (*spm1*$^-$) cells have a round cell shape and form long multiseptated and multinucleated filaments resembling pseudohyphae in conditions of osmotic stress (Toda et al., 1996; Zaitsevskaya-Carter and Cooper, 1997). *pmk1*$^-$ (*spm1*$^-$) mutants differ from *spc1*$^-$ (*sty1*$^-$) cells in that a much higher concentration of salt is required to inhibit growth. Also, loss of *pmk1*$^+$ (*spm1*$^+$) function results in a round cell shape, whereas, *spc1*$^-$ (*sty1*$^-$) mutants are highly elongated. It is still unclear as to what genes may be regulated by this pathway; however, the homology to Mpk1 and role in cell integrity may suggest that *S. pombe* homologs of the *S. cerevisiae* genes controlled by Mpk1 (see above) may be involved in this pathway.

MAP kinase pathways in mammals:

The best characterized MAPK signal cascade in mammals is the Raf-1 pathway (Figure 1.3) which is involved in growth and differentiation and has received the greatest amount of research attention (reviewed in Seger and Krebs, 1995; Blenis, 1993). Stimulation of the Raf-1 pathway results in proliferation or differentiation depending on cell type (Cowley, et al., 1994). Mutations in this pathway often lead to cellular transformation and altered proliferation. This pathway is stimulated by a growth factors such as epidermal

Figure 1.3. Representative MAP kinase pathways mammals. Bold text highlights the MAPKKK-MAPKK-MAPK cascade.


mammalian MAPK cascades
growth factor
growth factor receptor
?
?
?
GRB2
SOS
PKC
GCK
Ras
MEKK1
MEKK3
Raf-1
JNKK
MEK1/2
MKK3/6
JNK1/2/3
ERK1/2
p38
stress response
growth and differentiation
stress response

growth factor (EGF). The growth factor binds and activates a growth factor receptor such as the EGF receptor (EGFR). EGFR acts as a dimer and has intrinsic kinase activity, which results in autophosphorylation of tyrosine. This class of receptors containing kinase domains is refered to as receptor tyrosine kinase (RTK). Phophorylated RTKs will bind the adaptor protein Grb2 and the Ras guanine-nucleotide exchange factor, Sos (reviewed by Seger and Krebs, 1995; Blumer and Johnson, 1994). Sos catalyzes the dissociation of GDP from Ras allowing Ras to bind to GTP. Raf-1 is activated upon recruitment to the membrane by GTP-bound Ras (Stokoe, et al., 1994). The exact mechanism Raf-1 activation is not known; however, PKC has been shown to phosphorylate and activate Raf-1 *in vitro* and *in vivo* (Kolch, et al., 1993). Raf-1 is a serine/threonine kinase that phosphorylates and activates MEK1/2, redundant dual-specific serine/threonine and tyrosine kinases. MEK1/2 are MAPKKs which activate and phosphorylate the ERK1/2 serine/threonine kinases (MAPKs). ERK1/2 can activate transcription factors such as Elk-1, Fos and ATF-2, as well as others which are involved in cell proliferation (reviewed by Seger and Krebs, 1995). ERK1/2 can also activate kinases such as RSK (a MAPK-activated protein kinase; MAPKAPK) which are involved in phosphorylation of ribosomal protein S6, Fos, and others (reviewed by Seger and Krebs, 1995).

There are two other MAPK cascades identified in mammals. One is the Jun-N-terminal kinase (JNK) pathway (Figure 1.3) which is responsible for activation of the transcription factor c-Jun in response to stress (reviewed by Seger and Krebs, 1995). Stress signals include UV light or proinflammatory cytokines such as TNFα (Minden, et al., 1994; Sluss, et al., 1994). These signals result in the activation of a MAPKKK, MEKK1, which

shares structural and functional homology with *S. cerevisiae* Bck1 (Blumer, et al.. 1994). MEKK1 phosphorylates and activates human JNKK (SEK in rat), which in turn phosphorylates and activates three MAPK isoforms: JNK1/2/3 (Derijard, et al., 1994; Kallunki, et al., 1994; Gupta, et al., 1996). These human MAPKs are homologous to rat SAPKγ/α/β respectively. It has also been shown that a Ste20 homologue, germinal centre kinase (GCK) can activate JNK but not ERK1/2 or p38 (Pombo et al., 1995). Thus, GCK probably belongs upstream of MEKK1, however, this has not yet been shown directly. As with the *S. pombe* Wis1 pathway, the JNK pathway is responsible for transcription mediated by ATF2 (Gupta, et al., 1995).

The other MAPK pathway in mammals involves the MAPK p38, a Hog1 homologue (Han et al., 1994;Rouse et al., 1994). The p38 pathway is also implicated in stress response and is also activated by UV light and cytokines. No MAPKKK has been identified for this cascade, however, the MAPKKs MKK3 and MKK6 are responsible for p38 activation (Han, et al., 1996; Raingeaud, et al., 1996; Stein, et al., 1996).

The regulation of MAPK cascades in higher eukaryotes are extremely complex with crosstalk between the pathways (reviewed by Blenis, 1993). For example, JNKK has also been implicated in the activation of p38 (Lin, et al., 1995). Also, one cascade may be activated by several different upstream regulators that are activated by specific signals. For example, ERK1/2 can be activated by heterotrimeric G protein coupled receptors (acetylcholine receptor) through the action of phosphatidyl-inositol-specific phospholipase C (PI-PLC)(Qian, et al., 1993). ERK1/2 can also be activated by hetertrimeric G proteins in a PKC-independent manner by regulating GTP-bound Ras (reviewed in Blumer and

Johnson, 1994). To further complicate the matter, a novel MAPKKK has recently been identified, MEKK3, which has been shown to activate both the ERKs and the JNKs but not p38 (Ellinger-Ziegelbauer, et al., 1997). As one can see, there is still much to be learned about the regulation and activity of MAPK cascades.

Thesis objectives and rationale:

All of the *S. pombe* pathways are structurally and functionally homologous to existing *S. cerevisiae* pathways. With only one reported MAPKKK in *S. pombe* it is reasonable to assume that others may exist especially in lieu of the existence of several MAPKs of distinct function. Thus, the objective of this thesis was to identify potential genes. other than Byr2, that may encode a MAPKKK and describe its function and relationship to existing MAPKs in *S. pombe*. The specific aims of this thesis are as follows:

1. Use PCR technology to screen for putative MAPKKK homologues.
2. Determine the cellular roles of the novel MAPKKK using mutational analysis and molecular genetics..
3. Determine the genetic relationship between the novel MAPKKK and known *S. pombe* genes.

CHAPTER TWO: ISOLATION OF A NOVEL MAPKKK

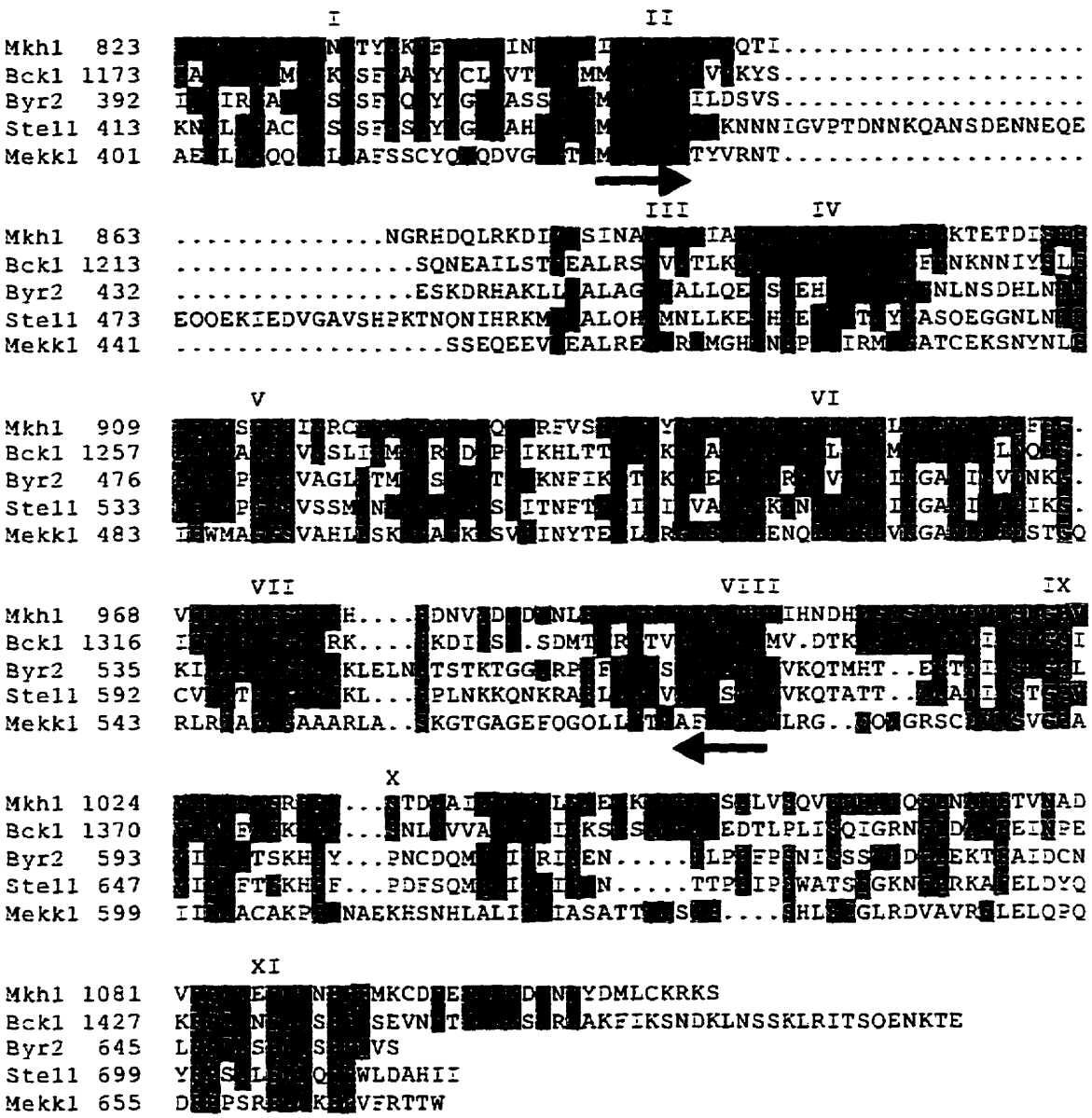
Introduction:

As mentioned in Chapter 1, *S. pombe* and *S. cerevisiae* signal through MAPKs that are both structurally and functionally similar. There are three identified MAPKKKs in *S. cerevisiae* whereas only one identified in *S. pombe*. Thus, it seemed quite likely that other MAPKKKs exist in *S. pombe*. Within the catalytic domain of all kinases there exists 11 conserved subdomains (Hanks, et al., 1988). Some of these domains have been shown to be critical for ATP binding and catalytic actions. However, within all kinases, their exists differences in sequence that allow classification of proteins into families (Hanks, et al., 1988). Thus, MAPKKKs can be differentiated from MAPKKs. Comparing amino acid sequences of homologous kinases between *S. pombe* and *S. cerevisiae* revealed that within the catalytic sequence, stretches of sequence identity exist (Hanks, et al., 1988; Figure 2.1). These are critical motifs that are conserved to maintain function.

The advent of polymerase chain reaction (PCR) has provided a powerful tool to analyse DNA. Regions of DNA can be readily amplifed using oligonucleotides complementary to conserved regions of a sequence of interest. This strategy has been used successfully to clone MAPK cascade proteins such as human MEKK3 (Ellinger-Ziegelbauer, et al., 1997) and *S. pombe* Spm1 (Zaitsevskaya-Carter and Cooper, 1997) and Shk1 (Marcus, et al., 1995).

Known yeast MAPKKKs share 35-55% identity in their carboxy-terminal catalytic domains, but there is very little homology among their N-terminal regulatory domains. These proteins range in size from 659 (*S. pombe* Byr2) to 1579 (*S. cerevisiae* Ssk2) amino acids in length. To identify MAPKKKs in *S. pombe*, degenerate oligonucleotides were designed

Figure 2.1. Comparison of catalytic domains of several MAPKKKs. Shaded amino acids represent identities to Mkh1. Arrows indicate sequences used to design degenerate oligonucleotides for the PCR screen of the *S. pombe* cDNA library.


I
II
III
IV
V
VI
VII
VIII
IX
X
XI
Mkh1 823
Bck1 1173
Byr2 392
Ste11 413
Mekk1 401
Mkh1 863
Bck1 1213
Byr2 432
Ste11 473
Mekk1 441
Mkh1 909
Bck1 1257
Byr2 476
Ste11 533
Mekk1 483
Mkh1 968
Bck1 1316
Byr2 535
Ste11 592
Mekk1 543
Mkh1 1024
Bck1 1370
Byr2 593
Ste11 647
Mekk1 599
Mkh1 1081
Bck1 1427
Byr2 645
Ste11 699
Mekk1 655

to regions of sequence that are highly conserved and characteristic of MAPKKK catalytic domains (Figure 2.1).

Methods and Materials:

A) Yeast strain:

The *S. pombe* strain used in this study (SP66 h^{90} *leu1-32 ade6-216*) was provided by D. Beach (Cold Spring Harbor Laboratory). Yeast culture was performed as previously described (Moreno et al., 1991).

B) Plasmids:

pM3K4b contains a 450 bp *Not*I fragment, with sequence homology to MAPKKKs, cloned into pBluescript II SK- (Stratagene). pMKH1 and pMKH2 contain independent clones of genomic *S. pombe Spe*I fragments containing the complete *mkh1*$^+$ coding region.

C) DNA manipulation and sequencing:

Procedures used for DNA manipulation and analysis (i.e., purification, restriction site mapping, electrophoresis, transformation, etc.) have been previously described (Sambrook et al., 1989). The DNA sequences of both strands of sequenced clones were determined by a modified dideoxy chain-terminating method (Sanger et al., 1977), using the *Taq* DyeDeoxy^{TM} Terminator Cycle Sequencing Kit (Perkin-Elmer).

D) Polymerase Chain Reaction:

The PCR method was used to amplify DNA fragments from a *S. pombe* cDNA library using the degenerate oligonucleotides 5'-TTGCGGCCGCTC[N]GG[N][GC][CAT]CATCCA[AG]AA-3' and 5'-TTGCGGCCGCATGGC[N]GT[N]AA[AG]CA[AG]GT-3' as primers. Construction of the *S. pombe* cDNA library has been previously described (Matviw et al., 1993). Reactions

of 100 µl reactions containing 100 pmol of each primer and 1 µg of *S. pombe* cDNA library were incubated for 30 cycles (1 min at 94°C, 2 min at 50°C, and 1 min at 72°C) on a Perkin Elmer Cetus 4800 DNA ThermoCycler, as previously described (Matviw et al., 1992). The 450 bp PCR product was purified and cloned into the *Not*I site of pBluescript II SK- (Stratagene).

Results:

A) Cloning the *S. pombe mkh1*$^+$ gene:

PCR amplification of a *S. pombe* cDNA library using degenerate oligonucleotides produced a 450 bp PCR product which was purified and cloned. The DNA sequences of seven independent clones were determined. As expected, three of the clones contained inserts that were derived from *byr2*$^+$. However, one clone, pM3K4b, differed from *byr2*$^+$, but encoded a peptide with strong homology to the catalytic domains of known MAPKKKs. This sequence was derived from a novel gene, which was named *mkh1*$^+$, for **MEKK** homolog one. Based on a restriction map of genomic DNA, it was estimated that this gene was contained within a 7.3 kb *Spe*I DNA fragment.

An *S. pombe* genomic DNA library was constructed as *Spe*I fragments in the λ ZAPII vector (Stratagene), and this library was screened by filter-hybridization using a probe derived from the cloned insert of pM3K4b. Several independent clones were isolated. The DNA sequence of two of these clones, pMKH1 and pMKH2, was determined and found to be identical (Figure 2.2). The sequence encoded a predicted protein of 1116 amino acid residues in length. There was an in-frame stop codon located 18 bp upstream of the putative start codon, and there are no consensus splicing sequences in the coding region (Mertins and

Figure 2.2. Genomic sequence of *mkh1*$^+$ and putative protein sequence of open reading frame. Genbank Accession No. U53872. *** Denotes an in-frame stop codon. The putative start codon (ATG) is represents codon number 1. The nucleotide sequence is numbered beginning at the ATG start codon.

```
-59 ACAAAAAAAAGGTTTTTTATCTTGAATTCTTTTCAAAGTAACTAATACTGTTCTGGGTAT
                                                    ***
  1 ATGGCTGCCGATATCGGATCGCAGTCATCAGGCTCTTTGGAAGAACGGTTTGAACAGTCT
  1  M  A  A  D  I  G  S  Q  S  S  G  S  L  E  E  R  F  E  Q  S
 61 CTTCATCTTCAGAATGTCGATAAGCAAGATTGGTCACTTAACAGTGTACTTCAGTTTTTA
 21  L  H  L  Q  N  V  D  K  Q  D  W  S  L  N  S  V  L  Q  F  L
121 AAACTATACAAATTTAACAAAGAATGGGAAGACGTTTTTATTAAAAGTCGAATCGAAATG
 41  K  L  Y  K  F  N  K  E  W  E  D  V  F  I  K  S  R  I  E  M
181 GATTTATTTATCAATTTGGCCGATCAATCAAAAGCCGAGGAATTCGCCTTTAAAAATAAA
 61  D  L  F  I  N  L  A  D  Q  S  K  A  E  E  F  A  F  K  N  K
241 TTGAGCAAGGAGTCTGCCATCCAATTGAGTAGCTGTATTCGCAAAACACTTTTAGCACCT
 81  L  S  K  E  S  A  I  Q  L  S  S  C  I  R  K  T  L  L  A  P
301 TCTTCGACTCGCGTACCTAGCAAAAACTCGTCTTACGAAACATTAACTTACAGCGCCAAA
101  S  S  T  R  V  P  S  K  N  S  S  Y  E  T  L  T  Y  S  A  K
361 GATAGTTCGGATGACGTTTTTACAGAAACTAACTCTGGTTTCCGCTCTTCAAATCAAAAT
121  D  S  S  D  D  V  F  T  E  T  N  S  G  F  R  S  S  N  Q  N
421 TCGTCCCTCAAAAGCTTTCAGAGTGTTCCTGATAGCAATGTGAACGTGTTTGGTGGCTTC
141  S  S  L  K  S  F  Q  S  V  P  D  S  N  V  N  V  F  G  G  F
481 GGTGGATCAGTAGTCGACAACAATGAGCTATTGAGCACAGGAAAAAACTCCCATCAAACA
161  G  G  S  V  V  D  N  N  E  L  L  S  T  G  K  N  S  H  Q  T
541 ACCTCTTTAAATTTGGAAGGCTCTCCTATAAACTTACACGCTTACAAAGGAACTGTCACT
181  T  S  L  N  L  E  G  S  P  I  N  L  H  A  Y  K  G  T  V  T
601 TCAATAATTAACGATGACAGCAGAAACATTAATAAAAAAACATTGTCGAAACAACCTGTA
201  S  I  I  N  D  D  S  R  N  I  N  K  K  T  L  S  K  Q  P  V
661 TCCGAACATAAAGAAAAACAAACTAGCTTTCTCCGTCGTTTCCGGGTACCTGGGTTTTCT
221  S  E  H  K  E  K  Q  T  S  F  L  R  R  F  R  V  P  G  F  S
721 CGTGACAAGGATAAAACTAAAGATTGCCCTTCTTCAAATTCGAACCCATTCCATTTAGCT
241  R  D  K  D  K  T  K  D  C  P  S  S  N  S  N  P  F  H  L  A
781 TCTTCAAATGTGAAAACATTAGACGCGTCTTTGGATCAAGGTGAGTGGGTACCTCGTATT
261  S  S  N  V  K  T  L  D  A  S  L  D  Q  G  E  W  V  P  R  I
841 CATCGTTTAGAAAGTCAAATTGGTTTAATATCCAAAAAGAAGTCATTTGTTCTTGCTACT
281  H  R  L  E  S  Q  I  G  L  I  S  K  K  K  S  F  V  L  A  T
901 ATGGATGATATGAAATTCACAGTAGTGGATATTACCAACGTCCAAAATGCTACTCAGCTA
301  M  D  D  M  K  F  T  V  V  D  I  T  N  V  Q  N  A  T  Q  L
961 CGTAAGCTAATAGCTAAGAGTATGTATTTAGACATTTCAATTGACCAGTTTGATTTGTTT
321  R  K  L  I  A  K  S  M  Y  L  D  I  S  I  D  Q  F  D  L  F
1021 CTCACGGAAGTCGGCGGGGCTCAATACATAGAAATATTAGATGATAGAAAGCTTGATATT
341  L  T  E  V  G  G  A  Q  Y  I  E  I  L  D  D  R  K  L  D  I
1081 GCAAGGCTTTATTCTGATGAATTTGGAACTATTAAATTTTTCGTAAAGCCATCACAAAAT
361  A  R  L  Y  S  D  E  F  G  T  I  K  F  F  V  K  P  S  Q  N
1141 GAAGAATCGGGTATGGATAGTGATACTTATTTATCTTTTGGCACAAAATCAAGTTCAACT
381  E  E  S  G  M  D  S  D  T  Y  L  S  F  G  T  K  S  S  S  T
1201 TATAAAGCTGATGATGACTCAATATATCATCGCAAGGAAGATTTTAAAAAGCAACCAAGT
```

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 401  Y  K  A  D  D  D  S  I  Y  H  R  K  E  D  F  K  K  Q  P  S
1261 TACCCTGTGCTTACTTCGGATTTTGAAATTACTGATGCAGGACCTAATTTATCATTATCA
 421  Y  P  V  L  T  S  D  F  E  I  T  D  A  G  P  N  L  S  L  S
1321 GGGCATCAACCTGATAATAAATACTACAAAGGTTTTAGTTCGGCACCGAATTTGGCAGTT
 441  G  H  Q  P  D  N  K  Y  Y  K  G  F  S  S  A  P  N  L  A  V
1381 GTTCCAGAATTACCATCTCGACGTTTTCGAGGGTTTGAAAAAATCCGTGGTGCTAAAGGA
 461  V  P  E  L  P  S  R  R  F  R  G  F  E  K  I  R  G  A  K  G
1441 GAAATGGCTACAAAAATTTTGGATGCCACTGAAGCCCAAAGTGAAAAAAACAAATTTACC
 481  E  M  A  T  K  I  L  D  A  T  E  A  Q  S  E  K  N  K  F  T
1501 GTTTGTAGACCTCACAAGAAGGTCACATTGAAAATGCCACTTAATTCCGGCTCTTCCGCT
 501  V  C  R  P  H  K  K  V  T  L  K  M  P  L  N  S  G  S  S  A
1561 CCCCAAAGTCCTTCATCTAATACTTCTGCTTCTGTTTTAACTAGAAATTTTGTGGCACAT
 521  P  Q  S  P  S  S  N  T  S  A  S  V  L  T  R  N  F  V  A  H
1621 AGAGATCCTCCACCCCCACCCACAGAGACATCTAGTTTACGTCGAAAAAATACATTGACT
 541  R  D  P  P  P  P  P  T  E  T  S  S  L  R  R  K  N  T  L  T
1681 CGTAGACCAAGTATTCGTCACGCTCGGTCCTCTCCTTACATTGATACCGGACATAACGAA
 561  R  R  P  S  I  R  H  A  R  S  S  P  Y  I  D  T  G  H  N  E
1741 GCTAGCAAATTTTCACATACGTCTTTTGACCCCAAAGCATCTAGTAAATCTTCTAATTCA
 581  A  S  K  F  S  H  T  S  F  D  P  K  A  S  S  K  S  S  N  S
1801 TTAAAGGAAAGTGTGGAAGCTTTATCAGAAATACCTTTTGAAGATGCGCCTGCACTAGAC
 601  L  K  E  S  V  E  A  L  S  E  I  P  F  E  D  A  P  A  L  D
1861 GAATCGGATCTTTCTGGGGATCCCTTTTGGGCTATACAGCCCAAACAATCTTCCTCCCAA
 621  E  S  D  L  S  G  D  P  F  W  A  I  Q  P  K  Q  S  S  S  Q
1921 GTACCTAAAGAAAATCATCACAACATTCAATCCAAACTTTCCATTAACACAGAGGCTGCT
 641  V  P  K  E  N  H  H  N  I  Q  S  K  L  S  I  N  T  E  A  A
1981 ACGGATTTGAAAGCAAATGAACTATCTTCGCCTAAAACTCCTGAATACTGTAGAGGTGAT
 661  T  D  L  K  A  N  E  L  S  S  P  K  T  P  E  Y  C  R  G  D
2041 GACAGATCCATTAGTTTATCACCGTTATCTTATCGTTTAAGAAAGTCCAAACATATTCGT
 681  D  R  S  I  S  L  S  P  L  S  Y  R  L  R  K  S  K  H  I  R
2101 GAATCCCCACCGTCTTCAAAGGTTATCAATTCTGGTAACTGGGAAGTTCGTCCATCTGCT
 701  E  S  P  P  S  S  K  V  I  N  S  G  N  W  E  V  R  P  S  A
2161 GATGATCTTTATGAGGATGTTGATCGATTTTTTCCCCGTTATGATTTGGATAAAGTACTT
 721  D  D  L  Y  E  D  V  D  R  F  F  P  R  Y  D  L  D  K  V  L
2221 GTAGTGGACCAAAGCCGCATGGTTTCTTCCCCTTCAAAGGTATCGATACGTCCGAAAATG
 741  V  V  D  Q  S  R  M  V  S  S  P  S  K  V  S  I  R  P  K  M
2281 AAGTCTGTACGATTATTAGCCCGCGAAGCATCTGAAGCTCGGAAAGAAATACGACATAAC
 761  K  S  V  R  L  L  A  R  E  A  S  E  A  R  K  E  I  R  H  N
2341 GCGAGACGCAATAAATCTGGAAATCTTCTACGTCGATCAAGTACGAAACTTTGGGGCTCT
 781  A  R  R  N  K  S  G  N  L  L  R  R  S  S  T  K  L  W  G  S
2401 AGGATTGTAGAACTAAAACCAGATACTACTATAACTTCTGGATCAGTTGTTTCACAAAAT
 801  R  I  V  E  L  K  P  D  T  T  I  T  S  G  S  V  V  S  Q  N
2461 GCCACGTTCAAATGGATGAAAGGAGAATTGATTGGAAATGGTACTTATGGTAAGGTATTT
 821  A  T  F  K  W  M  K  G  E  L  I  G  N  G  T  Y  G  K  V  F
```

```
2521 TTGGCTATGAACATTAATACGGGTGAATTGATTGCAGTAAAGCAAGTTGAAATACCACAA
 841  L  A  M  N  I  N  T  G  E  L  I  A  V  K  Q  V  E  I  P  Q
2581 ACTATTAATGGCCGTCATGACCAATTACGCAAAGATATCGTGGATTCCATTAATGCAGAA
 861  T  I  N  G  R  H  D  Q  L  R  K  D  I  V  D  S  I  N  A  E
2641 ATTTCTATGATTGCCGATTTGGATCACTTAAATATAGTGCAATATCTGGGTTTCGAAAAG
 881  I  S  M  I  A  D  L  D  H  L  N  I  V  Q  Y  L  G  F  E  K
2701 ACGGAAACGGATATAAGTATATTCCTGGAATATGTTTCAGGTGGTTCAATTGGTCGATGT
 901  T  E  T  D  I  S  I  F  L  E  Y  V  S  G  G  S  I  G  R  C
2761 TTGCGGAATTATGGTCCTTTCGAAGAGCAACTGGTCCGTTTTGTATCACGCCAGGTGCTC
 921  L  R  N  Y  G  P  F  E  E  Q  L  V  R  F  V  S  R  Q  V  L
2821 TACGGGTTGTCTTACTTACATTCTAAAGGTATTATACATCGAGATTTAAAGGCTGACAAT
 941  Y  G  L  S  Y  L  H  S  K  G  I  I  H  R  D  L  K  A  D  N
2881 TTGCTCATTGATTTTGATGGAGTTTGCAAAATTTCAGACTTTGGAATATCTAAGCATAGT
 961  L  L  I  D  F  D  G  V  C  K  I  S  D  F  G  I  S  K  H  S
2941 GATAATGTGTATGACAATGACGCAAACCTGTCCATGCAAGGATCCATCTTTTGGATGGCA
 981  D  N  V  Y  D  N  D  A  N  L  S  M  Q  G  S  I  F  W  M  A
3001 CCTGAAGTAATTCATAATGATCATCAAGGATATAGTGCTAAGGTCGACGTCTGGTCCTTG
1001  P  E  V  I  H  N  D  H  Q  G  Y  S  A  K  V  D  V  W  S  L
3061 GGATGTGTAGTGTTGGAAATGTTAGCTGGTCGTAGACCGTGGTCTACAGATGAGGCTATC
1021  G  C  V  V  L  E  M  L  A  G  R  R  P  W  S  T  D  E  A  I
3121 CAAGCTATGTTCAAGTTAGGTACCGAGAAAAAGGCGCCTCCTATTCCTAGTGAATTGGTG
1041  Q  A  M  F  K  L  G  T  E  K  K  A  P  P  I  P  S  E  L  V
3181 TCTCAGGTATCACCCGAAGCGATTCAATTTTTGAATGCATGCTTTACTGTGAATGCTGAT
1061  S  Q  V  S  P  E  A  I  Q  F  L  N  A  C  F  T  V  N  A  D
3241 GTAAGGCCAACCGCAGAGGAATTATTAAATCACCCGTTTATGAAATGTGACGAAGAATTC
1081  V  R  P  T  A  E  E  L  L  N  H  P  F  M  K  C  D  E  E  F
3301 AACTTTAAGGACACGAATCTTTACGACATGCTTTGTAAAAGAAAGAGCTAA
1101  N  F  K  D  T  N  L  Y  D  M  L  C  K  R  K  S ***
```

Gallwitz, 1987). Thus, the entire coding region of *mkh1*$^+$ appeared to be contained within a single exon.

B) Structure of *mkh1*$^+$ sequence:

The sequence of *mkh1*$^+$ was analysed for putative conserved motifs using the GCG sequence analysis software package version 8.1 (Genetics Computer Group Inc.). The MOTIFS command identified the protein kinase ATP binding site (aa 832 to 838) and the catalytic active site residue (Aspartic acid at aa 960). The position and identities of neighbouring amino acids of the active site residue belong to the serine/threonine class of protein kinases (Bairoch and Claveri, 1998). Mkh1 also has three putative tyrosine phosphorylation sites (aa 414, 429, and 989) that fit known consensus sequences (Cooper, et al., 1984).

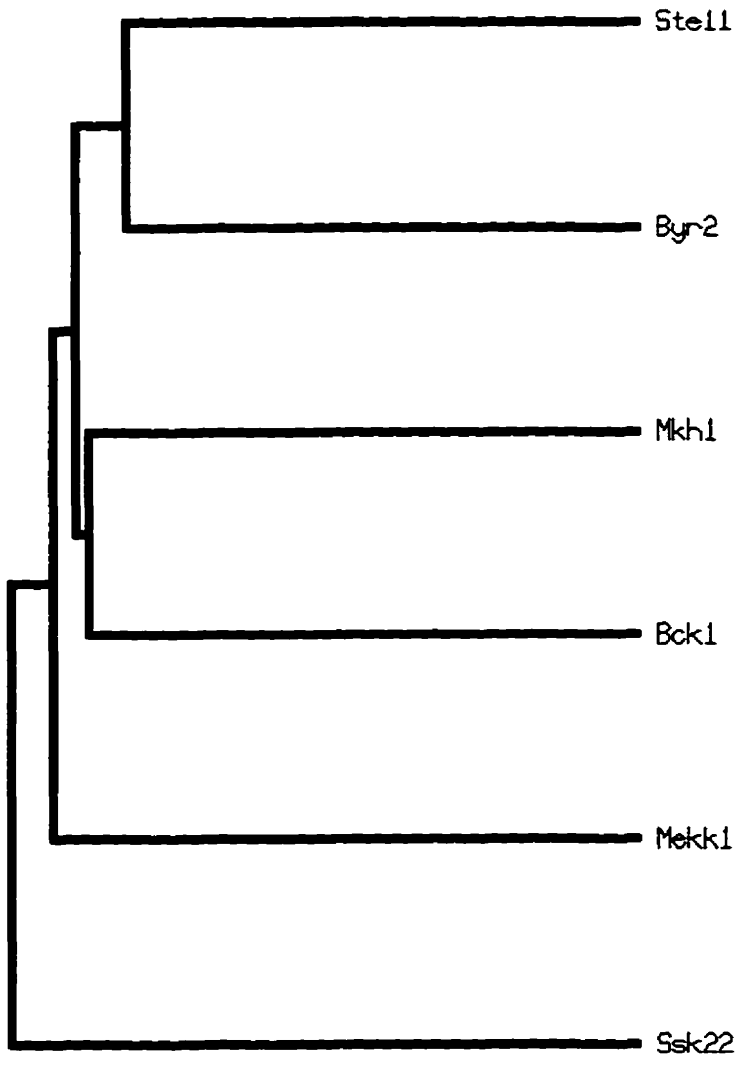
C) Comparison of Mkh1 with known MAPKKKs:

The putative catalytic domain of Mkh1 shares strong homology with other yeast and mammalian MAPKKK catalytic domains (Figure 2.1). Of known proteins, *S. cerevisiae* Bck1 shares the highest sequence homology with Mkh1. The putative catalytic domains of these proteins are 54% identical. A comparison of full-length sequence also puts Bck1 as the protein most closely related to Mkh1 (Figure 2.3). The most closely related mammalian protein to Mkh1 is MEKK1.

Discussion:

In this study, a putative MAPKKK was isolated. In *S. pombe*, Byr2 was the only other previously reported MAPKKK. According to sequence analysis, Mkh1 has motifs in the catalytic domain specific for the serine/threonine family of protein kinases. All MAPKKKs isolated thus far have been serine/threonine kinases (Blumer and Johnson, 1984;

Figure 2.3. Relationship between full-length Mkh1 and known MAPKKKs. This is a graphical respresentation of calculations performed using the GCG analytical software using the PILEUP command.


Ste11
Byr2
Mkh1
Bck1
Mekk1
Ssk22

Levin and Errede, 1995). Mkh1 also has three tyrosines that fit the tyrosine phosphorylation consensus sites (Cooper, et al., 1984). These sites may be phosphorylated by regulatory kinases to modify folding or function of Mkh1. However, one cannot say for sure, since the three-dimensional structure of Mkh1 is not known, these sites may not be exposed to the protein surface and, thus, may not be available for phosphorylation by other proteins.

There are three MAPK pathways that exist in *S. pombe*: for pheromone response, osmoregulation and cell wall integrity (Figure 1.1). The last two pathways have no identified MAPKKKs. Thus it is possible that Mkh1 is the putative upstream kinase to Wis1 or Spm1. However, Wis1 and Spc1 share strong homology to Pbs2 and Hog1, respectively (Warbrick and Fantes, 1991; Millar, et al., 1995; Shiozaki and Russell, 1995a,b). Mkh1, on the other hand, is more closely related to *S. cerevisiae* Bck1 which functions upstream of the MAPK Mpk1. Mpk1 has been shown to be both structurally and functionally homologous to *S. pombe* Spm1 (Toda, et al., 1996). Thus, from the sequence homology, it is highly possible that Mkh1 may be a member of the Spm1 pathway.

CHAPTER THREE: ANALYSIS OF THE *mkh1*⁻ MUTANT

Introduction:

S. cerevisaie and *S. pombe* are ideal eukaryotic organisms for studying cellular biochemistry and molecular genetics. They have similar technical advantages of bacteria yet have the complex genome of higher eukaryotes (reviewed in Sherman, 1991). Some major advantages are the ease of transformation, mutation and genetic analysis. Since, *S. cerevisaie*, *S. pombe* and higher eukaryotes have conserved MAPK cascades (Herskowitz, 1995; Seger and Krebs, 1995), proteins of structural and functional homology to mammalian cells can be readily studied in yeast. For example, the *S. pombe* MAPK Spc1 is homologous to *S. cerevisiae* Hog1 and mammalian p38. Both Spc1 and Hog1 regulate a stress-inducible gene, glycerol-3-phosphate dehydrogenase (Degols, et al., 1996; Albertyn, et al., 1994). Also, Spc1 and p38 both regulate ATF-2-like transcription factors in response to stress (Shiozaki and Russell, 1996; Wilkinson, et al., 1996; Raingeaud, et al., 1996). Thus, studying functions of proteins in one species can provide important clues to function of homologous proteins in other species.

Mutants provide significant insights to protein function. In fact, it is through mutational analysis of MAPK cascades in yeast that a large body of knowledge has been generated over the last decade (reviewed in Levin and Errede, 1995). Once a mutant is generated, the strain can be studied for morphological and growth defects. Mutations in MAPK pathways can result in changes in cell shape such as *wis1*⁻ cells which are highly elongated (Warbrick and Fantes, 1991). Mutations may result in functional defects such as *ste11* and *byr2*⁻ which are both sterile (Chaleff and Tatchell, 1985; Wang, et al., 1991). Still other mutants may have growth restrictions under certain conditions. For example, *bck1* cells

fail to grow at elevated temperature (35°C)(Lee and levin, 1992) and *pbs2* cells fail to grow on media containing high concentration of salt (Brewster, et al., 1993).

Thus, to study the function of Mkh1, the catalytic domain of *mkh1*$^+$ was replaced with the selectable marker *ura4*$^+$. The resulting deletion strain was analysed for phenotypic differences from that of the wild type strain.

Materials and Methods:

A) Yeast strains and genetic analysis:

The *S. pombe* strains used in this chapter were: SP870 (*h*^{90} *leu1-32 ade6-210 ura4-d18*), SP826 (*h*$^+$/*h*$^+$ *leu1-32/leu1-32 ade6-210/ade6-216 ura4-d18/ura4-d18*), AS6 (*mkh1*$^+$/*mkh1::ura4*$^+$; isogenic to SP826), AS1 (*h*^{90} *leu1-32 ade6-210 ura4-d18 mkh1::ura4*$^+$), AS2 (*h*^{90} *leu1-32 ade6-210 ura4-d18*) and AS7 (*h*^{90} *leu1-32 ade6-210*). SP870 and SP826 were provided by Dr. D. Beach, Cold Spring Harbor Lab. Yeast culture, transformation, iodine staining, tetrad analysis, and other genetic manipulations were performed as previously described (Moreno, et al., 1991).

B) Plasmids:

pMKH1 contains a 7.3 kb *Spe*I fragment, which includes the entire coding region of *mkh1*$^+$, cloned in pBluescript II SK- (Stratagene). pUMKΔ1 was derived from pMKH1 by replacing the 1.5 kb *Bam*HI-*Sph*I fragment encoding the catalytic domain of Mkh1 with a 1.8 kb fragment containing *ura4*$^+$. pAALN is a *S. pombe* expression vector containing *LEU2*, the *ars1*$^+$ sequence, and *adh1* promoter sequence flanking a polylinker site (Yu, et al., 1994; Xu, et al., 1990). pALMK1 contains the coding sequence of *mkh1*$^+$ cloned into the *Bam*HI-*Sac*I sites of pAALN. pALMK2 contains the catalytic domain (amino acid 788 to 1116) of Mkh1 cloned into the *Bam*HI-*Sac*I sites of pAALN.

C) Gene disruption:

The *mkh1*$^+$ gene was disrupted in the *S. pombe* haploid strain SP870 and the diploid strain SP826 by the gene replacement method (Rothstein, 1983; Russell and Nurse, 1986). SP826 was transformed with the 6.8 kb *Bss*HII fragment of pUMKΔ1, in which the catalytic domain of *mkh1*$^+$ (aa 625 to 1116) had been replaced with *ura4*$^+$, and Ura$^+$ transformants were selected on PMA + Leu media. Six independent transformants were tested for stability of the Ura+ phenotype, and they were analyzed by Southern blots to confirm that they contained the proper disruption in one copy of the endogenous *mkh1* genes. h^{90}/h^+ revertants of these strains, which occur at a frequency of approximately 10^{-3}, were detected by the iodine vapor staining test. The haploid strains AS1 (Ura$^+$) and AS2 (Ura$^-$) were derived from spores of a single ascus from one such revertant. AS7 (Ura$^+$) was derived from AS2 by replacing the *ura4-d18* with the wild-type *ura4*$^+$ allele.

D) Fluorescent staining:

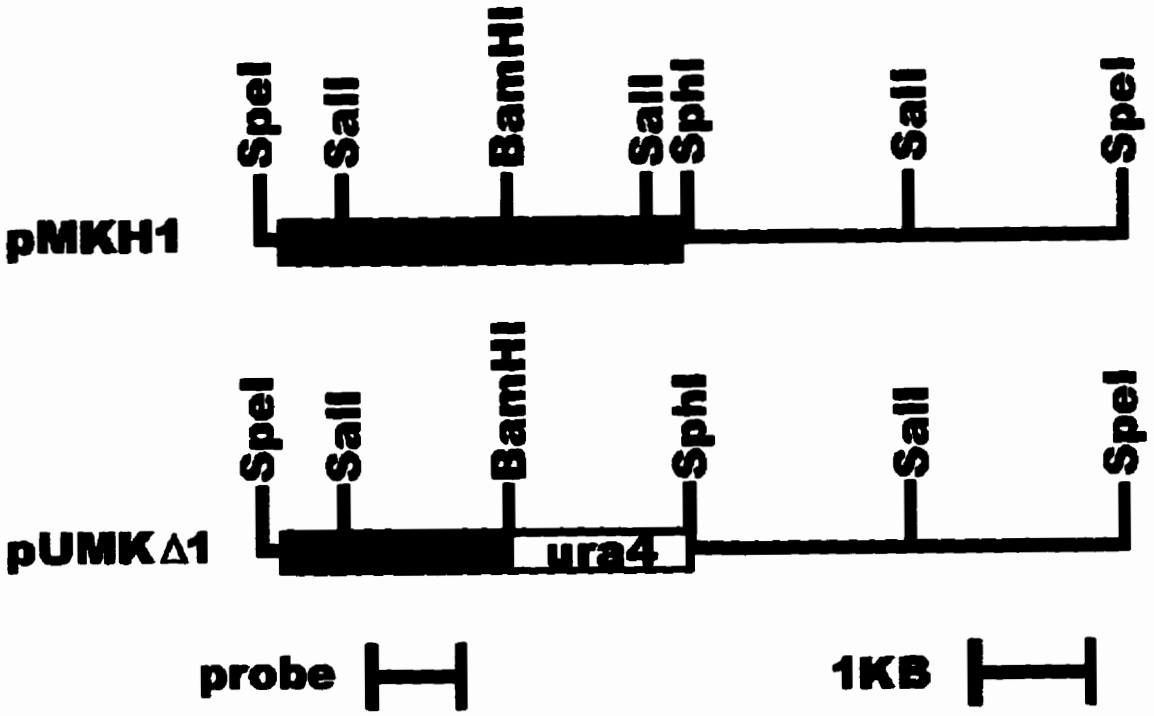
Cells were fixed in PBS containing 3.7% formaldehyde for 60 min at 37°C, washed 2X in PBS, stained with calcofluor (1 µg/ml) (Sigma), washed in PBS, and stained with DAPI (10 µg/ml) (Sigma) or Hoechst 33258 (20 ng/ml) (Aldrich), and examined by fluorescence microscopy using a UV filter (Alfa, et al., 1993).

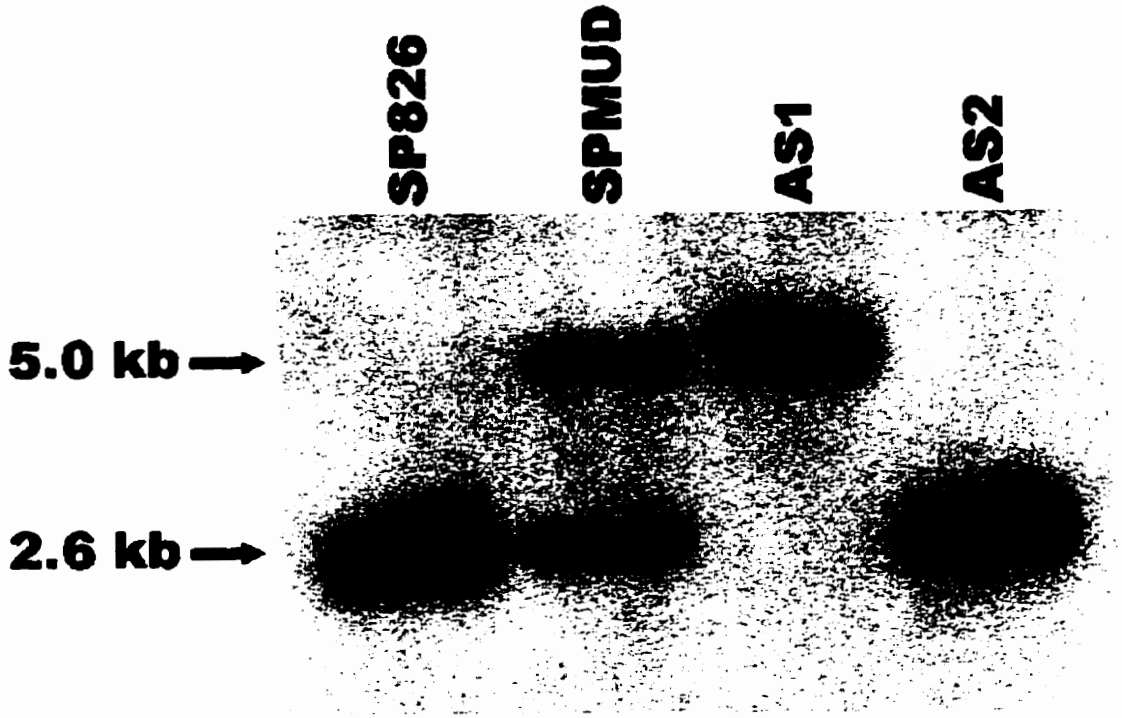
Results:

A) Role of Mkh1 in standard growth conditions:

To investigate the function of Mkh1, the coding sequence of the catalytic domain of *mkh1*$^+$ was replaced with *ura4*$^+$ in a diploid strain. Proper integration of the disrupted *mkh1::ura4*$^+$ gene was shown by Southern blot analysis (Figure 3.1). Five of six independent strains were deleted for a single copy of *mkh1*$^+$, while one strain had both copies

Figure 3.1. Genomic map of deletion construct and Southern blot analysis of deletion strain. The *mkh1*$^+$ genomic fragment was cloned into pMKH1 and the *mkh1*$^-$ integrating construct was cloned in pUMKΔ1. The coding region is indicated by the solid box. The 1.5 kb fragment encoding the catalytic domain of Mkh1 was replaced by a 1.8 kb *ura4*$^+$ gene represented by the open box. Southern blot of genomic DNA from a diploid *mkh1*$^+$ strain (SP826), a heterozygous *mkh1* deletion strain (SPMUD), and the Ura$^+$ (AS1) and Ura$^-$ (AS2) haploid segregants. The DNA was digested with *Sal*I and hybridized with a ^{32}P-labelled probe derived from the indicated *Sal*I-*Bam*HI fragment.


SpeI
SalI
BamHI
SalI
SphI
SalI
SpeI
pMKH1
SpeI
SalI
BamHI
SphI
SalI
SpeI
pUMKΔ1
ura4
probe
1KB


SP826
SPMUD
AS1
AS2
5.0 kb
2.6 kb

of *mkh1*$^+$ deleted. Tetrad analysis was performed on several transformants containing the *mkh1::ura4*$^+$ disruption. In most cases asci contained four viable spores, and two of the spores were Ura$^+$, indicating that they contained the *mkh1::ura4*$^+$ allele. *mkh1*$^-$ cells are capable of mating, but at a slightly lower efficiency than normal cells (data not shown).

Deletion of *mkh1*$^+$ also resulted in a subtle effect on cell shape. Most *mkh1*$^-$ cells were more rounded than *mkh1*$^+$ cells, which exhibited a normal cylinder shape at 23°C (Figure 3.2). Also, cells overexpressing full-length Mkh1 had a greater (~8%) average length than the control cells (data not shown). Overexpression of the catalytic domain of Mkh1 resulted in an average cell length increase of ~22% (data not shown). These phenotypes were only seen during late logarithmic or stationary phase cultures.

The *mkh1*$^-$ and *mkh1*$^+$ haploid strains grew at similar rates in standard growth conditions, as measured by both cell number and OD_{600} (Table 3.1). Also, overexpression of full length Mkh1 had no significant affect on growth rate. However, overexpression of the catalytic domain of Mkh1 resulted in significant growth inhibition (Table 3.1).

B) Effect of elevated temperature on *mkh1*$^-$ cells:

A more dramatic phenotype was observed when *mkh1*$^-$ cells were grown on minimal (PMA) media at elevated temperatures (35°C). Under such conditions a significant fraction (~10%) of *mkh1*$^-$ cells formed filaments containing multiple septa and nuclei (Figure 3.2). This pseudohyphal phenotype was observed much less frequently (1%) in *mkh1*$^-$ cells grown at 23°C or 30°C and not at all in wild type cells. Also, this phenotype was only seen after *mkh1*$^-$ cells reached stationary phase in liquid cultures, or after at least 3 days of growth on agar plates.

Figure 3.2. Morphology of *mkh1*$^{-}$ cells at 23°C and 35°C. Cells were grown for 5 days on YEA plates then stained with DAPI and calcofluor (left panels). Right panels show cell morphology as observed by differential contrast (DIC) microscopy.

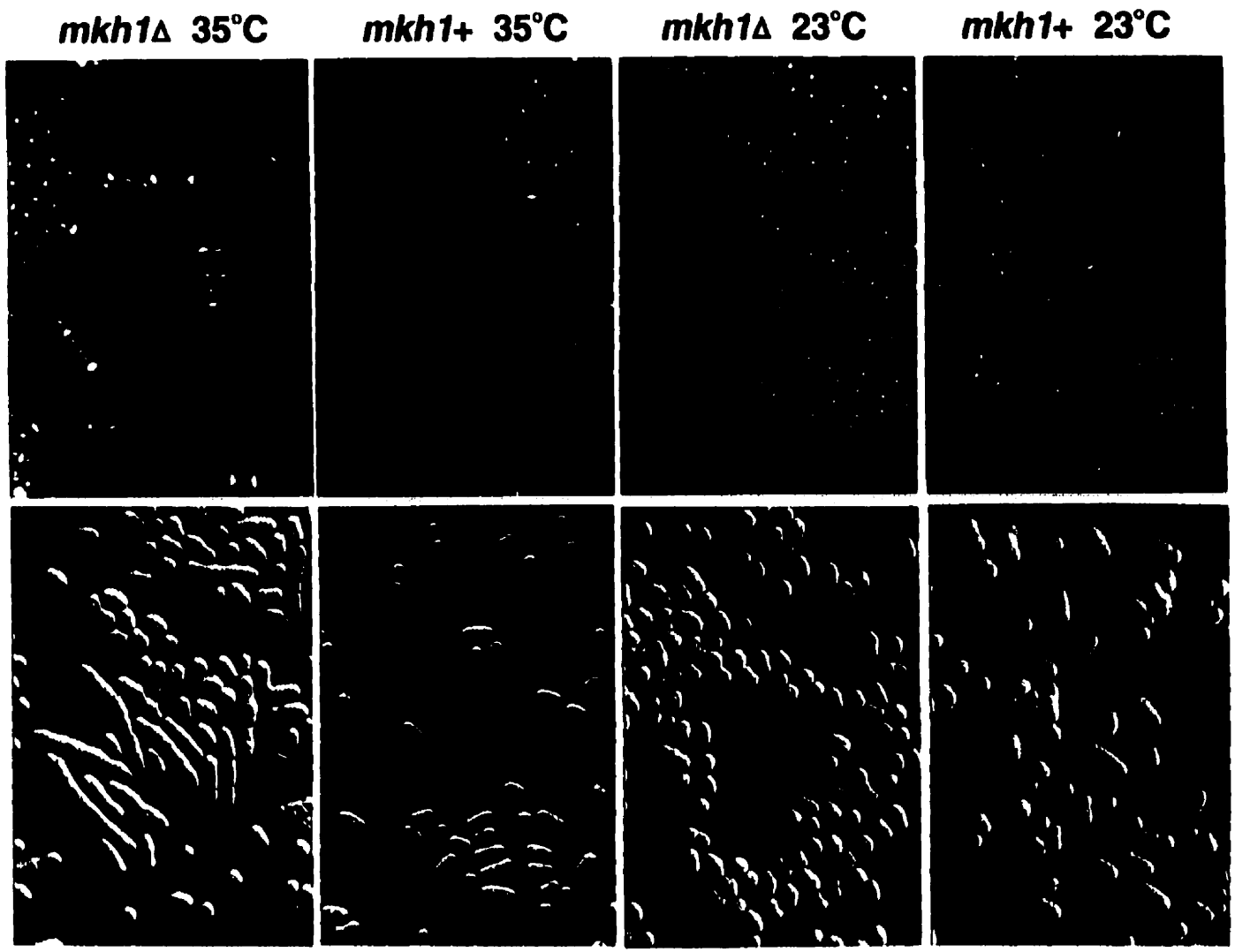

mkh1Δ 35°C
mkh1+ 35°C
mkh1Δ 23°C
mkh1+ 23°C

Table 3.1. Doubling times of various *mkh1*- strains. Growth rates of a wild type strain (SP870) overexpressing full-length Mkh1 (pALMK1) or the catalytic domain (pALMK2) were compared to empty plasmid (pAALN). *mkh1*$^-$ (AS1) was compared to a *mkh1*$^+$ strain (AS7) and a strain lacking the *ura4*$^+$ marker (AS2). Doubling times are measured in hours at 30°C by OD_{600} readings.

Strain + Plasmid[a]	PMA[b]
SP870 + pAALN	4.45 ± 0.07
SP870 + pALMK1	4.38 ± 0.08
SP870 + pALMK2	6.51 ± 0.05

Strain (Genotype)	YEA[b]
AS1 (*mkh1 ura4*$^+$)	2.04 ● 0.04
AS2 (*mkh1*$^+$ *ura4*)	2.32 ● 0.04
AS7 (*mkh1*$^+$ *ura4*$^+$)	2.11 ± 0.04

C) Role of Mkh1 in response to osmotic pressure:

mkh1$^-$ cells were tested for their ability to respond to osmotic stress. It was found that *mkh1*$^-$ cells were unable to grow in the presence of very high KCl concentrations (1.2 M), while growth of *mkh1*$^+$ cells was only partially inhibited (Figure 3.3). Similarly, *mkh1*$^-$ cell growth was inhibited by 0.9 M NaCl or 1.5 M sorbitol concentrations (data not shown). The pseudohyphal phenotype was much more apparent when *mkh1*$^-$ cells were grown on media containing 0.6 M KCL (Figure 3.4). Similar results were seen for cells grown on 1.0 M sorbitol (data not shown). Under such conditions, a larger fraction (~30%) of cells exhibited pseudohyphal growth, and cells appeared to be swollen. Also, the morphology of *mkh1*$^+$ and *mkh1*$^-$ colonies grown on media containing 0.6 M KCL were quite different. While *mkh1*$^+$ colonies exhibited a normal round shape, colonies of *mkh1*$^-$ cells exhibited an irregular shape with filamentous protrusions (Figure 3.4). Filamentous growth of *mkh1*$^-$ cells in hyperosmotic media was observed at low to high temperatures (23-35^{o}C), although it was enhanced at 35^{o}C. In contrast, *mkh1*$^+$ cells very rarely (0.1%) exhibited a pseudohyphal phenotype in response to high temperature or hyper-osmotic growth conditions after several days (Figures 3.2, 3.4).

D) Effect of stationary phase on *mkh1*$^-$ cells:

In collaboration with Dr. Nicholas Marini, it was determined that *mkh1*$^-$ cells required a significantly longer period of time to reenter the cell cycle upon reinoculation into fresh media following prolonged stationary phase arrest when compared to the *mkh1*$^+$ cells (Figure 3.5). This phenotype was not a function of cell viability as similar number of colonies grew from dilutions of stationary phase culture (data not shown).

Figure 3.3. KCl sensitivity of *mkh1*$^-$ cells. *mkh1*$^-$ cells expressing empty vector (pAALN), full-length Mkh1 (pALMK1) or GST-Spm1 fusion (pGSTSPM1) were compared to wild type *mkh1*$^+$ cells (AS7) carrying an empty vector (pAALN). Strains were streaked onto PMA and PMA + 1.2 M KCl plates and allowed to grow for 3 days at 30°C.

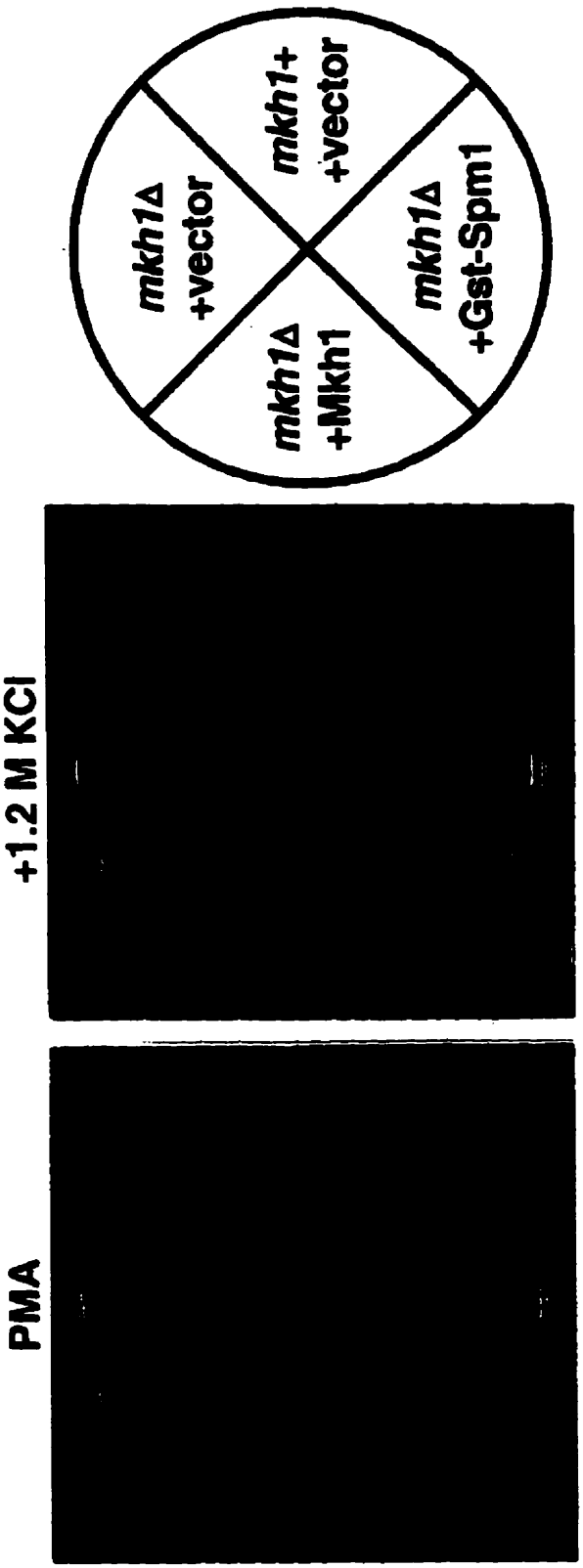

mkh1Δ +vector
mkh1+ +vector
mkh1Δ +Mkh1
mkh1Δ +Gst-Spm1
+1.2 M KCl
PMA

Figure 3.4. Morphology of *mkh1*$^-$ cells when grown in conditions of high salt. *mkh1*$^-$ and *mkh1*$^+$ cells were grown at 30°C on YEA plates containing 0.6 M KCl for 2 days. Cells were stained with DAPI and calcofluor (top panels) and observed by DIC microscopy (middle panels). Bottom panels reveal colony morphology as seen by light microscopy.

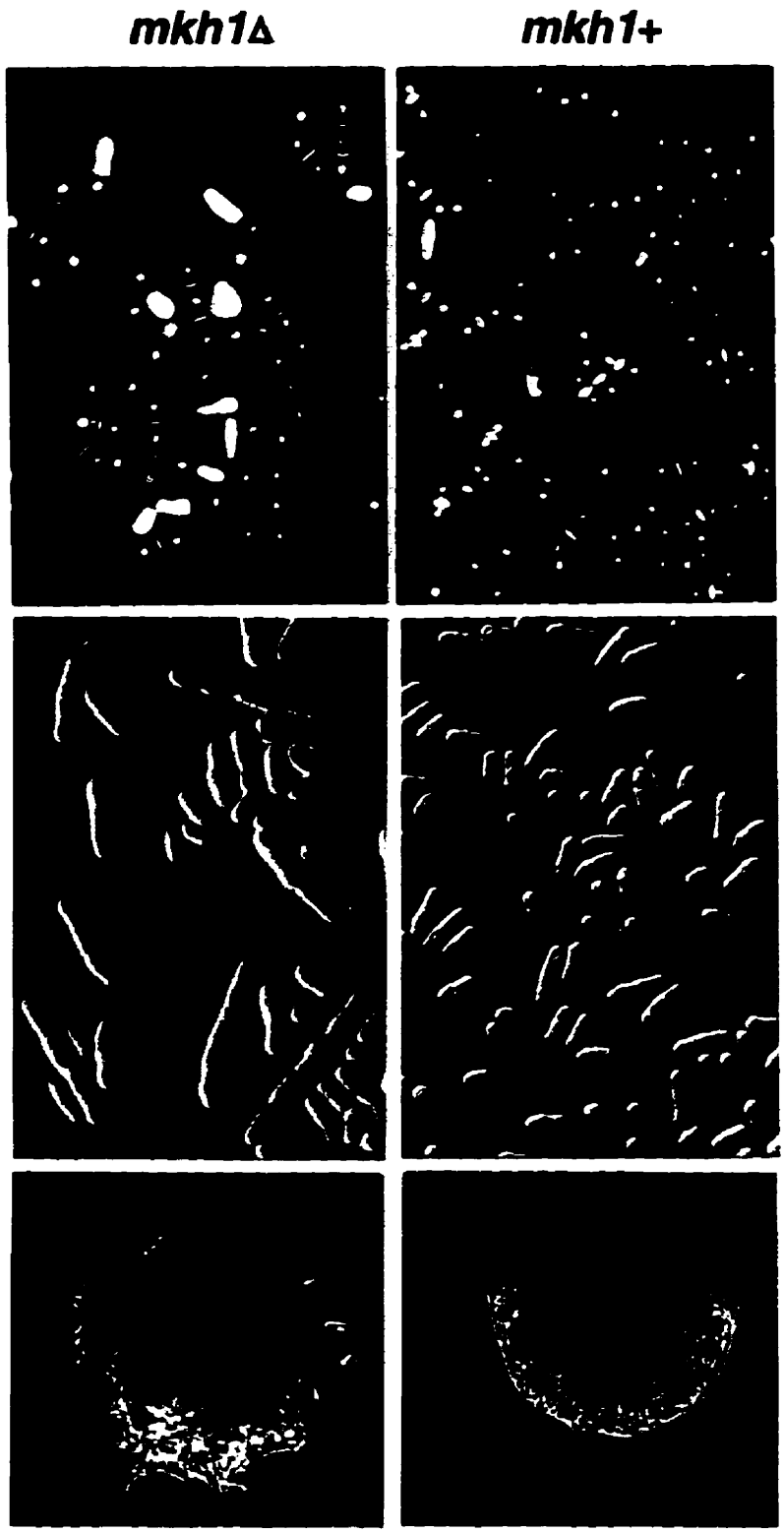
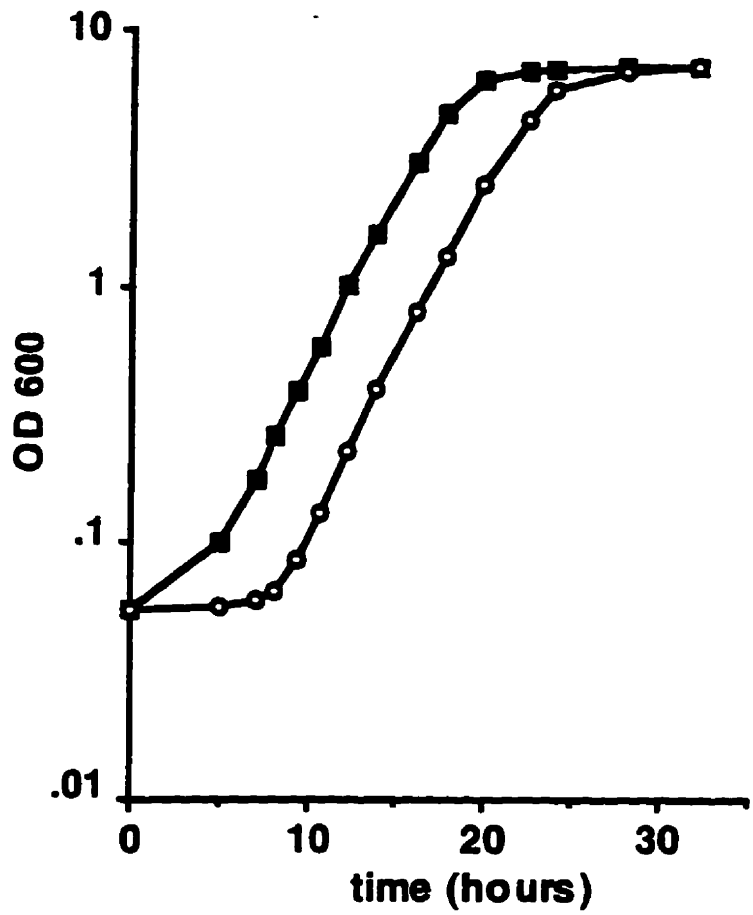

mkh1Δ
mkh1+

Figure 3.5. Recovery from stationary phase. *mkh1*$^-$ cells (AS1; open circles) and *mkh1*$^+$ cells (AS7; closed squares) were grown to mid-log phase and reinoculated into fresh liquid YEA media at a concentration of OD_{600}=0.05. After the cells reached stationary phase they were incubated for an additional 4 days. These were then used to inoculate fresh cultures in YEA at a concentration of OD_{600}=0.05, and growth curves were determined.


10
1
.1
.01
OD 600
0
10
20
30
time (hours)

E) Effect of low glucose and low nitrogen on *mkh1*$^-$ cells:

To test the effect of nutritional starvation, *mkh1*$^-$ cells were grown on media containing low glucose (0.5%) and low nitrogen (5 mM NH_4Cl). Normally agar plates contain 2% glucose and 93.5 mM NH_4Cl (Alfa, et al., 1993). *mkh1*$^-$ and *mkh1*$^+$ cells were grown on YEA agar plates for 3 days at 30°C. They were then replica plated onto agar plates severely restricted in either glucose or nitrogen and left at 30°C for 5 days. The strains were then replica plated on YEA plates and growth was monitored at 30°C. No difference in growth rate or viability was observed (data not shown). *mkh1*$^-$ cells did not display the pseudohyphal phenotype on either the glucose or nitrogen restrictive plates.

Discussion:

Haploid *mkh1*$^-$ spores were able to germinate and grow to form colonies. Thus, Mkh1 is not required for proper germination and growth. However, the *mkh1*$^-$ (AS1) strain grew much faster than the *mkh1*$^+$ (AS2) strain (Table 3.1). Since these strains were derived from a single ascus and should be identical in all other respects, there were two possible explanations for the results. One, deletion of the *mkh1*$^+$ allele resulted in an increased growth rate. Second, the AS2 strain was still auxotrophic for uracil and its growth could be limited by uracil. To test between these two possibilities, the AS7 strain was derived from AS2 by integrating a *ura4*$^+$ gene. The resulting strain showed a similar growth rate to the AS1 strain (data not shown). Thus, in *S. pombe* differences in selectable markers can have significant effects on growth. Henceforth, all strains used in experiments had identical markers, either integrated or plasmid borne.

Mkh1 was required for normal cell shape. Previously in this lab, Dr. Nicholas Marini showed, by comparing OD_{600} to cell numbers, that *mkh1*$^-$ cells were not smaller in volume

than *mkh1*$^+$ cells but were in fact just rounder and still maintained the same volume as the *mkh1*$^+$ cells. Several mutants in *S. pombe* have a round cell shape. *pck2*$^-$ cells have been shown to lack the ability to maintain cell polarity (Toda, et al., 1993). This phenotype is the result of failure to reorgnize actin (Kobori, et al., 1994) which is a requirement for cell wall formation and maintenance of normal morphology (Kobori, et al., 1989). *pmk1*$^-$ (*spm1*$^-$) cells have also been shown to display a round cell shape (Toda, et al., 1996). Ras1 is also required to maintain cell shape (Nadin-Davis, et al., 1986) through a Byr2 independent pathway (Wang, et al., 1991; Chang, et al, 1994). Thus, there may be a functional relationship between Mkh1 and these other proteins.

Overexpression of full-length or catalytic Mkh1 resulted in an elongated cell shape. Theoretically, the catalytic domain is constitutively active since any autoregulatory domains would have been removed with the amino-terminal domain (Blumer, et al., 1994). Thus, this could explain why the catalytic domain had a greater effect on cell shape than the full-length protein. The mechanism by which Mkh1 induces cell polarity and elongation is not known. Mkh1 may regulate actin reorganization such that excess cell wall materials are deposited causing elongation (Kobori, et al., 1989). Additionally, overexpression of Mkh1 may cause a mitotic delay. *wis1*$^-$ cells have an elongated cell shape phenotype resulting from a delay in initiating mitosis (Warbrick and Fantes, 1991).

The pseudohyphal phenotype of cells lacking Mkh1 indicates that they may be defective in cytokinesis. However, since only a percentage of cells have undergone pseudohyphal growth, perhaps there is only a partial block in cytokinesis. Genetic studies in yeast have helped to define several steps and mechanisms involved in cytokinesis (Fankhauser and Simanis, 1994). In *S. pombe*, an actin-based contractile ring forms in the

middle of the cell during early mitosis before anaphase. At the end of mitosis, the ring contracts as the septum forms at the site marked by the actin ring. Normally, the septum is then digested away, leading to the separation of the two daughter cells. However, under certain growth conditions, *S. pombe*, like other dimorphic yeast, exhibits pseudohyphal growth in which the daughter cells remain attached, resulting in pseudohyphae similar to those observed in *mkh1*$^-$ cells (Romano, 1966). Several genes involved in actin ring and septa formation in fission yeast have been defined (Chang et al., 1996; Marks et al., 1987; Nurse et al., 1976). However, very little is known about the mechanisms that regulate completion of cytokinesis after septa formation. The pseudohyphal phenotype of *mkh1*$^-$ cells could result from a defect in cell wall construction, septum structure, or in the regulation of enzymes that normally digest the septum away prior to cell separation. This phenotype was induced in Mkh1 defective cells by elevated temperature or high osmolarity in combination with several days of growth on agar plates, suggesting nutrient levels may also be involved in regulation of Mkh1 function.

In *S. pombe*, several genes have been implicated in pseudohyphal growth. Ppb1, a calcineurin-like protein, is required for regulating pseudohyphal growth. Deletion of *ppb1*$^+$ results in pseudohyphae at 23°C but not at 35°C (Yoshida, et al., 1994). Cells defective in the MAPK Spm1/Pmk1 also form pseudohyphae in hyperosmolar conditions (Zaitsevskaya-Carter and Cooper, 1997; Toda, et al., 1996). Recent studies have shown that pseudohyphal and invasive growth of *S. cerevisiae* require components of the mating pheromone-responsive MAPK cascade (Liu et al., 1993; Roberts and Fink, 1994). However, the proteins of the pheromone pathway in *S. pombe* have never been shown to be involved

in pseudohyphal formation. The filamentous growth of *mkh1*$^-$ cells suggests that Mkh1 may regulate pseudohyphal development in this yeast.

wis1$^-$ cells have been shown to greatly lose viability upon entry into stationary phase (Warbrick and Fantes, 1991). Loss of Mkh1 function had no effect on viability of *mkh1*$^-$ cells. However, *mkh1*$^-$ cells remained in a dormant state much longer than wild type cells. The mechanism of stationary phase exit is not well documented and, thus, the role of Mkh1 in stationary phase physiology is not known. However, considering all the above results, perhaps Mkh1 is required to adequately detect nutrient levels. Thus, *mkh1*$^-$ cells have failed to detect the fresh culture media, however, once detected, the cells are able to grow at the equivalent rate as that of the wild type cells.

The pseudohyphal phenotype was greatly enhanced the longer the cells were maintained at elevated temperature or high osmolarity. The cell shape phenotypes were also enhanced when cultures were grown to late logarithmic or stationary phase. *mkh1*$^-$ cells also had a defect in re-entering the cell cycle from stationary phase. These results imply that nutrient levels may be regulating the activity of Mkh1. However, glucose or nitrogen levels do not adversely impact *mkh1*$^-$ cells. In *S. cerevisiae*, low nitrogen levels result in pseudohyphal development (Gimeno, et al., 1992). Perhaps, low levels of a particular amino acid may be responsible for activation of Mkh1.

The deletion of *mkh1*$^+$ resulted in defects in the normal response to stresses such as high temperature and hyperosmolarity. These defects resulted in an abnormal morphology, delayed reentry from stationary phase into the cell cycle, sensitivity to high salt concentrations, and filamentous growth in response to stress. These observations suggest that Mkh1 may function in a MAPK pathway that responds to several stress conditions.

However, biochemical studies will be required to determine if Mkh1 is activated in response to each condition.

CHAPTER FOUR: RELATIONSHIP BETWEEN Mkh1 AND OTHER PATHWAYS

Introduction:

Another advantage of yeasts is the ability to examine the relationship of several genes to each other by studying their genetic behavior. Two strains mutated in different genes could be mated (provided neither is sterile) to generate strains that carry both mutations. Thus, by mating, strains carrying two or more mutated genes could be analyzed for phenotypic differences. Also, relationships between proteins could be determined by overexpression studies where plasmids carrying multiple copies of a wild type gene could be transformed into wild type or mutant strains to see what effects overexpression of that gene had on phenotypes.

Using the powerful genetic tools of yeasts, several genes of interest were examined for relationships to *mkh1*$^+$. The *S. pombe* MAPKK Wis1 was first identified as a mitotic inducer (Warbrick and Fantes, 1991); however, it had also been shown to be required for the regulation of mitosis and intracellular osmotic pressure in response to extracellular osmotic signals (Millar, et al., 1995; Shiozaki and Russell, 1995a,b). Deletion of *wis1*$^+$ caused a mitotic delay resulting in cell elongation, whereas overexpression of *wis1*$^+$ caused mitotic initiation to occur at a reduced cell size (Warbrick and Fantes, 1991). Defects in this pathway also resulted in growth inhibition at high temperature or hyperosmotic conditions (0.8 M KCl). As was shown in the previous chapter, overexpression of *mkh1*$^+$ resulted in an elongated cell shape, while *mkh1*$^-$ cells exhibited a round cell shape in standard growth conditions and pseudohyphal growth in hyper-osmotic media. Also, *mkh1*$^-$ growth was not temperature sensitive, but was shown to be inhibited by very high salt concentration (1.2 M

KCl). Considering these differences, it seemed unlikely that Mkh1 was an activator of the Wis1 pathway.

An *S. pombe* gene, *spm1*$^+$ (*pmk1*$^+$), encoding a novel MAPK was recently identified (Toda et al., 1996; Zaitsevskaya-Carter and Cooper, 1997). Deletion of *spm1*$^+$ resulted in phenotypes that are essentially identical to that of *mkh1*$^-$ cells, except that a larger fraction of *spm1*$^-$ cells exhibited pseudohyphal growth on synthetic media (PMA) at 30^{o}C. Spm1 was shown to function coordinately with Pck2 to maintain cell shape, cell wall integrity and response to conditions of high osmolarity (Toda, et al., 1996; Zaitsevskaya-Carter and Cooper, 1997). *spm1*$^-$ cells were also defective in exiting from stationary phase (Toda, et al., 1996). Thus, Spm1 seemed a likely candidate as the downstream MAPK component of the Mkh1 pathway.

Since Bck1, in *S. cerevisiae,* was the most closely related protein to Mkh1 in sequence, perhaps both proteins were also related in function. Many proteins that were structurally related have been shown to be functionally related as well. For example, *S. pombe* Pak1, a mating pathway protein kinase, was functionally homologous to *S. cerevisiae* Ste20 (Ottilie, et al., 1995), *S. pombe spk1*$^+$ could fully complement *S. cervisaie fus3* mutants (Neiman, et al., 1993) and mammalian MEKK1 can complement *S. cerevisiae bck1* cells (Blumer, et al., 1994). Since Bck1 and Mkh1 shared strong sequence homology, then perhaps their functions have also been conserved.

Bck1 has also been shown to act downstream of Pkc1 (Lee and Levin, 1992). In *S. pombe*, two protein kinase C homologs exist, Pck1 and Pck2 (Toda, et al., 1993; Mazzei, et al., 1993). While *pck1* mutants showed no distinct morphology, *pck2*$^-$ cells were round-shaped and similar to *mkh1*$^-$ cells (Toda, et al., 1993). Also, overexpression of *pck2*$^+$

resulted in two phenotypes: growth arrest (failure to form colonies) and pseudohyphal development (Toda, et al., 1993; Mazzei, et al., 1993). As mentioned above, Pck2 had already been shown to function coordinately with Spm1 (Toda, et al., 1996), while, in *S. cerevisiae*, Pkc1 functioned upstream of the Bck1 pathway (Lee and Levin, 1992). Thus, some divergence exists in the roles of protein kinase C homologs between the two species. Characterization of the relationship between Mkh1 and Spm1 and Pck2 should shed some light on the functions of these proteins in *S. pombe*.

Materials and Methods:

A) Yeast strains and genetic analysis:

The genotypes of *S. pombe* strains used in this study are: AS1 (*h90 leu1-32 ade6-210 ura4-d18 mkh1::ura4+*), AS7 (*h90 leu1-32 ade6-210*), TP47-2B (*h- leu1-32 pck2-8*), TP169-1C (*h- leu1-32 ura4-d18 pck2::LEU2*), AS13 (*h- leu1-32 ura4-d18 pck2::LEU2 mkh1::ura4+*), TZS69 (*h- leu1-32 ura4-d18 spm1::LEU2*), AS14 (*h? leu1-32 ura4-d18 spm1::LEU2 mkh1::ura4+*), ED904 (*h+ leu1-32 wis1::LEU2*), AS15 (*h? leu1-32 ura4-d18 wis1::LEU2 mkh1::ura4+*), 1788 (*MATa/MATα ura3/ura3 leu2/leu2 his4/his4 trp1/trp1 can1/can1*), and DL251 (*MATa/MATα bck1::URA3/bck1::URA3*; isogenic with 1788). TP47-2B and TP169-1C were provided by Dr. T. Toda (Toda, et al., 1993). TZS69 was provided by Dr. J. A. Cooper (Zaitsevskaya-Carter and Cooper, 1997). ED904 was provided by Dr. P. Fantes, University of Edinburgh. 1788 and DL251 were provided by Dr. D. E. Levin (Lee and Levin, 1992). Yeast culture, transformation, iodine staining, mating, tetrad analysis, and other genetic manipulations were performed as previously described (Moreno, et al., 1991).

B) Plasmids:

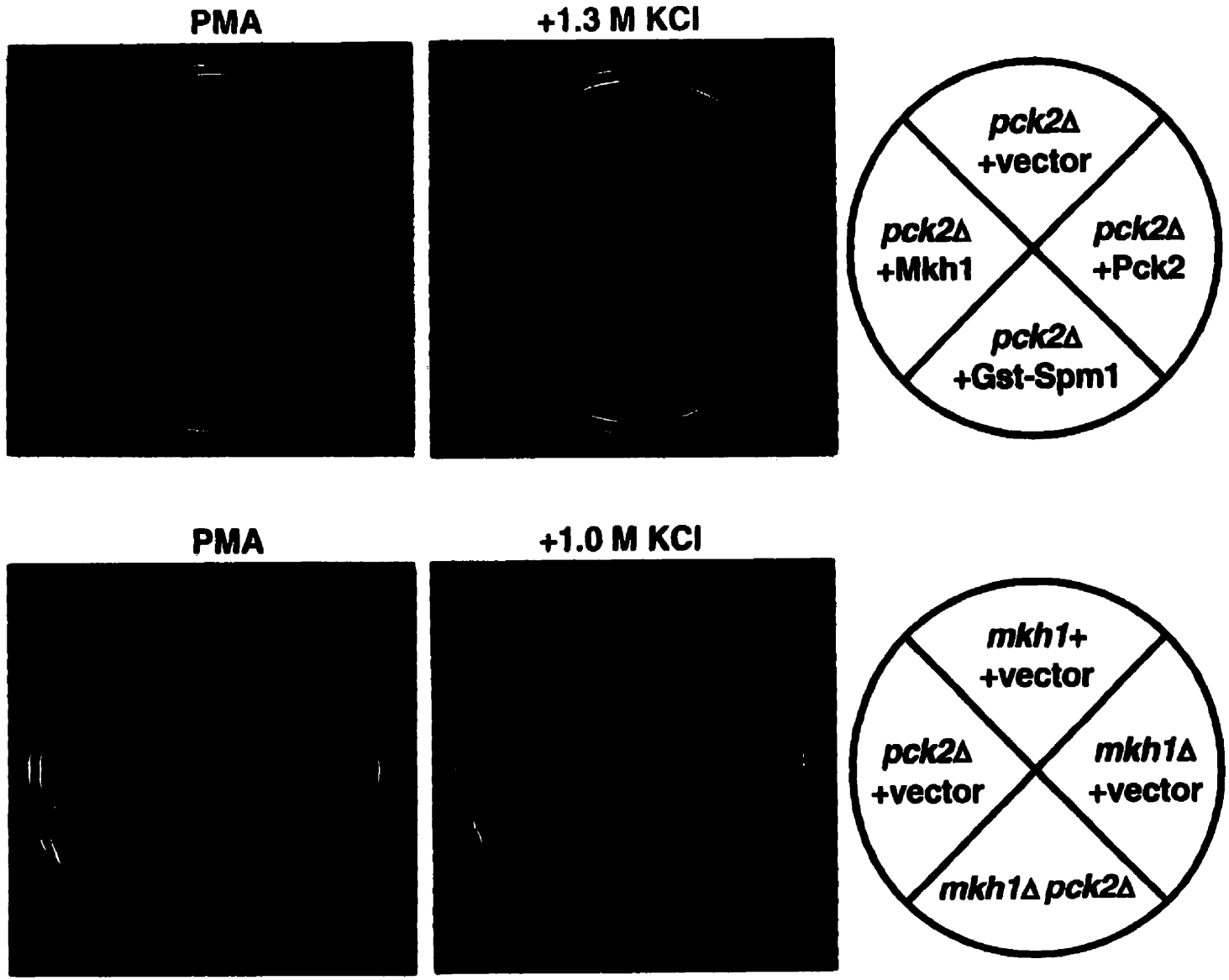
pAALN is a *S. pombe* expression vector containing *LEU2*, the *ars1*$^+$ sequence, and *adh1* promoter sequence flanking a polylinker site (Yu, et al., 1994; Xu, et al., 1990). pALMK1 contains the coding sequence of *mkh1*$^+$ cloned into the *Bam*HI-*Sac*I sites of pAALN. pREP1, pREP3 and pREP41X are *S. pombe* expression vectors containing the repressible *nmt1* promoter (Maundrell, 1993; Basi, et al., 1993). The pREP1 and pREP3 plasmids are high expressing whereas the pREP41X has an attenuated *nmt1* promoter. pR4L-GSPM1 contains the coding sequence for a GST-Spm1 fusion in pREP-41X (Zaitsevskaya-Carter and Cooper, 1997). pREP1-Wis1 and pREP1-GSTSpc1 contain the coding sequence for Wis1 and a GST-Spc1 fusion in pREP1, respectively (Shiozaki and Russell, 1995b). pR3L-PCK2 was constructed by inserting the coding sequence of Pck2 into the *Sal*I-*Bam*HI sites of pREP3. pPCK2-2B is a multicopy plasmid containing the *pck2*$^+$ gene (Toda, et al., 1993).

Results:

A) Roles of Mkh1 and Pck2 to maintain osmoresistance:

pck2$^-$ cells failed to grow on plates containing KCl, but they were not as sensitive as *mkh1*$^-$ cells since a higher concentration of KCl was required to completely inhibit *pck2*$^-$ cell growth (Figure 4.1; Figure 3.4). Overexpression of Mkh1 in *pck2*$^-$ cells failed to complement this defect (Figure 4.1). Similarly, overexpression of Pck2 failed to complement KCl sensitivity of *mkh1*$^-$ cells (data not shown). Furthermore, the double *pck2*$^-$*mkh1*$^-$ mutant was more sensitive to KCl than either single mutant strain (Figure 4.1).

Figure 4.1. KCl sensitivity of *pck2*$^-$ cells. Above) *pck2*$^-$ cells (TP47-2B) expressing full-length Mkh1 (pALMK1), GST-Spm1 fusion (pGSTSPM1) or carrying an empty vector (pAALN) or genomic fragment containing *pck2*$^+$ (pPCK2-2B) were streaked onto PMA and PMA + 1.3 M KCl plates. Below) *pck2*$^-$ cells (TP47-2B) carrying an empty vector (pAALN), *mkh1*$^-$ cells carrying an empty vector (pAALN), the double mutant *mkh1*$^-$*pck2*$^-$ (AS13) and *mkh1*$^+$ control strain carrying an empty plasmid (pAALN) were streaked onto PMA and PMA + 1.0 M KCl plates. In all panels, strains were grown for 3 days at 30°C.


PMA
+1.3 M KCl
pck2Δ
+vector
pck2Δ
+Mkh1
pck2Δ
+Pck2
pck2Δ
+Gst-Spm1
PMA
+1.0 M KCl
mkh1+
+vector
pck2Δ
+vector
mkh1Δ
+vector
mkh1Δ pck2Δ

B) Roles of Mkh1 and Pck2 to maintain cell morphology:

pck2$^-$ cells have a round-shaped morphology that is similar to that of *mkh1*$^-$ cells under normal growth conditions. The *pkc2*$^-$*mkh1*$^-$ double mutant also had a morphology similar to both *mkh1*$^-$ and *pck2*$^-$ cells (Figure 4.2). Also, the overexpression of Mkh1 in *pck2*$^-$ cells was able to restore a more normal, elongated cell shape in standard growth conditions (Figure 4.2).

C) Roles of Mkh1 and Pck2 in growth arrest and pseudohyphal growth:

As has been previously reported (Toda, et al., 1993), overexpression of Pck2 leads to cell growth arrest under normal growth conditions. Interestingly, it was observed that overexpression of Pck2 in *mkh1*$^-$ cells did not lead to growth arrest (Figure 4.3) in de-repressed conditions.

It has been shown previously that overexpression of Pck2 also results in the formation of multi-septated cells, very similar to those shown by *mkh1*$^-$ cells, in addition to growth arrest (Toda, et al., 1993; Mazzei, et al., 1993). Overexpression of Pck2 in either *mkh1*$^+$ or *mkh1*$^-$ cells resulted in a large fraction of pseudohyphal cells in standard growth conditions (Figure 4.3). The double *pck2*$^-$*mkh1*$^-$ mutant did not exhibit a pseudohyphal phenotype when grown at high temperature or on hyperosmotic media (Figure 4.2).

D) Relationship between Mkh1 and Spm1:

Overexpression of GST-Spm1 in *mkh1*$^-$ cells suppressed the sensitivities to KCl (Figure 3.3) but only partially suppressed the pseudohyphal growth phenotype (data not shown). However, it was also found that the GST-Spm1 fusion did not fully suppress the pseudohyphal phenotype of *spm1*$^-$ cells either (data not shown). In constrast, overexpression of Mkh1 in *spm1*$^-$ cells failed to complement any of the known mutant phenotypes, including

Figure 4.2. Morphology of *pck2⁻mkh1⁻* cells compared to *pck2⁻* cells. *pck2⁻* cells (TP47-2B) carrying empty vector (pAALN) or full-length Mkh1 (pALMK1) and *pck2⁻mkh1⁻* (AS13) cells were grown on PMA or PMA + 0.8 M KCl at 30°C for 3 days and examined by DIC microscopy.

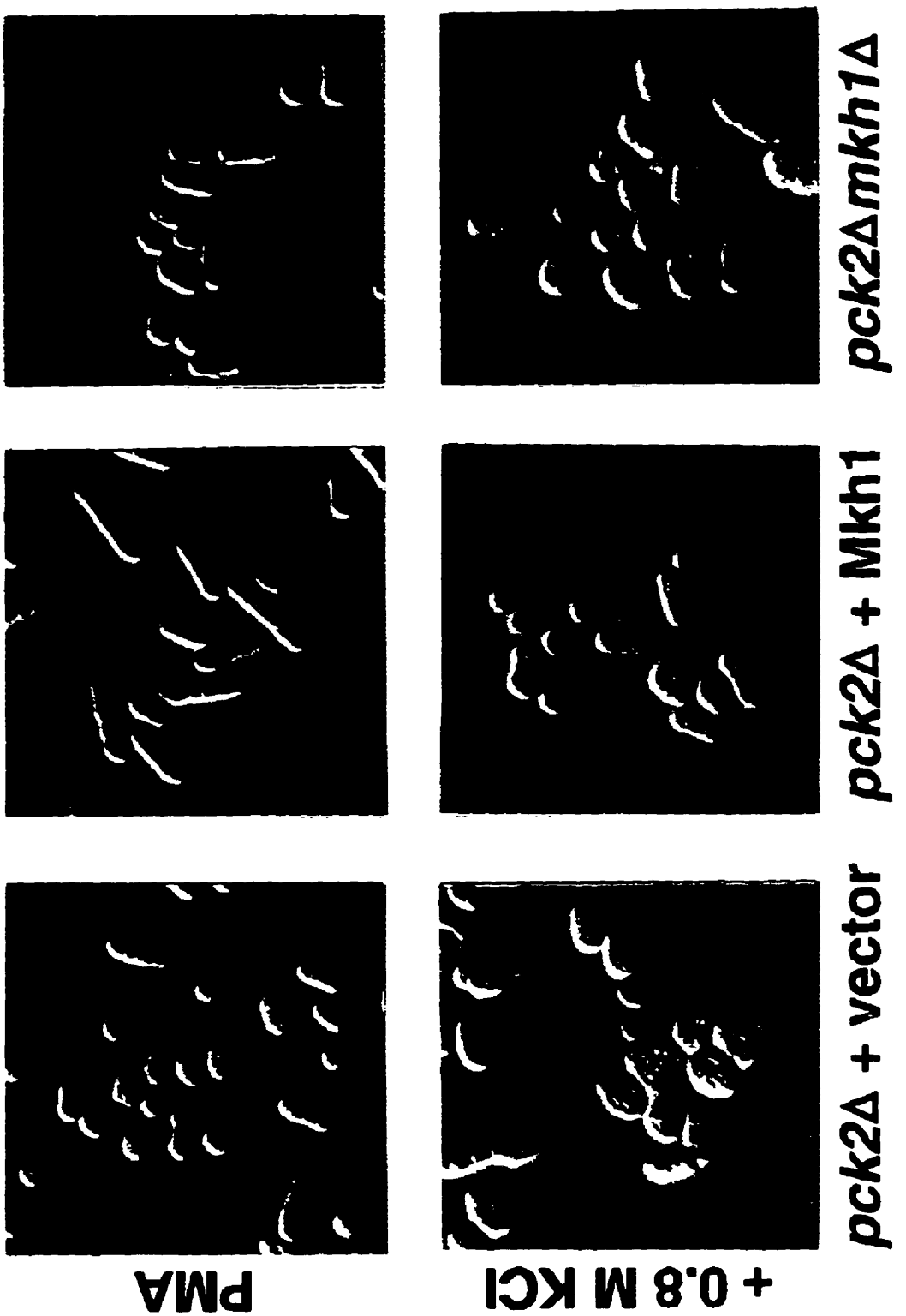
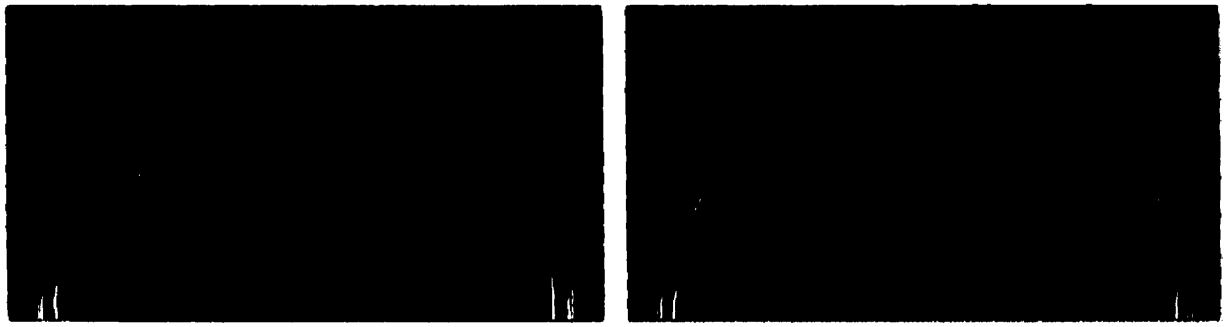
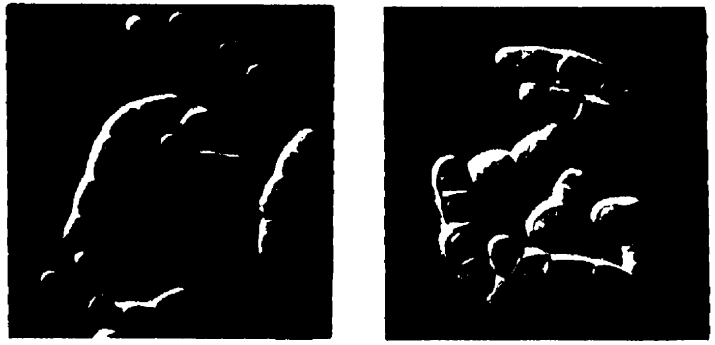

pck2Δ + vector
pck2Δ + Mkh1
pck2Δmkh1Δ
+ 0.8 M KCl
PMA

Figure 4.3. Inducible Pck2 overexpression (lack of thiamine) fails to inhibit growth in the absence of Mkh1. Panel A: *mkh1*$^{-}$ (AS1) and *mkh1*$^{+}$ (AS7) strains were transformed with a plasmid carrying a repressible *nmt* promoter driven *pck2*$^{+}$. These strains were grown on PMA plates with (to repress the *nmt* promoter) or without thiamine (to induce the *nmt* promoter) for 3 days at 30°C. Panel B: Morphology of cells grown on PMA, without thiamine, at 30°C for 3 days and examined by DIC microscopy.

A.


-thiamine
+thiamine
mkh1Δ +Pck2
mkh1+ +Pck2
mkh1Δ +Pck2
mkh1+ +Pck2

B.


mkh1Δ +Pck2
mkh1+ +Pck2

cell shape, pseudohyphal growth or KCl sensitivity (data not shown). A *mkh1⁻spm1⁻* double mutant strain was generated and it was found that the cell morphology of this strain was essentially identical to each single mutant (Figure 4.4). Also, the *mkh1⁻spm1⁻* strain displayed the pseudohyphal morphology on 0.6 M KCl and growth inhibition on 1.2 M KCl much like the *mkh1⁻* and *spm1⁻* strains.

E) Relationship between Spm1 and Pck2:

Expression of GST-Spm1 in *pck2⁻* cells partially restored normal cell morphology (data not shown) and resistance to KCl (Figure 4.1). Thus, Spm1 can partially compensate for some of the *pck2⁻* phenotypes, but deletion of either gene results in distinct phenotypes.

F) Function of Mkh1 in *S. cerevisaie*:

Overexpression of either the full length Mkh1 or the catalytic domain of Mkh1 failed to complement *bck1* mutants in *S. cerevisiae* (Figure 4.5). Expression of either of these constructs was unable to suppress cell lysis at elevated temperature. Surprisingly, expression of Mkh1 resulted in hypersensitivity to lysis in the *bck1* mutant (Figure 4.5) but not in the wild type *BCK1* strain (data not shown). Also, expression of the catalytic domain of Mkh1 failed to complement the lysis defect of *bck1* cells and also did not result in hypersensitivity to lysis (data not shown). *bck1* cells were more sensitive on plates containing 1% dextrose.

G) Relationship between Wis1 and Mkh1:

Since the Wis1-Sty1/Spc1 pathway is also osmoregulated, it seemed possible that Mkh1 may belong to this pathway. Overexpression of Wis1 or GST-Spc1 in *mkh1⁻* cells failed to complement the multi-septate phenotype or sensitivity to KCl (data not shown). To further investigate the relationship between Mkh1 and Wis1, *mkh1⁻wis1⁻* double mutants were generated. It was found that they had a morphology that appeared to reflect a

Figure 4.4. Morphology of *wis1⁻mkh1⁻* cells and *mkh1⁻spm1⁻* cells and compared to the single mutants. Above) Morphology of *wis1⁻* cells (ED904) compared to *wis1⁻mkh1⁻* cells (AS15). Below) *mkh1⁻* cells (AS1) carrying an empty vector (pAALN) and *spm1⁻* cells (TZS69) carrying an empty plasmid (pREP42X) were compared to *mkh1⁻spm1⁻* cells (AS14). All strains were grown on PMA at 30°C for 3 days and examined by DIC microscopy.

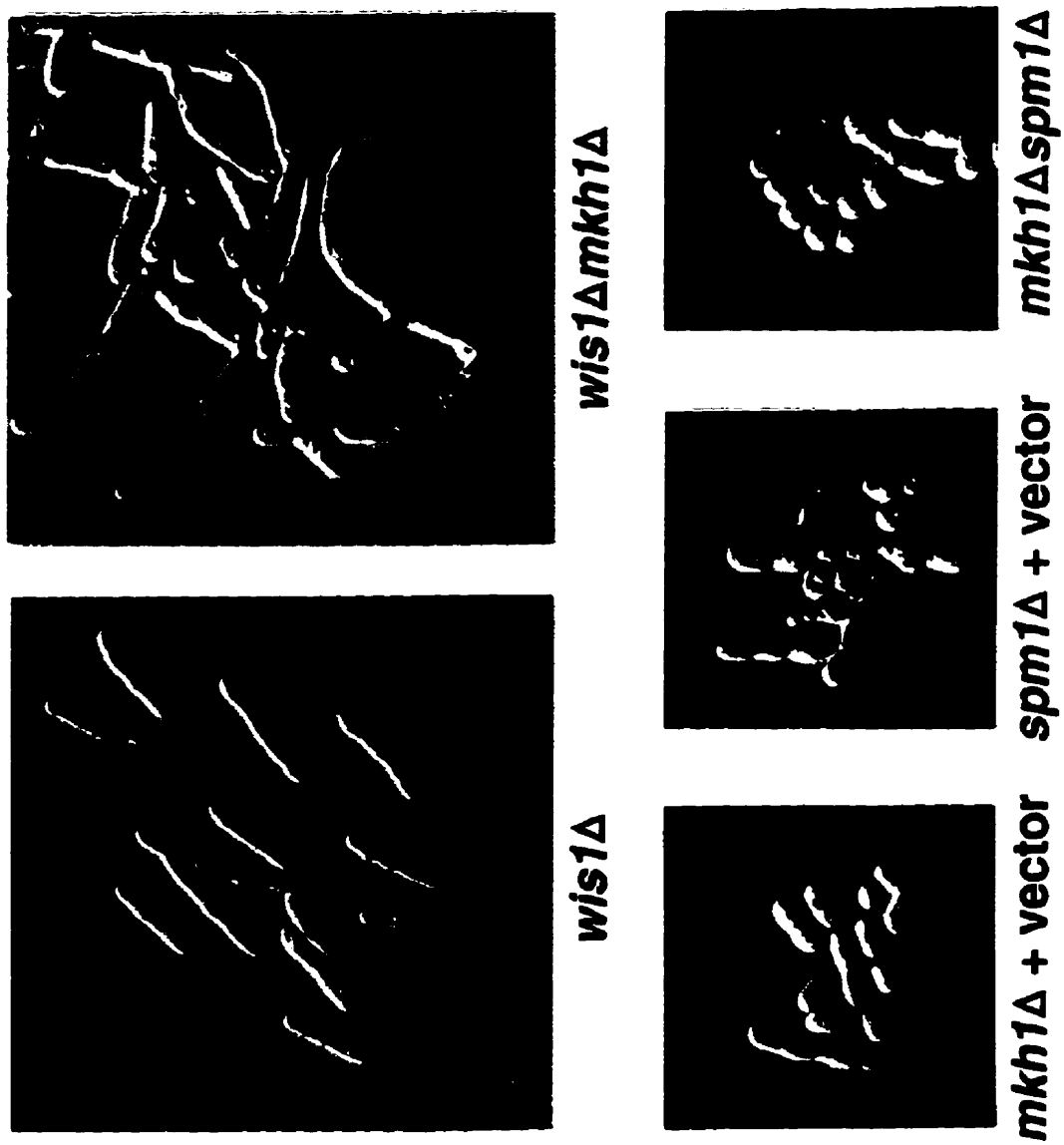
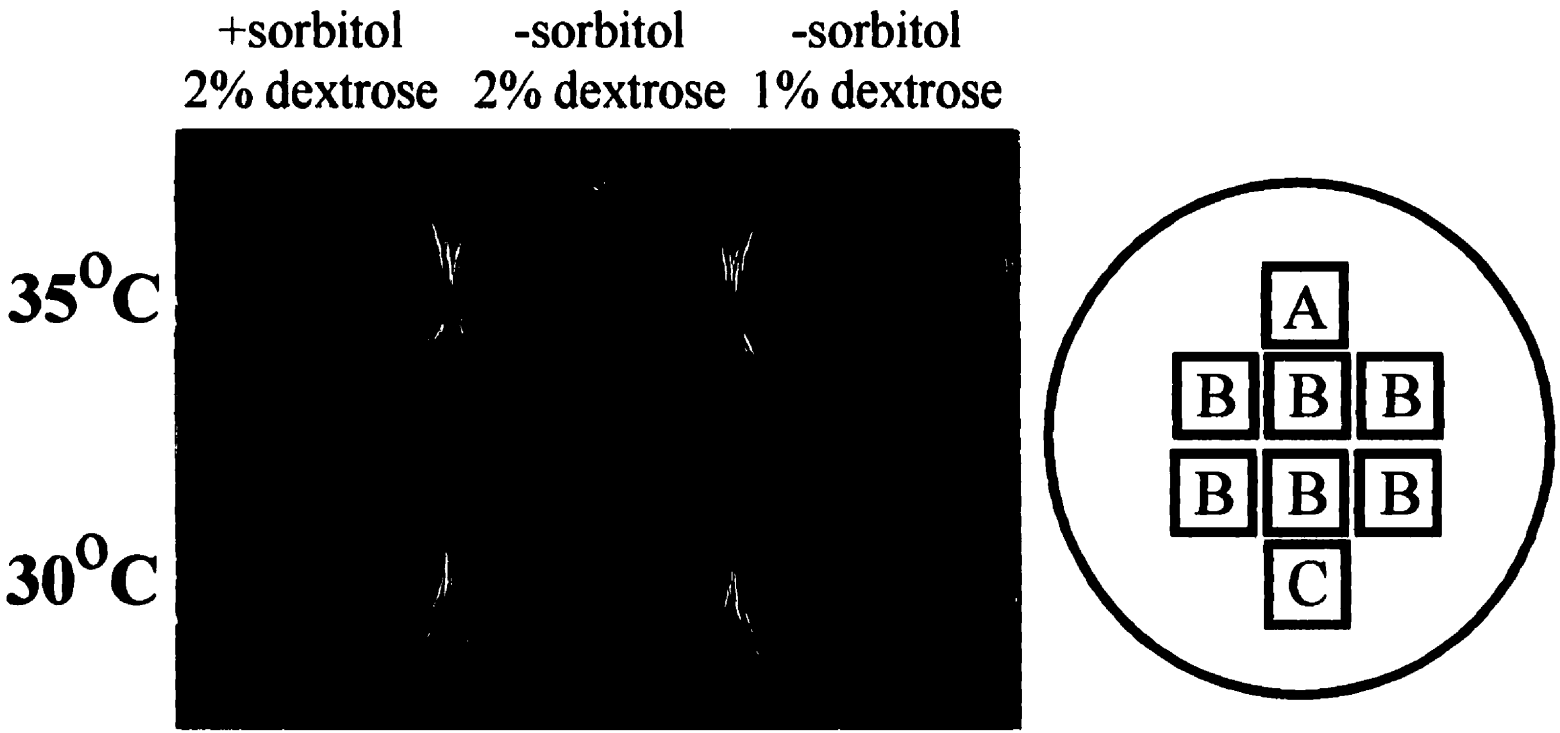

wis1Δ
wis1Δmkh1Δ
mkh1Δ + vector
spm1Δ + vector
mkh1Δspm1Δ

Figure 4.5. Expression of Mkh1 in *bck1* cells. A) *bck1* cells (DL251) carrying empty vector (pADANS). B) 6 independent clones of *bck1* cells carrying a vector expressing full-length Mkh1 (pADLMK2). C) Wild type *BCK1* cells (1788) carrying pADANS. Strains were patched onto SC - Leu plates containing 1.0 M sorbitol and grown for 2 days at 22°C. This plate was replica plated onto SC - Leu plates containing 1% or 2% dextrose, with or without sorbitol. These plates were grown at 30°C or 35°C for 2 days.


+sorbitol
2% dextrose
-sorbitol
2% dextrose
-sorbitol
1% dextrose
35°C
30°C
A
B
B
B
B
B
B
B
B
C

combination of the *wis1*$^-$ and *mkh1*$^-$ phenotypes. These cells appeared highly elongated and multiseptated and there was some branching (Figure 4.4).

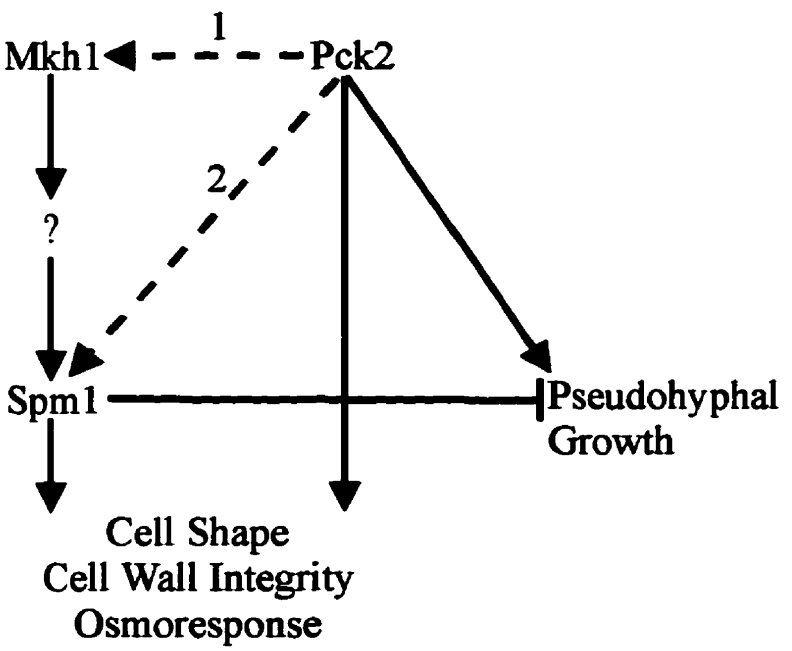
Discussion:

A) Relationship of Mkh1 to other *S. pombe* MAPK pathways:

Mkh1 appears to be important for a variety of cellular responses to stress and nutrient limitation. By analogy with known MAPK pathways, there are most likely two other kinases that act downstream from Mkh1, a MAPKK and a MAPK. The *S. pombe* Wis1 pathway is also responsive to hypertonic stress (Shiozaki and Russell, 1995a; Millar, et al., 1995), raising the possibility that Wis1 is activated by Mkh1. However, defects in the Wis1 pathway result in phenotypes that are very different from those exhibited by *mkh1*$^-$ cells (Warbrick and Fantes, 1991). The observation that deletion of both *mkh1*$^+$ and *wis1*$^+$ resulted in an additive phenotype suggests that these proteins act in different pathways.

The phenotypes resulting from *spm1*$^-$ cells are very similar to those of *mkh1*$^-$ cells (Toda, et al., 1996; Zaitsevskaya-Carter and Cooper, 1997). The *mkh1*$^-$*spm1*$^-$ mutant exhibits phenotypes very similar to the single mutants. Also, expression of GST-Spm1 complements most of the *mkh1*$^-$ phenotypes. Expression of GST-Spm1 was unable to complement the pseudohyphal phenotype in *mkh1*$^-$ cells, however, nor was the expression of that construct able to complement the pseudohyphal phenotype in *spm1*$^-$ cells either. Perhaps the GST moiety is interfering with the full activity of Spm1. In contrast, overexpression of Mkh1 did not rescue *spm1*$^-$ phenotypes. Additionally, the morphology of the *mkh1*$^-$*wis1*$^-$ double mutant is identical to that exhibited by *spm1*$^-$*wis1*$^-$ cells (Zaitsevskaya-Carter and Cooper, 1997). These results support the model that Spm1 acts downstream of Mkh1 (Figure 4.6).

Figure 4.6. Model of relationship between Mkh1, Spm1 and Pck2. [1]Pck2 may be acting upstream of Mkh1 with respect to growth inhibition by Pck2 overexpression. Spm1 acts downstream of Mkh1 and [2]may act downstream of Pck2 as well, however, results indicate that Spm1 and Pck2 are more likely acting independently to maintain cell shape, cell wall integrity and osmoresponse. The Mkh1-Spm1 pathway acts in opposition to Pck2 to regulate pseudohyphal growth.


Mkh1
1
Pck2
2
?
Spm1
Pseudohyphal
Growth
Cell Shape
Cell Wall Integrity
Osmoresponse

B) Relationship of Pck2 with Mkh1 and Spm1:

Many of the observations suggest that Pck2 and Mkh1 act to regulate similar cellular responses. On the one hand, the similar morphology and sensitivities to KCl shown by *pck2*$^-$ and *mkh1*$^-$ mutants suggest that they have related roles. On the other hand, the apparent opposite effect of these proteins on pseudohyphal growth suggests that they function antagonistically. Thus, it appears that Pck2 and Mkh1, in some cases, act cooperatively (cell shape and KCL resistance), and in another they act in opposition (pseudohyphal growth).

To complicate matters further, high levels of Pck2 did not inhibit growth in *mkh1*$^-$ cells as it does in a wild type background. Thus, it appears that Mkh1 can act downstream of Pck2 to inhibit growth. Recall from Chapter 3, expression of the catalytic domain of Mkh1 also resulted in growth inhibition. Thus, this is evidence that supports a model of Mkh1 acting downstream of Pck2 (Figure 4.6).

Overexpression of GST-Spm1 partially complements some of the *pck2*$^-$ mutant phenotypes and is consistent with model of Spm1 acting either downstream or independent of Pck2. Also, Mkh1 can complement the cell morphology of Pck2, returning the cells to a wild type cell shape. However, a previous report that *pck2*$^-$ and *pmk1*$^-$ (*spm1*$^-$) mutations have synergistic effects on cell wall integrity favors a model where these proteins act independently (Toda et al., 1996). Thus, perhaps Mkh1 and Spm1 can function coordinately with Pck2 to regulate cell shape and osmoresistance.

As one can see, the role of Pck2 in the Mkh1-Spm1 pathway appears very complex. In *S. cervisiae*, Pkc1 function bifurcates into two pathways, one being the Bck1 pathway (Lee and Levin, 1992). Recently the Swi4 transcription factor has been suggested as possibly acting in the second pathway. Swi4 has been shown to increase transcription of genes that

are also regulated by the Bck1 pathway, however, Swi4 and Bck1 act independently (Igual, et al., 1996). Thus, it is possible that Pkc1 is controlling two pathways that are both involved in cell integrity. Perhaps then, in *S. pombe*, Pck2 can function both upstream and independently of Mkh1, thus, mimicking the situation in *S. cerevisiae*.

C) Conservation of Mkh1 and Bck1 pathways:

The relatively strong conservation between Mkh1 and Bck1 suggested that these proteins may be functionally related. However, the deletion of these genes result in very distinct phenotypes. Deletion of *mkh1*$^+$ does not result in a temperature-sensitive cell lysis defect, like that exhibited by *bck1* cells (Lee and Levin, 1992). Furthermore, while *bck1* cells are stabilized by hyperosmotic conditions, *mkh1*$^-$ cell growth is sensitive to hyperosmotic conditions (Lee and Levin, 1992). It was also found, by Dr. Dallan Young, that deletion of *mkh1*$^+$ had no apparent effect on induced thermotolerance (data not shown), whereas *bck1* cells exhibit a reduced induced thermotolerance (Kamada et al., 1995). The observation that expression of Mkh1 fails to complement *bck1* indicates that the functional properties of these related MEKKs have not been highly conserved. In fact, the evidence suggests that Mkh1 may have a function that is antagonistic to Bck1. However, a previous report indicates that expression of Mpk1, the MAPK downstream from Bck1, can complement deletion of *pmk1*$^+$ (*spm1*$^+$) (Toda et al., 1996). This suggests that some parts of the pathway have been conserved in function. Perhaps then Mkh1 can interact with components upstream of Bck1, thus, generating a pseudo-dominant-negative phenotype. Such an upstream protein could be Pkc1, *pkc1* cells are more sensitive to lysis than *bck1* cells (Lee and Levin, 1992). Overexpression of the catalytic domain of Mkh1 was not able to induce hypersensitivity of *bck1* cells to temperature. This observation supports the notion

that Mkh1 may be able to bind upstream proteins, since, the catalytic domain would lack any regulatory motifs, such as a Pkc1 binding site. Mkh1 is unable to induce temperature sensitivity in *BCK1* cells perhaps due to a higher specificity Bck1 may have with upstream *S. cerevisiae* proteins.

CHAPTER FIVE: SUMMARY AND FUTURE DIRECTIONS

A novel member of the MAPKKK family of protein kinases, *mkh1*$^+$, was isolated and characterized from *S. pombe*. *mkh1*$^+$ encodes a 1116 amino acid serine/threonine kinase. Mutants of Mkh1 exhibit defects in cell shape, cytokinesis, cell cycle and osmolar response. These defects appeared to be enhanced in conditions of nutritional limitation. Thus, Mkh1 appears to be involved in a pathway responsive to various stresses. Mkh1 function is independent of Wis1 and Pck2, since, the double mutants with *mkh1*$^-$ show additive phenotypes. However, Mkh1 appears to be functioning upstream of the MAPK Spm1. The double mutant, *mkh1*$^-$*spm1*$^-$, behaves much like the single mutants. Also, expression of Spm1 is able to suppress most of the *mkh1*$^-$ phenotypes to nearly wild type levels. Table 5.1 summarizes the results presented in the earlier chapters.

Additionally, in this lab, Dr. Nancy Markley has peformed experiments that have further characterized the relationship between Mkh1 and Spm1. Pck2 has been implicated in maintenance of cell wall integrity (Kobori, et al., 1994). It has been shown that, upon digestion of the cell wall with β-glucanase, *pck2*$^-$ cells are unable to regenerate their cell walls (Kobori, et al., 1994). Also, growth of *pck2*$^-$ cells is sensitive to β-glucanase. Dr. Markley has shown in parallel experiments that *mkh1*$^-$ mutants are fully capable of regenerating their cell wall (Sengar, et al., 1997). However, like *pck2*$^-$ cells, *mkh1*$^-$ cells are growth sensitive to β-glucanase. This suggests Mkh1 has some role in maintaining cell wall integrity (Sengar, et al., 1997). The double mutant (*mkh1*$^-$*pck2*$^-$) was more sensitive to β-glucanase than either single mutant and Mkh1 expression was unable to rescue *pck2*$^-$ sensitivity. Together, these results support the model that Pck2 and Mkh1 are independent

Table 5.1. Summary of Results. $^{+}$ = denotes wild type gene. $^{-}$ = denotes mutant gene. ↑ = denotes overexpression of gene. ms = denotes multiseptate phenotype. slow = denotes delayed growth.

Strain	23 °C/30 °C	35 °C	0.6 M KCl	1.2 M KCl	Exit from G_0
mkh1$^+$	normal	normal	normal	normal	normal
mkh1$^-$	round	ms	ms	dead	delayed
mkh1 ↑	long				
Cat - *mkh1* ↑	very long				
pck2$^-$	round	round	round	dead (1.3 M)	
pck2 ↑	ms, dead				
pck2$^-$/*mkh1*$^-$	round			dead (1.0 M)	
pck2$^-$/*mkh1* ↑	normal			dead	
pck2 ↑/*mkh1*$^-$	ms				
pck2$^-$/*spm1* ↑	normal			slow	
spm1$^-$	round	ms	ms	dead	delayed
spm1$^-$/*mkh1* ↑	round	ms	ms	dead	
spm1$^-$/*mkh1*$^-$	round	ms	ms	dead	
spm1 ↑/*mkh1*$^-$	normal	ms	ms	normal	
wis1$^-$	long				
wis1 ↑/*mkh1*$^-$	round		dead	dead	
wis1$^-$/*mkh1*$^-$	long, ms				

in their functions; however, they act coordinately to maintain cell wall integrity (Sengar, et al., 1997).

Interestingly, while *spm1*$^-$ phenotypes and *mkh1*$^-$ phenotypes are virtually identical, there was one discrepancy reported. Toda, et al (1996), have reported that *pmk1*$^-$ (*spm1*$^-$) cells confer resistance to high NaCl concentrations. This result conflicts with experiments performed in this lab and the results from Zaitsevskaya-Carter and Cooper (1997). It has been shown here that *mkh1*$^-$ cells are sensitive to KCl, NaCl and sorbitol. Also, Spm1 has been shown to be activated by high salt (Zaitsevskaya-Carter and Cooper, 1997). The reasons for this discrepancy are not yet known.

Much has been learned about the role of Mkh1, however, there are several questions about the Mkh1 pathway that are still unresolved. Even though the genetic evidence suggests that Spm1 is downstream of Mkh1, biochemical studies are required to really provide conclusive proof. For example, the kinase activity and phosphorylation of Spm1 will have to be compared in *mkh1*$^-$ and *mkh1*$^+$ backgrounds. Myelin basic protein (MBP) would be used as a substrate to measure Spm1 kinase activity (Zaitsevskaya-Carter and Cooper, 1997). If Mkh1 and Spm1 belong to a linear pathway, then one would expect that Spm1 activity would be significantly reduced in the absence of Mkh1. These experiments could be performed on cultures grown in various conditions. For example, the effects of elevated temperature and hyperosmolarity can be measured in terms of Spm1 activity.

Similarly, biochemical studies could be performed in *pck2*$^-$ mutants as well. Pck2 and Mkh1 appeared to function independently to regulate cell shape and response to hyperosmolar conditions. Biochemical evidence should reveal Spm1 activity unchanged in

pck2$^-$ cells. However, Pck2 and Mkh1 function in opposition to regulate pseudohyphal growth. Thus, perhaps Spm1 activity would be inhibited in strains overexpressing Pck2.

Traditionally, MAPK pathways have a trio of sequentially acting protein kinases. Thus, to further characterize the Mkh1 pathway, other components, such as a MAPKK, will have to be identified. Proteins that interact with Mkh1 may be identified using the yeast two-hybrid screen. Other proteins that function downstream of Mkh1 may be identified using a genetic screen. *mkh1*$^-$ cells fail to grow at high salt. Thus, a genomic library can be transformed into *mkh1*$^-$ cells and grown in conditions of high salt. Colonies that are able to grow on high salt may be expressing proteins that are functioning downstream, such as a MAPKK, Spm1 or transcription factors. Additionally, proteins that interact with Mkh1 may be isolated and identified by immunoprecipitation of Mkh1 from stimulated cultures. Complete MAPK cascades could be reconstituted *in vitro* and analysed for induction of Spm1 activity.

As mentioned before, the round cell shape phenotype of *mkh1*$^-$ cells resembles *pck2*$^-$ cells. Pck2 has been implicated in the reorganization of actin (Toda, et al., 1993). Thus, actin staining studies may reveal disrupted actin structures in *mkh1*$^-$ cells as well.

mkh1$^-$ cells are also sensitive to hyperosmolar conditions. In *S. cerevisiae*, the Hog1 pathway increases internal glycerol levels to counter increased osmolar pressure (Brewster, et al., 1993). Similarly, the Wis1-Spc1 pathway in *S. pombe* increases intracellular glycerol levels (Wilkinson, et al., 1996). This is accomplished by increasing the expression of glycerol-3-phosphate dehydrogenase, *gpd1*$^+$. Thus, if Mkh1 has a role in osmotic response, then perhaps it too regulates *gpd1*$^+$. Northern blot analysis would be performed to analyse *gpd1*$^+$ mRNA levels in *mkh1*$^-$ and *mkh1*$^+$ cells in response to high KCl levels. Alternatively,

Mkh1 may induce osmotic response through a Spc1 dependent pathway. Thus, to test this, *gpd1*$^{\bullet}$ levels could be measured by Northern blot analysis of *spc1*$^{-}$ strains overexpressing full-length Mkh1 or the constitutively active catalytic domain of Mkh1.

ATF-2-like transcription factors are involved in stress response in *S. pombe* (Shiozaki and Russell, 1996; Wilkinson, et al., 1996) and in mammals (Gupta, et al., 1995). Thus, Mkh1 may be regulating stress response through the actions of the *S. pombe* ATF-2-like proteins, Atf1 and Atf21. Phophorylation of these proteins could be measured in *mkh1*$^{-}$, *mkh1*$^{+}$ and Mkh1 overexpressing strains.

Even though Mkh1 was unable to suppress *bck1* mutant in *S. cerevisiae*, expression of Bck1 in *mkh1*$^{-}$ cells could be examined to see if the proteins are functionally homologous in *S. pombe*. Also, it has been shown that mammalian MEKK1 is functionally homologous to Bck1 (Blumer, et al., 1993). Since, MEKK1 is the most structurally homologous mammalian protein to Mkh1, it would be very interesting to see if expression of MEKK1 can suppress any of the *mkh1*$^{-}$ phenotypes.

Additionally, if Mkh1 expression in *bck1* cells is indeed interacting with upstream proteins, as the induced hypersensitivity to lysis may indicate, and this effect is not dependent on the catalytic domain, then the expression of the regulatory domain only in *bck1* cells should also induce temperature sensitivity. If this should be true, then perhaps Mkh1 is able to interact with upstream proteins. Thus, it would be very interesting to test if Mkh1 can physically interact with Pck2. This could be assayed using the yeast two-hybrid test or by co-immunoprecipitation and subsequent Western blot analysis.

To determine the role of Mkh1 in stationary phase exit, *mkh1*$^{-}$ and *mkh1*$^{+}$ cells can be stained with propidium iodide and anaylsed by flow fluorocytometry (Alfa, et al., 1993).

Propidium iodide is a DNA stain and can be used to measure DNA content of cells at various times of the cell cycle. Thus, strains could be allowed to enter and exit from stationary phase and analysed for differences in the way *mkh1*$^-$ cells may be behaving.

These are some experiments that could be performed in the near future and may provide further insight into the function of Mkh1. Given the amount of sequence and functional conservation that exists amoung MAPK cascades in various eukaryotes, the results obtained in this study and future studies of Mkh1 may provide valuable clues to the regulation and role of MAPK cascades in other organisms including humans.

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