Studies on the regulation and role of the cell integrity pathway of *Saccharomyces cerevisiae*

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Dedicated to my parents Jafar and Layla, for giving me strength and courage to carry on

Declaration

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Sahar Z. S. Sabetnia

"All things I thought I knew;
but now confess the more I know I know,
I know the less."

John Owen

Abstract

The Pkc1p-MAPK pathway of *S. cerevisiae* is required for proper cell surface construction and is hence referred to as the cell integrity pathway. The cell integrity pathway is known to be activated in response to cell surface damage, such as heat, hypo-osmotic shock, mating pheromone treatment, calcofluor white, zymolyase, secretion blocks/tunicamycin treatment and addition of membrane deforming compound, chlorpromazine. *HCS77* and *MID2* encode type-I transmembrane proteins that are putative receptors of cell surface damage. Hcs77p is known to be required for activation of the Pkc1p-MAPK pathway during heat shock. In this study we identify Mid2p as an upstream regulator of Pkc1p-MAPK pathway during cell surface stress. Furthermore, we find that Mid2p and Hcs77p are redundant activators of the cell integrity pathway. First, it we find that *MID2* genetically interacts with the Pkc1p-MAPK pathway, functioning upstream of *PKC1* and the MAPK cascade. Second, *MID2* is a suppressor of the cell lysis defect of *hcs77Δ* cells at elevated temperatures. Third, *mid2Δhcs77Δ* cells are inviable in the S288C strain background and viable only on osmotically stabilised medium in the EG123 strain background. Fourth, Mpk1p activation assays show that Mid2p and Hcs77p function redundantly to activate the cell integrity pathway in response to heat shock and mating pheromone.

Mid2p and indeed the whole of the Pkc1p-MAPK pathway are required to maintain viability of cells during the mating response. First, *mid2Δ* cells display a pheromone induced death. Second, death of *mid2Δ* cells in presence of mating pheromone is suppressed by over-expression of Pkc1p-MAPK pathway components. Third, *HCS77* and *MPK1* are also involved in the mating response to maintain cell integrity, as null mutants of these components show loss of cell integrity during pheromone treatment. Fourth, the pheromone-induced death of *hcs77Δ* cells can be suppressed by over-expression of the Pkc1p-MAPK pathway components. Fifth, Mid2p and Hcs77p function as redundant activators of the Pkc1p-MAPK pathway in response to mating pheromone treatment. Sixth, death of Pkc1p-MAPK pathway mutants is as a result of cell lysis, probably due to defects in the cell wall. Seventh, the death of Pkc1p-MAPK pathway mutants in α-factor is not osmotically remedial. Eighth, the defect in the cell wall is not one associated with

improper chitin deposition during pheromone treatment. Ninth, Pkc1p-MAPK pathway mutants are not defective in actin localisation. Tenth, the pheromone-induced death of these mutants is not a consequence of cell polarisation. All the data presented indicate that the Pkc1p-MAPK pathway has an important role to play in maintaining cellular integrity during the mating response, independent of actin localisation and chitin deposition. However, the role that Pkc1p pathway plays during the mating response appears to be not associated with regulation of cell-cell fusion.

In this study, we also find that the Pkc1p-MAPK pathway and the calcineurin pathway are co-regulated in response to cell surface stresses, by independent sensing mechanisms. Calcineurin is a Ca^{2+}/calmodulin regulated protein phosphatase that is conserved from yeast to mammalian cells. Calcineurin activity is stimulated during pheromone treatment, heat shock comparable to the Pkc1p-MAPK pathway. First, Using a calcineurin reporter construct, the calcineurin pathway is found to be activated in response to cell surface stress associated with tunicamycin, chlorpromazine, calcofluor white and zymolyase. Second, activation of the calcineruin pathway in response to cell surface stress is mediated by an increase in calcium influx, partly *via* the Mid1p putative mechanosensitive calcium channel. Third, regulation of calcineurin activity in response to cell surface stress is not dependent upon the Pkc1p-MAPK pathway. Fourth, the whole of the Pkc1p-MAPK pathway is involved in modulation of basal calcineurin activity, as *mpk1Δ* and *mid2Δhcs77Δ* cells display a reduced basal calcineurin activity. Fifth, the Pkc1p-MAPK pathway modulates calcineurin activity by regulating calcium efflux and hence maintaining cellular calcium homeostasis. Sixth, the Pkc1p-MAPK pathway regulates calcium efflux by a mechanism, which is independent of the transcription factor Rlm1p. Our data suggest that the Pkc1p-MAPK pathway functions upstream of the calcium-signaling pathway. To support this proposal, our Mpk1p activation assays, show that calcium is not required for activation of Mpk1p in response to cell surface stress.

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"I gained it so,
By climbing slow,
By catching at the twigs that grow
Between the bliss and me.
It hung so high,
As well the sky,
Attempt by strategy.

I said I gained it,-
This was all.
Look, how I clutch it,
Lest it fall,
And I a pauper go;
Unfitted by an instant's grace
For the contended beggar's face
I wore an hour ago."

Emily Dickenson

Abbreviations

CEN:	Centromere
CLAP:	Chymostatin Leupetin Aprotinins Pepstatin-A
CPM:	Counts per minute
DEPC:	Diethylpyrocarbonate
dNTP:	Deoxy-nucleoside-5'-triphosphate
ECL:	Enhanced chemi-luminescence
EDTA:	Ethylene diamene tetra acetic acid
HCl:	Hydrogen Chloride
MOPS:	4-Morpholine-propanesulfonic acid
NaCl:	Sodium Chloride
OD:	Optical density
PAGE:	Polyacrylamide gel electrophoresis
PBS:	Phosphate buffered saline
PCR:	Polymerase chain reaction
PMSF:	Phenylmethylsulphonyl fluoride
SDS:	Sodium dodecyl sulphate
SSC:	Sodium-Chloride Sodium Citrate
TAE:	Tris acetate EDTA
TBE:	Tris boric EDTA
V/V:	Volume per volume
W/V:	Weight per volume
YEp:	Yeast episomal plasmid

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Chapter 1: Introduction

1.1. Background

Fungi are eukaryotic organisms that share a common ancestor with multicellular eukaryotic organisms. Despite their evolutionary divergence, fungi are most closely related to animals than to plants, algae or bacteria and thus share important features with mammalian cells (Lengeler *et al.* 2000). The fundamental processes of growth control, cell cycle regulation and signal transduction are similar in all eukaryotes. The increased tractability of simpler eukaryotes to genetic analysis offers gene discovery based on function. Homologues of genes identified in one organism can be rapidly isolated in others, and conservation of function allows examination of the properties of one component in a heterologous system.

Saccharomyces cerevisiae (budding yeast), is a unicellular fungi which has been used as a model organism because of technical advantages such as rapid growth, ease of mutant isolation, a well defined genetic system and a highly versatile DNA transformation system. Budding yeast has a stable haploid and diploid states. The haploid cells are of two mating types, designated **a** and α cells. Fusion of **a** and α cells by sexual reproduction yields **a**/α diploid cells (Fig. 1.1). When starved, the diploid cells go through meiosis to form haploid spores, which germinate when growth conditions improve to become haploid cells that can either proliferate or fuse sexually (Fig. 1.1). Under optimal nutritional conditions, yeast cells reproduce asexually by budding every 90 minutes. Each cell does this by forming a single bud-like appendage into which a full complement of daughter chromosomes moves during a mitotic division. The bud is separated to yield a smaller daughter cell, which increases in size until it becomes as large as the parent cell before initiating another round of cell division (Alberts *et al.* 1994; Watson *et al.* 1987).

Budding yeast are ovoid or spheroid shape cells bounded by a plasma membrane and a thick cell wall. The cell wall of budding yeast consists of four classes of macromolecules (Montijn *et al.* 1999). About 40% of the cell wall is composed of mannoproteins, 55% consists of β1,3-glucan, 5% of β1,6-glucan and about 2% of chitin (Montijn *et al.* 1999).

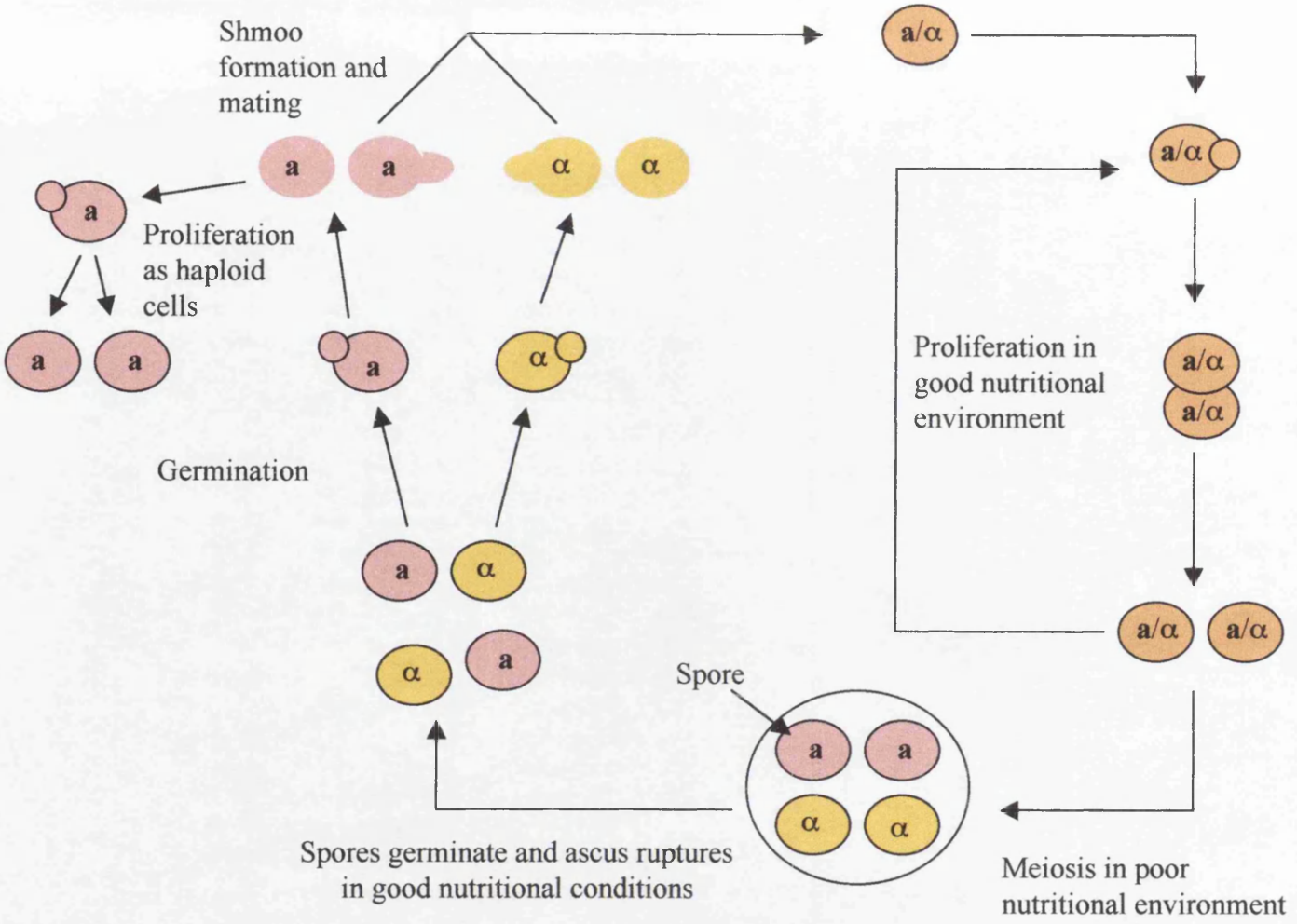

Figure 1.1. Life cycle of *S. cerevisiae*. In good nutritional environment the haploid and diploid cells will proliferate by budding, except when opposing mating types meet to form **a**/α diploid cells. In poor nutritional environment the diploid cells will undergo meiosis to form haploid spores. When environmental conditions are favourable, the spores will germinate to proliferate either as haploid cells (asexually; **a**-cells are shown as an example, but α-cells also undergo proliferation), or fuse sexually to proliferate as diploid cells.

The β1,3-glucan chains constitute a three dimensional resilient framework, that together with chitin, is believed to confer cell shape and physical strength to the cell surface (Kapteyn *et al.* 2001). The molecular architecture of the budding yeast cell wall is highly dynamic. This dynamic nature is illustrated in yeast cells challenged with cell wall de-stabilising agents. Such stresses lead to cell wall compensatory responses, such as enrichment in chitin or augmentation of linkages between chitin and β1,6-glucans (De Nobel *et al.* 2000; Kapteyn *et al.* 2001; Ketela *et al.* 1999; Roncero and Duran 1985; Turchini *et al.* 2000). Deletion of the 1,3-β-glucan-synthase gene *FKS1*, also leads to high levels of compensatory chitin synthesis (Garcia-Rodriguez *et al.* 2000). These data demonstrate the ability of yeast cells to adapt the architecture of their cell wall as a protective mechanism against cell wall weakening conditions.

In eukaryotic cells, the Mitogen Activated Protein Kinase (MAPK) cascades are key elements in mediating the transduction of many signals generated at the cell surface to the nucleus. Three protein kinases that are highly conserved in all eukaryotes make up this module: MAPK (also known as extracellular signal regulated kinase-ERK), MAPK kinase (MAPKK-also known as mitogen-activated ERK activating kinase-MEK), and MAPKK kinase (MAPKKK- also known as MEK kinase-MEKK). Sequential activation of these kinases is central to the transduction of signal through this module. The signals that lead to activation of MAPK cascades in budding yeast are perceived by a variety of receptor types: G-protein coupled seven transmembrane receptors, His-Asp phosphorelay sensors and type-I transmembrane proteins. Inactivation of MAPK cascade by dual specificity phosphatases is one mechanism for attenuation of the signal and adaptation to the response (Hunter 1995; Mattison *et al.* 1999; Neel and Tonks 1997).

The high osmolarity-response MAP-kinase pathway in yeast will be first described in this chapter.

1.2. High osmolarity response in *S. cerevisiae*

Glycerol plays an important role in adaptation of yeast to increased external osmolarity and is the main osmolyte in yeast. In hyper-osmotic conditions, cells increase their synthesis of glycerol, which leads to an increased internal osmolarity, compensating for

the elevated external osmolarity. Cells, which are unable to produce glycerol, are unable to grow on hyper-osmotic medium (Albertyn *et al.* 1994). Glycerol accumulation is stimulated in part by increased activity of glycerol-3-phosphate dehydrogenase, encoded by *GPD1* (Albertyne *et al.* 1994). This increased activity results from activation of a MAPK cascade. Adaptation to high osmolarity is mediated by the High Osmolarity Glycerol (HOG) pathway in yeast.

The Hog1p-MAPK cascade (Fig. 1.2) consists of Ssk1p and Ssk2p (MAPKKK), Pbs2p (MAPKK) and Hog1p (MAPK). Hog1p is activated by phosphorylation by Pbs2p, and Pbs2p is activated by two different branches, both sensing hyper-osmolarity, and each acting independently of the other (Maeda *et al.* 1994, 1995).

1.2.1. Sho1p osmosensor activates the HOG pathway

One branch (Sln1p branch) involves a His-Asp phosphorelay system (Fig. 1.2); the other (Sho1p branch) involves a putative transmembrane osmosensor (Fig. 1.2; Maeda *et al.* 1994; Posas *et al.* 1996). The Sln1p branch activates two redundant MAPKKK (Ssk2p and Ssk22p). The other branch for activation of Pbs2p is mediated by the Sho1p osmosensor. Sho1p has four transmembrane domains at its N-terminus and a cytoplasmic SH3 (Src homology) domain at its C-terminus (Maeda *et al.* 1994). The SH3 domain is known to interact with other proteins by binding to proline-rich motifs (Cohen *et al.* 1995; Schlessinger 1994). The SH3 domain of Sho1p mediates interaction with Pbs2p (Maeda *et al.* 1994). Sho1p also activates Ste11p, which is the MAPKKK for the pheromone response pathway.

The ability of Ste11p to function in separate pathways requires stable associations with pathway-specific proteins. In the HOG pathway, Pbs2p serves as a scaffold protein, interacting with Ste11p, Hog1p and Sho1p (Maeda *et al.* 1995; Posas *et al.* 1996; Posas and Saito 1997). O'Rourke and Herskowitz (1998) have proposed that Hog1p prevents osmolarity-induced cross talk (with the mating pathway) by inhibiting Sho1p, as part of a feedback control mechanism. Deletion of the N-terminus of Ste11p leads to constitutive

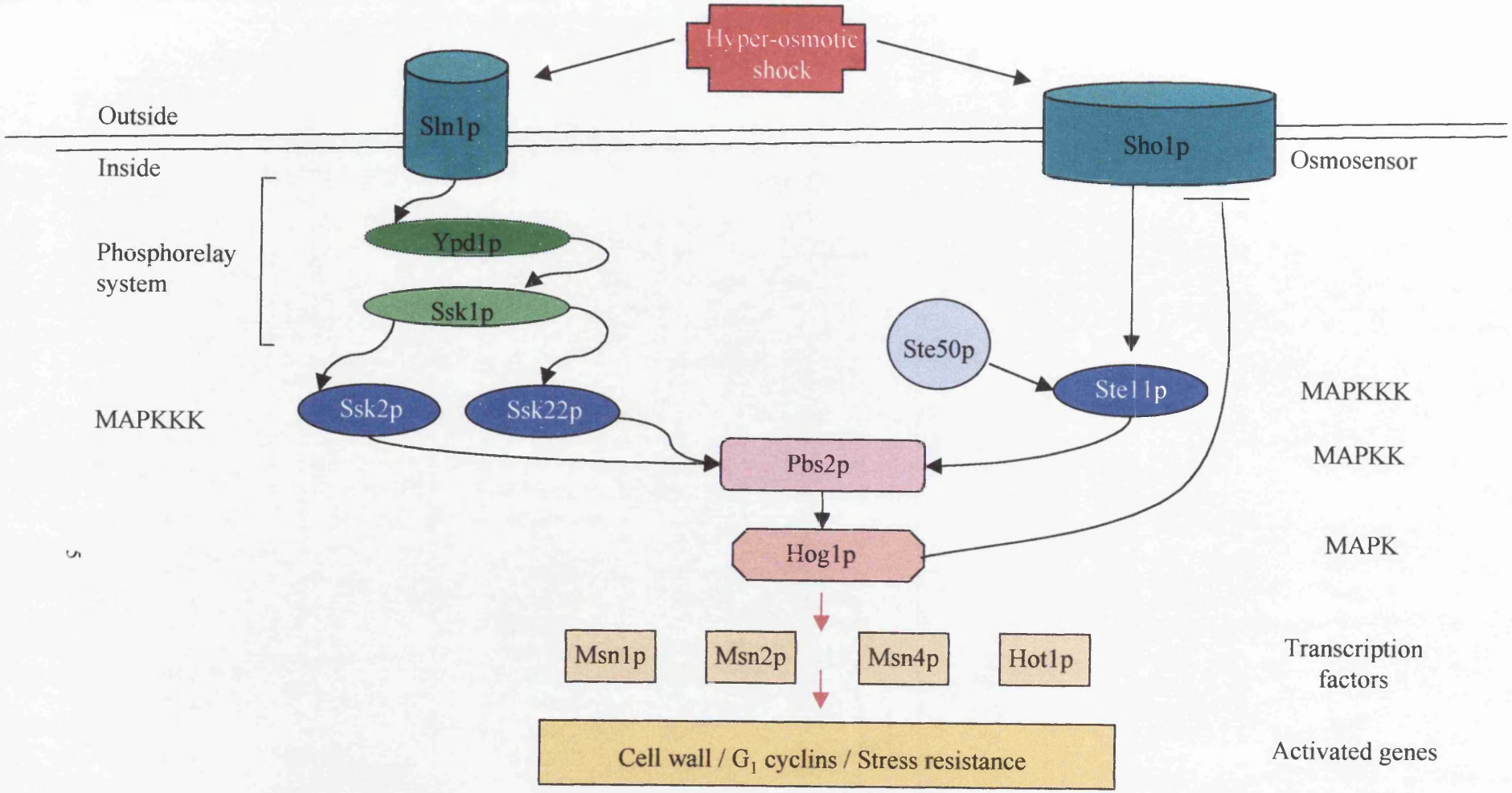

Figure 1.2. Hyper-osmotic stress response in *S. cerevisiae*. Response to osmolarity is mediated by the HOG pathway. Arrows indicate activation, lines with bars indicate inhibition, red arrows indicate that other components may be involved, and dashed elipse around Ypd1p indicates that the protein is present but not phosphorylated. See text for details.

activation of the HOG pathway, and is toxic to the cell (Cairns *et al.* 1992; Posas and Saito 1997; Stevenson *et al.* 1992). Deletion of the downstream pheromone response

pathway MAPKKK Ste7p or the deletion of the downstream HOG pathway MAPKKK Pbs2p does not suppress the lethality of constitutively-active Ste11p. However, deletion of both MAPKKKs, Ste7p and Pbs2p, completely suppresses the lethality of the constitutively-active Ste11p (Posas and Saito 1997). Another regulator of the HOG pathway, appears to be Ste50p, which binds to the N-terminal regulatory domain of Ste11p, relieving the inhibitory activity of Ste11p N-terminus (Wu *et al.* 1999). This interaction appears to be essential for activation of the HOG pathway (Posas *et al.* 1998).

1.2.2. Regulation of HOG pathway by a three component system

The phosphorolay system that governs the HOG pathway consists of three different proteins: Sln1p, Ypd1p and Ssk1p (Maeda *et al.* 1994; Posas *et al.* 1996). Sln1p contains two putative transmembrane domains that flank an extracellular domain, a cytoplasmic His-kinase domain and a receiver domain (Ota and Varshavsky 1993; Posas *et al.* 1996). The postulated role of Sln1p as an osmosensor for the HOG pathway can be traced in part to the similarity between Sln1p and the *Escherichia coli* osmosensor EnvZ. The overall structure of EnvZ is similar to that of Sln1p, except that EnvZ lacks a COOH-terminal receiver domain (Gustin *et al.* 1998).

In the HOG pathway, hyperosmolarity inhibits the protein kinase activity of Sln1p, the first protein in the phosphorelay system (Posas and Saito 1997). Thus no phosphate transfer occurs among the phosphorelay components, which leads to Ssk1p being unphosphorylated and able to activate Ssk2p and Ssk22p by inducing their auto-phosphorylation (Posas and Saito 1998). Activated Ssk2p and Ssk22p, phosphorylate and turn on Pbs2p, which in turn activates Hog1p by phosphorylation. When osmolarity conditions are returned to normal, the Sln1p kinase is activated, phospho-transfer occurs and the phosphorylated Ssk1p can not activate Ssk2p and Ssk22p. This response provides evidence that this three-component pathway is actually sensing a change in osmolarity. Therefore, these three proteins form a phosphorelay system that moves phosphate from

ATP to Sln1p-His to Sln1p-Asp to Ypd1-His to Ssk1p-Asp (Posas *et al.* 1996). Since the same phosphate is transferred, there is no signal amplification in this process.

Observations of bacterial two component signalling pathways shows that both phosphorylation and dephosphorylation reactions are potential targets of regulation (Parkinson 1993; Wurgler-Murphy and Saito 1997). Mutation of any one of the four conserved amino acids in the three proteins, completely blocks the negative regulation of the HOG pathway resulting in the hyper-activation of the MAPK cascade and subsequent toxicity (Posas *et al.* 1996; Ota and Varshavsky 1993; Maeda *et al.* 1994, 1995). Deletion of *SLN1* or *YPD1* is lethal unless HOG pathway signalling is blocked by deletion of *SSK1*, *SSK2*, *PBS2* or *HOG1* (Maeda *et al.* 1994; Posas *et al.* 1996). Sln1p and Ypd1p are therefore negative regulators of the HOG pathway. Moreover, the HOG pathway is also negatively regulated by the action of the protein phosphatases Ptp2p and Ptp3p (Guan *et al.* 1992; James *et al.* 1992; Ota and Varshavsky 1992). Another negative regulator of the HOG pathway is the protein phosphatase Ptc1p (Jiang *et al.* 1995; Maeda *et al.* 1993, 1994). Removal or inactivation of these negative regulators causes reduced cell growth through hyperactivation of the HOG pathway (Maeda *et al.* 1993).

Sln1p and Ypd1p appear to have more complex roles, than mere regulation of the HOG pathway. Sln1p regulates the expression of Mcm1p-dependent genes, a function that is independent of the HOG pathway (Fassler *et al.* 1997; Yu *et al.* 1995). Mcm1p is a transcription factor that is essential for cells. Mcm1p appears to form complexes with other transcription factors to regulate a variety of genes related to cell wall/membrane structure, pheromone response, the cell cycle and cell type specificity (Elble and Tye 1991; Errede and Ammerer 1989; Kuo and Grayhack 1994; Oehlen *et al.* 1996).

The mechanism responsible for Sln1p activation of *MCM1*-regulated genes involves the two component proteins Skn7p and Ypd1p (Gustin *et al.* 1998; Ketela *et al.* 1998). Skn7p is a non-essential protein that contains a receiver domain with a conserved aspartate residue that is a target for phosphorylation by histidine kinases (Brown *et al.* 1993). Over-expression of *SKN7* activates expression of *MCM1*-dependent reporter gene (Gustin *et al.* 1998). Increasing osmolarity would inactivate Sln1p, turning off Skn7p functions related to growth; turning on Ssk1p functions such as the HOG pathway activation that are related

to stress resistance, and mediating expression of G_1 cyclins and cell wall genes (Brown *et al.* 1993,1994; Krems *et al.* 1996; Morgan *et al.* 1995).

1.2.3. Regulation of gene expression by the HOG pathway

An increase in external osmolarity is stressful to the yeast cells and leads to many physiological changes (Mager and Varela 1993). These changes include loss of an organised actin cytoskeleton, temporary decrease in protein synthesis and induction of a subset of heat shock proteins (Blomberg 1995; Chowdhury *et al.* 1992; Mager and Valera 1993; Schuller *et al.* 1994; Varela *et al.* 1995). In response to an osmotic stimulus, different genes show different time-dependent patterns of expression and extent of induction (Blomberg 1995; Hirayama *et al.* 1995). The HOG pathway is required for the increased activity of many but not all of these genes (Gustin *et al.* 1998).

One class of genes that is induced by hypo-osmotic shock are those that have a DNA sequence called STRE (stress response element) in their promoter (Evangelista *et al.* 1996; Gustin *et al.* 1998). Genes with STRE elements are also induced by other stresses such as heat shock and have therefore a more general role in the protection from stress induced damage (Gustin *et al.* 1998; Rep *et al.* 1999-a). The cytoplasmic catalase gene *CTT1* which catalyses the breakdown of hydrogen peroxide, a chaperonin (*HSP104*) and *HSP12* whose precise molecular function is unknown are members of the STRE-regulated class of stress genes (Lindquist and Kim 1996; Schuller *et al.* 1994; Siderius *et al.* 1997; Varela *et al.* 1995). Another class consists of genes are induced specifically in response to hyper-osmotic stress. This class includes genes encoding enzymes involved in glycerol synthesis, such as *GPD1* and *GPP2* (Abertyn *et al.* 1994; Hirayama *et al.* 1995; Norbeck *et al.* 1996; Rep *et al.* 1999-b).

STRE-mediated gene expression appears to be mediated in part by the two redundant transcription factors Msn2p and Msn4p (Martinez-Pastor *et al.* 1996; Scmitt and McEntee 1996). In response to a large variety of stresses, including osmotic stress, these factors are translocated to the nucleus, where they bind to and activate expression from target promoters (Rep *et al.* 1999a). Osmotic stress-induced nuclear translocation of Msn2p to the nucleus is unaffected by deletion of Hog1p. Instead, translocation of Msn2p to the

nucleus is negatively regulated by cyclic-AMP and protein kinase-A (Gorner *et al.* 1998). Other transcriptional regulators such as Msn1p and Hot1p that control responses to high osmolarity stress have been also been identified (Rep *et al.* 1999a). Cells lacking Msn1p, Msn2p, Msn4p and Hot1p lack short-term transcriptional responses of the genes *GPD1*, *GPP2*, *CTT1* and *HSP12* to osmotic stress (Rep *et al.* 1999b). The regulation of gene expression in osmotically stressed cells is clearly complex, with the HOG pathway cooperating with other pathways to regulate gene expression.

1.3. Low osmolarity response in *S. cerevisiae*

In contrast to the HOG pathway, which responds to hyperosmolarity, the protein kinase C (PKC) pathway is activated by low osmolarity. It is also activated in response to a variety of stimuli such as thermal stress and pheromones (Costigan *et al.* 1992; Davenport *et al.* 1995; Kamada *et al.* 1995; Mazzoni *et al.* 1993). A major role of the PKC pathway is believed to be maintenance of cellular integrity by controlling cell wall and membrane assembly. Changes in cell wall composition can occur in response to changes in growth medium, the presence of pheromones and during cell-cell fusion (Cid *et al.* 1995; Mazzoni *et al.* 1993; Philips and Herskowitz 1997; Zarzov *et al.* 1996).

During apical growth of a yeast bud, the cell wall is in a dynamic state of change, as new material is added at the growing point and subsequently modified to produce a structure capable of sustaining mechanical and chemical stress (Cid *et al.* 1995; Cabib *et al.* 1997). These modifications of the cell wall in response to diverse conditions requires activation of cell wall-synthesising enzymes resident in the cell membrane, vectorial transport and exocytosis of vesicles that carry other wall and membrane components (Cid *et al.* 1995). The inability of a cell to modify its cell wall accordingly may result in a fragile cell wall and may lead to cell lysis, misshapen cells or altered patterns of growth.

1.3.1. The Pkc1p-MAPK cascade of *S. cerevisiae*

The MAPK cascade that mediates the transduction of the signal generated by low osmolarity and heat stress consists of the MAPK Mpk1p, two redundant MAPKKs Mkk1p and Mkk2p, and the MAPKKK Bck1p (Fig. 1.3; Costigan *et al.* 1992; Irie *et al.* 1993; Lee

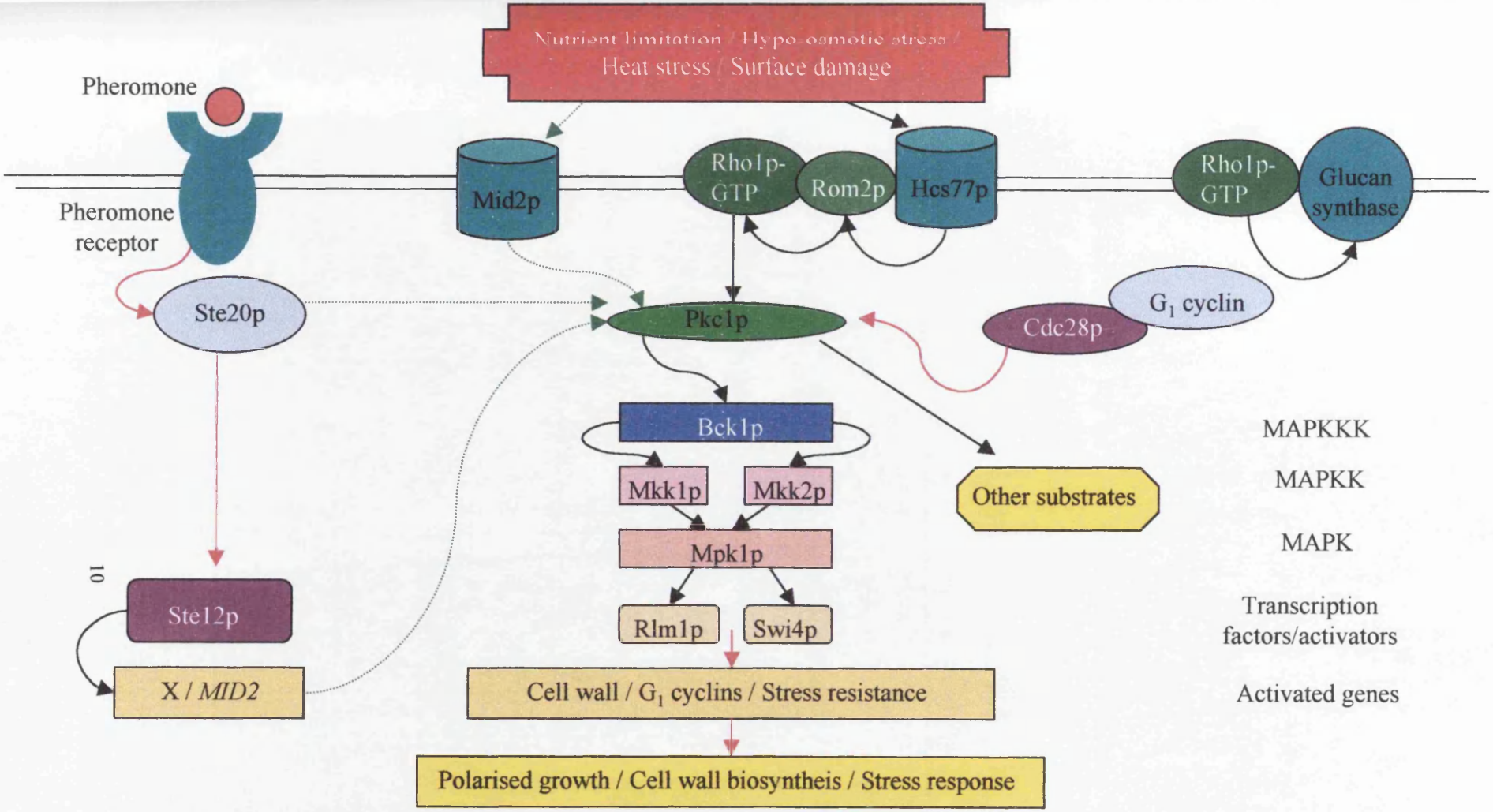

Figure 1.3. The Pkc1p-MAPK pathway regulates cell integrity. The cell integrity pathway is activated in response to heat, hypo-osmotic shock, nutrient limitation, and cell surface damage. Hcs77p is a putative mechano-sensor that may detect membrane stretch. Pkc1p is proposed to activate a branched pathway, independently of the MAPK cacade. Rlm1p and Swi4p are targets of Mpk1p. Solid arrows indicate activation, dashed arrows are speculative, red arrows indicate that other components may be involved. See text for details.

et al. 1993; Lee and Levin 1992; Mazzoni *et al.* 1993; Torres *et al.* 1991). The proposal for sequential activation by phosphorylation of the MAPK cascade is based on *in vivo* epistasis analysis and on structural relatedness to kinases in other pathways for which *in vitro* function has been established. The MAPK Mpk1p is threonine/tyrosine phosphorylated and activated in response to heat shock and hypotonic conditions in a Mkk1p-Mkk2p-Bck1p and Pkc1p dependent manner (Davenport *et al.* 1995; Kamada *et al.* 1995; Zarzov *et al.* 1996).

The MAPK cascade is activated by the serine/threonine protein kinase Pkc1p. Deletion of any of the genes encoding components of the MAPK cascade (*bck1Δ*, *mkk1Δ*, *mkk2Δ* or *mpk1Δ*) results in cell lysis at elevated temperatures, which is osmotically remedial. In contrast to this temperature sensitive growth defect, *PKC1* and *RHO1* are essential for growth at all temperatures. Cells lacking *PKC1* can only proliferate in osmotically-stabilised medium. These cells undergo rapid lysis after transfer to medium lacking osmotic stabiliser at all temperatures (Levin and Bartlett-Heubusch 1992; Paravicini *et al.* 1992). Since deletion of *PKC1* causes a more severe phenotype than does deletion of *MPK1*, it has been proposed that Pkc1p governs a branched pathway, the components of which remains unknown (Lee and Levin 1992). Some mutants of this pathway are also sensitive to caffeine, staurosporin (a specific inhibitor of PKC isozymes) and calcofluor white (Heinisch *et al.* 1999). Sensitivity to these drugs may be caused by changes in the carbohydrate content of the cell surface (Lussier *et al.* 1997).

The cell integrity MAPK cascade is most similar to the classical mitogen-activated ERK1-ERK2 MAPK cascade in animal cells in functional tests of the pathway components (Blumer *et al.* 1994; Lee *et al.* 1993; Lim *et al.* 1997). Furthermore, both the mammalian and the yeast cell integrity pathway have the same general function in their respective systems. This general function is positive regulation of growth and cell proliferation (Gray *et al.* 1997; Gustin *et al.* 1998; Madden *et al.* 1997; Marini *et al.* 1996; Marshal 1995; Zarzov *et al.* 1996). The human ERK1 expressed in yeast can function as an activator of cell wall remodeling in these cells (Atienza *et al.* 2000).

The Pkc1p in the cell integrity pathway, is the only homolog of the mammalian protein kinase C. The mammalian PKC-η can complement the *pkc1Δ* in yeast cells (Nomoto *et*

al. 1997). The yeast Pkc1p has a variety of functional domains, as deduced from their homology to the mammalian isozymes (Heinisch *et al.* 1999). Pkc1p has two C1 domain-like sequences containing zinc fingers that may serve as a binding site for diacylglycerol (DAG); a C2 domain involved in calcium-dependent phospholipid binding; the kinase domain; a pseudo-substrate site; the V5 region located at the C-terminal end, which is thought to control intracellular localisation and two HR1 domains in the N-terminal region that interact with small G-proteins (Mellor and Parker 1998). Most members of the PKC family rely on diacylglycerol (DAG), phosphatidyl-serine (PS) and calcium (Sim and Scott 1999). Other PKC families do not require calcium for activation and hence have an altered C2 domain (Sim and Scott 1999). Those PKC isozymes that lack the C2 domain, do not require calcium or DAG. An activated mammalian C-kinase can phosphorylate serine or threonine residues on target proteins. C-kinase activation can lead to activation of a MAPK cascade and hence transcriptional regulation of target genes. Activation of mammalian C-kinase may also lead to phosphorylation of inhibitory proteins (Iκ-B) which release cytoplasmic regulatory proteins (NF-κB), that migrates to the nucleus and activate transcription of target genes.

1.3.2. Activation of the Pkc1p-MAPK pathway by hypo-osmotic shock

Yeast cells like plants and other organisms with a cell wall maintain an osmotic gradient across their plasma membrane to create turgor pressure (Blomberg and Adler 1992). Lowering the external osmolarity is believed to increase this osmotic gradient and turgor pressure, therefore creating stress on the plasma membrane and cell wall (Davenport *et al.* 1995; Kamada *et al.* 1995). Hypo-osmotic stress increases tyrosine phosphorylation of Mpk1p and hence activates the cell integrity pathway. This response requires Pkc1p, Bck1p, Mkk1p and Mkk2p.

Studies on cell fusion of yeast during mating have suggested that the magnitude of the osmotic gradient across the plasma membrane is a signal that regulates the cell integrity pathway which may in turn regulate cell-cell fusion (Philips and Herskowitz 1997). This study has shown that expression of a hyper-activated allele of *PKC1* blocks the fusion of mating cells (Philips and Herskowitz 1997). The same phenotype is observed in cells lacking the glycerol transporter Fps1p (Luyten *et al.* 1995, Philips and Herskowitz 1997;

Sutherland *et al.* 1997). These authors therefore proposed a model in which an elevated osmotic gradient causes hyper-activation of the cell integrity pathway, which in turn blocks cell fusion (Philips and Herskowitz 1997). This model suggests that secretion of glycerol at the shmoo tip leads to hyper-osmotic state between mating cells. This high osmolarity condition may then inactivate the Pkc1p pathway in the mating partners, leading to cell wall degradation at point of contact and hence fusion. However, deletion of *GPD1* (the enzyme required for glycerol synthesis) suppresses the cell fusion defect of *fps1Δ* cells, but not that induced by expression of activated Pkc1p. The cell integrity pathway seems to be required under conditions of high turgor pressure, but the role that Pkc1p pathway may play during cell-cell fusion requires further investigation (Gustin *et al.* 1998).

1.3.3. Activation of the Pkc1p-MAPK pathway by heat-shock

The cell integrity pathway is required for growth of yeast cells at high temperatures and for acquired thermo-tolerance (Kamada *et al.* 1995; Lee and Levin 1992; Levin *et al.* 1990). Thermo-tolerance is the ability of cells to better survive severe heat shock if they are first exposed to mild heat shock. The cell integrity pathway is also activated by heat stress and chlorpromazine (a membrane deforming drug) treatment (Kamada *et al.* 1995; Zarzov *et al.* 1996). This activation of the cell integrity pathway appears to be delayed in osmotically stabilised cells (Kamada *et al.* 1995). Phosphorylation of Mpk1p in response to heat shock requires Pkc1p and Bck1p (Kamada *et al.* 1995).

Mutants of the cell integrity pathway are defective for growth at high temperatures (Irie *et al.* 1993; Lee *et al.* 1993; Lee and Levin 1992; Levin and Bartlett-Heubusch 1992; Mazzoni *et al.* 1993; Paravicini *et al.* 1992). The temperature sensitivity of the mutants suggests that the cell integrity pathway has a physiological function that is required for growth at higher temperatures (Gustin *et al.* 1998). This temperature sensitivity is associated with weak cell walls, as heat shock of these mutants leads to cell lysis (Levin and Bartlett-Heubusch 1992; Levin *et al.* 1994; Paravicini *et al.* 1992; Roemer *et al.* 1994). This phenotype suggests that an increase in growth temperature creates a stress on the cell wall or the plasma membrane, which then activates the cell integrity pathway, thereby increasing cell wall gene expression and cell wall synthesis to relieve the stress

(Gustin *et al.* 1998; Igual *et al.* 1996; Kamada *et al.* 1995). Increasing the osmolarity of the medium suppresses the temperature sensitivity and cell lysis defect of the cell integrity pathway mutants (Costigan *et al.* 1992; Lee and Levin 1992; Levin and Bartlett-Heubusch 1992; Paravicini *et al.* 1992). Increasing the external osmolarity is proposed to collapse the osmotic gradient across the plasma membrane and reduce mechanical pressure on the cell wall (Gustin *et al.* 1998).

1.3.4. Activation of the Pkc1p-MAPK pathway by mating pheromone

Pheromone treatment and pheromone-induced cell polarisation increase the tyrosine phosphorylation and kinase activity of Mpk1p (Zarzov *et al.* 1996). Pheromone activation of Mpk1p requires a functional Pkc1p, Mkk1p and Mkk2p (Buehrer and Errede 1997). One research group investigating pheromone activation of the cell integrity pathway found that Bck1p is required (Zarzov *et al.* 1996). However, a second group has found that Bck1p is only partially required, suggesting that another MAPKKK may help to mediate activation of the pathway in response to pheromone (Buehrer and Errede 1997).

Zarzov *et al.* (1996) have proposed that Ste20p, and not Ste11p or Ste12p, is involved in regulating activation of Mpk1p in response to mating pheromone, suggesting that the pheromone response pathway is not involved in this regulation. However, Buehrer and Errede (1997) have found that in the *cdc28* mutant background, Ste12p, and protein synthesis are required for pheromone activation of Mpk1p. Therefore, suggesting that pheromone activation of the cell integrity pathway, is mediated by proteins whose expression is induced by pheromone. Further investigation is required to clarify the role of the mating response pathway in Mpk1p activation during the mating response.

1.3.5. Cell cycle regulation of the Pkc1p-MAPK pathway

Formation of a new bud occurs at the G_1/S transition, after START (Lew and Reed 1993, 1995). The cyclin-dependent kinase Cdc28p in complex with the G_1 cyclins Cln1p, Cln2p and Cln3p are required for this process. Increasing or decreasing the activity of Cdc28p in cells induces corresponding increases or decreases in the tyrosine phosphorylation and hence activation of Mpk1p (Gustin *et al.* 1998; Marini *et al.* 1996; Zarzov *et al.* 1996).

Mutations in the cell integrity pathway kinases show synthetic lethality with *cdc28* mutations (Marini *et al.* 1996; Mazzoni *et al.* 1993). Mpk1p phosphorylation peaks at the G_1/S transition (Mazzoni *et al.* 1993). Peak Mpk1p phosphorylation correlates with the time of polarisation of growth towards the bud tip (Lew and Reed 1995). Furthermore, this peak period of Mpk1p phosphorylation corresponds with increase in cell wall gene expression (Igual *et al.* 1996). *PKC1* is required for cell cycle-dependent expression of a subset of cell wall gene such as *FKS1* and *MNN1* (Igual *et al.* 1996).

It has been proposed, that the mechanism by which G_1 cyclin-Cdc28p stimulates the cell integrity pathway, involves the hydrolysis of phosphatidylcholine to choline phosphate and diacylglycerol by Cdc28p (Gustin *et al.* 1998; Marini *et al.* 1996). The increase in the amount of diacylglycerol is proposed to activate Pkc1p similar to the activation of mammalian cell protein kinase C (Gustin *et al.* 1998; Nishizuka 1992). Mutants defective in a putative diacylglycerol binding site of Pkc1p display a partial loss of Pkc1p function (Gustin *et al.* 1998; Jacoby *et al.* 1997). However, attempts to show regulation of the cell integrity pathway with diacylglycerol or related phorbol esters have been unsuccessful (Antonsson *et al.* 1994; Davenport *et al.* 1995; Gustin *et al.* 1998; Watanabe *et al.* 1994).

1.3.6. Upstream regulators of the Pkc1p-MAPK pathway

Rho1p belongs to the family of small GTP binding proteins (G-proteins), which includes the Rho, Rac and the Ras subfamilies (Madaule *et al.* 1987). Rho1p binds and is required for the activity of Pkc1p (Kamada *et al.* 1996; Nonaka *et al.* 1995). Rho1p is essential for yeast growth, but a set of temperature sensitive *rho1* mutants are defective in heat-induced activation of Mpk1p activity (Kamada *et al.* 1996; Qadota *et al.* 1994). These *rho1* mutants also have a temperature-sensitive lysis phenotype. This phenotype can be suppressed by increasing osmolarity of the medium, expression of an activated form of Pkc1p, or over-expression of different downstream components of the MAPK branch of the cell integrity pathway (Kamada *et al.* 1996; Nonaka *et al.* 1995; Qadota *et al.* 1994). The GTP bound form of Rho1p rather than the Rho1p-GDP, has been shown to preferentially interact with Pkc1p (Kamada *et al.* 1996; Nonaka *et al.* 1995).

Rom1p and Rom2p are guanine nucleotide exchange factors (GEF) that convert Rho1p-GDP to Rho1p-GTP (Ozaki *et al.* 1996; Schmidt *et al.* 1997). GTPase-activating proteins (GAPs) enhance the intrinsic GTPase activity of the G-proteins (Cabib *et al.* 1998). Therefore, these proteins stimulate the transition from GTP- to GDP-bound state (Cabib *et al.* 1998). Bem2p and Sac7p are Rho1p-specific GAPs that convert Rho1p-GTP to Rho1p-GDP (Kim *et al.* 1994; Peterson *et al.* 1994; Schmidt *et al.* 1997; Wang and Bretscher 1995). Bem2p and Sac7p have been proposed to be negative regulators of the cell integrity pathway (Martin *et al.* 2000). Gdi1p is a Rho-GDP dissociation inhibitor that binds the GDP-bound inactive state of Rho1p and inhibits its activation (Garrett *et al.* 1994; Koch *et al.* 1997; Masuda *et al.* 1994). Therefore, in the small G-protein system, GAPs function as negative regulators, GEFs as activators and GDIs keep the G-proteins in the cytoplasm in an inactive state (Cabib *et al.* 1998).

Rom1p and Rom2p are regulated by the phosphatidyl-inositol kinase homologue Tor2p (Schmidt *et al.* 1997). The Tor proteins, Tor1p and Tor2p, respond to nutrient availability and are positive regulators of translation initiation and progression through G_1 (Barbet *et al.* 1996; Di Como and Arndt 1996; Gustin *et al.* 1998). Tor2p also promotes the organisation of the actin cytoskeleton in G_1 phase (Helliwell *et al.* 1998-b; Schmidt *et al.* 1996). Temperature sensitivity of *tor2*^{ts} mutants can be suppressed by increasing osmolarity of the growth medium or over-expression of *PKC1*, suggesting a role for Tor2p in the cell integrity pathway (Helliwell *et al.* 1998-a).

Synthesis of the cell wall β-1,3-glucan requires Rho1p (Gustin *et al.* 1998). The cell wall polysaccharide is synthesised at the cell surface by two differentially expressed glucan synthases, Fks1p and Fks2p (Castro *et al.* 1995; Douglas *et al.* 1994; Garrett-Engele *et al.* 1995; Shematek *et al.* 1980; Shematek and Cabib 1980). Rho1p-GTP is required for the activity of the plasma membrane bound Fks1p (Drgonova *et al.* 1996; Mazur and Baginsky 1996; Mol *et al.* 1994; Qadota *et al.* 1996). Pkc1p does not seem to be involved in the Fks1p-Rho1p complexes, suggesting that Rho1p forms separate complexes with glucan-synthase and Pkc1p (Drgonova *et al.* 1996, Gustin *et al.* 1998). However, Pkc1p is localised at sites of polarised growth and this localisation is dependent on presence of Rho1p (Andrews and Stark 2000-a). The cell integrity pathway is required for the cell cycle regulation of *FKS1* expression and for regulation *FKS2* expression (Igual *et al.*

1996; Kamada *et al.* 1996). Therefore, Rho1p regulates cell wall synthesis both directly and through the Pkc1p-MAPK pathway. Furthermore, Rho1p, Pkc1p, Rom2p and Hcs77p regulate *FKS1* and actin cytoskeleton depolarisation in response to cell surface damage, presumably as a means of a repair mechanism (Delley and Hall 1999).

Rho1p-GTP and other Rho family proteins, Cdc42p and Rho3p, bind to Bni1p, which is a cytoskeletal protein needed for proper bud site selection and rearrangement of the actin cytoskeleton during mating projection formation (Evangelista *et al.* 1996; Kohno *et al.* 1996). Over-expression of *ROM1*, *ROM2* and *RHO2* suppress the profilin deficient (*pfy1Δ*) phenotype. Profilin is a small actin monomer binding protein that binds Bni1p and is involved in actin polymerisation (Marcoux *et al.* 2000). Rom2p has been localised to sites of polarised growth and has been proposed to have a role in microtubule function (Manning *et al.* 1997). However, *rho1* mutants show normal localisation of actin patches and cables but lyse at small bud stage (Yamochi *et al.* 1994). Therefore, the regulatory role of Rho1p-Bni1p appears to be complicated and may involve other components (Gustin *et al.* 1998).

1.3.7. Targets of the Pkc1p-MAPK pathway

A downstream substrate of Mpk1p is the Swi4p/Swi6p complex (Madden *et al.* 1997). Swi4p is the DNA binding subunit and transcriptional activator of this complex (Koch *et al.* 1996; Ogas *et al.* 1991). Swi4p is required for normal expression of the G_1 cyclin genes *CLN1*, *CLN2*, *PCL1* and *PCL2* at the G_1/S transition (Cross *et al.* 1994; Gustin *et al.* 1998; Nasmyth and Dirick 1991). The cell integrity pathway appears to regulate Swi4p/Swi6p complex through Mpk1p-catalysed phosphorylation of Swi4p and Swi6p (Madden *et al.* 1997). Suprisingly, over-expression of *PKC1* or *HCS77* suppress the *swi4Δ* temperature-sensitive growth defect (Gray *et al.* 1997). Cells lacking the *SWI4* gene have a defect in bud emergence that can be suppressed by an activated Pkc1p pathway (Gray *et al.* 1997). Therefore, the Pkc1p pathway appears to be a positive regulator of bud emergence, independent of Swi4p. Harrison *et al.* (2001) have demonstrated that the kinases of the cell integrity pathway are required for the morphogenesis check point which delays mitotic activation of Cdc28p (G_2 arrest) during environmental stress. The Swi4p/Swi6p complex and the Pkc1p-MAPK cascade appear

to also have some non-overlapping functions, since the cell integrity pathway mutations are lethal when combined with *swi4Δ* (Gray *et al.* 1997; Gustin *et al.* 1998; Igual *et al.* 1997).

Another target of the Pkc1p-MAPK pathway is Rlm1p, a member of the MADS box family of transcription factors of which the mammalian serum response factor is a member (Shore and Sharrocks 1995; Watanabe *et al.* 1995). Rlm1p is a downstream substrate for Mpk1p and displays a heat stressed induced Mpk1p-dependent phosphorylation (Watanabe *et al.* 1997). A genome-wide analysis of genes regulated by the cell integrity pathway has revealed that most of transcriptional regulation is mediated through Rlm1p (Jung and Levin 1999). Moreover, deletion of *RLM1* does not lead to the cell lysis phenotype, characteristic of the Pkc1p-MAPK pathway mutants, indicating that the Pkc1p-MAPK pathway may have other downstream targets which are independent of Rlm1p (Dodou and Treisman 1997, Watanabe *et al.* 1997).

1.3.8. Upstream sensors of the Pkc1p-MAPK pathway

Cell wall or plasma membrane stress, induced by heat or hypotonic shock, is a signal that must be transduced from the plasma membrane to Pkc1p-MAPK cascade. Such stress sensors at the plasma membrane have been proposed to include the Wsc family of proteins (Gray *et al.* 1997; Verna *et al.* 1997). Wsc1p, Wsc2p, Wsc3p and Wsc4p have a single putative transmembrane domain as predicted from their amino acid sequences. Wsc1p/Hcs77p/Slg1p is localised to the plasma membrane of the cell (Verna *et al.* 1997). Deletion of *HCS77* results in a phenotype that is similar to the phenotype observed for deletion of the Pkc1p-MAPK pathway components, a temperature sensitive lysis phenotype that can be remedied by osmotic stabilisers (Gray *et al.* 1997). Heat activation of Mpk1p is severely reduced in *hcs77Δ* cells (Gray *et al.* 1997). Assay of Mpk1p activity in response to stress, and genetic analysis, have indicated that Hcs77p is a sensor of the Pkc1p-MAPK pathway (Gray *et al.* 1997). The phenotype associated with a *wsc1Δwsc2Δwsc3Δ* is a cell lysis defect, which can be suppressed by over-expression of *RHO1* or *PKC1* (Verna *et al.* 1997). In a recent two hybrid analysis, it has been shown that Hcs77p interacts with Rom2p, suggesting that Hcs77p activates Pkc1p *via* Rho1p, by stimulating binding of GTP to Rho1p (Philip and Levin 2001).

Another possible cell surface sensor Mid2p encodes an integral membrane protein, which is similar to Hcs77p in that it possesses a single small cytoplasmic domain, a single transmembrane region and its extracellular region is rich in serine/threonine residues (Ketela *et al.* 1999; Ono *et al.* 1994; Rajavel *et al.* 1999). The C-terminal domain of Mid2p contains a short charged domain rich in aspartic residues, suggested to resemble a calcium-binding domain (Ono *et al.* 1994). *MID2* was originally identified in a screen for mutants that died in presence of mating pheromone, after differentiating into shmoos (Ono *et al.* 1994).

Buehrer and Errede (1997) have shown that *ste12Δ* cells display a defect in increased Mpk1p activity in response to mating pheromone. Furthermore, in the absence of protein synthesis cells failed to stimulate Mpk1p activity when exposed to mating pheromone (Buehrer and Errede 1997). These results suggest that activation of Mpk1p in presence of mating pheromone may specifically require the synthesis of a Ste12p dependent gene product. The *MID2* gene contains two pheromone response elements in the 5' upstream region, and its expression is stimulated by mating pheromone (Ono *et al.* 1994). Mid2p therefore, is an excellent gene product that may activate the Pkc1p-MAPK pathway in response to the activation of the mating pathway.

1.3.9. Interactions of the Pkc1p-MAPK pathway with protein phosphatases

The mechanism by which the Pkc1p-MAPK pathway is down-regulated is not well characterised. However, recent work has suggested that the protein tyrosine phosphatases Ptp2p and Ptp3p inactivate Mpk1p (Mattison *et al.* 1999). These authors showed that transcription of *PTP2* is increased in response to heat shock in a Mpk1p dependent manner (Mattison *et al.* 1999). Their data suggests that Ptp2 acts in a negative feedback loop to inactivate Mpk1p (Mattison *et al.* 1999). These two protein phosphatases have also been shown to inactivate the MAPKs of the HOG and the mating pathway Hog1p and Fus3p respectively (Jacoby *et al.* 1997; Wurgler-Murphy *et al.* 1997; Zhan *et al.* 1997). Therefore, Ptp2p and Ptp3p may function as global regulators of the MAPK pathways in yeast, similar to the role of some mammalian phosphatases (MKP-1, MKP-2 and MKP-4; Mattison *et al.* 1999).

Another dual protein phosphatase Msg5p, has been shown to dephosphorylate Mpk1p (Martin *et al.* 2000). Msg5p appears to be involved in maintaining a low basal level of phosphorylated Mpk1p (Martin *et al.* 2000). This latter data suggests that Ptp2p and Ptp3p, may be involved in inactivating Mpk1p after cessation of the stimulus. Msg5p appears to also regulate the activity of the mating pathway MAPK Fus3p (Martin *et al.* 2000). In the mating pathway, Msg5p appears to participate in the adaptive response of cells after pheromone stimulation.

GLC7, which encodes the catalytic subunit of type-I protein phosphatase in yeast, appears to be required for the cell integrity pathway, for proper bud morphology and polarisation of the actin cytoskeleton (Andrews and Stark 2000-a). Hence, this type-I protein phosphatase appears to be a positive regulator of the cell integrity pathway. However, the mechanism by which this phosphatase exerts its effect remains unknown.

1.3.10. Interactions of the Pkc1p-MAPK pathway with the calcium-signalling pathway

The protein phosphatase calcineurin appears to perform a vital cellular function in conjunction with the cell integrity pathway. Calcineurin is not required for vegetative cell growth, however a calcineurin null mutation is lethal in combination with *pkc1Δ* or *mpk1Δ* (Garrett-Engele *et al.* 1995, Nakamura *et al.* 1996). Expression of a constitutively-active form of calcineurin partially suppresses the temperature sensitive, cell lysis phenotype of *pkc1Δ* or *mpk1Δ* cells (Garrett-Engele *et al.* 1995). Calcineurin mutants display a pheromone-induced death, which is suppressed by over-expression of *MPK1* (Nakamura *et al.* 1996).

Cells with reduced calcineurin activity are sensitive to the loss of the glucan-synthase gene *FKS1* (Eng *et al.* 1994;Garrett-Engele *et al.* 1995; Parent *et al.* 1993). Thus in the absence of *FKS1*, *FKS2* becomes essential, and its expression is dependent on calcineurin (Mazur *et al.* 1995). Therefore, the reduced expression of *FKS1* and *FKS2* in cell integrity pathway mutants may account for their synthetic lethality with calcineurin mutants that have a low *FKS2* expression levels (Igual *et al.* 1996; Zhao *et al.* 1998). The calcineurin regulation of *FKS2* expression is mediated through the transcription factor Crz1p (Matheos *et al.* 1997; Stathopoulos and Cyert 1997).

Activation of the cell integrity pathway, during times of pheromone exposure correlates with an increase in the cytosolic calcium concentration (Buehrer and Errede 1997; Iida *et al.* 1990; Ohsumi and Anraku1985; Zarzov *et al.* 1996). Cells deprived of calcium, like calcineurin mutants, lose viability after pheromone treatment (Cyert *et al.* 1991; Cyert and Thorner 1992; Iida *et al.* 1990, 1994; Paidhungat and Garrett 1997). Cells lacking Mpk1p also lose viability after pheromone treatment, suggesting that this pathway may function parallel to or as part of the calcium induced response required for survival after pheromone treatment (Errede *et al.* 1995; Gustin *et al.* 1998).

The intracellular calcium concentration has been suggested to increase during the G_1/S transition, since calcium or manganese ions are needed to allow yeast to progress beyond the minibud stage of growth (Gustin *et al.* 1998; Loukin and Kung 1995). However, it has not yet been clearly demonstrated that the cytosolic calcium concentration increases in the late G_1 or early S phase. Note: the cell integrity pathway is activated at the G_1/S transition, which leads to expression of genes required for cell wall construction in the bud (Igual *et al.* 1996; Zarzov *et al.* 1996).

Finally, when cells are exposed to hypotonic shock, there is an increase in cytosolic calcium concentrations (Batiza *et al.* 1996; Beeler *et al.* 1997). Moreover, the cell integrity pathway becomes activated in times of hypotonic stress, providing another correlation between the two signalling pathways (Davenport *et al.* 1995; Kamada *et al.* 1995).

1.4. The calcium-calcineurin signalling pathway

Ionised calcium (Ca^{2+}) is one of the most important signal-transduction elements in cells ranging from bacteria to specialised neurons. Ca^{2+}, unlike other second messenger molecules, cannot be metabolised and cells therefore tightly regulate the intracellular Ca^{2+} levels. Calmodulin is a trigger protein that upon binding of calcium changes its conformation and modulates effector molecules such as enzymes and ion channels (Fig. 1.4). One such effector enzyme is the Ca^{2+}/calmodulin-dependent serine/threonine phosphatase, calcineurin (Fig. 1.4; Klee *et al.* 1979). Calcineurin is a heterodimeric phosphatase formed by the association of phosphatase catalytic subunit (Calcineurin-A;

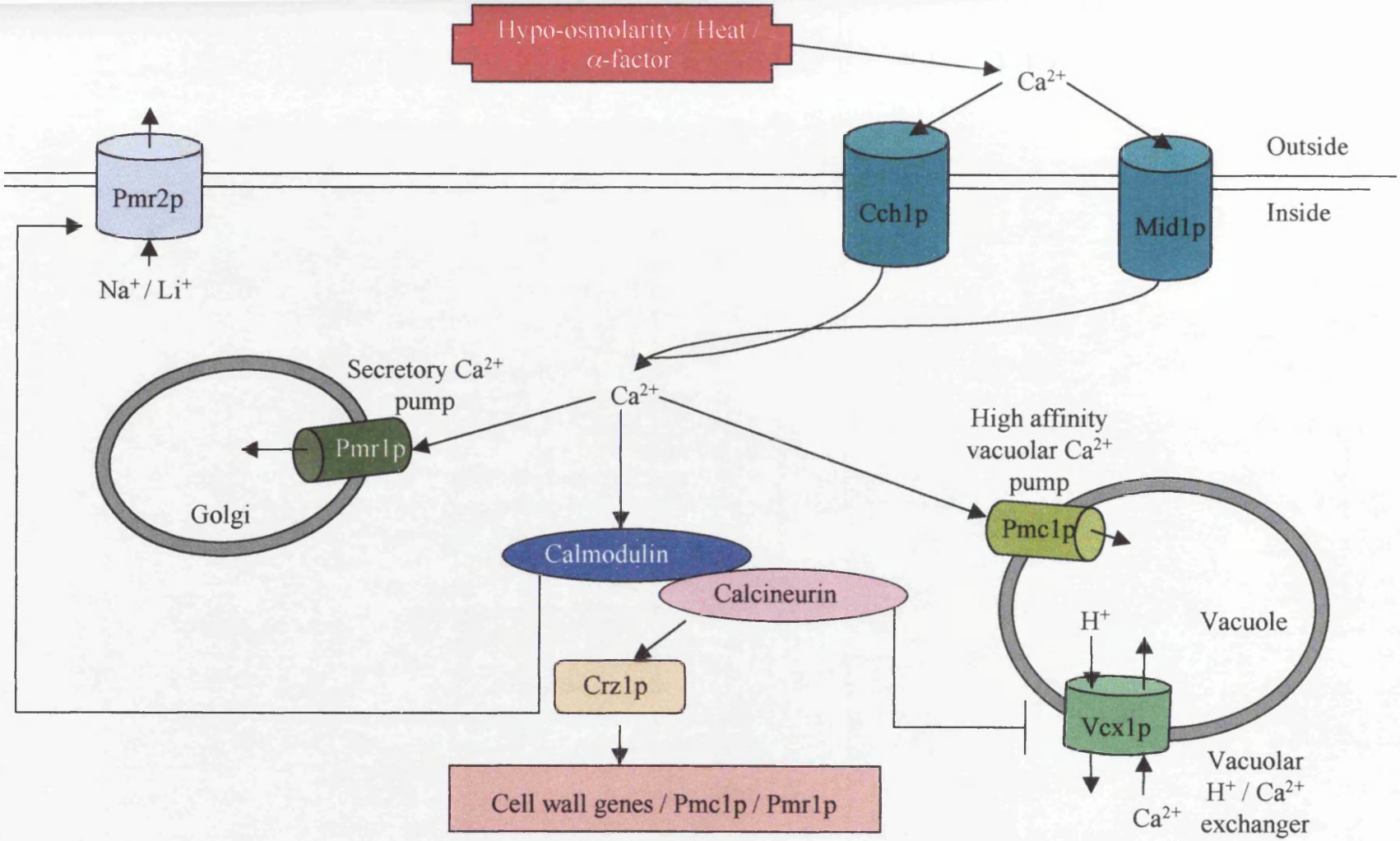

Figure 1.4. The Ca^{2+} / calmodulin / calcineurin signaling pathway in *S. cerevisiae*. Upstream of the calcineurin pathway, two putative Ca^{2+} channels Mid1p and Cch1p operate, to allow influx of Ca^{2+}. Calcineurin has many roles, many of which are regulated by the transcription factor Crz1p. Arrows indicate activation and lines with bars indicate inhibition. See text for details.

CNA) and a high-affinity calcium-binding regulatory subunit (Calcineurin-B; CNB). Calcineurin dephosphorylates NFAT transcription factor family members, allowing them to translocate into the nucleus and activate gene expression (Flanagan *et al.* 1991; Northrop *et al.* 1994; Timmerman *et al.* 1996).

In mammalian cells calcineurin regulates the transcription of the T-cell growth factor Interleukin-2 (Klee *et al.* 1998). The translocation of the transcription factor NF-ATp, in response to an increase of intracellular Ca^{2+} induced by the occupancy of the T-cell receptor, is dependent upon its dephosphorylation by calcineurin (Klee *et al.* 1998). Other functions of calcineurin in mammalian cells include, control of neuronal signalling, muscular hypertrophy, muscle contraction and apoptosis (Baksh and Burakoff 2000). Apart from mammalian cells, genes of calcineurin subunits have been identified in yeast, protozoa, plants and insects (Rusnak and Mertz 2000). The conserved structural features of calcineurin are responsible for the unique ability of calcineurin to interact specifically with and to be inhibited by two classes of immunosuppressive drugs cyclosporin-A (CsA) and FK506 (Klee *et al.* 1998).

1.4.1 The Ca^{2+}/calmodulin regulated calcineurin of *S. cerevisiae*

There are two genes for the catalytic subunit of calcineurin in yeast, *CNA1* and *CNA2*, and one gene for the regulatory B-subunit, *CNB1* (Cyert *et al.* 1991; Cyert and Thorner 1992; Kuno *et al.* 1991; Liu *et al.* 1991). The yeast calcineurin is involved in the regulation of various cellular processes including recovery from pheromone-induced growth arrest, adaptation to salt stress, cellular Ca^{2+} homeostasis and resistance to vanadate (Cunningham and Fink 1994-a; Cyert and Thorner 1992; Farcasanu *et al.* 1995; Hemenway *et al.* 1995; Nakamura *et al.* 1992, 1993, 1995). Calcineurin, is also implicated in the regulation of cell wall synthesis (Douglas *et al.* 1994; Garrett-Engele *et al.* 1995; Mazur *et al.* 1995).

1.4.2. Putative calcium channels involved in Ca^{2+} influx

MID1 was originally identified in a screen for mutants that died as shmoos after pheromone exposure (Iida *et al.* 1994). *MID1* was subsequently found to encode a plasma

membrane protein, which is required for efficient calcium influx upon pheromone treatment (Iida *et al.* 1994). Kanzaki *et al.* (1999), have shown that Mid1p is a putative stretch-activated non-selective cation channel (SA-Cat). SA-Cat channels are suggested to act as mechano-transducers in various biological functions such as sensation and hearing (Kanzaki *et al.* 1999). Functional expression of Mid1p in Chinese hamster ovary (CHO) cells, conferred sensitivity to mechanical stress that results in increase of cytosolic [Ca^{2+}] (Kanzaki *et al.* 1999). These increases in cytosolic [Ca^{2+}] were dependent on the presence of external [Ca^{2+}]. Ca^{2+} influx during mating in yeast cells may therefore rely on the remodeling of the cell wall and the stretch imposed on the plasma membrane because of turgor pressure, leading to activation of Mid1p channel activity.

The *CCH1* gene has been found to encode a second putative calcium channel in yeast, with homologies to the mammalian voltage gated calcium channel α1-subunit genes (Fischer *et al.* 1997). Cch1p has been found to mediate Ca^{2+} influx during the late stages of the mating response (Fischer *et al.* 1997). Deletion of both *MID1* and *CCH1* leads to similar phenotypes as the single deletions. This result indicates that the two gene products act in the same pathway. Moreover, since cells deficient for both *MID1* and *CCH1* display calcium influx which is reduced but not diminished, it is possible that other calcium channels operate during the pheromone response. Locke *et al.* (2000) have suggested that Cch1p and Mid1p function to provide adequate levels of Ca^{2+} to Pmr1p to sustain secretion and growth. This calcium influx is proposed to be due to a capacitative calcium entry (CCE) mechanism. CCE is a regulatory mechanism which stimulates Ca^{2+} influx specifically in response to depletion of calcium from the endoplasmic reticulum (Putney 1986, Putney and Mckay 1999). Depletion of secretory Ca^{2+} pools leads to stimulated Ca^{2+} influx *via* Mid1p and Cch1p (Locke *et al.* 2000). It remains unclear, however, if CCE is involved in Ca^{2+} entry after pheromone treatment.

1.4.3. Ca^{2+} homeostasis by the calcineurin signalling pathway

Calcineurin is involved in the regulation of Ca^{2+} pumps and exchangers responsible for Ca^{2+} homeostasis in yeast (Rusnak and Mertz 2000). These maintain cytoplasmic [Ca^{2+}] in the range of 100-300nM (Cunningham and Fink 1994-b). The vacuolar H^+-ATPase, provides the driving force for Ca^{2+} sequestration by the Ca^{2+}/H^+ exchanger Vcx1p (Fig.

1.4; Garrett-Engele *et al.* 1995; Hemenway *et al.* 1995; Tanida *et al.* 1995). Two Ca^{2+}-ATPases, Pmc1p and Pmr1p, are responsible for depleting the cytosol of Ca^{2+} (Fig. 1.4; Rusnak and Mertz 2000). Pmc1p is a homolog of the mammalian Plasma membrane Ca^{2+}-ATPase (PMCA) family (Cunningham and Fink 1994-a). Pmr1p is proposed to be a member of the sarco/endoplasmic reticulum (SERCA) family of Ca^{2+}-ATPase (Cunningham and Fink 1994-a). However, the inhibitor sensitivity of Pmr1p, indicates that it is a Ca^{2+} pump, which is distinct from the SERCA and PMCA pumps (Sorin *et al.* 1997).

Pmc1p is localised to the vacuole and Pmr1p is localised to the golgi, with important roles in the secretory pathway (Cunningham and Fink 1994-a; Rudolph *et al.* 1989). Proteins secreted from cells which are *pmr1Δ*, lack the outer chain glycosylation that results from passage through the golgi (Rudolph *et al.* 1989). Furthermore, loss of Pmr1p function suppresses the lethality of secretion blocks (Rudolph *et al.* 1989). Pmr1p has also been associated with regulatory events of protein degradation in the endoplasmic reticulum (Durr *et al.* 1998). In the absence of Pmr1p, there is an increase in cytosolic Ca^{2+} and increased accumulation of Ca^{2+} in the vacuole *via* Pmc1p (Forster and Kane 2000; Halachmi and Eilam 1996; Marchi *et al.* 1999). This accumulation of Ca^{2+} in the vacuole is independent of Vcx1p and is dependent on a functional calcineurin (Forster and Kane 2000; Halachmi and Eilam 1996; Marchi *et al.* 1999). Cells lacking Pmc1p or Pmr1p cannot grow in media containing high Ca^{2+}. Deletion of either of the calcineurin subunits, or treatment of cells with CsA or FK506, restores growth to *pmc1Δ* or *pmc1Δpmr1Δ* cells in high calcium (Cunningham and Fink 1994-a). These data suggesting that calcineurin activation can have a negative effect on growth. Calcineurin also inhibits the vacuolar H^+-ATPase Vcx1p, post-transcriptionally (Cunningham and Fink 1996).

1.4.4. Downstream targets of the calcineurin pathway

A downstream signalling component regulated by calcineurin is the zinc-finger transcription factor Crz1p/Tcn1p (Matheos *et al.* 1997; Stathopoulos and Cyert 1997). Activated Crz1p binds to a promoter element called the calcineurin-dependent response element (CDRE), and turns on a variety of genes (Stathopoulos and Cyert 1997). The transcriptional regulation of *PMC1*, *PMR1*, *PMR2A* and *FKS2*, which confer tolerance to

high Ca^{2+}, Mn^{2+}, Na^{+}, and cell wall damage respectively, require Crz1p (Matheos *et al.* 1997; Stathopoulos and Cyert 1997). Calcineurin regulates Crz1p by dephosphorylation, which leads to translocation of Crz1p to the nucleus (Stathopoulos-Gerontides *et al.* 1999). Crz1p displays some sequence similarities to the mammalian transcription factor NF-AT, which is also dephosphorylated by calcineurin for translocation to the nucleus (Stathopoulos-Gerontides *et al.* 1999). The translocation of Crz1p to the nucleus requires the karyopherin Nmd5p which binds to the dephosphorylated form of Crz1p (Polizotto and Cyert 2001).

1.4.5. Regulators of calcineurin

The DNA-binding domain of Skn7p (response regulator of the HOG pathway) is required for binding of Skn7p to Crz1p and calcineurin *in vitro* (Williams and Cyert 2001). Skn7p appears to modulate calcineurin-dependent transcriptional output, by affecting the rate of Crz1p turnover (Williams and Cyert 2001). By binding to Crz1p and calcineurin, Skn7p has been proposed to protect Crz1p from degradation.

Rcn1p, is a member of the calcineurin inhibitor family, of which the mammalian DSCR1 is a member (Kingsbury and Cunningham 2000). Rcn1p has been found to bind calcineurin and inhibit its phosphatase activity *in vitro*; moreover, it prevents activation of Crz1p *in vivo* (Kingsbury and Cunningham 2000). Calcineurin signalling induces Rcn1p expression through Crz1p, indicating that Rcn1p is a feedback inhibitor of calcineurin signalling (Kingsbury and Cunningham 2000). Calcium or a calcium-dependent mechanism has been proposed to down-regulate calcineurin expression and accumulation, since in presence of FK506 and high calcium Cna1p levels decreased within cells (Kingsbury and Cunningham 2000). Rcn1p has also been proposed to regulate calcineurin expression, since in *rcn1Δ* cells, the level of Cna1p is reduced (Kingsbury and Cunningham 2000). These conflicting roles of Rcn1p have lead to the proposal that Rcn1p functions to "fine tune" the activity of calcineurin (Kingsbury and Cunningham 2000).

1.4.6. Ca^{2+}/calmodulin regulation of vacuole fusion

Transport from the endoplasmic reticulum to the golgi-apparatus, endosome fusion and assembly of the nuclear envelope in the cell depends on Ca^{2+} (Pryer *et al.* 1992). Membrane fusion involves several subreactions which include, priming, tethering and docking. It has been shown that Ca^{2+} is released from the vacuolar lumen following the completion of the docking step (Peters and Mayer 1998). Calmodulin is the putative calcium sensor, and is the first component required for the post-docking phase of vacuole fusion (Peters and Mayer 1998). Calmodulin has been found to actively promote bilayer mixing (Peters and Mayer 1998).

1.4.7. Cell cycle regulation by calcineurin

Calcium has been implicated in regulating the cell cycle progression in G_2 phase (Mizunuma *et al.* 1998). The Swe1p kinase inhibits a G_2 form of Cdc28p by phosphorylation, and hence delays the onset of mitosis (Morphogenetic check-point; Booher *et al.* 1993). The regulation of cell cycle is proposed to occur through activation of calcineurin and Mpk1p by Ca^{2+}, leading to activation of Swe1p by these two pathways (Mizunuma *et al.* 1998). Mck1p, a member of the glycogen synthetase kinase-3 (GSK-3) family, has been suggested to be a downstream regulator of Mpk1p pathway branch of calcium-induced G_2 delay/arrest (Mizunuma *et al.* 2001). Together, Mck1p and calcineurin have been found to down-regulate the Swe1p inhibitor Hsl1p in response to high calcium levels (Mizunuma *et al.* 2001).

1.4.8. Salt tolerance and the calcineurin signalling pathway

Calcineurin deficient yeast display decreased tolerance to the monovalent cations Li^+ and Na^+ (Mendoza *et al.* 1996; Nakamura *et al.* 1993). Adaptation to high salt stress requires the presence of a plasma membrane Na^+-ATPase involved in Na^+ and Li^+ efflux, Pmr2p. Cells deficient in calcineurin accumulate Na^+ and Li^+ due to decreased expression of Pmr2p (Mendoza *et al.* 1994). Ca^{2+}, *via* calmodulin activation of calcineurin, regulates adaptation to high salt stress by induced expression of Pmr2p (Danielsson *et al.* 1996; Hirata *et al.* 1995; Mendoza *et al.* 1996). The activity of Pmr2p is also stimulated by

Ca^{2+}/calmodulin; hence regulating Na^{+} efflux transcriptionally and post-translationally by Ca^{2+} (Rudolph *et al.* 1989; Weiland *et al.* 1995).

1.4.9. Ca^{2+}/calcineurin regulation of the mating response

Exposure of haploid cells to mating pheromone leads to an increase in intracellular calcium and activation of calcineurin (Withee *et al.* 1997). External Ca^{2+} is required for increased cytoplasmic Ca^{2+} (Iida *et al.* 1990). This external cytoplasmic Ca^{2+} is also required for survival, because cells treated with pheromone in Ca^{2+} depleted medium lose viability (Iida *et al.* 1990). Cells deficient for either of the calcineurin subunits fail to recover from mating pheromone-induced growth arrest (Cyert *et al.* 1991; Cyert and Thorner 1992). Addition of cyclosporin-A or FK506 to α-factor treated cells also renders cells defective for recovery from pheromone-induced growth arrest, most likely due to cell death (Foor *et al.* 1992). The activator protein of calcineurin, calmodulin, has been shown to be required for recovery from cell cycle arrest after pheromone treatment (Moser *et al.* 1996). Ca^{2+}-calmodulin and its targets are required for maintenance of cell viability in the continuous presence of pheromone (Moser *et al.* 1996).

1.5. Mating pheromone response pathway

The pheromone response pathway of *S. cerevisiae* mediates mating of two haploid cells. This process is stimulated by the release of small peptide mating pheromones, **a**-factor from *MAT***a** cells and α-factor from *MAT*α cells. These mating pheromones act on cells of the opposite mating type to prepare that cell for mating. Cellular responses to mating pheromone include polarised growth towards a mating partner, cell cycle arrest in G_1, and increased expression of proteins needed for cell-adhesion, cell-fusion and nuclear-fusion (Gustin *et al.* 1998).

1.5.1. Activation of the mating pheromone MAPK cascade

Pheromone binds to and activates a seven transmembrane domain receptor that in turn induces the dissociation of a heterotrimeric G protein (Blinder *et al.* 1989; Dietzel and Kurjan 1987; Jahng *et al.* 1988; Miyajima *et al.* 1987). The receptor for **a**-factor is

encoded by *STE3* and that for α-factor is encoded by *STE2* (Fig. 1.5; Herskowitz 1988). The G protein consists of Gα, Gβ and Gγ subunits, encoded by *GPA1*, *STE4* and *STE18* genes respectively (Kurjan 1993; Marsh *et al.* 1991). Gβγ subunit is the activator of the downstream components, and binding of Gα to the Gβγ subunit prevents this activation in the absence of mating pheromone (Herskowitz 1995; Leberer *et al.* 1997; Marsh *et al.* 1991). Sst2p is proposed to be the GAP for the Gα-GTP, and hence it is involved in the adaptation response to mating pheromone (Berman *et al.* 1996; Dietzel and Kurjan 1987; Dohlman *et al.* 1996).

1.5.2. The mating pheromone MAPK cascade and the Ste5p Scaffold

The MAPK cascade consists of MAPKKK Ste11p, which phosphorylates and activates the MAPKK Ste7p (Fig. 1.4; Neiman and Herskowitz 1994; Zhou *et al.* 1993). Ste7p in turn phosphorylates and activates the redundant MAPKs Fus3p and Kss1p (Fig. 1.4; Errede *et al.* 1993; Gartner *et al.* 1992; Ma *et al.* 1995). Fus3p has been the more critical kinase for most of the pheromone response outputs (Farley *et al.* 1999). Downstream of the MAPKs is the transcriptional activator Ste12p, which governs the expression of many genes for the pheromone response pathway, cell and nuclear fusion (Fig. 1.5). It binds to a pheromone response element (PRE) located in the 5' regulatory region of target genes (Kurjan 1993; Marsh *et al.* 1991).

Two proteins, Rst1p and Rst2p interact with and appear to act as inhibitors of Ste12p activation in the absence of pheromone (Cook *et al.* 1996; Tedford *et al.* 1997). Both Fus3p and Kss1p interact with these proteins and phosphorylate them (Cook *et al.* 1996; Tedford *et al.* 1997). Rst1p and Rst2p are proposed to form a complex with Ste12p at specific target sites, preventing transcriptional activation. Upon pheromone stimulation, Fus3p and Kss1p phosphorylate Rst1p, Rst2p and Ste12p, resulting in dissociation of the complex and allowing Ste12p to activate transcription. Fus3p also promotes G_1 arrest by phosphorylating Far1p (Elion *et al.* 1993; Peter *et al.* 1993). This phosphorylation stabilises Far1p and allows its association with the three different Clnp/Cdc28p complexes leading to the inhibition of their activity (Peter *et al.* 1993; Tyers and Futcher 1993; Jeoung *et al.* 1998). Kss1p promotes recovery from G_1 arrest by inhibiting the MAPK cascade at or below Ste11p (Cherkasova *et al.* 1999).

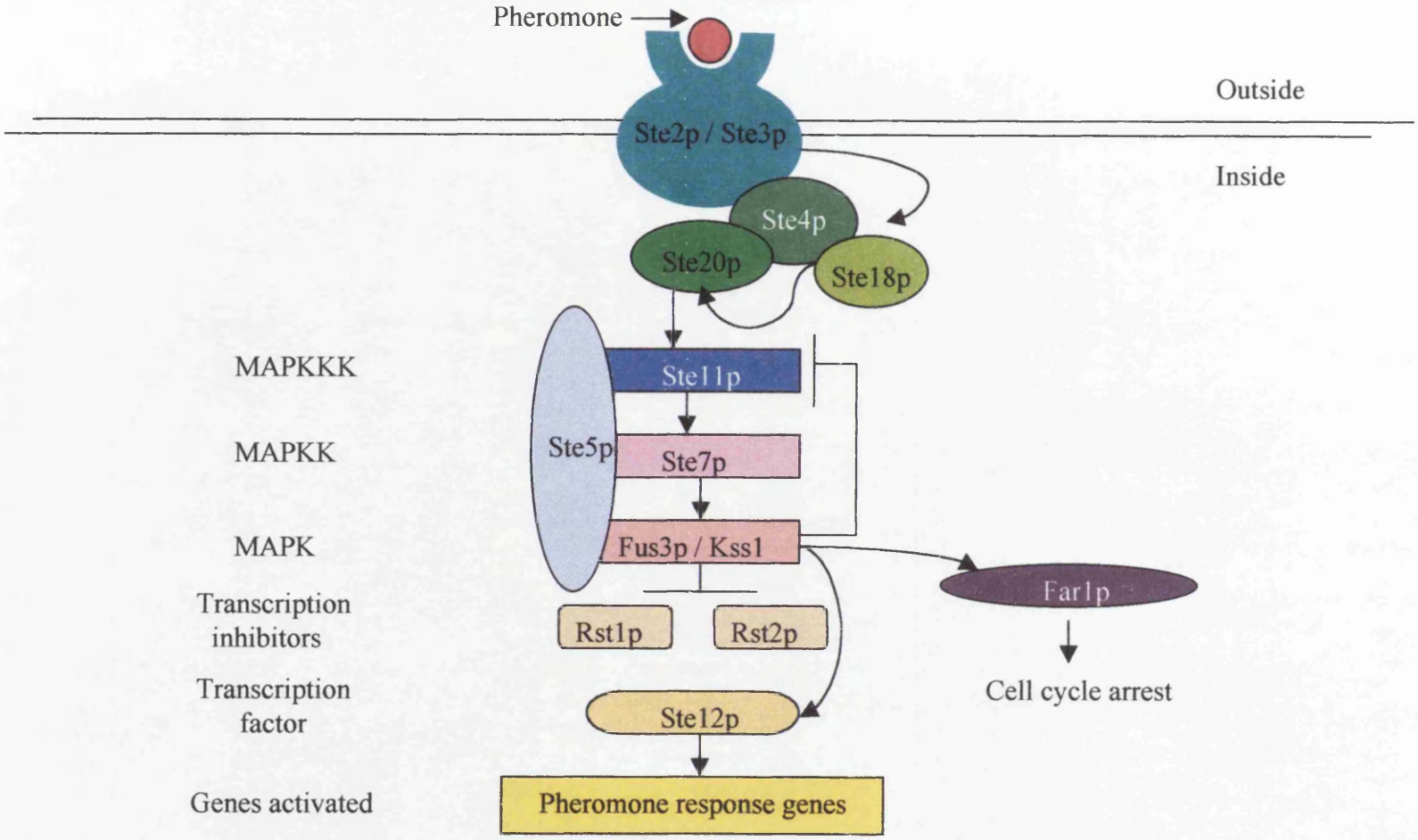

Figure 1.5. The pheromone response pathway of *S. cerevisiae*. The pheromone response pathway is activated upon binding of **a**-factor or α-factor to the receptors Ste3p and Ste2p respectively. Ste5p acts as a scaffolding that keeps the cascade together. Arrows indicate activation and lines with bars indicate inhibition. See text for details.

STE5 encodes a protein with a LIM-like motif, which appears to mediate protein-protein interactions in other systems (Leberer *et al.* 1993). Ste5p interacts with all components of the MAPK cascade: Ste11p, Ste7p, Fus3p and Kss1p (Fig. 1.5; Yablonski *et al.* 1996). Each of the MAPK components interacts with different region of Ste5p (Choi *et al.* 1994; Inouye *et al.* 1997). Specifically, Ste5p interacts with the amino-terminal inhibitory domain of Ste11p, so that it may regulate the activity of this kinase (Choi *et al.* 1994; Printen and Sprague 1994). Ste5p has therefore been proposed to act as a scaffold that brings the MAPK components together to promote sequential phosphorylation during activation of the pathway, and also for attenuation of response by Fus3p (Elion 1995; Yablonski *et al.* 1996). Ste5p may undergo some conformational changes to fulfill its role as a scaffold protein (Sette *et al.* 2000). In its role as a scaffold, Ste5p may also prevent cross talk between pathway by confining the components of the pheromone response MAPK cascade in a complex. The MAPKKK Ste11p also interacts with the HOG pathway (Posas and Saito 1997). If Ste11p was free to cross-talk then pheromone might activate the HOG pathway.

1.5.3. Ste20p regulation of the mating pheromone MAPK cascade

Ste20p is a serine/threonine protein kinase of the p21-activated kinase (PAK) family found in different eucaryotic organisms and is shown to be activated *in vitro* by GTP-bound Cdc42p (Burbello *et al.* 1995; Sells and Chernoff 1997). Ste20p, contains a C-terminal catalytic domain and a N-terminal non-catalytic region, within which is a conserved motif, the Cdc42/Rac interactive binding motif (CRIB), necessary for binding by activated Cdc42p (Burbello *et al.* 1995). However, the binding of Cdc42p to Ste20p is not required for transcriptional induction, mating projection formation or G_1 arrest (Burbello *et al.* 1995). Deletion of the CRIB motif, induces reduced zygote formation, and cells fail to localise Ste20p to the tip of the shmoo, implying that the Cdc42p-Ste20p interaction may be required for cell-cell fusion (Leberer *et al.* 1997; Peter *et al.* 1996). However, Ste20p localisation to the shmoo tip may also be required for polarised growth (Sheu *et al.* 2000).

At its C-terminal non-kinase domain, Ste20p interacts with Ste4p (Gβ; Leeuw *et al.* 1998). Phosphorylation of Ste20p and association of Ste4p with Ste20p is stimulated by

pheromone (Leeuw *et al.* 1998; Wu *et al.* 1995). Signal transduction from the Gβ protein Ste4p to the downstream MAPK cascade requires the protein kinase Ste20p and Ste5p (Leberer *et al.* 1992; Ramer and Davis 1993). Ste20p also activates Ste11p by direct phosphorylation to mediate signal from the pheromone receptor to the MAPK pathway (Dan *et al.* 2001).

1.5.4. The cytoskeleton and the mating pheromone MAPK cascade

Cdc42p and Bem1p, connect the pheromone response pathway to the cytoskeleton (Gustin *et al.* 1998). Cdc42p is proposed to collaborate with Bem1p to facilitate signal transduction (Moskow *et al.* 2000). Cdc42p is required to orient the actin cytoskeleton to form mating projections (Adams *et al.* 1990; Drubin and Nelson 1996; Li *et al.* 1995). The growth of mating projections in response to pheromone involves activation of Cdc42p but not the MAPK cascade kinases (Nern and Arkowitz 1998; Schrick *et al.* 1997). Bem1p associates with actin and with the pheromone response pathway-signalling proteins, Ste5p and Ste20p (Leeuw *et al.* 1995; Lyons *et al.* 1996). Moskow *et al.* (2000) have suggested that Cdc42p-Bem1p act as a scaffold that concentrates the signalling kinases, hence promoting activation of the kinase cascade by Ste20p. The Bem1p-bound Ste5p is complexed to the Ste11p-Ste7p-Fus3p MAPK cascade (Lyons *et al.* 1996). Interaction of Ste20p with Bem1p is required for the association of Ste20p with actin (Leeuw *et al.* 1995).

1.5.5. Transcriptional regulation of the mating pheromone MAPK cascade

Pheromone stimulation activates transcription of many different genes. Among the products of these genes are proteins that activate (Fus3p) or inhibit (Msg5p) signalling in the pathway (Doi *et al.* 1994; Elion *et al.* 1990; Zhan *et al.* 1997). Other gene products include proteins needed for cell fusion (Fus1p), nuclear fusion (Kar4p) and other mating related functions (Kurihara *et al.* 1996; McCaffrey *et al.* 1987; Trueheart *et al.* 1987). All these genes have one thing in common, in that they contain in their promoter region repeats of a pheromone response element (PRE) that is necessary and sufficient for pheromone regulated transcription (Hagen *et al.* 1991). The MAPK cascade mediates transcription of PRE-containing genes through phosphorylation and activation of at least

three nuclear proteins (Gustin *et al.* 1998). These nuclear proteins are Rst1p, Rst2p and Ste12p (Song *et al.* 1991; Tedford *et al.* 1997). Rst1p and Rst2p are negative regulators of the transcription factor Ste12p.

1.6. Outline of the project aims

MID2, identified in a screen for mutants that die in presence of mating pheromone, has no known function (Ono *et al.* 1994). As *MID2* contains PRE elements in its 5' region, then Mid2p may be a good candidate that may be involved in activation of the Pkc1p-MAPK pathway during the mating response and also during vegetative growth (section 1.3.8). This project work will analyse the possible role of *MID2* in the Pkc1p-MAPK pathway. Furthermore, the role of Mid2p and components of the Pkc1p-MAPK pathway during the mating pheromone response will be examined.

The role of calcineurin during cell surface stress will be investigated. As the calcineurin and the Pkc1p-MAPK pathway appear to be co-activated when cells are heat shocked or exposed to mating pheromone, the possible co-regulation of these two pathways during cell surface stress would also be assessed.

Chapter 2: Materials and Methods

2.1. Reagents and Equipment

All the chemicals used were of higher grade and quality available. The main suppliers of chemicals were SIGMA and BDH chemicals Ltd, unless otherwise stated. Solid chemicals were weighed by a top loading balance with a range of 1g to 1 Kg. Smaller quantities were measured by the Oertling analytical balance (model R20) for weight less than 1g. Centrifugation was carried out in a BECKMAN (model J2-21) refrigerated super-speed centrifuge or BECKMAN Benchtop centrifuge and for samples less than 1.5 ml, the HERMLE (model Z 230 M) minifuge was used. For absorbance readings, the MILTON ROY SPECTRONIC spectrophotometer (model 601) was used. The New Brunswick Scientific shaker incubator (model G76) was used for growing bacterial or yeast cultures. The BIBBY Stuart Scientific incubator was used for growing bacteria or yeast on solid media. The MJ Research thermal cycler (model PTC-200) PCR machine was used for DNA amplifications. Measurement of 5μl to 1ml were measured using Gilson pipettes. Millipore filter units used had pore size of 0.2μm with Luer fitting.

Anti-Mpk1p antibodies and secondary antibodies used in the western blots were supplied by Santa Cruz Biotechnology. Antibodies against the dually phosphorylated Mpk1p were supplied by Upstate Biotechnology. Pre-cast 10% Bis-Tris NuPAGE gels were obtained from Invitrogen (Novex). Radioactivity was supplied by Amersham. HYBAID hybridisation oven was used for blot hybridisation. The Leitz WEYZLER microscope and a Zeiss AXIOSKOP were used for light microscopy and micro-dissection respectively. Fluorescent cells were viewed with an olympus BX-60 fluorescent microscope with a 100 W mercury lamp and an olympus 100× Plan-NeoFluor oil-immersion objective. Images were captured using a Roper scientific MicroMax 1401E cooled CCD camera using Scananalytics IP lab software on an apple Macintosh 7300 computer. The Olumpus UMW filter was used for calcofluor stained cells and teh U-M41 W070 filter was used for Rhodamine stained cells.

2.2. Oligonucleotide sequences and genotypes of plasmids, yeast and bacterial strains

2.2.1. Bacterial strain

Table. 2.1.

Strain	Genotype	Source/reference
E. coli DH5α	*SupE*44 Δ*lacU*169 (ϕ80*lacZ*ΔM15) *hsdR*17 *recA*1 *endA*1 *gyrA*96	Hanahan (1983)

2.2.2. Plasmid and deletion construct descriptions

Table 2.2.

Plasmid	Description	Source/reference
pG6	*YEp*24	JVG
pG5	*YCP*50	JVG
p*RLM1* : : *LEU2*	*rlm1Δ* : : *LEU2* (pBluescript)	Watanabe *et al.* (1995)
pDS147	*mid2Δ* : : *URA3* (pGEM-T Amp^r)	Stirling D. A.
pDS143	2μ *MID2* (*YEp*24 Amp^r)	Stirling D. A.
pDL289	2μ *PKC1*	Levin D. E.
pDL582	2μ *MPK1*(*YEp*24)	Kamada Y.
pJO36	2μ *HCS77* (*YEp*24)	JVG
pG20	2μ *RHO1*	Hall M.
pDL636	*CEN BCK1-20*	Levin D. E.
pUL9	*LEU2*-KanR (pUC19-*URA3*) Amp^r Kan^r	Cross R. C.
pFA6-kanMX4	KanMX	Ayscough K.
pAMS347	4×CDRE : : *lacZ* (pBluescript)	Cyert M. S.
pVT-L[CNA2]	*CNA2*	Cyert M. S.
pVT-L[CNA2Δ]	*CNA2Δ*	Cyert M. S.
pDL468	*pGal*1/10 [*PKC1* : : HA] (pBM743)	Levin D.
PDL469	*pGal*1/10 [*PKC1*-K853R : : HA] (pBM743)	Levin D.
pDL242	*pGal*1/10 [*PKC1*-R398A] (pBM743)	Boone C.
PDL293	*pGal*1/10 [*PKC1* : : HA] (pBM743)	Boone C.

2.2.3. Yeast Strains

Genetic background that is common to all of a particular strain type is indicated in brackets.

Table 2.3.

Strain Background	Genotype	Source/ Strain Number
EG123	*MAT***a** *(trp1-1 leu2-3,112 ura3 his4 can1)*	JVG 718
EG123	*MATα*	JVG 719
EG123	*MAT***a**/*MATα*	JVG 720
EG123	*MAT***a** *mid2::URA3*	This study JVG 1237
EG123	*MAT***a** *mid2::LEU2*	This study JVG 1282
EG123	*MAT***a** *hcs77::LEU2*	JVG 337
EG123	*MATα hcs77::LEU2*	JVG 338
EG123	*MAT***a**/*MATα mid2::URA3/MID2 hcs77::LEU2/HCS77*	This study JVG 1329
EG123	*MAT* **a** *mid2::URA3 hcs77::LEU2*	This study JVG 1331
EG123	*MAT***a**/*MATα mid2::URA3/MID2 mpk1::TRP1/MPK1*	This study JVG 1284
EG123	*MAT***a** *mpk1::TRP1*	JVG 219
EG123	*MAT***a** *rlm1::LEU2*	This study JVG 1361
EG123	*MAT***a** *bck1::URA3*	JVG 208
EG123	*MAT***a** *mid1::KanMX*	This study JVG 1360
Y53-6D	*MATa stt1-1 (pkc1^{ts}) leu2 ura3 his3 ade8 met3*	Yoshida S. JVG300
S288C	*MAT***a** *(trp1-Δ63 leu2-Δ1 ura3-52 his3-Δ200 ade2-101 lys2-801)*	JVG 964
S288C	*MATα*	JVG 963
S288C	*MAT***a**/MATα	JVG 967
S288C	*MAT***a** *mid2::URA3*	This study JVG 1236
S288C	*MAT***a** *hcs77::LEU2*	JVG 1079
S288C	*MAT***a**/*MATα hcs77::LEU2/hcs77::LEU2 TRP1/TRP1*	JVG 1081
S288C	*MAT***a**/*MATα mid2::URA3/MID2 hcs77::LEU2/HCS77*	This study JVG 1246
S288C	*MATα bar1::LEU2*	JVG 983
S288C	*MAT***a** *bar1::LEU2*	JVG 984
S288C	*MAT***a** *mid2::URA3 bar1::LEU2*	This study JVG 1279

S288C	*MAT***a** *swi4::HIS3 TRP1*	JVG 970
S288C	*MAT***a**/**MAT**α *swi4::TRP1/ swi4^{ts} bar1::LEU2/BAR1 TRP1/TRP1*	JVG 998
SP1	*MAT***a** (*leu2 his3 ura3 trp1ade8)*	Ballester R. JVG 1336
SP1	*MAT***a** *wsc1::ADE8 wsc2::URA3 wsc3::TRP1*	Ballester R. JVG 1335
W303	*MAT***a** (*ura3 leu2 his3 trp1 ade2 can1*)	JVG 1312
W303	*MAT***a** *mid2::URA3*	This study JVG 1367
W303	*MAT***a** *bni1::KanMX*	Boone C. JVG1304
W303	*MAT***a** *mid2::URA3 bni1::KanMX*	This study JVG 1451
W303	*MAT***a** *mpk1::TRP1*	This study JVG 1369

Note: All strains with no source indicated above are from the Gray laboratory strain collection.

2.2.4. Oligonucleotide sequences

Table 2.4.

Name	Sequence 5'-3'
mid1Δ forward	CGTCATTTTGGGCATTGTGATGTTAAAGACAGGTCGCCGTCATAATAAAGAT TCCCGGATCCCCGGGTTAATTAA
mid1Δ reverse	ATATATTAATGTCCAACTCATCAGTCACAGGTATATCTTTAACATTGAAACTA TTGAATTCGAGCTCGTTTAAAC
KanMX6 internal forward	GGCTGACGGAATTTATGCCT
MID1 downstream reverse	TGGTTGCGGAAAATTTCCCT
MID1 forward	ACCAACGCCTTTTAAAGGGA
MID1 reverse	CATGAATACGTAAATTTAGC
FKS2 forward	ATGTCCTACAACGATCCAAA
FKS2 reverse	TATCTATCTTTGGAATACAA
ACT1 forward	TCTAAGATCTTGGCTACTAC
ACT1 reverse	GTTCATGTGGTTTAAACTTG

2.3. Yeast and bacterial growth media

All media were autoclaved for 20 minutes at 20 lb/sq.in. (120°C). Osmotically stabilised media contained 10% w/v sorbitol.

2.3.1. YPD medium

2% w/v bacto-agar (for YPD agar plates only - use 4% for dissection purposes)
2% w/v bacto-peptone
1% w/v yeast extract
2% w/v glucose (2% raffinose or 2% w/v galactose and 0.1% w/v sucrose replace glucose for galactose induction)

2.3.2. SD medium

2% w/v bacto-agar (for SD agar plates only)
1% w/v yeast nitrogen base
4% w/v glucose (2% w/v galactose and 0.1% w/v sucrose replace glucose for galactose induction)
1 × amino acids mixture - omit the amino acid selected for, in each experiment (section 2.4)

2.3.3. Calcium-deficient chemically-defined medium

2% w/v glucose
0.35% w/v ammonium sulphate
0.1% w/v potassium dihydrogen-orthophosphate (KH_2PO_4)
0.05% w/v magnesium sulphate ($MgSO_4.7H_2O$)
0.05% w/v sodium chloride (NaCl)
1 × amino acids mixture - omit the amino acid selected for, in each experiment (section 2.4)
1 × trace elements (section 2.5)
1 × growth factors (section 2.6)

0.001% biotin

0.001% folic acid

2.3.4. Sporulation medium

2% w/v bacto-agar (for sporulation agar plates only)

0.1% w/v bacto-yeast extract

0.05% dextrose

1% w/v potassium acetate

1 × amino acids (section 2.4)

2.3.5. LB medium

2% w/v bacto-agar (for LB agar plates only)

1% w/v bacto-tryptone

0.5% w/v bacto-yeast extract

1% w/v sodium chloride (NaCl)

2.4. 10 × amino acid mixture

The amino acid mixture were autoclaved for 20 minutes at 20 lb/sq.in. (120°C).

Table 2.5.

Amino acids	
0.015% w/v isoleucine	0.015% w/v lysine
0.075% w/v valine	0.01% w/v methionine
0.02% w/v adenine	0.025% w/v phenylalanine
0.01% w/v arginine	0.1% w/v threonine
0.01% w/v histidine	0.02% w/v tryptophan
0.01% w/v uracil	0.015% w/v tyrosine
0.05% w/v leucine	

2.5. 100 × trace elements

0.005% w/v boric acid (H_3BO_3)

0.0036% w/v manganese chloride ($MnCl_2.4H_2O$)

0.004% w/v zinc sulphate ($ZnSO_4.7H_2O$)

0.001% w/v ferric chloride ($FeCl_3.6H_2O$)

0.001% w/v sodium molybdate ($Na_2MoO_4.2H_2O$)

0.0005% w/v potassium iodide (KI)

0.00025% w/v copper sulphate ($CuSO_4$)

2.6. 100 × growth factors

0.1% w/v myo-inositol

0.02% w/v sodium pantothenate

0.004% w/v nicotinic acid

0.004% w/v pyridoxine hydrochloride

0.004% w/v thiamine

0.002% w/v p-aminobenzoic acid

0.002% w/v riboflavin

2.7. Antibiotics

Table 2.6.

Antibiotic	Stock solution	Working concentration (μg/ml)
Ampicilin	100 mg/ml in H_2O	100
Carbenicilin	35 mg/ml in ethanol	35
Chloramphenicol	30 mg/ml in ethanol	50
Kanamycin	30 mg/ml in H_2O	50
Tetracyclin	16 mg/ml in 50% v/v ethanol	50
G-418	200 mg/ml in H_2O	200

2.8. Growth and monitoring of liquid cultures

Liquid cultures of *E. coli* or *S. cerevisiae* were grown in a shaking incubator (typically at 37°C for *E. coli* and 25°C for *S. cerevisiae*). Cultures were typically grown to mid-logarithmic phase (0.2-0.4 apparent optical density), unless otherwise stated. Growth was monitored by aseptic removal of 1 ml culture sample and measurement of apparent optical density at a wavelength of 600 nm using a UV/Vis spectrophotometer.

2.9. Cryopreservation of *E. coli* and *S. cerevisiae*

Stabilates of yeast and bactrial cells were made by mixing equal volumes of cell cultures in mid-logaithmic phase with 50% glycerol. These stabilates were stored at -80°C, and could be thawed and streaked directly on to agar plates.

2.10. DNA manipulations

2.10.1. Restriction analysis

Restriction analysis of plasmids was carried out intotal volume of 20 μl. Appropriate buffer (10×, supplied by manufacturer; 2 μl) was added to the DNA (approximately 0.5-1 μg; 17 μl), enzyme (5 U; 1μl) was then added and the reaction mixture was incubated at 37°C for 3 hours.

2.10.2. Purification of DNA from agarose gels

QIAquick gel extraction kit from QIAGEN was used to purify individual DNA fragments from low-melt agarose gels. Manufacturers instructions were followed as described herein. The DNA fragment was excised from the agarose gel and placed in an Eppendorf tube, and its weight and approximate volume (1 g ≈ 1 ml) were determined. QG buffer at 3 × gel volume was added and the mixture was incubated at 50°C for 10 minutes, until the gel had completely dissolved. To the dissolved agarose mixture, 1 × volume of isopropanol was added to the sample and mixed. This sample was then applied to a QIAquick spin column and centrifuges at 14000 rpm for 1 minute. The column was then

washed with 0.5 ml of QG buffer and centrifuged at 14000 rpm for 1 minute. A second wash was carried out by addition of 0.75 ml of PE buffer to the column and centrifuged for 1 minute at 14000 rpm. The DNA was eluted into a new tube by addition of 50 μl of EB buffer, and centrifugation at 14000 rpm for 1 minute.

2.10.3. Purification of plasmids from *E. coli*

QIAGEN maxi-prep kit was used for purification of plasmid DNA from *E. coli*. Manufacturers instructions were followed as described herein. 100 ml of mid-logarithmic phase culture was centrifuged at 6000 rpm for 15 minutes at 4°C. The bacterial pellet was resuspended in 10 ml of P1 buffer. To this suspension, 10 ml of P2 buffer was added while gently mixing, and was incubated at room temperature for 5 minutes. 10 ml of P3 buffer was added, mixed and incubated on ice for 20 minutes. The suspension was then centrifuged at 12000 rpm for 30 minutes at 4°C. The supernatant was recentrifuged at 12000 rpm for 15 minutes at 4°C. The supernatant was then applied to a pre-equilibrated (with 10 ml of QBT buffer:) QIAGEN-tip. The column was washed with 2 × 30 ml of QC buffer. The DNA was subsequently eluted with 15 ml of QF buffer. DNA was precipitated by addition of 10.5 ml of isopropanol to the eluent, mixed and centrifuged at 9500 rpm at 4°C for 30 minutes. The DNA pellet was washed with 5 ml of ethanol, centrifuged at 9500 rpm for 10 minutes. The pellet was air-dried and resuspended in 50 μl of water.

2.11. Agarose electrophoresis

Agarose electrophoresis was carried out using a HORIZON (11.4) electrophoresis apparatus. Agarose gels (0.8-1%) were prepared in, and run with TAE buffer (0.04 M Tris-acetate, 1 mM EDTA). DNA samples were mixed with 0.2 volumes of loading buffer (0.25% w/v bromophenol blue and 40% w/v sucrose), loaded on the gel and electrophoresed at a constant voltage (100 V) over 1.5-2 hours.

2.12. Polymerase chain reactions (PCR)

The DNA for amplification of pFA6-kanMX4 plasmid for deletion of the *MID1* coding sequence, or amplification of *FKS2*, *MPK1* or *ACT1* coding sequences for probing purposes was carried out using the primers listed in section 2.2.4. Oligonucleotide primers were synthesised commercially by MWG Biotechnology group. The components listed below were added to a 0.5 ml microfuge tube and were maintained on ice.

20 ng of template (Genomic DNA or plasmid) 1 μl
25 ρmoles of forward primer 2 μl
25 ρmoles of reverse primer 2 μl
2 mM dNTPs 4 μl (containing 2 mM of each of dATP, dTTP, dCTP and d GTP)
25 mM $MgCl_2$ 2.5 μl
10 × Taq buffer 10 μl
2.5 U of Taq polymerase 1 μl
77.5 μl of nuclease free sterile, deionised and distilled H_2O

The tubes were then transferred to a programmable thermal cycler with a heated lid to undergo the following program

Step1: Denaturation at 95°C for 5 minutes
Step2: Denaturation at 94°C for 1 minute
Step3: Annealing at 47.5°C for 1 minute
Step4: Elongation at 72°C for 1.5 minutes
Step5: To step 2 for 10 cycles
Step6: Denaturation at 94°C for 1 minute
Step7: Annealing at 52.5°C for 1 minute
Step8: Elongation at 72°C for 1.5 minutes
Step9: To step 6 for 20 cycles
Step10: Elongation at 70°C for 5 minutes
Step11: Storage at 4°C

2.13. Disruption of *MID1* loci

The *MID1* reading frame was completely replaced by the kanMX knockout cassette. All primer sequences used for PCR of kanMX were homologous to the target gene sequence immediately downstream of the start codon and upstream of the stop codon. PCR reaction were set up as described in section 2.12 with primers listed in table 2.4. Yeast cells were transformed (as described in section 2.15) with 10 μl of the kanMX PCR reaction (without any purification of the PCR product). Transformant colonies were picked and streaked onto YPD plates containing G418 (200 μg/ml). These colonies were resuspended in 50 μl of SPZ buffer (1 M sorbitol, 10 mM phosphate buffer, 10 U Zymolyase), boiled for 5 minutes, cooled briefly on ice and then centrifuged at 14000 rpm for 30 seconds at room temperature. Supernatant (5 μl) was added to PCR reaction with primers internal to the *MID1* coding region, or one primer internal to the transformation module and a second primer outside of the *MID1* coding sequence were used to test efficiency of marker integration. Colonies with efficient disruption of *MID1* loci displayed no PCR product with the first set of primers and showed a PCR product of expected size (1059 bp) with the second set of primers, after running samples on agarose gels.

2.14. Preparation of competent *E. coli*

Mid-logarithmic cell cultures were harvested by centrifugation at 4000 rpm for 10 minutes at 4°C. The pellet was resuspended in ice-cold 100 mM $CaCl_2$ (10 ml). The suspension was centrifuged at 4000 rpm for 10 minutes at 4°C. The pellet was resuspended in 5 ml of ice cold 100 mM $CaCl_2$ and 20% glycerol. Cells were aliquoted into 50 μl samples and stored at -80°C.

2.15. Transformation of *E. coli* by the calcium chloride method

Purified DNA (10 ng - 100 ng) was added to 50 μl of competent cells and was incubated on ice for 30 minutes. Cells were heat shocked for 2 minutes at 37°C. LB broth (500 μl) was added to the cells and the culture was incubated at 37°C for 1 hour. Cells were then harvested by centrifugation at 14000 rpm for 1 minute, and resuspended in 100 μL of LB

broth. The cell culture was then plated onto selective medium and incubated at 37°C over-night.

2.16. Transformation of *S. cerevisiae* by the lithium acetate method

Mid-logarithmic phase cells were harvested by centrifugation at 3000 rpm for 10 minutes at room temperature. Cell pellet was resuspended in 10 ml of lithium-acetate / TE mix (10 mM lithium-acetate, 10 mM Tris-HCl pH 7.5, 1 mM EDTA). The suspension was centrifuged for 10 minutes at 3000 rpm at room temperature. The resulting pellet was resuspended in 1 ml of lithium-acetate / TE mix. 15 μl of boiled salmon sperm DNA (20 mg/ml) was added to the cell suspension, along with 2 μl of DNA sample (plasmid etc.), and 700 μl of 40% PEG solution (4 g of polyethyleneglycol, 6 ml of lithium-acetate/TE mix). The cell suspension was then first incubated at 30°C for 30 minutes and then incubated at 42°C for 15 minutes. The cells were harvested by centrifugation at 14000 rpm for 10 seconds at room temperature. The pellet was resuspended in 1 ml of YPDbroth and centrifuged for 10 seconds at 14000 rpm at room temperature. The pellet was then resuspended in 1 ml of YPD broth and incubated at 30°C for 1 hour. The cells were harvested by centrifugation at 14000 rpm for 10 seconds at room temperature. The pellet was resuspended in 100 μl of YPD and plated on selective medium.

2.17. Mating of *S. cerevisiae* cells

One colony of *MAT* **a** cells was mixed on a YPD plate with one colony of *MAT* α cells and was incubated at 25°C for 5 hours. The resulting mated *MAT* **a**/α cells were replica plated on to selective medium (selecting for markers present in the mated cells only) and incubated over-night at 25°C. The resulting colonies were streaked onto selective medium and incubated at 25°C.

2.18. Sporulation of diploid *S.cerevisiae* cells

MAT a/α cells were patched onto YPD plates and incubated over-night at 25°C. The resulting colonies were replica plated onto sporulation plates and incubated for 3 days at 25°C.

2.19. Dissection of sporulated cells

Sporulated diploid cells were mixed with 10 U of zymolyase (20 μl) and incubated at 37°C for 15 minutes. 180 μl of ice cold YPD 10% w/v sorbitol was gently added to the tetrad/zymolyase mixture and the resulting suspension was stored on ice. 6 μl of this cell suspension was overlaid onto dissection medium and the tetrads were dissected using a micro-dissection microscope. The resulting dissected tetrads were incubated at 25°C for 3 days. Single colonies were streaked onto YPD plates and incubated over-night at 25°C.

2.20. Determination of viability of yeast cells

To determine the number of viable cells, methylene blue staining was performed by adding equal volumes of culture and methylene blue (0.02%)/sodium citrate (4%). Thenumber of methylene blue negative and positive cells was determined by using bright field microscopy. More than 200 cells were counted for each sample. Dark-blue stained cells are dead and are thus incapable of metabolising the dye to a colourless derivative.

2.21. Determination of cell lysis in yeast cells

Lysis of cells was determined by propidium iodide staining. Cells were harvested by centrifugation at 14000 rpm for 30 seconds at room temperature. Cell pellet was resuspended in propidium iodide (20 μl; 50 μg/ml in PBS), and was incubated for 2 minutes at room temperature in the dark. The number of propidium iodide negative and positive cells was determined by fluorescent microscopy. More than 200 cells were counted for each sample. Bright orange fluorescent cells were indicative of lysed cells.

2.22. β-galactosidase assay

The β-galactosidase assay was carried out by a modification of the method of Miller (1972). The apparent optical density of cells at 600 nm was measured by using a spectrophotometer and 1 ml of cell culture was harvested by centrifugation at 14000 rpm for 10 seconds at room temperature. The resulting pellet was resuspended in 1 ml of Z-buffer (60 mM $Na_2HPO_4.7H_2O$, 40 mM $NaH_2PO_4.H_2O$, 10 mM KCl, and 1 mM

$MgSO_4.7H_2O$; pH 7.0). The cells were harvested by centrifugation at 14000 rpm for 10 seconds at room temperature. The cells were lysed by addition of 150 μl Z-buffer with mercaptoethanol (50 mM), 50 μl of chloroform, 20 μl of 0.1% w/v SDS and vortexing for 30 seconds. The sample tubes were incubated at 30°C. 700 μl of pre-warmed (30°C) Z-buffer/ONPG (o-nitrophenyl β-D-galactopyranoside; 1 mg/ml) was added to the sample tubes, vortexed and maintained at 30°C. Control tubes contained all solutions but without the cells. When a yellow colour had developed in the reaction tubes, the reaction was stopped by addition of 500 μl of 1M sodium carbonbate. The time taken for development of the yellow colour was noted. The samples were centrifuged for 10 minutes at 14000 rpm at room temperature. The upper phase was removed from the tubes and the optical density of the supernatant was measured at 420 nm. All reactions were carried out in triplicates. The specific activity of β-galactosidase was calculated using the following equation:

β-galactosidase activity (Miller units) = $(A_{420} \times 1000) \div (A_{600} \times$ development time $\times$ milliliters of culture used)

Fold activation in response to various treatments of cells (with various chemicals) was measured by calculating relative activity of treated cells to non-treated cells. Miller units of non-treated cells (basal) was represented as 1 fold activity, and treated cells were represented as fold increases over basal.

Comparison of basal activity between wild-type and mutant cells was carried out in the following manner. The basal activity of wild-type cells represented 100% basal activity. The basal activity of mutant cells (in Miller units) was divided by the basal activity of wild-type cells (in Miller units), and multiplied by 100 to represent percentage increase or decrease in basal activity.

2.23. Preparation of yeast total RNA

All solutions and glassware used in the manipulations of RNA were treated with diethylpyrocarbonate (DEPC) to inhibit RNase activity, where possible. DEPC was added to a final volume of 0.1% (v/v), the solution was shaken vigorously, placed at room-temperature overnight and then autoclaved.

20 ml of cell culture with an optical density in the range of 0.05 and 0.1 was centrifuged at 3000 rpm for 10 minutes at room temperature. The pellet was resuspended in 1 ml of H_2O and centrifuged at 14000 rpm for 10 seconds at room temperature (if RNA preparation is not required immediately, cells may be frozen at this stage in liquid nitrogen). Resuspend pellet in 200 μl of lysis buffer (0.5 M NaCl, 0.2 M Tris-HCl, 0.01 M EDTA, 1% SDS). The cell suspension was transferred to a new tube containing 0.4 g of glass beads. 200 μl of 1:1 v/v phenol:chloroform (containing 0.5% w/v 8-hydroxyquinoline, equilibrated with 10 mM sodium acetate, pH 6.0) was added to the cell suspension. The cell suspension was vortexed for 30 seconds, then incubated on ice for 30 seconds, and this step was repeated 5 times. Lysis buffer (300 μl) was added to the suspension, along with 300 μl of 1:1 v/v phenol:chloroform. The mixture was vortexed for 30 seconds and centrifuged for 2 minutes at 14000 rpm at room temperature. The aqueous top layer was removed into a new tube, and 1:1 phenol:chlorofrom (300 μl) was added. The mixture was vortexed for 30 seconds and centrifuged at 14000 rpm for 2 minutes at room temperature. The aqueous layer was removed to a new tube and the last two steps were repeated. The resulting aqueous layer was transferred to a new tube, ice-cold ethanol (2.5 volume), and sodium acetate (0.3 M, 0.1 volume, pH 5.3) were added to the aqueous phase and was incubated at -80°C for 1 hour. The total RNA was recovered by centrifugation at 14000 rpm for 10 minutes at 4°C and washed with 70% ethanol. The RNA pellet was resuspended in DEPC-treated, distilled and deionised H_2O. The concentration was estimated by measuring the absorbance at 260 nm, and the RNA was stored in aliquots at -80°C.

2.24. RNA formaldehyde-agarose gel electrophoresis

Formaldehyde agarose gels were prepared by a modification of that described by Lehrach *et al.* (1977). Solutions, glassware and plastic-ware were treated with DEPC, where possible.

Agarose (1.5 g) was melted in 123 ml of 1 × MOPS buffer (10×; 0.2 M MOPS, 50 mM sodium acetate pH 8.0, 10 mM EDTA; pH 7.0 was used for the purpose of running buffer) and cooled to 55°C. Formaldehyde (27 ml; 37% w/v) was added to the melted agarose mixture and the gel was poured in cast and allowed to set at room temperature for 45 minutes.

Samples were prepared by addition of 5 × sample buffer (2 μl; 0.2 M MOPS, 50 mM sodium acetate pH 7.0, 10 mM EDTA), formamide (10 μl) and formaldehyde (37% w/v; 3.5 μl) to the RNA sample (10 μg; 5 μl). The sample was incubated at 65°C for 5 minutes and then was placed on ice. RNA loading dye (25% v/v glycerol, 0.025% w/v bromophenol blue, 5 mM EDTA pH 8.0; 2 μl) and ethidium bromide (0.1% w/v; 1 μl) were added and samples were loaded onto gel. Gels were run at constant voltage of 100 V for 2 hours in 1 × MOPS buffer.

2.25. Northern blotting

RNA samples submitted to formaldehyde/agarose electrophoresis (section 2.24) were transferred onto nylon membrane (GeneScreen Hybridisation transfer membrane).

Denhardt's reagent (50×; 1% w/v Ficoll 400, 1% w/v polyvinylpyrrolidone and 1% w/v bovine serum albumin) and 20 × SSC (3 M sodium chloride, 0.2 M trisodium citrate dihydrate) were prepared. The nylon membrane was wet in distilled, deionised H_2O and then soaked in 5 × SSC for 5 minutes. A capillary blot transfer pyramid was contructed consisting of (from the bottom up) a Whatman paper wick, the gel, nylon membrane, whatman paper (3 layers), tissues (10 cm in height, tightly packed) and a 1 kg weight, with 5 × SSC as the transfer solution. The gel was blotted onto membrane for 12 hours. The membrane was then fixed at 80°C for 2 hours. Pre-hybridisation solution (5 × SSC, 50% w/v formamide, 5 × Denhardt's reagent, 1% w/v SDS, 0.1 mg/ml salmon sperm DNA; 20 ml) was placed at 42°C for 30 minutes and then added to the fixed nylon membrane. The blot was prehybridised at 42°C for 2 hours with gentle agitation.

DNA fragments were labelled with [α-^{32}P]dCTP by means of a random priming method (Strategene Prime-It II Random primer labelling kit). Probe (25 ng; 10 μl) and random oligonucleotide primers (containing dATP, dGTP, and dTTP and random hexa-deoxyribonucleotides; 10 μl) made up to 34 μl with H_2O was incubated at 100°C for 5 minutes. Primer buffer (10 μl), [α-^{32}P]dCTP (50 μCi; 5 μl) and Exo(-)Klenow enzyme (5 U; 1 μl), was added and the reaction mixture was incubated at 37°C for 10 minutes. NucTrap purification column was washed with STE buffer (100 mM NaCl, 20 mM Tris-

HCl pH 7.5, 10mM EDTA; 80 μl). Probe was added to column and pushed down the column. The remainder of probe was washed down with STE buffer (160 μl). The purified probe was incubated for 10 minutes at 100°C, added to the pre-hybridisation mix/membrane and incubated over-night at 42°C with gentle agitation. The hybridisation solution was removed and the blot was washed in 0.25 × SSC and 0.1 SDS for 15 minutes at room temperature and this procedure was then repeated. The blot was then washed once in 0.1 × SSC and 0.1% SDS at room temperature for 15 minutes. A final wash was carried out at 65°C in 0.1 × SSC and 0.1% SDS. The blot was sealed in plastic and was then exposed for 12-16 hours at -80°C to Kodak X-Omat LS film.

2.26. Stripping of RNA membranes

Probes can be removed from membranes to allow reprobing with a different probe. Blots used in this study were stripped once to allow reprobing with a control probe, however as a general procedure membranes may be stripped three or four times for further reprobing. Boiling water containing SDS (0.1%) was added to membranes and gently agitate solution until temperature reaches 37°C. This step is repeated three times. The membrane can be immediately prehybridised (section 2.25) or sealed and stored at -20°C.

2.27. Preparation of yeast total protein

Cell cultures between the optical density of 0.2-0.5 (A_{600}), were centrifuged at 3000 rpm for 10 minutes at 4°C. Cell pellets were washed with 1 ml of ice-cold H_2O, and centrifuged at 14000 rpm for 30 seconds at 4°C. The resulting pellet was resuspended in 100 μl of lysis buffer (50 mM Tris-HCl pH 7.5, 1% v/v Triton X-100, 0.1% w/v SDS, 1% w/v sodium deoxycholate, [2 μg/ml CLAP, 1 mM PMSF, 5 mM sodium pyrophosphate, 15 mM *p*-nitro-phenylphosphate, 10 mM sodium orthovanadate, 1% v/v phosphatase inhibitor cocktail; prepared on the day]). Glass beads (0.4 g) were added to the cell suspension and the mixture was vortexed for 30 seconds, then incubated on ice for 30 seconds and the procedure was repeated four times. The resulting cell lysate was centrifuged at 14000 rpm for 10 minutes at 4°C. Supernatant (80 μl) was transferred to a new tube, containing sample buffer (25 mM Tris-HCl pH 6.8, 10% glycerol, 0.006% w/v bromophenol blue, 2% w/v SDS, 0.1M dithiothreitol).

2.28. 10% Bis-Tris-PAGE

The NuPAGE Bis-Tris pre-cast gels (10%) were supplied by Invitrogen (Novex). Samples from section 2.27 were incubated at 100°C for 10 minutes, and centrifuged at 14000 rpm for 10 seconds. Precast gels were washed with 1 × NuPAGE MOPS running buffer (20×; 50 mM MOPS, 50 mM Tris-base, 3.5 mM SDS, 1 mM EDTA; pH 7.7). Set up gel in supplied electrophoresis apparatus. NuPAGE running buffer (1×; 200 ml) was added to the inner chamber along with 500 μl of NuPAGE anti-oxidant, and the samples and molecular weights (Novex pre-stained standards) were loaded into wells. Running buffer (50 ml) was then added to the outer chamber. The gel was electrophoresed in a Novex (Invitrogen) module XCell II electrophoresis module at a constant voltage (200 V) for 1 hour until the tracker dye reached within 5 mm of bottom of the gel. Using the tools provided by supplier, the gel was then removed from its cast.

2.29. Western blotting

Following Bis-Tris-PAGE, resolved proteins were blotted onto nitrocellulose Immoblin membranes using the Novex (Invitrogen) XCell II blot. Membrane was soaked in methanol and then placed in transfer buffer (100 ml methanol, 50 ml of 20 × NuPAGE transfer buffer [25 mM Bicine, 25 mM Bis-Tris, 1 mM EDTA; pH 7.2], 500 μl NuPAGE anti-oxidant). The gel/membrane sandwich was made in the following manner (from bottom up): two sponges (provided by manufacturer), Whatman paper, gel, membrane and two sponges. Sandwich was placed into transfer box, and 1 × tranfer buffer was added to the transfer box. The proteins were blotted onto the membrane at 30 constant volts for 1 hour.

The blot was blocked for 30 minutes with blocking buffer (TBS [10 mM Tris-base, 0.15 M NaCl], 10% non-fat dried milk, 0.1% v/v Tween-20). The membrane was then incubated over-night at 4°C, with primary antibody diluted (anti-dual phospho 1:2000 [mouse]; anti-Mpk1p 1:500 [goat]) in blocking buffer (only 5% non-fat dried milk). The blot was then washed with TBS containing 0.1% Tween-20 (50 ml; 3 times), for 10 minutes. The blot was then incubated for 2 hours at room temperature, with secondary antibody diluted (anti-mouse 1:5000; anti-goat 1:5000; HRP conjugated) in blocking

buffer (only 2% non-fat milk). The blot was then washed with TBS containing 0.1% Tween-20 (50 ml; 3 times), for 10 minutes. Membrane was then placed onto cling-film and excess buffer was removed by blotting using Whatman paper. NuPAGE ECL solutions A and B were mixed (2 ml of solution A: 50 μl of solution B) and overlaid onto the membrane, incubated for 5 minutes and blotted off with Whatman paper. The membrane was wrapped in cling-film and exposed to X-ray film (1 hour for anti-phospho antibody; 10 minutes for anti-Mpk1p antibody).

2.30. Stripping of protein blots

Protein membrane blots in this study were stripped once to allow reprobing with a control antibody. Membranes were submerged in stripping buffer (100 mM 2-mercaptoethanol, 2% w/v SDS, 62.5 mM Tris-HCl pH 6.7), and were incubated at 50°C for 30 minutes, while gently agitated. The membranes were washed in TBS:Tween-20 (200 ml) at room temperature for 10 minutes, while gently agitated. The wash procedure was repeated once. The membranes were blocked in blocking buffer (section 2.26) for 1 hour at room temperature, while gently agitated.

2.31. Rhodamine phaloidin and calcofluor white staining of yeast cells

Mid-logarithmic cell culture (1 ml) was centrifuged at 14000 rpm for 30 seconds at room temperature. The Pellet was washed with PBS (0.14 M NaCl, 8 mM Na_2HPO_4, 3 mM KCl, 1 mM KH_2PO_4; 1 ml), and the step was repeated once. The cell pellet was resuspended in calcofluor white (1 mg/ml; 10 μl) or rhodamine phaloidin (10 μl), along with 50 μl of PBS, and was incubated for 5 minutes or 30 minutes respectively at room temperature. The cells were washed with PBS (1 ml), and centrifuged at 14000 rpm for 30 seconds at room temperature. The washing procedure was repeated once. The cells were incubated at room temperature for 10 minutes and then resuspended in 200 μl of PBS and observed by fluorescent microscopy. As both calcofluor white and rhodamine phaloidin are light sensitive, all incubations were carried out in the dark. More than 300 cells were photographed for each time point sample.

2.32. Assay of [^{45}Ca] influx and accumulation into yeast cells

Calcium influx and accumulation experiments were done by a modified method described by Iida *et al.* (1990). Mid-logarithmic phase cells (A_{600} between 0.7-0.9) were incubated at room temperature with $^{45}CaCl_2$ (Amersham) to a final specific activity of 33 μCi/μmol in YPD and 11 μCi/μmol in selective media. These two media types contain different final concentrations of calcium [final concentration of Ca^{2+} in YPD is 0.3 mM and 0.1 mM in selective media as determined by Dunn *et al.* (1994)], and hence the final specific activity of radioactivity was adjusted to accommodate this difference. Aliquots (100 µl) were removed at time intervals from the cell cultures and added to 1 ml of ice-cold YPD. The cells were harvested by centrifugation at 14000 rpm for 10 seconds. The cell pellet was resuspended in ice-cold YPD and centrifuged for 10 seconds at 14000 rpm. The wash step was repeated two times. The tubes with pellets were placed into vials containing Ecoscint™ scintillation fluid (10 ml) for liquid scintillation counting.

2.33. Determination of $^{45}CaCl_2$ efflux from yeast cells

Mid-logarithmic phase cells (A_{600} between 0.02-0.05) were incubated at room temperature, overnight, with $^{45}CaCl_2$ (Amersham) to a final specific activity of 33 µCi/µmol in YPD and 11 µCi/µmol in selective media. Cells were harvested by centrifugation at 14000 rpm for 10 seconds. Cells were washed with ice-cold media and centrifuged at 14000 rpm for 10 seconds. The wash step was repeated two times. The cell pellet was resuspended in room temperature equilibrated media (1 ml). Aliquots (100 μl) were removed at time intervals and put into ice-cold tubes. The cells were harvested by centrifugation at 14000 rpm for 10 seconds. Supernatant was removed into a new tube. Both pellet and supernatant were placed into separate vials containing Ecoscint™ scintillation fluid (10 ml) for liquid scintillation counting. Percentage efflux was calculated as the amount of radioactivity in the supernatants relative to the amount of radioactivity in the cell pellet at time zero minutes, multiplied by 100.

% efflux = (CPM in supernatant ÷ CPM in time zero pellet) × 100

"Success in whatever shape, and to whatever extent, must ultimately depend on the willingness to identify and pursue the important questions, and in that pursuit willingly to place at risk all current reassurances of reality."

D'Arcy B. and Jayaratna N.

Chapter 3: *MID2* is an upstream activator of the Pkc1p-MAPK pathway

3.1. Introduction

The cell wall of the budding yeast *Saccaromyces cerevisiae* is required to maintain cell shape and integrity (Cid *et al.* 1995; Klis *et al.* 1994). The Pkc1p-mediated MAPK pathway is one of the signal transduction pathways that operates in the budding yeast to control its cellular integrity. Mpk1p is specifically activated during periods of polarised cell growth, for example during bud emergence or mating projection formation (Mazzoni *et al.* 1993; Zarzov *et al.* 1996). In addition, heat and hypo-osmotic shocks also lead to phosphorylation and activation of Mpk1p (Davenport *et al.* 1995; Kamada *et al.* 1995). It has been argued that cell surface stress causes activation of the Pkc1p-MAPK pathway. Several phenotypes are associated with genetic defects in this pathway. For example, mutants defective in any of the Pkc1p-MAPK pathway components, show a tendency to lyse with small buds at higher temperatures. This cell lysis phenotype is rescued by osmotic stabilisation using high salt media or 1M sorbitol (Levin *et al.* 1994).

If the Pkc1p pathway provides a way of responding to perturbations at the cell surface, one expects to find sensors associated with the membrane and the cell wall as upstream components in the pathway. A possible cell surface sensor has been identified as Hcs77p/Wsc1p/Slg1, encoding a type I transmembrane protein of the plasma membrane (Gray *et al.* 1997; Jacoby *et al.* 1998; Verna *et al.* 1997). Hcs77p was proposed to be an upstream component of the Pkc1p pathway since phosphorylation of Mpk1p in *hcs77Δ* cells is severely compromised during heat shock (Gray *et al.* 1997). Furthermore, *HCS77* over-expression suppresses the growth defects associated with a *swi4* mutant at high temperatures, as does over-expression of *PKC1* itself (Gray *et al.* 1997). Swi4p is a DNA binding protein and a transcriptional activator. Swi4p is required for the normal expression of the G_1 cyclin genes at the G_1/S transition (Cross *et al.* 1994; Koch *et al.* 1996; Nasmyth and Dirik 1991; Ogas *et al.* 1991).

WSC2, *WSC3* and *WSC4*, are all members of the *HCS77* family of putative cell surface sensors. However, their contribution to the regulation of the Pkc1-MAPK pathway appears to be minor (Verna *et al.* 1997). Cells, which are *wsc2Δ*, *wsc3Δ* or *wsc4Δ*, do not display any vegetative phenotype, whereas *wsc1Δwsc2Δ* or *wsc1Δwsc3Δ* cells show an exacerbation of the *wsc1Δ* phenotype. This suggests redundant roles among the *WSC* family members (Verna *et al.* 1997). Deletion of all *WSC* genes leads to inability to grow at any temperature on YPD, a phenotype that is osmotically remedial. The primary structures of Wsc1p-Wsc4p suggest that they are type I (single) trans-membrane proteins (Verna *et al.* 1997).

In this chapter, the role of another possible sensor for detection of cell wall disturbance, encoded by *MID2* will be analysed. The *MID2* gene was initially identified in a genetic screen for mutants that died after exposure to mating pheromone (Iida *et al.* 1994). Death in mating pheromone occurred when cells differentiated into shmoos. Calcium influx is essential for maintaining viability of shmoos when cells have been exposed to α-factor (Iida *et al.* 1990). Furthermore, wild-type cells grown in calcium deficient medium die in presence of α-factor after differentiating in shmoos (Iida *et al.* 1990). Therefore, the mutants identified in the screen were proposed to have a defect either with calcium influx or calcium signal transduction. Cells deficient for *MID2* gene showed no defect in calcium influx (Iida *et al.* 1994). Since Mid2p contains a putative calcium-binding domain, Mid2p has therefore been proposed to have a role in calcium-signalling (Iida *et al.* 1994). It has been suggested that Mid2p ensures cell viability by maintaining calcium homeostasis (Ono *et al.* 1994). Its deduced amino acid sequence displays similarities to type I transmembrane class of proteins, similar to Hcs77p, and predicts that Mid2p would be localised to the periplasmic space. Over-expression of *MID2* in *hcs77Δ* cells suppresses the temperature-sensitive growth defect of these mutants (Stirling, D.A.; personal communication). These data suggest that Mid2p may act as another sensor for the Pkc1p-MAPK pathway. The possible role of *MID2* in the Pkc1p-MAPK pathway will be analysed within this chapter.

3.2. Results

3.2.1. *MID2* over-expression suppresses the *swi4* growth defect

Swi4p is the DNA binding protein and transcriptional activator of the cell cycle regulated transcription factor, the Swi4p/Swi6p complex. Like *mpk1Δ* mutants, haploid *swi4Δ* mutants are temperature sensitive for growth and this growth defect is osmotically remedial (Madden *et al.* 1997; Ogas *et al.* 1991). In contrast, *swi4Δ* mutants do not lyse at high temperatures but display a specific defect in bud emergence. This growth defect at high temperatures and the bud emergence defect of *swi4Δ* cells at high temperatures is relieved by over-expression of known Pkc1p-MAPK pathway components, such as *HCS77* and *PKC1* (Gray *et al.* 1997).

If Mid2p is an activator of Pkc1p, then one would expect that over-expression of *MID2* would suppress the growth defect of *swi4* mutants, as do *HCS77* and *PKC1*. For this experiment, *MAT* **a** *swi4Δ* cells in the S288C background were transformed with either a high-copy plasmid containing *MID2*, or the *YEP24* vector control. Transformants were streaked onto selective media. Single colonies, were streaked on to selective media and incubated at 25°C or 37°C. *MID2* over-production, from a multi-copy plasmid, suppressed the temperature sensitivity defect of *swi4Δ* cells at 37°C (Fig. 3.1). This result is consistent with *MID2* acting within the Pkc1p-MAPK pathway.

Over-expression of *HCS77* or *PKC1* suppresses the *swi4Δ* defect, independently of cell type. Therefore, over-expression of *MID2* in the diploid *swi4* mutation was analysed, to test if suppression of the *swi4Δ* growth defect by *MID2* was dependent on cell type. Since the diploid *MAT* **a**/α *swi4Δ/swi4Δ* cells are inviable, *MAT* **a**/α $swi4^{ts}$/*swi4Δ* cells in the S288C background, were transformed with the high-copy plasmids containing *MID2* or *YEP24* vector control. As before, transformants were streaked onto selective media. The resulting colonies were streaked onto selective media, and were incubated at 25°C or 37°C (Fig. 3.1). At 37°C *MID2* over-expression suppressed the temperature sensitive growth defect of the diploid $swi4^{ts}$/*swi4Δ* mutant. These results confirm that *MID2* suppresses the growth defect of cells lacking swi4p function, and that this suppression is independent of

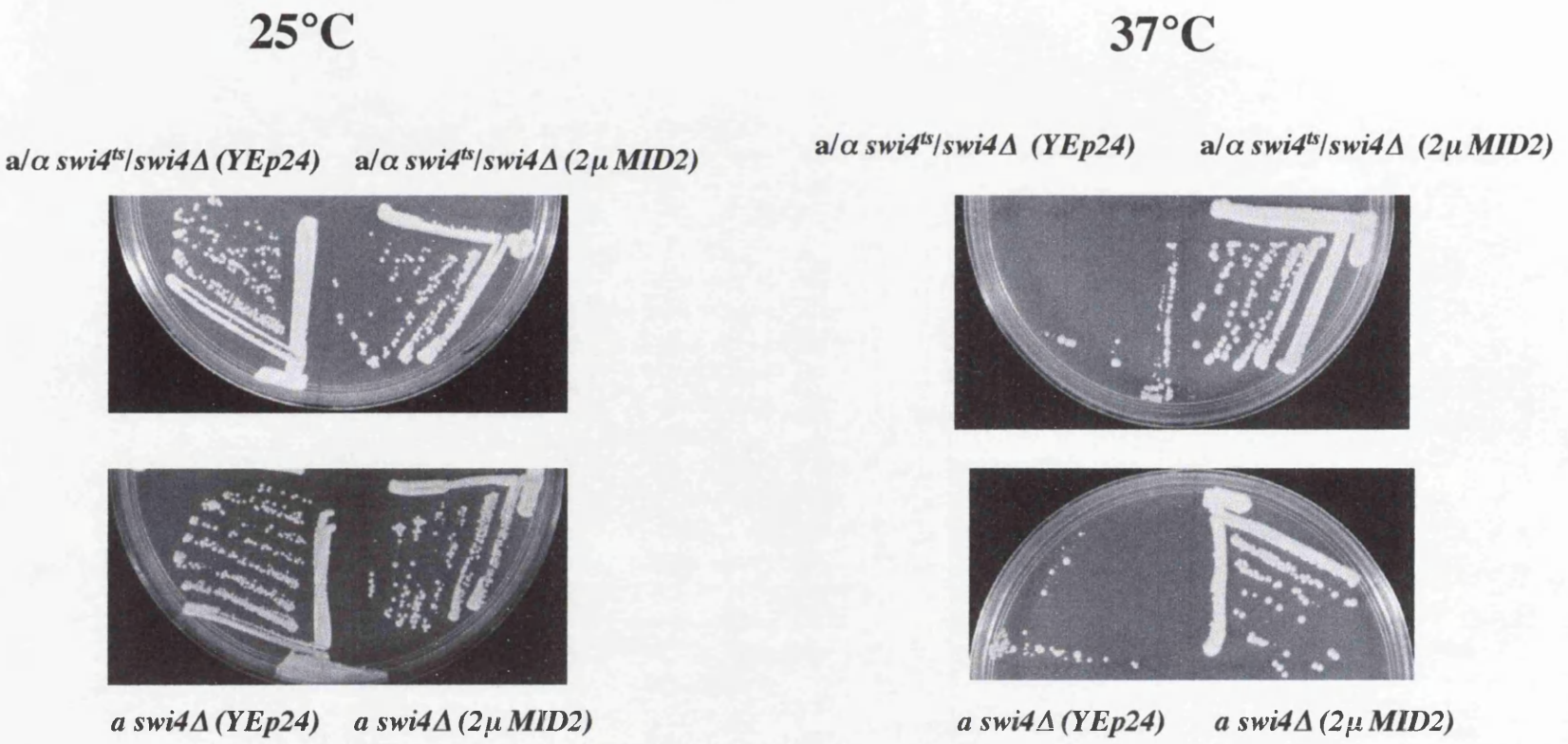

Figure 3.1. *MID2* over-expression suppresses the growth defects of *swi4* mutants. *MAT* **a**/α *swi4*^{ts}/*swi4Δ* and *MAT* **a** *swi4Δ* cells were transformed with high copy number plasmids of *MID2* or the vector control *YEp24*. The transformants were selected on Ura drop-out medium at 25°C. The resulting colonies were streaked onto fresh selective medium and incubated at 25°C or 37°C for three days.

cell type or ploidy. These results are consistent with Mid2p functioning in the Pkc1p-MAPK pathway.

3.2.2. Over-expression of *MID2* suppresses the growth defect of *hcs77Δ* mutants

Hcs77p is a putative cell surface protein that functions upstream of Pkc1p in the Pkc1p-MAPK pathway, and is required for heat activation of Mpk1p (Gray *et al.* 1997). Cells lacking the *HCS77* gene display a growth defect at 37°C, which is a phenotype similar to other mutants of the Pkc1p-MAPK pathway (Gray *et al.* 1997).

To further assess the possible role of Mid2p in the Pkc1-MAPK pathway, the effect of *MID2* over-expression in *hcs77Δ* cells was examined. *MAT* **a**/α *hcs77Δ/hcs77Δ* cells were transformed with high-copy plasmid containing *MID2* or with the empty *YEp24* vector. Transformants were streaked onto selective media and the resulting single colonies were tested for growth at 25°C and 37°C, on selective media. Homozygous diploid *hcs77Δ/hcs77Δ* mutants were used in these experiments to eliminate the occurrence of recessive mutations that could suppress the growth defect of *hcs77Δ* cells. Furthermore, the homozygous diploid mutants have a more severe phenotype than the congenic haploid mutants. The temperature sensitive growth defect of *MAT* **a**/α *hcs77Δ/hcs77Δ* at 37°C, was efficiently suppressed by over-expression of *MID2* (Fig. 3.2). This result is consistent with *MID2* playing a role downstream or parallel to *HCS77* within the Pkc1p-MAPK pathway.

3.2.3. Over-expression of *MID2* does not suppress growth defect of *mpk1Δ*, *bck1Δ* or *pkc1^{ts}* mutants

Since over-expression of *MID2* suppresses the *hcs77Δ* growth defect, can over-expression of *MID2* suppress the growth defect of other Pkc1p-MAPK pathway mutants? To assess this question, *MAT* **a** *mpk1Δ*, *MAT* **a** *pkc1^{ts}* and *MAT* **a** *bck1Δ* haploid cells in the EG123 strain background were transformed with high-copy plasmids containing *MID2* or the empty *YEP24* vector. Transformants were streaked onto selective media and the resulting single colonies were tested for growth at 25°C and 37°C, on selective media. *MID2* over-

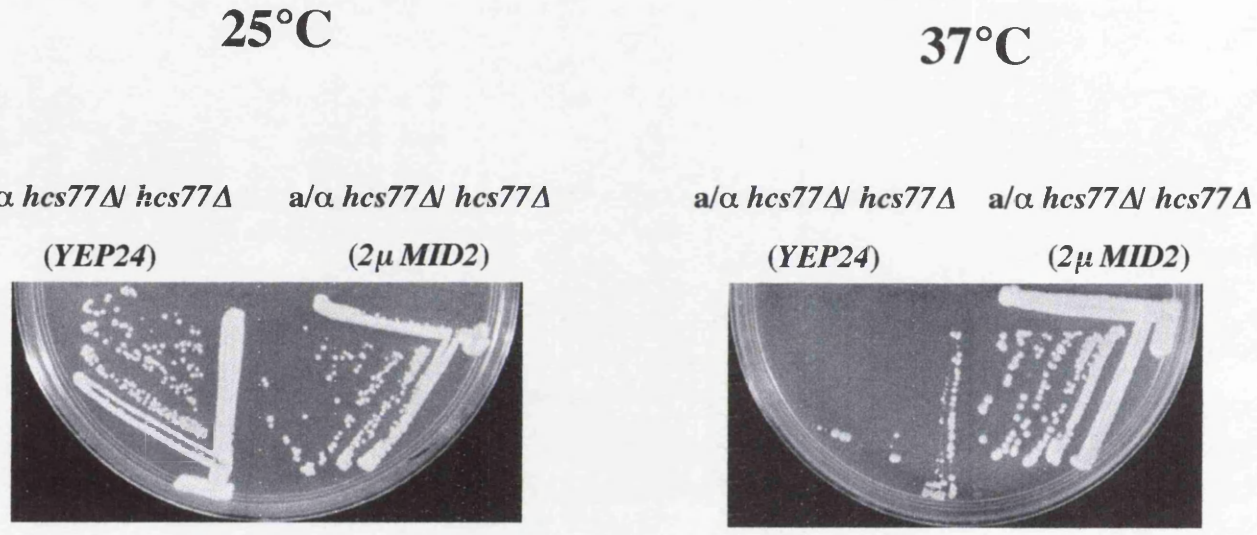

Figure 3.2. Over-expression of *MID2* suppresses the growth defect of *hcs77* mutants. *MAT* **a**/α *hcs77Δ/hcs77Δ* cells were transformed with high copy number plasmids of *MID2* or the vector control *YEp24*. The transformants were selected on Ura drop out medium at 25°C. The resulting colonies were streaked on fresh selective medium and incubated at 25°C or 37°C for three days.

expression from a high-copy plasmid did not suppress the growth defects of *mpk1Δ*, *pkc1*^{ts} or *bck1Δ* in the EG123 strain background, despite its suppression of the *swi4Δ* and *hcs77Δ* growth defects (Fig. 3.3). If Mid2p functions downstream of the Pkc1p-MAPK pathway, then suppression of the growth defects associated with these mutations would have been expected. However, the failure of *MID2* to suppress these growth defects suggests that Mid2p may function upstream of the Pkc1p-MAPK cascade, as we would expect, for a cell surface sensor acting within this pathway.

3.2.4. *mid2Δ* cells do not display a growth defect at high temperatures

Mutants lacking many components of the Pkc1 pathway for example *hcs77Δ*, *bck1Δ* or *mpk1Δ* cells display a vegetative growth defect at high temperatures. This phenotype results from cell lysis. Furthermore, both the cell lysis phenotype and the growth defect are osmotically remedial. If Mid2p is a component of the Pkc1p pathway, then mutant-lacking Mid2p may display a similar vegetative phenotype.

Single *MAT* **a** *mid2Δ* colonies were streaked onto YPD plates, and were incubated at 25°C, 30°C or 37°C. *MAT* **a** *mid2Δ* cells formed colonies at 25°C, 30°C and 37°C, similar to wild-type cells at the same temperature (Fig. 3.4). This result shows that *mid2Δ* cells do not have a growth defect at any of these temperatures. Furthermore, this result suggests that in vegetatively growing cells *HCS77* may play a more important role than *MID2* in the Pkc1p-MAPK pathway.

3.2.5. Hcs77p and Mid2p are essential for the Pkc1p-MAPK pathway and vegetative growth

If Hcs77p and Mid2p are both functioning in the Pkc1p-MAPK pathway and are partially redundant in sensing cell surface stress, then deletion of both *HCS77* and *MID2* should result in a severe vegetative growth defect. To test this hypothesis, *MAT* α *hcs77Δ* : : *LEU2* cells were mated to *MAT* **a** *mid2Δ* : : *URA3* cell in the S288C strain background. The resulting diploids were sporulated and the tetrads were dissected on 10% sorbitol YPD. High osmolarity medium was used to stabilise the resulting spores, since Pkc1p pathway mutants show growth defects that are osmotically remedial. From dissection of

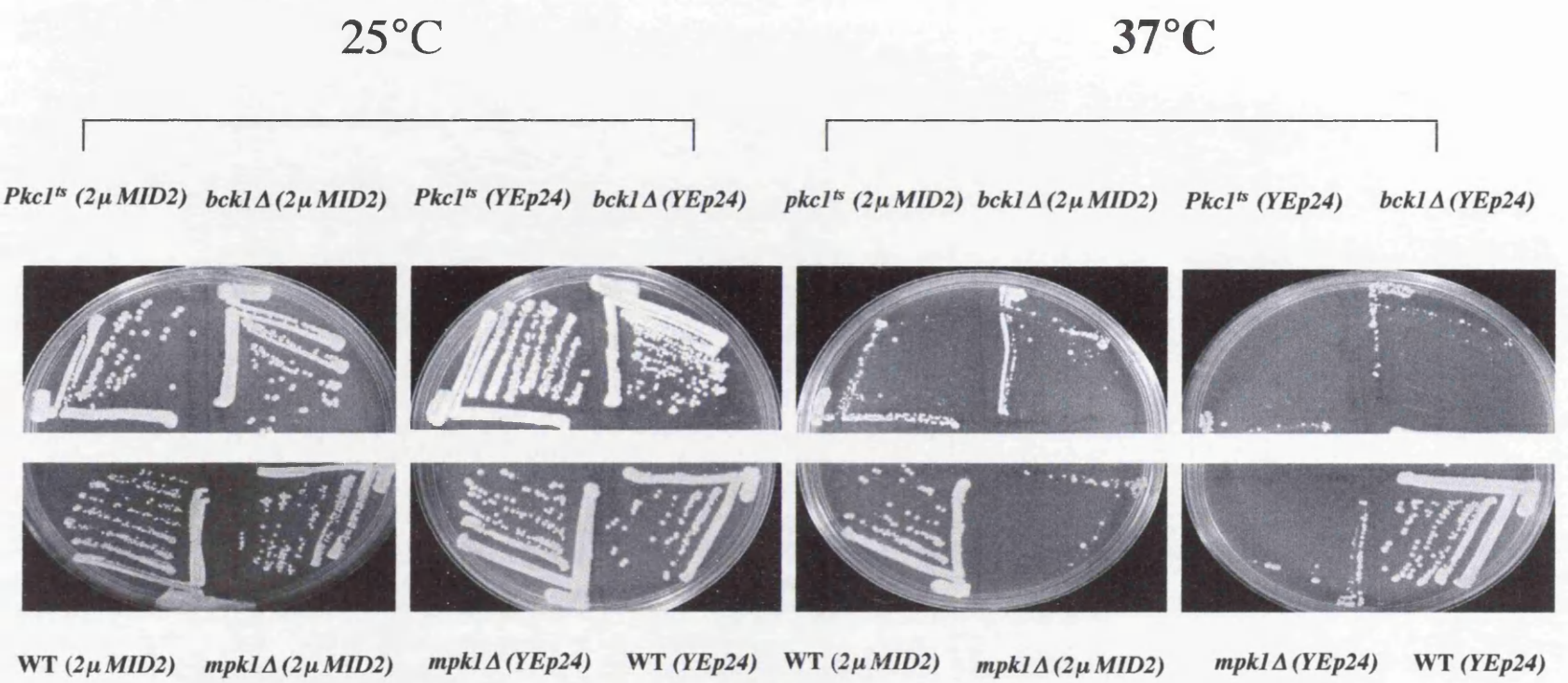

Figure 3.3. *MID2* over-expression does not suppress the growth defects of *pkc1*^{ts}, *bck1Δ*, or *mpk1Δ* cells. *MAT* **a** *pkc1*^{ts}, *bck1Δ* or *mpk1Δ* cells were transformed with high copy number plasmids of *MID2* or the vector control *YEp24*. The transformants were selected on Ura drop out medium at 25°C. The resulting colonies were streaked on fresh selective medium and incubated at 25°C or 37°C.

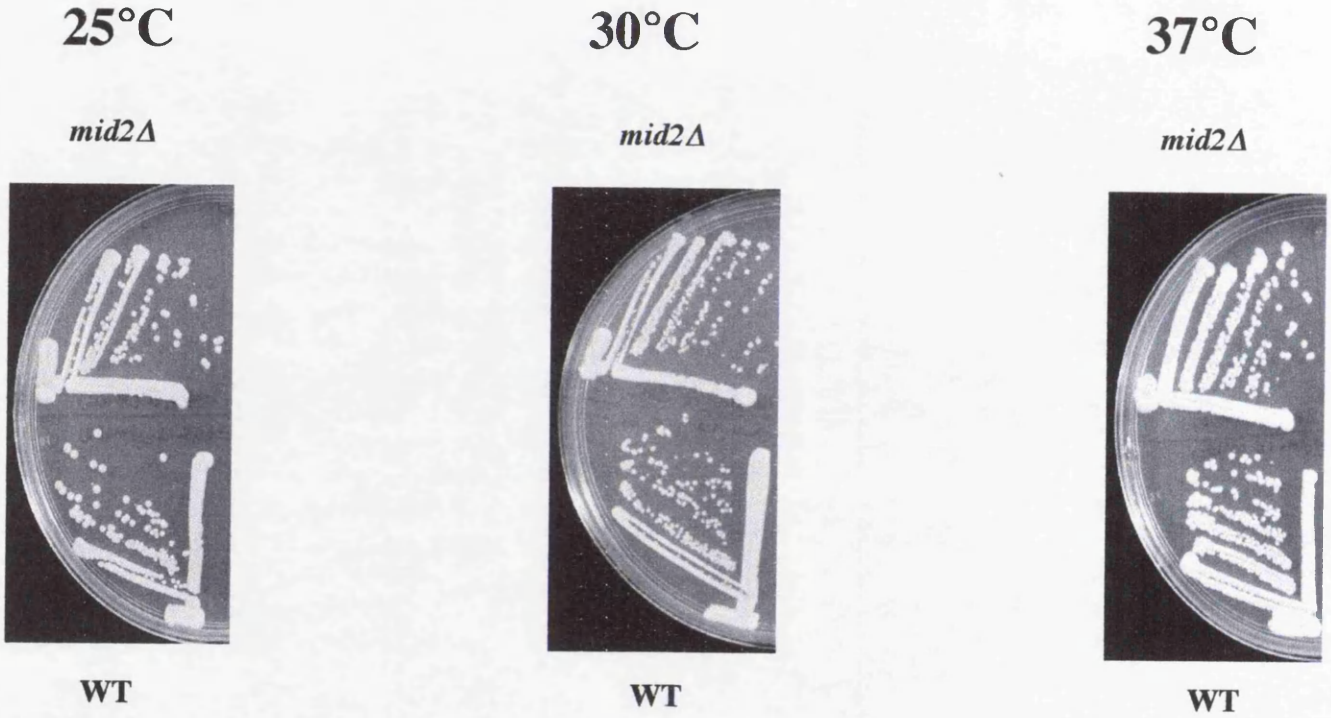

Figure 3.4. *MID2* is not required for vegetative growth even at high temperatures. *MAT* **a** *mid2Δ* cells were streaked onto YPD plates and were incubated at 25°C, 30°C or 37°C for three days.

22 tetrads, the resulting spores from 17 tetrads did not all form colonies. In each of those 17 tetrads either 1/4 or 2/4 spores did not grow. The colonies that were obtained were further streaked onto 10% sorbitol YPD plates and resulting colonies were streaked onto selective media with 10% sorbitol. The distribution of the markers showed that the spores, which did not form colonies were *LEU2*$^+$*URA3*$^+$, *i.e.* the *mid2Δhcs77Δ* cells (Table 3.1). Therefore, the absence of both *HCS77* and *MID2* is lethal to cells in the S288C background, and this lethality is not osmotically remedial. This defect is more severe than the *pkc1Δ* in the S288C background, which has a growth defect that can be remedied by growth in high osmolarity medium.

Since in the S288C background the double *mid2Δhcs77Δ* cells are dead, the deletion of both cell surface sensors from the EG123 background was examined next to assess whether this is a phenotype shared among other strain backgrounds. Therefore, *MAT* α *hcs77Δ* : : *LEU2* cells were mated to *MAT* **a** *mid2Δ* : : *URA3* cells in the EG123 background. The resulting diploids were sporulated and tetrads were dissected onto 10% sorbitol YPD. Spore colonies formed were streaked onto YPD 10% sorbitol and were further selected on osmotically stabilised selective media. From 22 tetrads 20 contained spores, which were able to grow on both selective media, and therefore were *mid2Δhcs77Δ* cells (Data not shown). Hence, the deletion of both *HCS77* and *MID2* in the EG123 background is not lethal in the presence of sorbitol. However, when *mid2Δhcs77Δ* cells were streaked onto YPD with no osmotic stabilisation, no colony growth was observed at 25°C (Fig. 3.5). This result shows that *mid2Δhcs77Δ* cells in the EG123 strain background are incapable of growth in absence of osmotic stabilisation; this also a phenotype shared by *pkc1Δ* cells (Fig. 3.5). Furthermore, *mid2Δhcs77Δ* cells are inviable at high temperatures (data not shown), similar to *pkc1Δ* cells (Levin *et al.* 1994). Note: in the W303 strain background the *mid2Δhcs77Δ* cells are temperature sensitive but are viable in the absence of osmotic support - this report underlying the difference in the genetic background of the strains used (Stirling and Stark 2000).

These results are consistent with Mid2p and Hcs77p functioning within the same pathway and sharing a common vegetative function. Furthermore, the double deletion of *MID2* and *HCS77* in the EG123 background is similar to the deletion of *PKC1*. Both these sensors may therefore be required for the functionality of the Pkc1p-MAPK pathway. The

Tetrad no.	Spore 1 (growth)	Spore 2 (growth)	Spore 3 (growth)	Spore 4 (growth)
1	*hcs77Δ* (+)	*mid2Δ* (+)	*mid2Δ* (+)	*hcs77Δ* (+)
2	*mid2Δ* (+)	*hcs77Δmid2Δ* (-)	*hcs77Δ* (+)	WT (+)
3	*hcs77Δ* (+)	*mid2Δ* (+)	*hcs77Δmid2Δ* (-)	WT (+)
4	WT (+)	*hcs77Δ* (+)	*mid2Δ* (+)	*hcs77Δmid2Δ* (-)
5	WT (+)	*hcs77Δmid2Δ* (-)	*hcs77Δmid2Δ* (-)	WT (+)
6	WT (+)	WT (+)	*hcs77Δmid2Δ* (-)	*hcs77Δmid2Δ* (-)
7	*hcs77Δ* (+)	*mid2Δ* (+)	*hcs77Δ* (+)	*mid2Δ* (+)
8	*mid2Δ* (+)	*hcs77Δ* (+)	WT (+)	*hcs77Δmid2Δ* (-)
9	WT (+)	*mid2Δ* (+)	*hcs77Δ* (+)	*hcs77Δmid2Δ* (-)
10	WT (+)	*hcs77Δ* (+)	*mid2Δ* (+)	*hcs77Δmid2Δ* (-)
11	WT (+)	WT (+)	*hcs77Δmid2Δ* (-)	*hcs77Δmid2Δ* (-)
12	*mid2Δ* (+)	*hcs77Δmid2Δ* (-)	*hcs77Δ* (+)	WT (+)
13	WT (+)	*hcs77Δmid2Δ* (-)	*hcs77Δmid2Δ* (-)	WT (+)
14	WT (+)	*hcs77Δmid2Δ* (-)	*hcs77Δmid2Δ* (-)	WT (+)
15	*hcs77Δ* (+)	WT (+)	*mid2Δ* (+)	*hcs77Δmid2Δ* (-)
16	WT (+)	*hcs77Δ* (+)	*hcs77Δmid2Δ* (-)	*mid2Δ* (+)
17	WT (+)	*hcs77Δ* (+)	*mid2Δ* (+)	*hcs77Δmid2Δ* (-)
18	WT (+)	*hcs77Δ* (+)	*mid2Δ* (+)	*hcs77Δmid2Δ* (-)
19	WT (+)	*hcs77Δmid2Δ* (-)	*hcs77Δ* (+)	*mid2Δ* (+)
20	*hcs77Δmid2Δ* (-)	WT (+)	*hcs77Δ* (+)	*mid2Δ* (+)
21	WT (+)	WT (+)	*hcs77Δmid2Δ* (-)	*hcs77Δmid2Δ* (-)
22	WT (+)	WT (+)	*hcs77Δmid2Δ* (-)	*hcs77Δmid2Δ* (-)

Table 3.1. Tetrad dissection of **a**/α *hcs77Δ/HCS77 mid2Δ/MID2* cells in the S288C strain background. A +/- in brackets indicates spore growth or no growth respectively, after dissection. Presence of each mutant was detected by growth of spores on to selective media, Leu$^-$ for *hcs77Δ* selection and Ura$^-$ for *mid2Δ* selection.

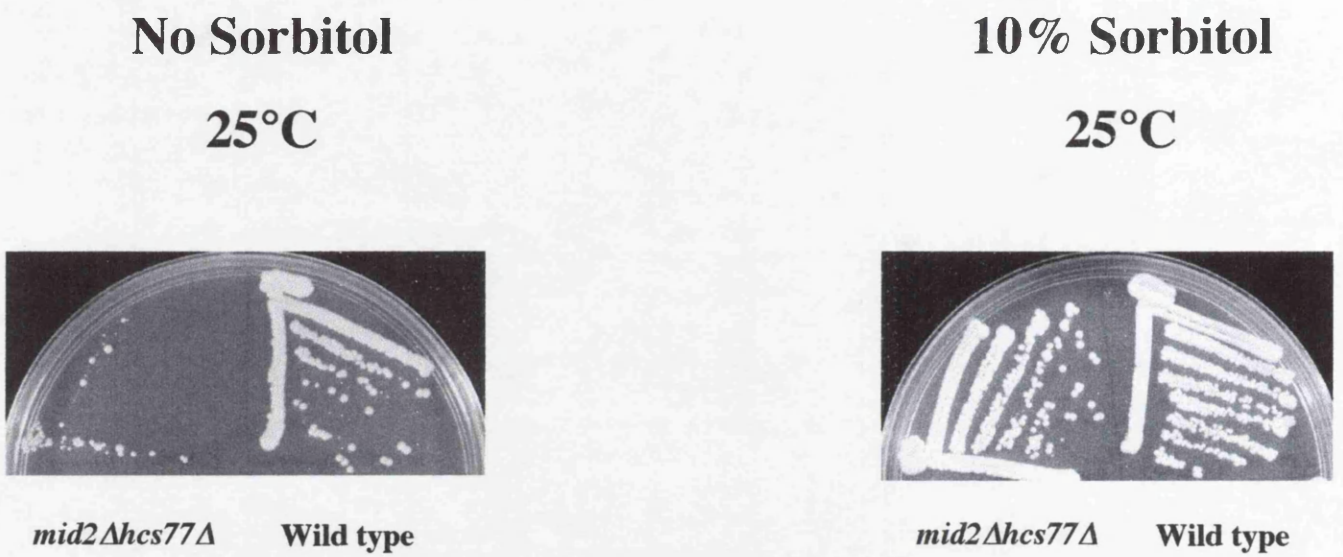

Figure 3.5. *MID2* and *HCS77* share a redundant role, and are required during vegetative growth. A *mid2Δhcs77Δ* mutant displays a cell lysis defect that is osmotically remediable. *MAT* **a** *mid2Δhcs77Δ* cells in the EG123 strain background were streaked onto YPD or YPD supplemented with 10% sorbitol, and were incubated at 25°C for three days.

difference seen between the two different strain backgrounds, may result from other genetic modifiers.

3.2.6. *MID2* appears to have a minor role in the Pkc1p-MAPK pathway during vegetative growth

If Mid2p functions in the Pkc1p-MAPK pathway and has a minor role to play during vegetative growth, then deletion of both *MID2* and *MPK1* should not be additive. However, if *MID2* function is required for a pathway parallel to the MAPK pathway, downstream of Pkc1p, then deletion of *MPK1* and *MID2* should be additive, since the functionality of two redundant pathways would be lost. To test this hypothesis, *MAT* α *mpk1Δ* : : *TRP1* cells were mated to *MAT* **a** *mid2Δ* : : *URA3* S288C cells. The resulting diploids were sporulated and the tetrads were dissected onto 10% sorbitol YPD. All resulting spore colonies were streaked onto 10% sorbitol YPD, and the resulting colonies were further streaked onto osmotically stabilised selective medium. From 20 tetrads, 19 contained spores, which formed colonies on both the *TRP1* and *URA3* selective media and were therefore, *mid2Δmpk1Δ* cells (Data not shown). These results show that deletion of both *MID2* and *MPK1* is not lethal to vegetatively growing cells. Furthermore, this result indicates that during vegetative growth *MID2* functions with in the Pkc1p-MAPK pathway.

mpk1Δ cells are normally defective for growth at 37°C, and this defect is osmotically remedial. *mid2Δmpk1Δ* cells, formed colonies at 25°C without osmotic stabilisation, however at 30°C and 37°C *mid2Δmpk1Δ* did not form colonies on YPD (Fig. 3.6A). This growth defect is restored by the presence of 10% sorbitol in the medium (Fig. 3.6B). Although at 30°C the *mid2Δmpk1Δ* cells show a more severe growth defect than the *mpk1Δ* cells, the deletion of *MID2* and *MPK1* is not lethal. This observation supports a minor role for Mid2p upstream of the Pkc1p-MAPK pathway.

If Mid2p is required for optimal activation of Pkc1p, then *mid2Δ* mutants should be very sensitive to reduction in Pkc1p activity. Therefore, the sensitivity of *mid2Δ* cells in the EG123 background, to the reduction of Pkc1p activity was examined. *MAT* **a** *mid2Δ* cells

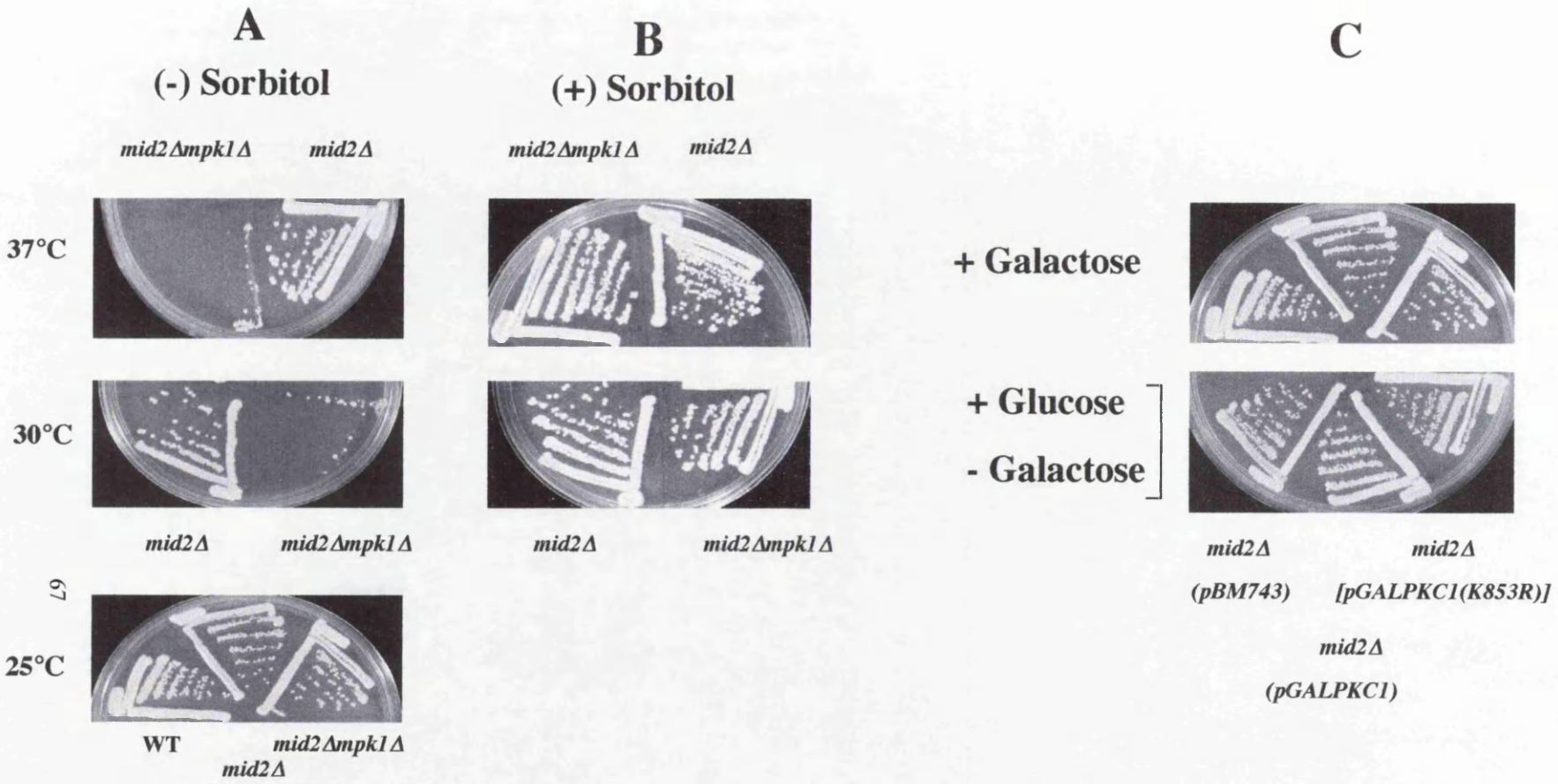

Figure 3.6. *MID2* appears to play a minor role in the Pkc1p-MAPK pathway during vegetative growth. **(A and B)** *mid2Δmpk1Δ* cells are viable and only display an osmotically remedial temperature sensitive defect. **(C)** Loss of Pkc1p function and over-production of Pkc1p does not affect growth of *mid2Δ* cells. **(A and B)** *mid2Δ* and *mid2Δmpk1Δ* cells were streaked on YPD+/- sorbitol and grown at 25°C, 30°C, or 37°C for three days. **(C)** *MAT* **a** *mid2Δ* cells were transformed with pBM743 (vector control), pDL468 (pGAL*PKC1*) or pDL469 [pGAL*PKC1* (K853R)]. Transformants were selected on Ura dropout plates containing glucose at 25°C for three days. The resulting colonies were then streaked onto Ura dropout plates containing galactose and incubated at 25°C, 30°C or 37°C; one representative temperature is shown.

were transformed with pBM743 (vector control), pDL468 (pGAL*PKC1*) or pDL469 [pGAL*PKC1* (K853R)], which is the catalytically inactive form of *PKC1* (Gray *et al.* 1997). The transformants were streaked onto selective medium and the resulting colonies were further streaked on galactose-selective medium and were incubated at 25°C.

Expression of the catalytically inactive *PKC1* from the *GAL1-10* promoter does not inhibit the growth of wild-type cells (Fig. 3.6C; Gray *et al.* 1997; Watanabe *et al.* 1994). Wild-type cells are not sensitive to over-expression of *PKC1*-K853R, as this dominant negative allele is not potent enough to fully inactivate the Pkc1p signalling in these cells. Furthermore, over-expression of *PKC1* itself has no effect on growth of wild-type cells (Fig. 3.6C). Expression of the catalytically inactive Pkc1p in *hcs77Δ* cells is lethal (Gray *et al.* 1997). *mid2Δ* cells expressing *PKC1*-K853R form colonies at all temperature, as do the cells expressing *PKC1* and the vector control cells (Fig. 3.6C). Therefore, partial inhibition of Pkc1p activity does not inhibit the growth of the *mid2Δ* cells. This result is consistent with *MID2* playing a minor role in the Pkc1p-MAPK pathway during vegetative growth.

3.2.7. *MID2* functions upstream of *MPK1*

The cell integrity pathway is required for growth of yeast at elevated temperatures and the pathway is activated by heat stress (Kamada *et al.* 1995; Lee *et al.* 1992; Levin *et al.* 1990). Increasing the growth temperature from 23°C to 39°C induces phosphorylation of Mpk1p (Kamada *et al.* 1995, Zarzov *et al.* 1996). Mpk1p phosphorylation, and hence its activation positively regulates the expression of the *MPK1* gene (Jung and Levin 1999). Thus monitoring the expression of *MPK1* serves as an assay of the Pkc1p-MAPK pathway activation.

If Mid2p is an upstream activator of Pkc1p during vegetative growth, then loss of *MID2* should compromise heat activation of *MPK1* transcription. Therefore, the extent of transcriptional induction of *MPK1* in response to heat shock in *MAT* **a** wild-type, *mid2Δ*, *hcs77Δ*, and *mid2Δhcs77Δ* EG123 cells was examined. Cells were grown to mid-logarithmic phase in YPD 10% sorbitol at 25°C. Sorbitol was present to maintain the viability of *mid2Δhcs77Δ* cells during the experiment. Cell cultures were shifted to 42°C

(to mimic the temperature shift performed by previous heat shock studies) by transfer of the cultures into pre-heated flasks. Cell aliquots were removed at 0, 10 and 20 minutes following shift to 42°C. Total RNA prepared from these aliquots was analysed by northern blotting. The northern blots were probed for *MPK1* and *ACT1* transcripts. The *ACT1* transcript encoding actin was used as a loading control for the samples.

It was observed that the *mid2Δ* and *hcs77Δ* mutants showed both delayed and reduced induction of *MPK1* transcripts in comparison to the wild-type cells (Fig. 3.7). Therefore, *MID2* is required for the full activation of *MPK1* in response to heat shock. Interestingly, *mid2Δhcs77Δ* cells showed no activation of *MPK1* transcription in response to heat shock (Fig. 3.7). Hence, *MID2* and *HCS77* are required to activate the Pkc1p-MAPK pathway in response to heat shock. Furthermore, *MID2* and *HCS77* have a partially redundant role in the activation of the Pkc1p-MAPK pathway.

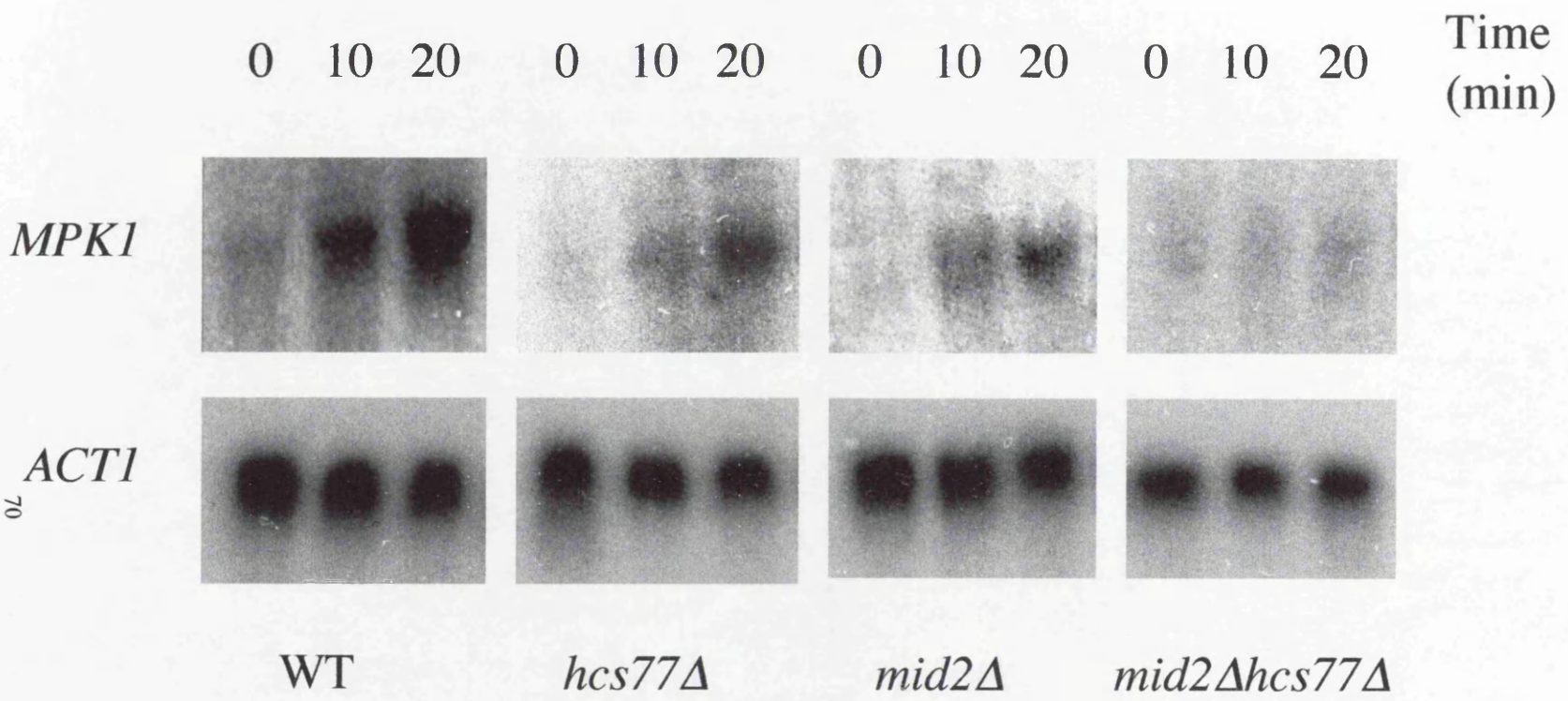

Figure 3.7. Mid2p and Hcs77p are required to activate Mpk1p in response to heat shock. Autoradiograph showing *MPK1* and *ACT1* levels in cell extracts. *MAT* **a** wild-type, *mid2Δ*, *hcs77Δ* and *mid2Δhcs77Δ* cells in the EG123 strain background were grown to mid-logarithmic phase in YPD at 25°C. The cultures were then shifted to 42°C for 0, 10 and 20 minutes. Total RNA was prepared from aliquots removed at time intervals. The resulting northern blots were probed with ^{32}P labeled *ACT1* (as loading control) or the *MPK1* gene.

3.3. Discussion

HCS77 is an upstream regulator of the Pkc1p-MAPK pathway that encodes a trans-membrane protein, localised to the plasma membrane, which acts as a cell surface sensor of stress (Gray *et al.* 1997; Rajavel *et al.* 1999; Verna *et al.* 1997). Cells deficient in the *HCS77* gene display a cell lysis phenotype at high temperatures, which is osmotically remedial (Gray *et al.* 1997). *MID2*, which also encodes a trans-membrane protein localised to the plasma membrane, seems to be a likely candidate for a second sensor of stress for the Pkc1p-MAPK pathway (Ketela *et al.* 1999; Ono *et al.* 1994; Rajavel *et al.* 1999). However unlike *HCS77*, *MID2* appeared not to be required during vegetative growth, since the *mid2Δ* strain displayed no growth defects at any temperature, a result consistent with recent independent work (Rajavel *et al.* 1999; and Ketela *et al.* 1999). However, *MID2* over-expression suppressed the *hcs77Δ* growth defect at 37°C, suggesting that Mid2p may function parallel or downstream of Hcs77p as a cell surface sensor in the Pkc1p-MAPK pathway. This role in the Pkc1p-MAPK pathway appeared to be minor, since over-expression of the catalytically inactive *PKC1* in *mid2Δ* did not inhibit growth, a result similar to that seen in wild-type cells. However, the significance of these two cell surface sensors became apparent in the *mid2Δhcs77Δ* double deletion. In the EG123 strain background *mid2Δhcs77Δ* cells displayed a phenotype that was similar to that of *pkc1Δ* cells. Without osmotic stabilisation, these double mutant cells could not grow at any temperature. This finding supports the work published recently, in which *mid2Δhcs77Δ* cells in the EG123 strain background were found to be inviable at all temperatures without osmotic stabilisation (Rajavel *et al.* 1999; and Ketela *et al.* 1999). In the S288C background, the double *mid2Δhcs77Δ* mutant is not viable even in high osmolarity, a phenotype that is more severe than that seen in *pkc1Δ* mutants. This may be due to the different genetic modifiers that exist in the S288C and EG123 strain backgrounds. These results showed that *MID2* and *HCS77* share a redundant function during vegetative growth. Furthermore, these results support an important role for Hcs77p and Mid2p in maintaining cell viability during vegetative growth, within the Pkc1p-MAPK pathway.

MID2 over-expression also relieved the temperature sensitivity phenotype of the *swi4Δ* and *swi4^{ts}/swi4Δ* cells. This result is similar to those obtained for *HCS77* and *PKC1* over-

expression in these mutants, and further supports a role for *MID2* within the Pkc1p-MAPK pathway (Gray *et al.* 1997).

If *MID2* plays a similar role to *HCS77*, then it may also be involved in signalling stress to the Pkc1p-MAPK pathway, which would lead to the activation of the pathway. Activation of *MPK1* transcription in response to heat-shock, is delayed and reduced, in *mid2Δ* cells when compared to wild-type cells. Therefore, activation of *MPK1* transcription and hence activity of Mpk1p after heat shock is partially dependent on the presence of *MID2* in vegetatively growing cells. This result supports the recent findings, which showed that *mid2Δ* cells are defective in heat activation of Mpk1p (Rajavel *et al.* 1999; Ketela *et al.* 1999). *HCS77* is also required for full activation of Mpk1p, since *hcs77Δ* cells show a reduced and delayed activation of *MPK1* transcription in heat-shocked cells. This latter result is consistent with results obtained by Gray *et al.* (1997). Most significantly, *mid2Δhcs77Δ* cells do not activate the transcription of *MPK1* following heat-shock. This result shows that Mid2p and Hcs77p have an essential and redundant role in the activation of Pkc1p-MAP kinase pathway, in response to stress.

MID2 has been implicated in recent reports to function in many different cellular processes. It has been proposed that Mid2p is an activator of the transcription factor Skn7p (Ketela *et al.* 1999). Skn7p is involved in a number of cellular processes, including cell wall biosynthesis (Ketela *et al.* 1999). Skn7p has been proposed to act parallel to the Pkc1p pathway, since *skn7Δpkc1Δ* cells are inviable and *SKN7* over-expression suppresses the *pkc1Δ* cell lysis phenotype (Brown *et al.* 1994). Activation of Skn7p through Mid2p may involve the binding of Mid2p to Rom2p (the guanine nucleotide exchange factor (GEF) for Rho1p (Philip *et al.* 2001)), thereby activating Rho1p. Rho1p activation would in turn lead to activation of Skn7p, which may then regulate cell wall gene expression and G_1 cyclin expression (Ketela *et al.* 1998).

Chapter 4: The Pkc1p-MAPK pathway is required to maintain the integrity of mating pheromone-treated cells

4.1. Introduction

In chapter three Mid2p was confirmed to be a component of the Pkc1p-MAPK pathway. Specifically, Mid2p is an activator of the Pkc1p-MAPK pathway during cell surface stress of heat shock. These results are consistent with published data suggesting a role for Mid2p as a cell surface sensor for the Pkc1p-MAPK pathway (Ketela *et al.* 1999; Rajavel *et al.* 1999). *mid2Δ* cells display no obvious vegetative defect. However, Ono *et al.* (1994), have shown that *mid2Δ* cells die in presence of mating pheromone. It is known that the Pkc1p-MAPK pathway is activated in response to mating pheromone and that *mpk1Δ* mutants die upon shmoo formation (Errede *et al.* 1995; Zarzov *et al.* 1996). Mid2p may therefore be required for activation of the Pkc1p-MAPK pathway during shmoo formation. Buehrer *et al.* (1997) have suggested that the protein product of an unknown Ste12p-activated gene is required to activate Mpk1p in response to mating pheromone. *MID2* has a potential pheromone response element in its putative promoter region, and expression of *MID2* is stimulated by α-factor (Ono *et al.* 1994). If Mid2p is this unidentified factor, required for activating the Pkc1p-MAPK pathway during mating, then there are three possibilities for the role of Mid2p. One possibility involves Mid2p playing a redundant role with Hcs77p in activating the Pkc1p-MAPK pathway during shmoo formation. This would be similar to the function these two sensors have during vegetative growth and heat shock (Chapter 3). The second possibility is that Mid2p is selectively required to activate the Pkc1p-MAPK pathway during mating either dependent or independent of cell surface damage. The third possibility may be that increased expression of *MID2* during mating is sufficient to activate the Pkc1p-MAPK pathway.

Philips and Herskowitz (1997) have suggested that Pkc1p regulates cell-cell fusion during mating. Glycerol is the main osmolyte in yeast. One possibility raised by the work of Philips and Herskowitz (1997) is that the secretion of glycerol at the shmoo tip leads to hyper-osmotic state between two mating partners in close proximity. This high osmolarity

condition may then inactivate the Pkc1p pathway in the mating partners, allowing breakdown of the cell wall material, leading to cell-cell fusion. In this view, the Pkc1p pathway is an inhibitor and key regulator of cell-cell fusion.

The role of Mid2p during shmoo formation may not be restricted to the regulation of Pkc1p-MAPK pathway. Marcoux *et al.* (1998) found *MID2* to be a high copy suppressor of the profilin deficient phenotype (Marcoux *et al.* 1998). Profilin is one of the proteins involved in actin cytoskeletal organisation (Goldschmidt-Clermont *et al.* 1992). Profilin also binds to Bni1p, which is a target of Rho1p. Bni1p is involved in cell wall synthesis and organisation of the actin cytoskeleton (Evangelista et al. 1997; Kohno *et al.* 1996; Yamochi *et al.* 1994). Philip *et al.* (2001) have suggested that Mid2p may activate Rho1p by binding to Rom2p the guanine exchange factor of Rho1p which localises to sites of polarised growth (Manning *et al.* 1997). As an activator of Rho1p, one would expect *MID2* to be involved in many events that may include the regulation of the actin cytoskeleton.

During periods of polarised growth, and specifically during mating pheromone-induced polarised growth, new cell wall is synthesised (Cabib *et al.* 1971; Schekman and Brawley 1979). It has been shown that during shmoo formation, the rate of chitin synthesis is increased and furthermore chitin is deposited at the neck of the polarised projection (Schekman and Brawley 1979). Errede *et al.* (1995), have shown that mutants of the cell integrity MAPK cascade, die in presence of mating pheromone. This death in mating pheromone has been proposed to be due to a defective cell wall. Furthermore, Ketela *et al.* (1999), have suggested that *mid2Δ* cells may have a defect in chitin synthesis in response to mating pheromone.

In this chapter, the role of Mid2p, Hcs77p and other components of the Pkc1p-MAPK pathway during mating will be examined.

4.2. Results

4.2.1. Cells lacking *MID2* show an α-factor-induced death

The *MID2* gene was initially identified during a screen for cells that die in pheromone, after differentiating into shmoos (Ono *et al.* 1994.). Ono *et al.* (1994), have also shown that expression of *MID2* is activated during periods of exposure to α-factor. Here, the death of *mid2Δ* cells in our EG123 and S288C strain backgrounds is examined to verify these results. *MAT* **a** *mid2Δ* and *MAT* **a** wild-type cells were grown to mid-logarithmic phase in pH 4.0 YPD at 25°C and were then exposed to α-factor for five hours. To keep a continuous supply of α-factor in the cell cultures, the media used was at pH 4.0, because Bar1p, the enzyme that degrades α-factor in yeast, is less active at this pH. Samples removed at several time point intervals were stained with methylene blue, and observed under the microscope. Dark-blue stained cells are dead and are thus incapable of metabolising the dye to a colourless derivative. In our *mid2Δ* strain backgrounds, after five hours of pheromone exposure, most of the cell population had stained blue, and had thus died, in comparison to approximately 10% death in wild-type cells (Fig. 4.1A/B). These results confirm those obtained by Ono *et al.* (1994).

To rule out the possibility that the high percentage of *mid2Δ* cells which die in α-factor is a consequence of growing cells in low pH medium, a double mutant lacking both *MID2* and *BAR1* was constructed. *MAT* **a** *mid2Δ* : : *URA3* cells were mated to *MAT* α *bar1Δ* : : *LEU2* cells in the S288C strain background, and the resulting diploids were sporulated. From 12 tetrads, seven contained spores, which were capable of growth on both *URA3* and *LEU2* selective media, indicating that they were *mid2Δbar1Δ* cells (Data not shown). *MAT* **a** *mid2Δbar1Δ* cells were grown to mid-logarithmic phase in YPD, and were exposed to α-factor. Samples were removed at time intervals and stained with methylene-blue. Approximately 95% of the *mid2Δbar1Δ* cell population stained dark blue after five hours of pheromone exposure indicating that they had died, in comparison to approximately 10% of the *bar1Δ* cells staining dark blue (Fig. 4.1C). Thus, the death of *mid2Δ* cells in α-factor is not dependent on the pH of the medium. These results confirm

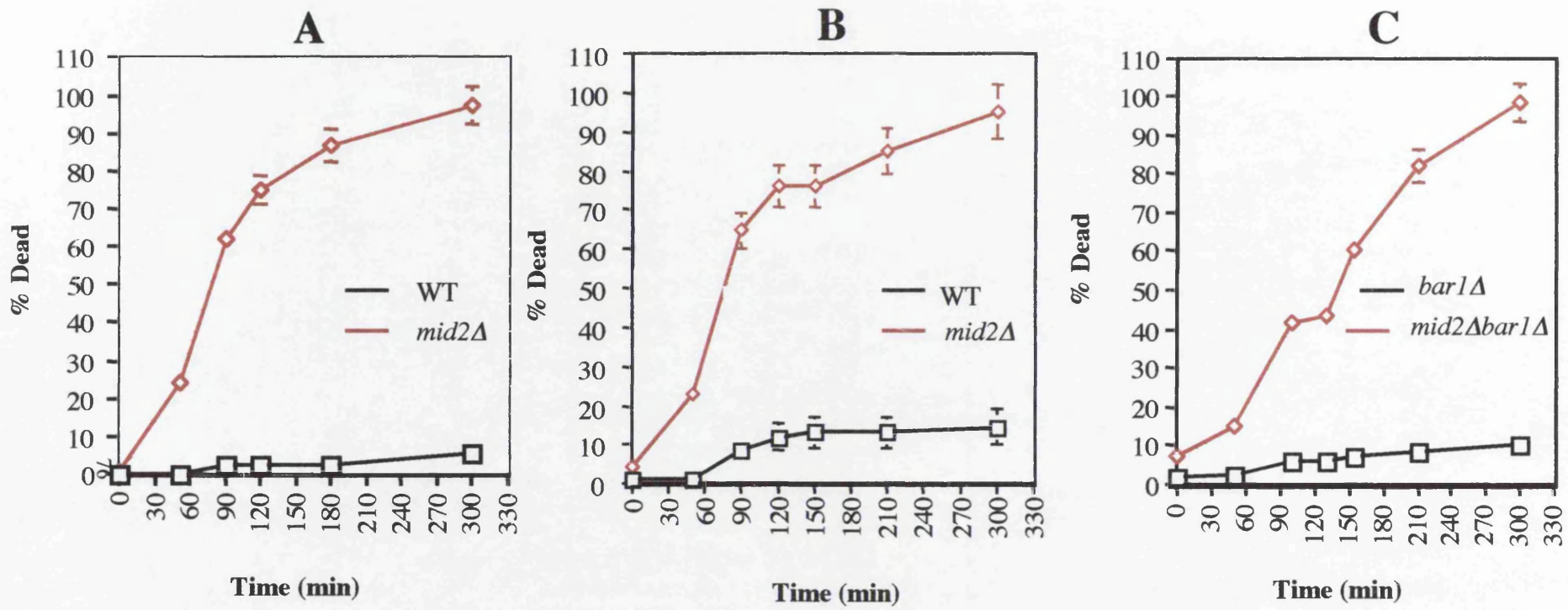

Figure 4.1. Cells deficient for *MID2* die in presence of mating pheromone. *MAT* **a** Wild type and *mid2Δ* cells in the grown to mid-logarithmic phase in pH 4.0 YPD at 25°C. Mating pheromone (α-factor; 12μM) was added to the cultures. Samples removed at time intervals were stained with methylene blue and were tested for viability under the microscope. (**A**) S288C strain background. (**B**) EG123 strain background. (**C**) S288C strain background *MAT* **a** *mid2Δbar1Δ* and *MAT* **a** *bar1Δ* cells grown to mid-logarithmic phase in YPD at 25°C. Viability of cells was assessed at time intervals under the microscope with samples stained with methylene-blue. In the data presented n=3 and error is +/-SEM.

that in our strain backgrounds *mid2Δ* cells die in presence of mating pheromone (Ketela *et al.* 1999; Rajavel *et al.* 1999).

4.2.2. Cells lacking *MPK1* display a mating pheromone-induced death

Errede *et al.* (1995), have shown that *mpk1Δ* cells and other mutants of the MAPK cascade die in mating pheromone. Since Mid2p functions in the Pkc1p-MAPK pathway during vegetative growth, then death of *mpk1Δ* cells in α-factor suggests that mating pheromone-induced death is a defect common to all Pkc1p-MAPK pathway components. Here, the death of *mpk1Δ* cells in our strain background was verified. Mid-logarithmic phase *MAT* **a** *mpk1Δ* and *MAT* **a** wild-type cells in the EG123 strain in pH 4.0 YPD were exposed to α-factor. Samples were removed at time intervals, stained with methylene blue and were observed under the microscope. The *mpk1Δ* cells die in presence of α-factor such that after five hours exposure to mating pheromone, almost 90% of the cell population had died in comparsion to 8% of wild-type cells (Fig. 4.2A). This result is consistent with results obtained by Errede *et al.* (1995). This result indicates that death in α-factor is a defect likely shared by all components of the Pkc1p-MAPK pathway. Note: in absence of sorbitol, *mpk1Δ* cell population shows about 70% viability at zero time.

If death of *mid2Δ* cells in mating pheromone is a consequence of a defective Pkc1p-MAPK pathway then one would expect that the phenotype of *mid2Δmpk1Δ* cells in α-factor would not be more severe than that of either deletion alone. However, if death of *mid2Δ* cells is due to a defect in a parallel pathway to the Pkc1p-MAPK pathway, then *mid2Δmpk1Δ* cells should display a more severe defect in α-factor than a single deletion. To clarify this situation, mating pheromone-induced death in *mid2Δmpk1Δ* cells was assessed. Mid-logarithmic phase *MAT* **a** *mid2Δmpk1Δ* and *MAT* **a** *mid2Δ* cells in the S288C strain background were exposed to α-factor and samples were removed at time intervals. As before, all samples were stained with methylene blue and observed under the microscope. The *mid2Δmpk1Δ* cells do not display a more severe phenotype than the *mid2Δ* cells in presence of α-factor (Fig. 4.2B). These results indicate that the death of *mid2Δ* cells in mating pheromone may be dependent on the Pkc1p-MAPK pathway and not on a parallel pathway.

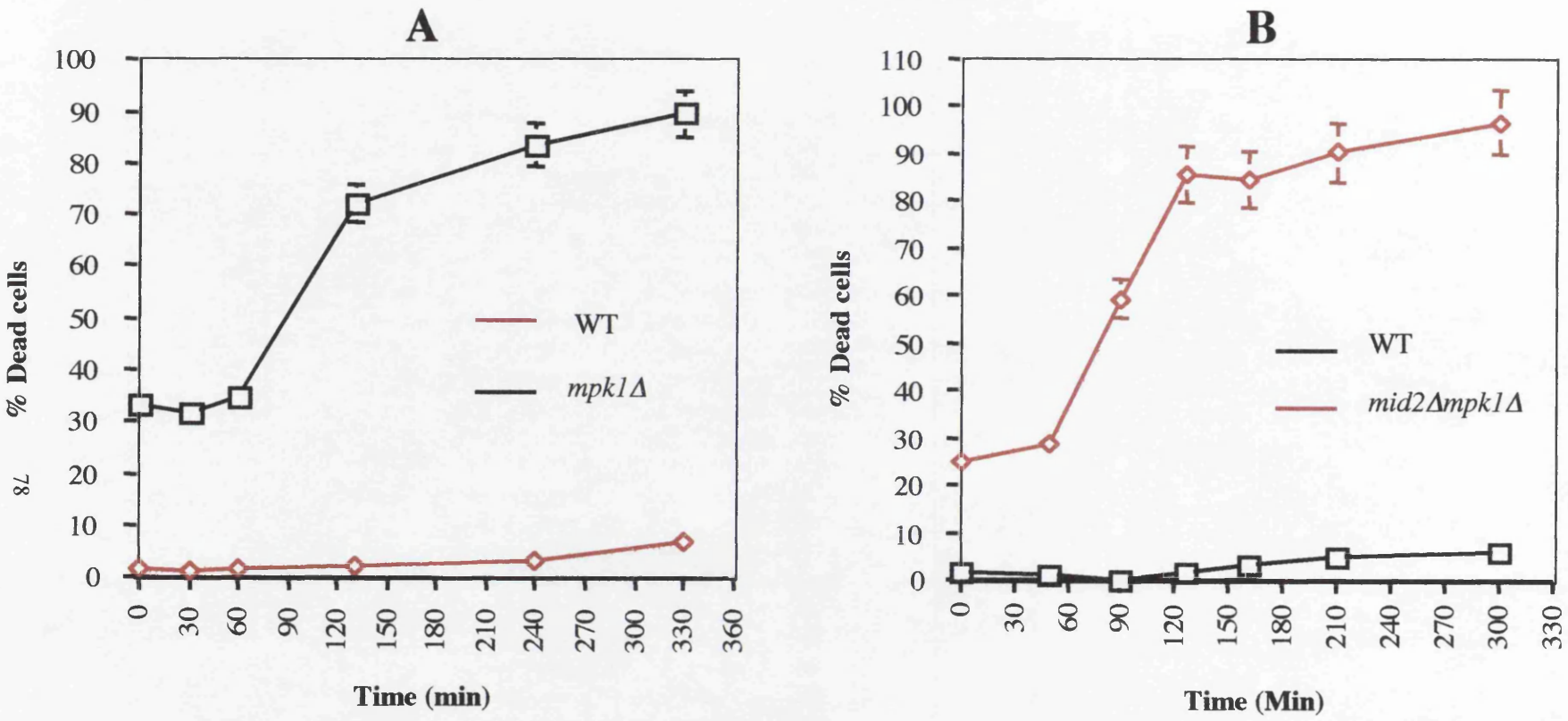

Figure 4.2. Mpk1p and Mid2p appear to function in the same pathway in response to mating pheromone (**A**) *MAT* **a** wild-type and *mpk1Δ* EG123 cells or (**B**) *MAT* **a** *mid2Δmpk1Δ* and *mid2Δ* cells in the EG123 strain background were grown to mid-logarithmic phase in pH 4.0 YPD at 25°C. Mating pheromone (α-factor; 12μM) was added to the cultures. Viability of samples, removed at time intervals, was assessed by microscopy of methylene-blue stained cells. In the data presented n=3 and error is +/-SEM.

4.2.3. Over-expression of Pkc1p-MAPK pathway components suppress the pheromone-induced death of *mid2Δ* cells

If death of *mid2Δ* cells is due to an inactive or partially inactive Pkc1p-MAPK pathway, then over-expression of other Pkc1p-MAPK pathway components may suppress the death of *mid2Δ* cells in α-factor. To test this hypothesis, *MAT* **a** *mid2Δ* cells in the EG123 strain background were transformed with *YEp24* or *HCS77* multi-copy plasmids. The transformants were streaked onto selective media, and the resulting colonies were grown in pH 4.0 selective liquid media. Mid-logarithmic phase cultures were exposed to α-factor, and samples were removed at time intervals. All samples were stained with methylene blue and their viability was assessed by microscopy. Cells over-expressing *HCS77* displayed a mating pheromone-induced death which was significantly less than that of cells transformed with the vector control (Fig. 4.3A). These results show that over-expression of *HCS77* partially suppresses the α-factor-induced death of the *mid2Δ* mutant (Fig. 4.3A). This result is consistent with the death of *mid2Δ* cells in α-factor being at least partly due to a defective Pkc1p-MAPK pathway. This result also indicates that Hcs77p shares a common function with Mid2p in the mating pheromone response. Note: over-expression of *MID2* in *mid2Δ* cells as a multi-copy plasmid does not fully restore the wild-type phenotype. This may be because over-expression of *MID2* is itself lethal to the cells.

Consistent with the proposal that death of *mid2Δ* cells is due to a defective Pkc1p-MAPK pathway, one expects that activation of other components of the Pkc1p-MAPK pathway would also suppress the pheromone-induced death of *mid2Δ* cells. To further assess this possibility, *MAT* **a** *mid2Δ* cells in the EG123 strain background were transformed with *BCK1-20* (which encodes a partially activated version of the MEK kinase) or *RHO1* high-copy plasmids. The transformants were streaked onto selective media and the resulting colonies were grown in pH 4.0 liquid selective media. Mid-logarithmic phase cultures, were exposed to α-factor and samples were removed at time intervals. The samples were stained with methylene blue and their viability was assessed by microscopy. Cells transformed with *BCK1-20* or *RHO1* showed 10-15% more viable cells after five hours of α-factor treatment in comparison to cells containing the vector control (Fig. 4.3B). *BCK1-20* and *RHO1* over-expression partially suppressed the α-factor-induced death of

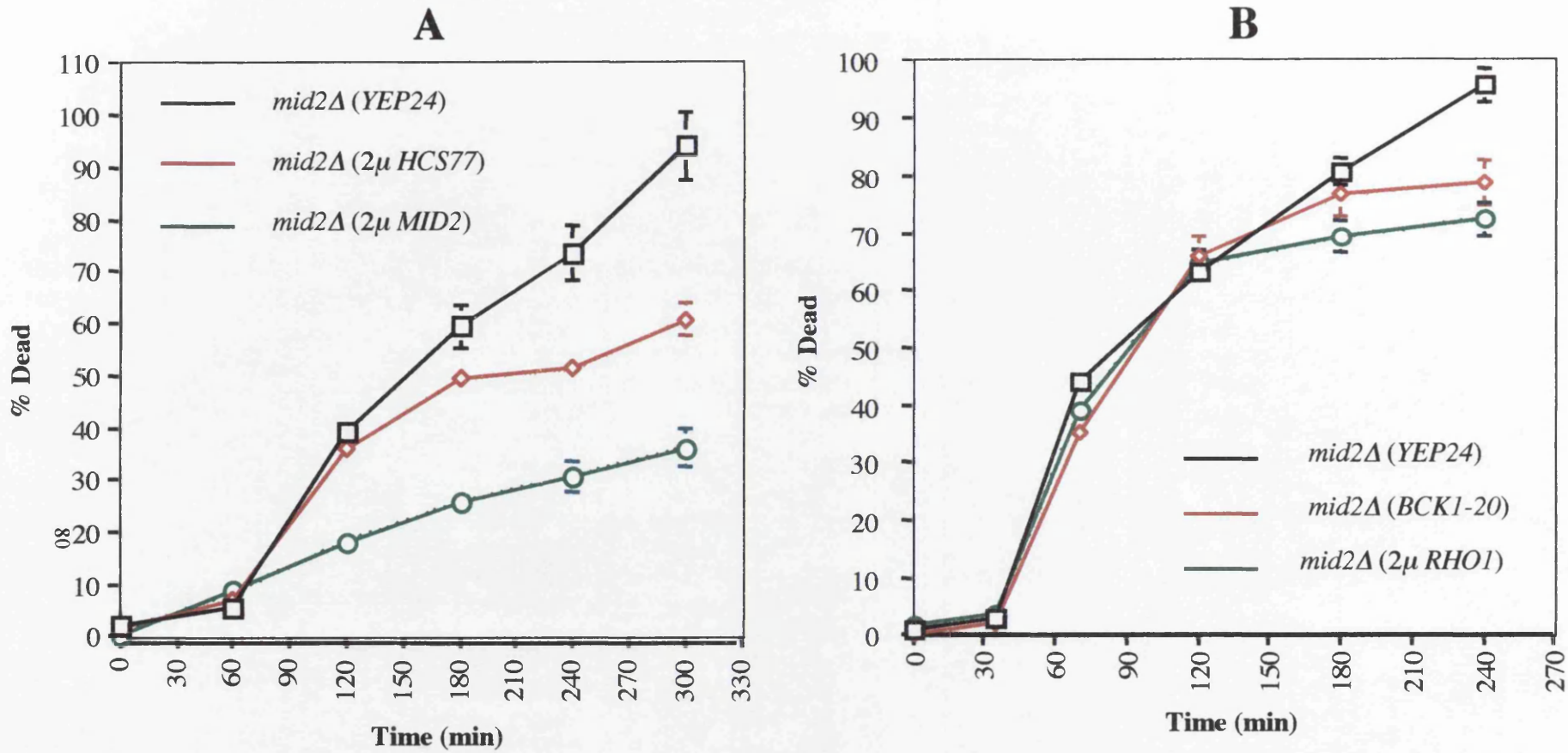

Figure 4.3. Over-expression of Pkc1p-MAPK pathway components partially suppresses the mating pheromone induced death of *mid2Δ* cells. *MAT* **a** *mid2Δ* cells in the EG123 strain background transformed with (**A**) *YEp24* or *HCS77* (**B**) *BCK1-20*, *RHO1* or *YEp24* were grown to mid logarithmic phase in pH 4.0 Ura$^-$ selective media at 25°C. The cultures were exposed to mating pheromone (α-factor; 12μM). The viability of samples removed at time intervals was assessed by microscopy of methylene-blue stained cells. In the data presented n=3 and error is +/-SEM.

mid2Δ cells (Fig. 4.3B). These results further support a role for the Pkc1p-MAPK pathway in the pheromone-induced death of *mid2Δ* mutants.

4.2.4. *MID2* is required for full activation of Mpk1p during shmoo formation

During periods of polarised growth, Mpk1p becomes phosphorylated and hence activated (Buehrer *et al.* 1997, Zarzov *et al.* 1996). Mpk1p activation leads to transcriptional activation of *MPK1* (Jung and Levin 1999), and monitoring of *MPK1* expression, serves as an assay of Pkc1p-MAPK activation. Mid2p is required for stress signalling to Mpk1p when cells are heat shocked (section 3.2.7). Mid2p may also be required to activate Mpk1p during α-factor treatment.

To test this hypothesis, the following experiments were carried out. Mid-logarithmic phase *MAT* **a** wild-type and *MAT* **a** *mid2Δ* cells in the EG123 strain background in pH 4.0 YPD were exposed to α-factor and samples were removed at time intervals. Total RNA was prepared from samples. Expression of *MPK1* was monitored by northern blotting and *ACT1* was used as an internal control of expression. In comparison to wild-type cells *mid2Δ* cells showed a reduced and delayed activation of *MPK1* transcription in presence of mating pheromone (Fig. 4.4A). This result shows that *mid2Δ* cells are deficient for activation of Mpk1p in response to mating pheromone. Furthermore, this result is consistent with the hypothesis that *mid2Δ* cells die in mating pheromone because of a reduced Pkc1p-MAPK pathway activity.

4.2.5. *hcs77Δ* cells die in mating pheromone

Over-expression of *HCS77* can suppress the α-factor-induced death of *mid2Δ* cells. This observation suggests that *HCS77* may also have a role in the α-factor response. To assess this hypothesis, *MAT* **a** *hcs77Δ* and *MAT* **a** wild-type cells in the EG123 strain background were grown to mid-logarithmic phase in pH 4.0 YPD. These cell cultures were exposed to α-factor and samples were removed at time intervals. Samples were stained with methylene blue and their viability was assessed by microscopy. After five hours of

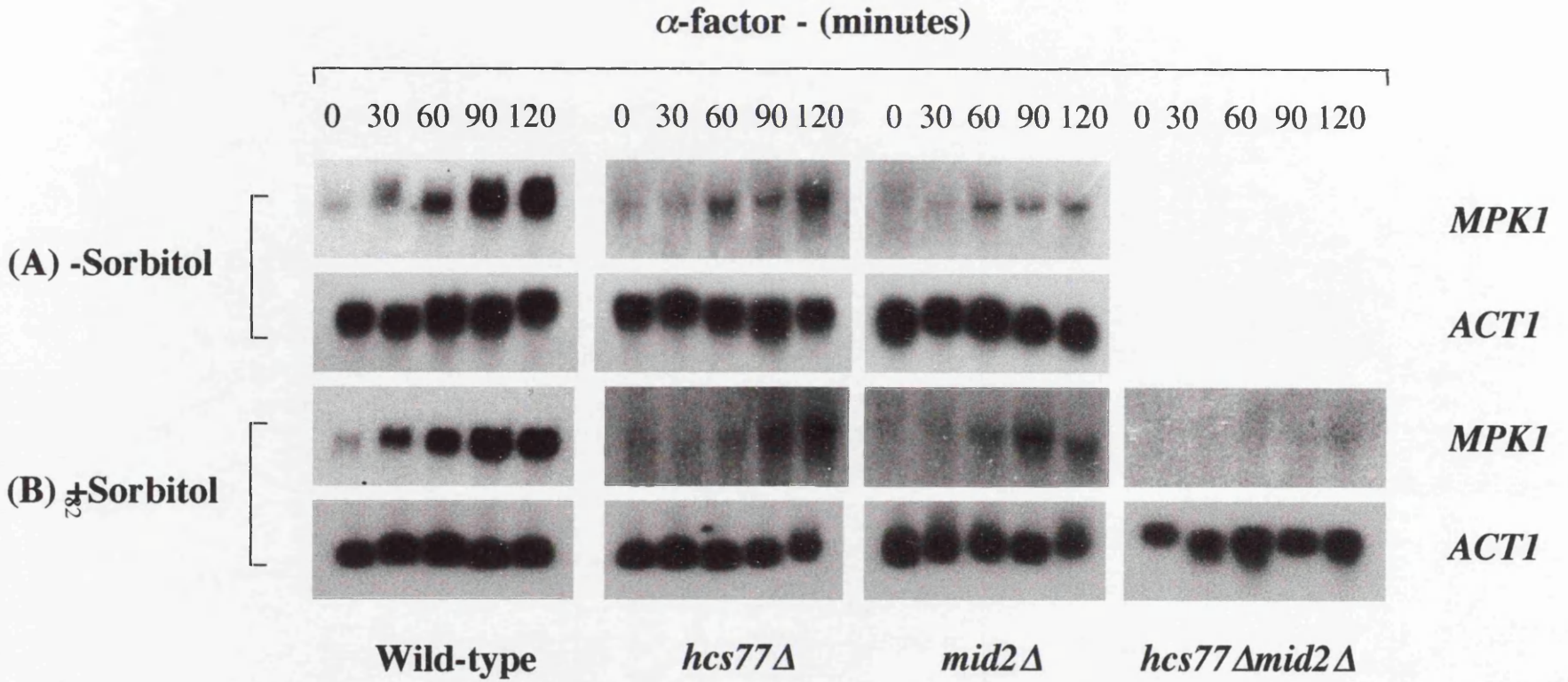

Figure 4.4. Mid2p and Hcs77p share a redundant role in activation of Mpk1p in response to mating pheromone. Autoradiograph showing *MPK1* and *ACT1* levels in cell extracts. *MAT* **a** wild type, *mid2Δ*, *hcs77Δ* and *mid2Δhcs77Δ* cells in the EG123 strain background were grown to mid-logarithmic phase in pH 4.0 (**A**) YPD (**B**) 10% sorbitol YPD, at 25°C. The cell cultures were exposed to mating pheromone (α-factor; 12μM). Total RNA was prepared from samples removed at time intervals. The resulting northern blots were probed with radio-labeled *ACT1* or *MPK1* DNA. The results shown are representatives of triplicate experiments.

exposure to α-factor, up to 60% of the *hcs77Δ* cell population die, in comparison to 7% of wild-type cells (Fig. 4.5A). This result suggests that Hcs77p has a role to play in the mating response. These results are in contrast to reports by Rajavel *et al.* (1999), suggesting that Hcs77p has only a vegetative function as cell surface sensor.

4.2.6. Mating pheromone-induced death of *hcs77Δ* cells is partially suppressed by over-expression of Pkc1p-MAPK pathway components

Since *HCS77* over-expression can partially compensate for loss of *MID2* in response to α-factor, then one would expect that *MID2* would compensate for the loss of *HCS77* during the mating response. To assess this possibility *MAT* **a** wild-type and *MAT* **a** *hcs77Δ* cells in the EG123 strain background were transformed with high copy plasmids containing *MID2* or the empty vector. The transformants were streaked onto selective plates and the resulting colonies were grown to mid-logarithmic phase in pH 4.0 selective media. These cell cultures were exposed to α-factor and samples were removed at time intervals. Samples were stained with methylene blue and their viability was assessed by microscopy. After five hours of exposure to mating pheromone, *hcs77Δ* cells over-expressing *MID2* showed 25% death in comparison to 60% death in cells containing the empty vector (Fig. 4.5B). Over-expression of *MID2* therefore partially suppresses the death of *hcs77Δ* cells in mating pheromone. This result shows that Mid2p and Hcs77p share a common function during the mating response and that Mid2p can partially compensate for the loss of Hcs77p in response to mating pheromone.

Since *MID2* can compensate for the loss of *HCS77* during mating, then one would expect that over-expression of other downstream components of the Pkc1p-MAPK pathway may also suppress the *hcs77Δ* phenotype in mating pheromone. To examine this possibility, *MAT* **a** *hcs77Δ* cells in the EG123 strain background were transformed with high-copy plasmids of *PKC1* or the empty vector. The transformants were streaked onto selective plates and the resulting colonies were grown to mid-logarithmic phase in pH 4.0 selective media. These cells were then exposed to α-factor and samples were removed at time intervals. Samples were stained with methylene blue and their viability was assessed by microscopy. After five hours of α-factor exposure cells over-expressing *PKC1* showed 40% death in comparison to 60% death in cells transformed with the empty vector (Fig.

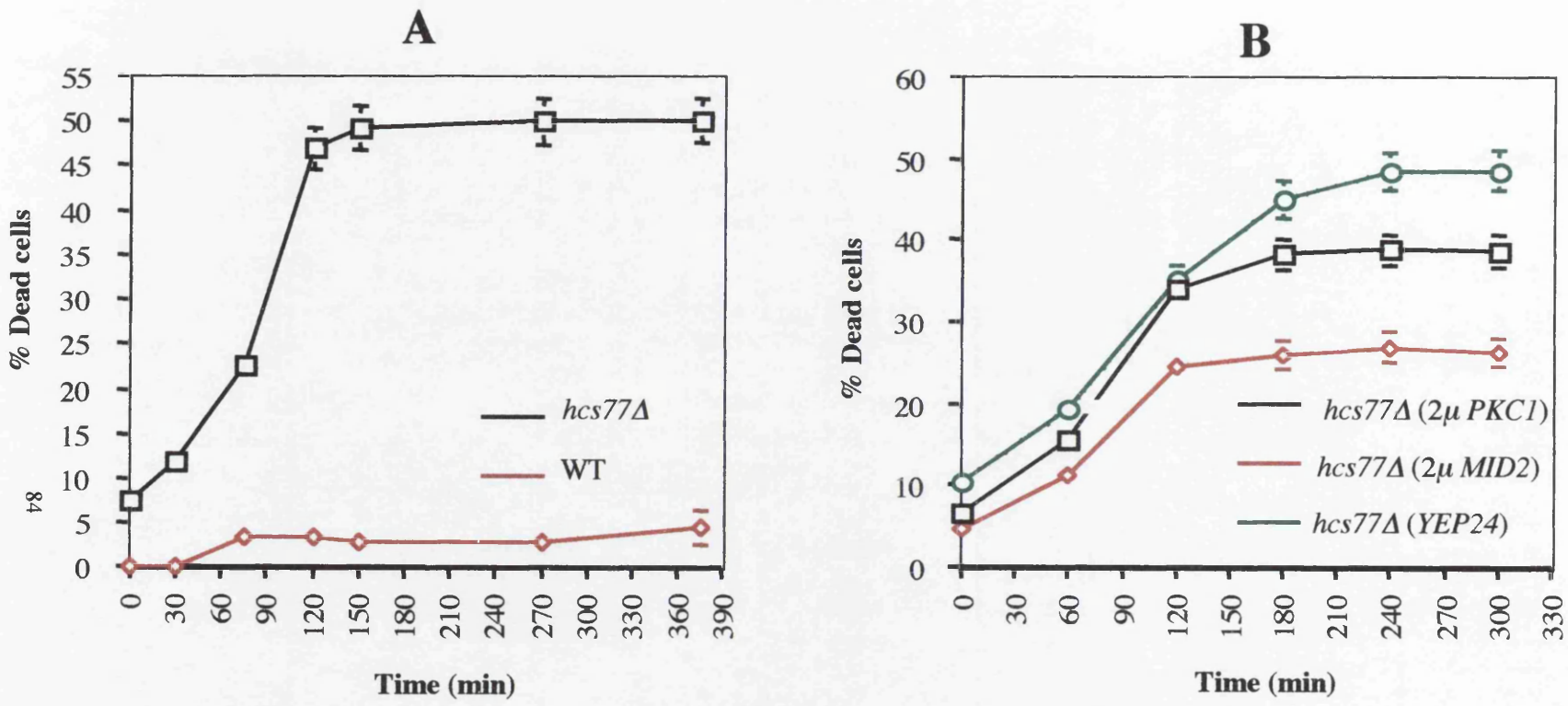

Figure 4.5. Cells mutant for *HCS77* display a pheromone-induced death that is suppressed by over-expression of Pkc1p-MAPK pathway components. (**A**) *MAT* **a** wild type and *hcs77Δ* cells in the EG123 strain background; (**B**) *MAT* **a** *hcs77Δ* cells, transformed with high-copy number *YEp24*, *MID2* or *PKC1*, were grown to mid-logarithmic phase in pH 4.0 YPD at 25°C. Cell cultures were exposed to mating pheromone (α-factor; 12μM). Viability of samples removed at time intervals was assessed by microscopy of methylene blue stained cells. In the data presented n=3 and error is +/-SEM.

4.5B). Over-expression of *PKC1* partially suppresses the death of *hcs77Δ* cells in α-factor. Therefore, this result suggests that *hcs77Δ* cells die in α-factor because of a defective Pkc1p-MAPK pathway.

4.2.7. *HCS77* is required for activation of Mpk1p in response to mating pheromone

Since Hcs77p is an upstream activator of Mpk1p in response to heat shock, it is possible that Hcs77p also functions to activate Mpk1p in response to mating pheromone. If Hcs77p is an upstream activator of Mpk1p when exposed to α-factor, then death of *hcs77Δ* cells may be as a result of reduced Mpk1p activity. To assess this hypothesis, mid-logarithmic phase *MAT* **a** *hcs77Δ* and *MAT* **a** wild-type cells in the EG123 strain background in pH 4.0 YPD were exposed to α-factor and samples were removed at time intervals. Total RNA was prepared from all samples. The resulting northern blots of samples were probed with *MPK1* or *ACT1* DNA. The *hcs77Δ* cells showed a delayed and reduced activation of *MPK1* transcription in comparison to wild-type cells (Fig. 4.4A). This result shows that Hcs77p is required for the full activation of Mpk1p in presence of mating pheromone.

4.2.8. Mid2p and Hcs77p have a redundant role in activation of Mpk1p during pheromone treatment

Since Hcs77p and Mid2p have a redundant role in activation of Mpk1p during vegetative growth, it is possible that these two cell surface sensors share a redundant role in activation of the Pkc1p-MAPK pathway, in response to α-factor. However, *mid2Δhcs77Δ* cells are only viable in osmotically-stabilised medium. To assess the activatibility of Mpk1p in the *mid2Δhcs77Δ* mutant, it was necessary to determine if the pathway is activated by pheromone treatment in the presence of high osmolarity. Therefore, activation of the Pkc1p-MAPK pathway in presence of sorbitol in response to mating pheromone was analysed. *MAT* **a** wild-type cells in the EG123 strain background were grown to mid-logarithmic phase and were then exposed to α-factor. Samples were removed at time intervals and total RNA was prepared from all samples. *MPK1* expression was monitored by northern blotting and *ACT1* was used as an internal control of expression. Wild-type cells showed activation of *MPK1* transcription in response to

mating pheromone (Fig. 4.4B). This result shows that in high osmolarity, wild-type cells activate Mpk1p in response to mating pheromone.

Since Mpk1p is activated, in high osmolarity when cells are treated with pheromone, then activation of the pathway in absence of both cell surface sensors can be analysed. Therefore, mid-logarithmic phase *MAT* **a** *mid2Δ*, *MAT* **a** *hcs77Δ* and *MAT* **a** *mid2Δhcs77Δ*, cells in the EG123 strain background in 10% sorbitol pH 4.0 YPD were exposed to α-factor. Samples were removed at time intervals. Total RNA prepared from all samples. *MPK1* expression was monitored by northern blotting and *ACT1* was used as an internal control of expression. In presence of sorbitol *hcs77Δ* and *mid2Δ* cells exposed to α-factor, displayed a reduced and delayed activation of *MPK1* transcription (Fig. 4.4B). The *hcs77Δmid2Δ* cells showed no activation of transcription of *MPK1* in presence of mating pheromone when compared to wild-type cells (Fig. 4.4B). These results show that Hcs77p and Mid2p share a redundant function in activation of the Pkc1p-MAPK pathway during shmoo formation.

4.2.9. Death of Pkc1p-MAPK pathway mutants in α-factor is not a consequence of cell polarisation

The Pkc1p-MAPK pathway mutants die in α-factor as shmoos. If the Pkc1p-MAPK pathway mutants die in α-factor, because of an underlying event associated with polarisation, then prevention of polarisation should rescue these cells from pheromone-induced death. To test this hypothesis, mid-logarithmic phase *MAT* **a** wild-type and *MAT* **a** *mid2Δ* cells in the EG123 strain background in pH 4.0 YPD, were exposed to Latrunculin-A, for one hour before addition of α-factor. Latrunculin-A prevents polymerisation of actin filaments (Ayscough *et al.* 1997). Samples were removed at time intervals after α-factor addition. Samples were stained with methylene blue and their viability was assessed by microscopy. Prevention of actin polymerisation and hence polarisation did not suppress the death of *mid2Δ* cells in α-factor (Fig. 4.6A). This result indicates that polarisation may not be the underlying cause of death in *mid2Δ* cells exposed to α-factor. It was observed that at the end time point of the experiment 50% of wild-type cells died when exposed to Latrunculin-A and α-factor in comparison to only

10% death in wild-type cells that were only exposed to pheromone (Fig. 4.6A). In presence of Latrunculin-A therefore, wild-type cells died at a more rapid rate than that observed in non-treated cells (Fig. 4.6A).

Note however, that when wild-type and *mid2Δ* cells were only exposed to Latrunculin-A, some death of cell population was observed. In presence of only Latrunculin-A, approximately 10% of wild-type cells and 25% of *mid2Δ* cells were dead (Fig. 4.6B). These results indicate that prevention of actin polarisation may prevent the basic shuttling of essential components with in the cells, which especially in times of pheromone response would be lethal to the cells.

To further analyse the role of polarisation in pheromone-induced death of *mid2Δ* cells the following experiments were undertaken. Cells, which are *bni1Δ*, are defective for the re-organisation of actin that is required for cell polarisation (Evangelista *et al.* 1997). Therefore, *bni1Δ* cells are defective for polarised growth that is required for shmoo formation. If cells are deficient in both *MID2* and *BNI1* genes then effects of polarisation on death of these cells could be analysed. Hence, *MAT* **a** *mid2Δ* : : *URA3* and *MAT* α *bni1Δ* : : Kan^R cells in the W303 strain background were mated and sporulated. The resulting spores were streaked onto selective plates. Double mutants were selected from spores that formed single colonies on both ura^- and Kan^+ selective plates. From 12 tetrads dissected, nine contained the *mid2Δbni1Δ* double mutants. Mid-logarithmic phase *MAT* **a** *mid2Δbni1Δ* and *MAT* **a** *mid2Δ* cells in pH 4.0 YPD were exposed to α-factor. Samples were stained with methylene blue and their viability was assessed by microscopy. After five hours exposure to α-factor, nearly all of the *mid2Δbni1Δ* cell population had died, similar to *mid2Δ* cells (Fig. 4.6C). This result confirms that prevention of cell polarisation does not suppress death of *mid2Δ* cells. Furthermore, this result confirms above results and suggests that death of *mid2Δ* cells in α-factor, may be the result of cell wall defects, that are independent of polarisation (see discussion). Note: *bni1Δ* cells also die in presence of α-factor, although to a lesser extent than *mid2Δ* cells.

Since *hcs77Δ* cells also die in presence of mating pheromone, the effect of polarisation on mating pheromone-induced death of *hcs77Δ* cells was also analaysed. Mid-logarithmic

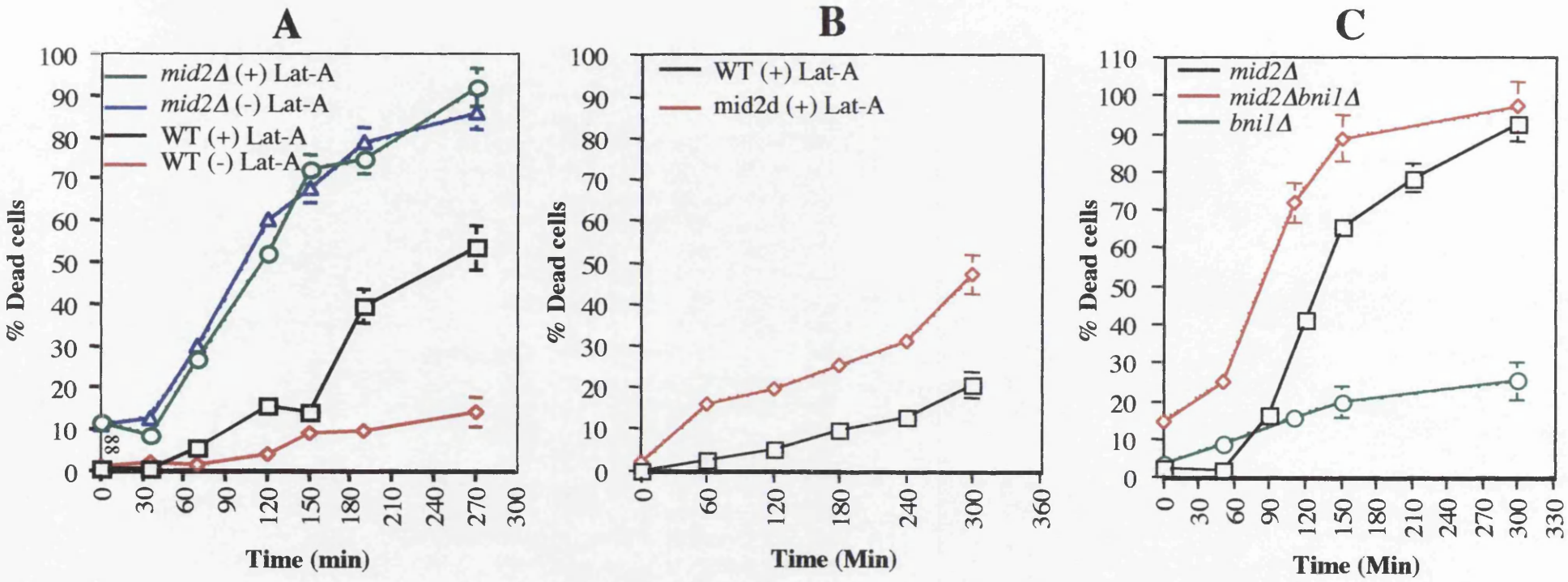

Figure 4.6. Death of *mid2Δ* cells in mating pheromone is not a consequence of cell polarisation. (**A**) *MAT* **a** wild-type and *mid2Δ* cells in the EG123 strain background were grown to mid-logarithmic phase in pH 4.0 YPD at 25°C. Cell cultures were pretreated with Latrunculin-A for one hour before addition of mating pheromone (α-factor; 12μM). Samples removed at time intervals were stained with methylene blue, and their viability was assessed by microscopy. (**B**) Samples removed from cultures treated with only Latrunculin-A were stained with methylene blue and their viability assessed by microscopy. (**C**) Cells which are *mid2Δbni1Δ* in the W303 strain background, were grown to mid-logarithmic phase in pH 4.0 YPD at 25°C. Cell cultures were exposed to mating pheromone (α-factor; 12μM). Samples removed at time intervals were stained with methylene-blue and their viability assessed by microscopy. In the data presented n=3 and error is +/-SEM.

phase *MAT* **a** wild-type and *MAT* **a** *hcs77Δ* cells in the EG123 strain background were exposed to Latrunculin-A, for one hour before addition of α-factor. Samples were stained with methylene blue and their viability was assessed by microscopy. Prevention of actin polymerisation did not suppress the pheromone-induced death of *hcs77Δ* cells (Fig. 4.7). This result indicates that polarisation may not be the underlying cause leading to death of *hcs77Δ* cells in α-factor. However, *hcs77Δ* cells exposed to Latrunculin-A and α-factor, showed a more severe death phenotype than non-Latrunculin treated cells. After five hours of exposure to pheromone, Latrunculin-A treated cells displayed approximately 80% death in comparison to 55% of non-treated cells (Fig. 4.7). Note that similar to *mid2Δ* cells, *hcs77Δ* cells exposed to just Latrunculin-A, after five hours, displayed up to 20% death in comparison to 5% death in wild-type cells (data not shown).

If cell polarisation is not the underlying defect that leads to death of cells deficient in the cell surface sensors Mid2p and Hcs77p, then one would expect that death of *mpk1Δ* cells in α-factor would also be independent of polarisation. To test this hypothesis, mid-logarithmic phase *MAT* **a** wild-type and *MAT* **a** *mpk1Δ* cells in EG123 strain background in 10% sorbitol YPD pH 4.0 were exposed to Latrunculin-A, for one hour before addition of α-factor. Cells were cultured in 10% sorbitol, as there is a higher population of *mpk1Δ* cells viable at start of experiments. Samples were stained with methylene blue and their viability was assessed by microscopy. The presence of Latrunculin-A did not suppress the death of *mpk1Δ* cells in α-factor (Fig. 4.8). This result indicates that polarisation is not the underlying cause of death in *mpk1Δ* cells in α-factor. Furthermore, Latrunculin-A treated *mpk1Δ* cells display a more severe phenotype in α-factor than non-treated cells (Fig. 4.8). Nearly 95% of the *mpk1Δ* population had died in presence of Latrunculin-A and α-factor in comparison to 40% death in non-treated cells. Note that, *mpk1Δ* cells display a Latrunculin-A induced death, such that up to 55% of the population dies after five hours of exposure to the drug, in comparison to 5% of wild-type cells (data not shown).

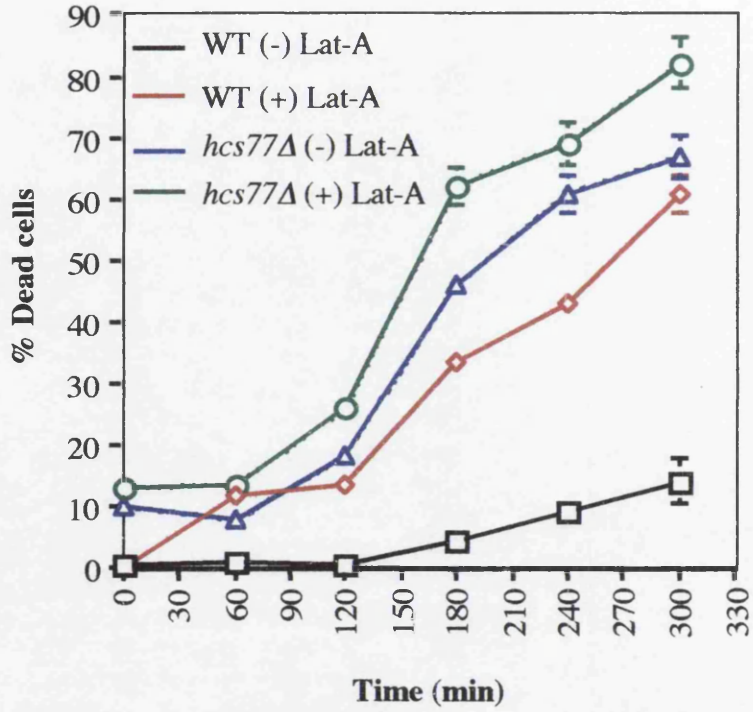

Figure 4.7. Death of *hcs77Δ* cells in mating pheromone is not a consequence of cell polarisation. *MAT* **a** wild-type and *hcs77Δ* cells in the EG123 strain background were grown to mid-logarithmic phase in pH 4.0 YPD at 25°C. Cell cultures were pretreated with Latrunculin-A for one hour before addition of mating pheromone (α-factor; 12μM). Samples removed at time intervals were stained with methylene-blue, and their viability was assessed by microscopy. In the data presented n=3 and error is +/-SEM.

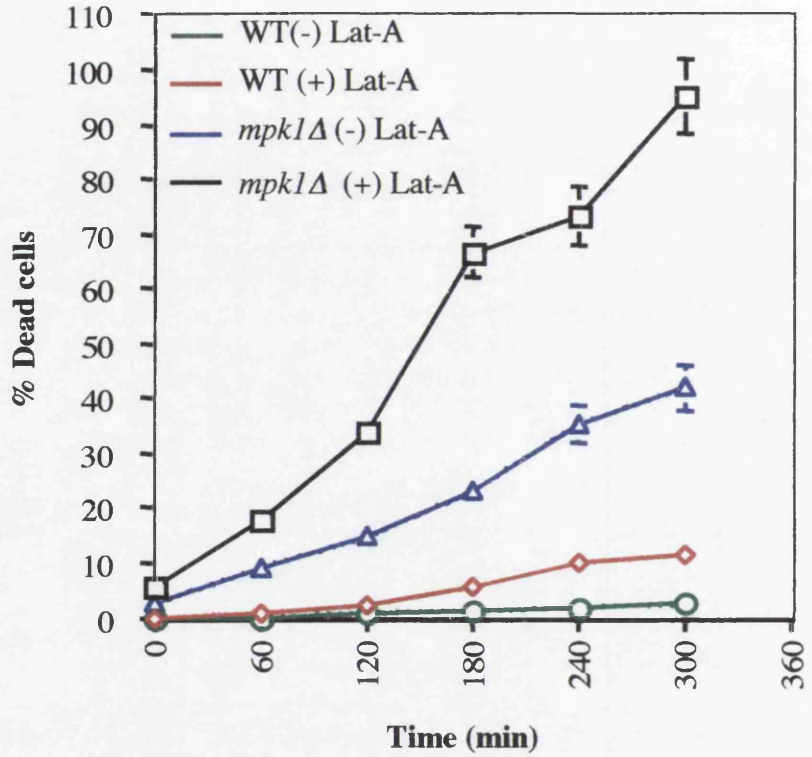

Figure 4.8. Death of *mpk1Δ* cells in mating pheromone is not a consequence of cell polarisation. *MAT* **a** wild-type and *mpk1Δ* cells in the EG123 strain background were grown to mid-logarithmic phase in pH 4.0 YPD at 25°C. Cell cultures were pretreated with Latrunculin-A for one hour before addition of mating pheromone (α-factor; 12μM). Samples removed at time intervals were stained with methylene-blue, and their viability was assessed by microscopy. In the data presented n=3 and error is +/-SEM.

4.2.10. *mid2Δ* cells do not appear to be defective for calcineurin signalling when exposed to mating pheromone

Calcineurin mutants are similar to *mid2Δ* cells as they also die in response to mating pheromone (Cyert *et al.* 1991; 1992). In chapter five the role of the Pkc1p-MAPK pathway in regulation of the calcineurin-signalling pathway is discussed in detail. It is possible that death of *mid2Δ* cells in mating pheromone is due to a defect in calcineurin activation. To examine this possibility the following experiments were carried out. *MAT* **a** *mid2Δ* and *MAT* **a** wild-type cells in the EG123 strain background were transformed plasmid that contained the constitutively-active calcineurin-A2 (*CNA*, the catalytic subunit of calcineurin; pVT-L[*CNA2Δ*]), or plasmid encoding full-length calcineurin (pVT-L[*CNA2*]). *CNA2Δ* lacks the calmodulin binding site and the C-terminal auto-inhibitory domain that renders the activity of the full-length enzyme dependent on Ca^{2+}/calmodulin (Hubbard and Klee 1989; Cyert *et al.* 1991; O'Keefe *et al.* 1992; Withee *et al.* 1997). Transformants were streaked onto selective plates. Single colonies were grown to mid-logarithmic phase in pH 4.0 selective media and were then exposed to α-factor. Samples were removed at time intervals, stained with methylene blue and their viability was assessed by microscopy. The *mid2Δ* cells over-expressing the full-length calcineurin and those over-expressing the constitutively-active calcineurin showed up to 95% death in comparison to about 10% of wild-type cells (Fig. 4.9). Therefore, over-expression of the constitutively active calcineurin does not dramatically suppress the death of *mid2Δ* cells in mating pheromone (Fig. 4.9). This result suggests that *mid2Δ* cells die in mating pheromone, as a results of defects that may not be directly associated with calcineurin activation.

4.2.11. Mutants of the Pkc1p-MAPK pathway display normal chitin deposition in mating pheromone

The mutants of the Pkc1p-MAPK pathway die in the presence of α-factor. Cells deficient for both cell surface sensors, Hcs77p and Mid2p, are defective for activation of Mpk1p in response to mating pheromone. These results suggest that maintaining cellular viability during the mating response, may be one function of the Pkc1p-MAPK pathway, and that this function requires full activation of the Pkc1p-MAPK. Schekman and Brawley (1979)

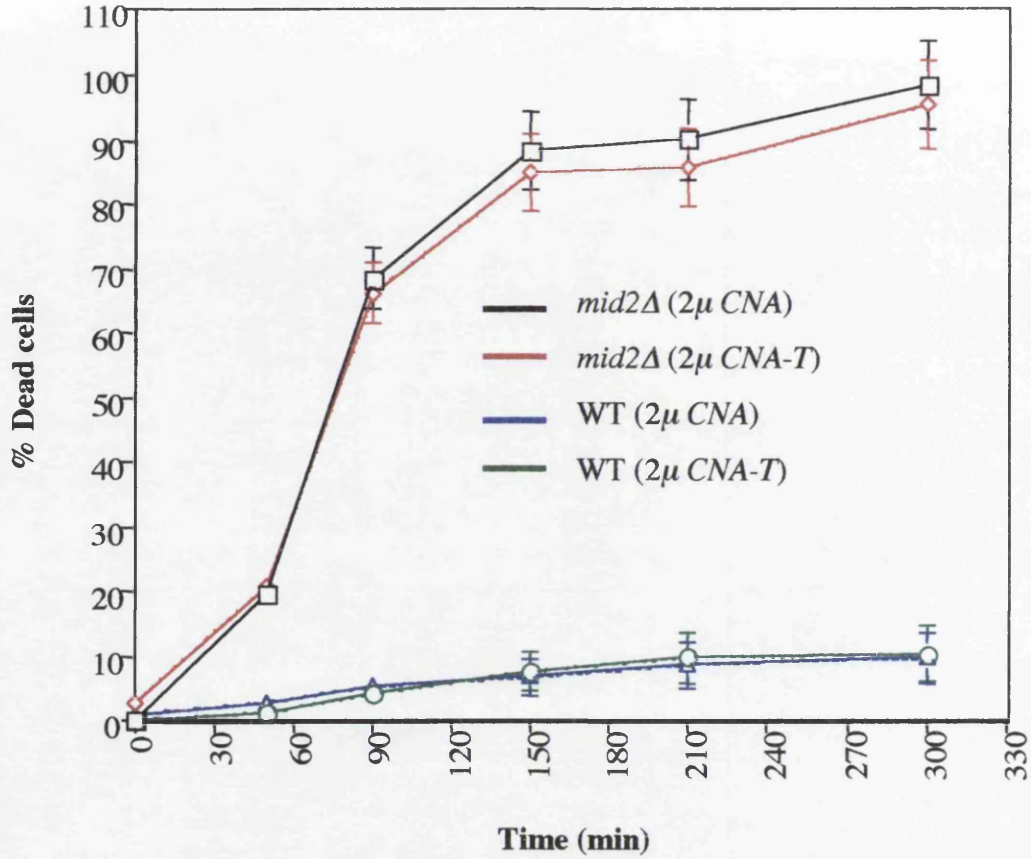

Figure 4.9. Cells which are *mid2Δ*, are not defective in calcineurin signaling when exposed to mating pheromone. *MAT* **a** wild-type and *mid2Δ* cells in the EG123 strain background, transformed with plasmids of *CNA2* or *CNA2ΔT* (constitutively active), were grown to mid-logarithmic phase in pH 4.0 Ura$^-$ selective media. These cell cultures were exposed to mating pheromone (α-factor; 12μM). Samples removed at time intervals were stained with methylene blue and assessed for viability by microscopy. In the data presented n=3 and error is +/-SEM.

have shown that α-factor-treated cells show chitin deposition at sites of polarised growth (Schekman and Brawley 1979). Chitin represents up to 2% of the yeast cell wall, and is located in division septa and bud scars (Cabib *et al.* 1971). Ketela *et al.* (1999) have suggested that *MID2* regulates chitin synthesis during mating, and hence cells defective in the *MID2* gene have lower chitin levels. This proposal indicates that the Pkc1p-MAPK pathway mutants may die in α-factor because of chitin deficiency and hence a less stable cell wall. To further assess the cause of mating pheromone-induced death in Pkc1p-MAPK mutants; chitin deposition during time course experiments was monitored.

Mid-logarithmic phase *MAT* **a** wild-type and *MAT* **a** *mid2Δ* cells in the EG123 strain background in pH 4.0 YPD were exposed to α-factor. Samples removed at time intervals, were stained with calcofluor white and examined by fluorescent microscopy. Calcofluor white is a fluorescent dye that intercalates into nascent chitin chains (Elorza *et al.* 1983). Chitin deposition can be seen under the fluorescent microscope. When compared with wild-type cells, *mid2Δ* cells displayed normal chitin deposition at sites of polarised growth (Fig. 4.10A and B). This result indicates that *mid2Δ* cells are not deficient for chitin deposition and that pheromone-induced death is not the result of improper chitin deposition. Ketela *et al.* (1999) have measured chitin levels in *mid2Δ* cells that were exposed to mating pheromone for three hours. However at three hours of α-factor treatment up to 70% of *mid2Δ* cells die, as demonstrated in section 4.2.1. This may explain the low level of chitin these authors observed in total *mid2Δ* cells exposed to α-factor. However, further studies into the amount of chitin synthesised in *mid2Δ* cells in comparison to wild-type cells should provide more insight into this proposed function of Mid2p. It should be noted that chitin deposition is not a direct measurement of chitin synthesis and experiments described in this section only demonstrate chitin deposition. Furthermore, *mid2Δ* cells show normal cellular morphology in vegetative growth and during periods of polarised growth, in comparison to wild-type cells (Fig. 4.10A and B). These results indicate that *mid2Δ* cells have no visible morphological defect that may lead to death in α-factor.

Hcs77p shares a redundant role with Mid2p during both vegetative and the mating response. Chitin deposition was analysed *hcs77Δ* cells to assess if chitin deposition was

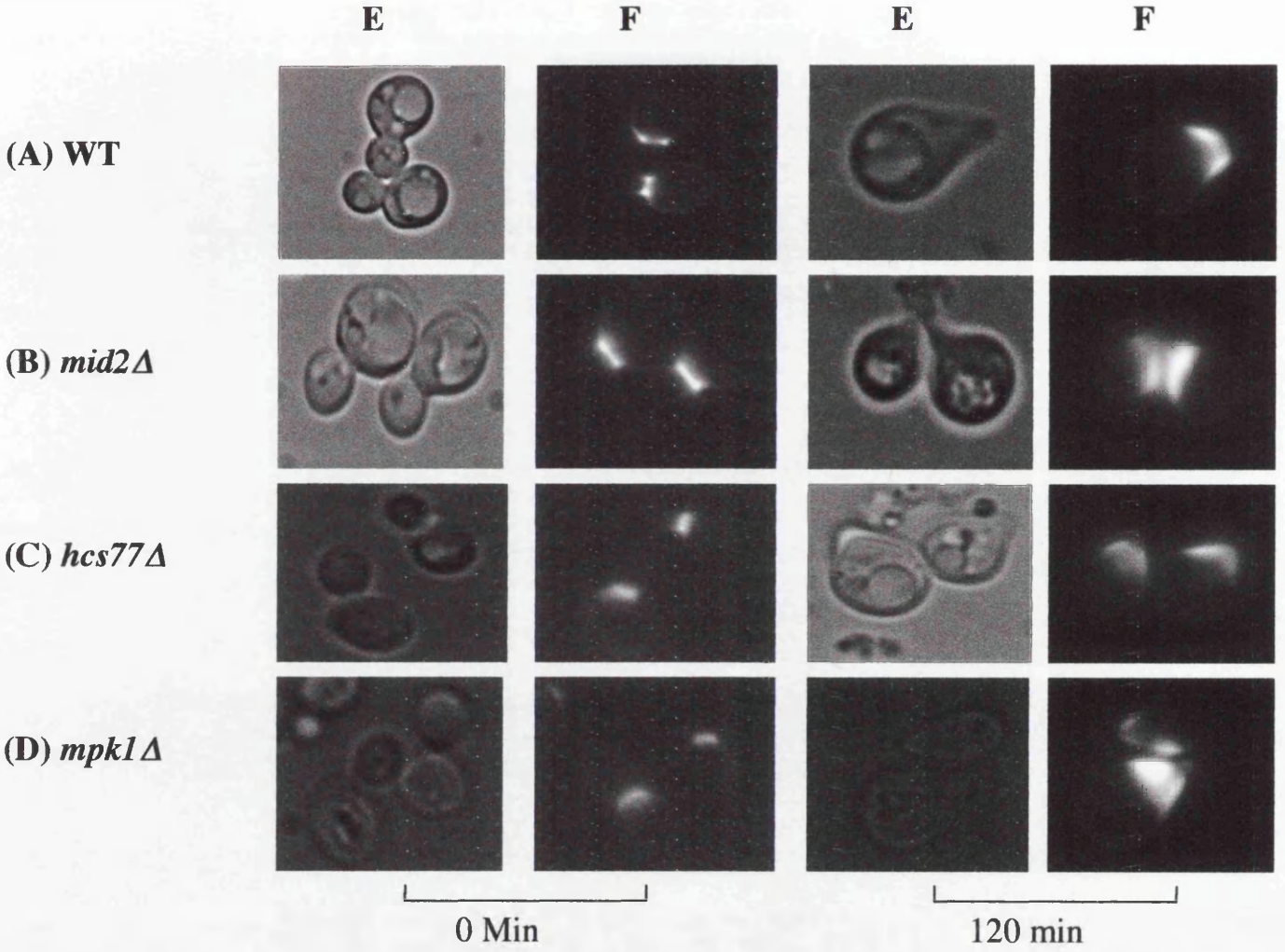

Figure 4.10. Pkc1p-MAPK pathway mutants display normal cellular morphology and chitin deposition when exposed to mating pheromone. *MAT* **a** (**A**) wild-type, (**B**) *mid2Δ*, (**C**) *hcs77Δ* and (**D**) *mpk1Δ* cells in the EG123 strain background, were grown to mid-logarithmic phase in pH 4.0 YPD at 25°C. The cell cultures were exposed to mating pheromone (α-factor; 12μM). Samples removed at time intervals were stained with calcofluor white and were examined by fluorescent microscopy. Panel **E** represents the Nomarski images and panel **F** represents the calcofluor white stained cells. Note that live cells were in motion while images were taken and orientation of some corresponding images changed as a result.

normal, similar to *mid2Δ* cells, in mating pheromone. When mid-logarithmic phase *MAT* **a** wild-type and *MAT* **a** *hcs77Δ* cells in the EG123 strain background in pH 4.0 YPD were exposed to α-factor, *hcs77Δ* cells displayed normal chitin deposition in comparison to wild-type cells (Fig. 4.10A and C). This result indicates that, similar to *mid2Δ* cells, pheromone-induced death in *hcs77Δ* cell is not the result of improper chitin deposition, and may be dependant on other factors associated with the cell wall.

Cells deficient for *MPK1* also die in presence of mating pheromone. If the Pkc1p-MAPK pathway mutants were defective for chitin deposition during mating, then one would expect to observe defective chitin deposition in these mutants. Mid-logarithmic phase *MAT* **a** wild-type and *MAT* **a** *mpk1Δ* cells in the EG123 strain background in pH 4.0 YPD, were exposed to α-factor. Samples removed at time intervals, were stained with calcofluor white and examined by fluorescent microscopy. The *mpk1Δ* cells showed no defect in chitin deposition in mating pheromone when compared to wild-type cells (Fig. 4.10A and D). This result indicates that death in α-factor in *mpk1Δ* cells is not associated with improper chitin deposition.

4.2.12. Pkc1p-MAPK pathway mutants do not display actin polarisation defects in pheromone

Delley and Hall (1999) have suggested a role for the Pkc1p pathway in polarisation of actin cytoskeleton. If Pkc1p is required during polarised growth, then mutants of the Pkc1p-MAPK pathway may have defects in actin polarisation in presence of mating pheromone. This proposed defect may therefore be the cause of the α-factor-induced death of Pkc1p-MAPK pathway mutants. This hypothesis was tested by growing *MAT* **a** wild-type and *MAT* **a** *mid2Δ* cells in the EG123 strain background to mid-logarithmic phase in pH 4.0 selective media. These cells were then exposed to α-factor and samples were removed at time intervals. All cell samples were stained with rhodamine phaloidin and examined by fluorescent microscopy. The *mid2Δ* cells displayed actin patch localisation similar to that of wild-type cells in response to mating pheromone (Fig. 4.11A and B). These results show that *mid2Δ* cells have no actin polarisation defect in response to mating pheromone and hence death in α-factor is not attributable to actin polarisation defects.

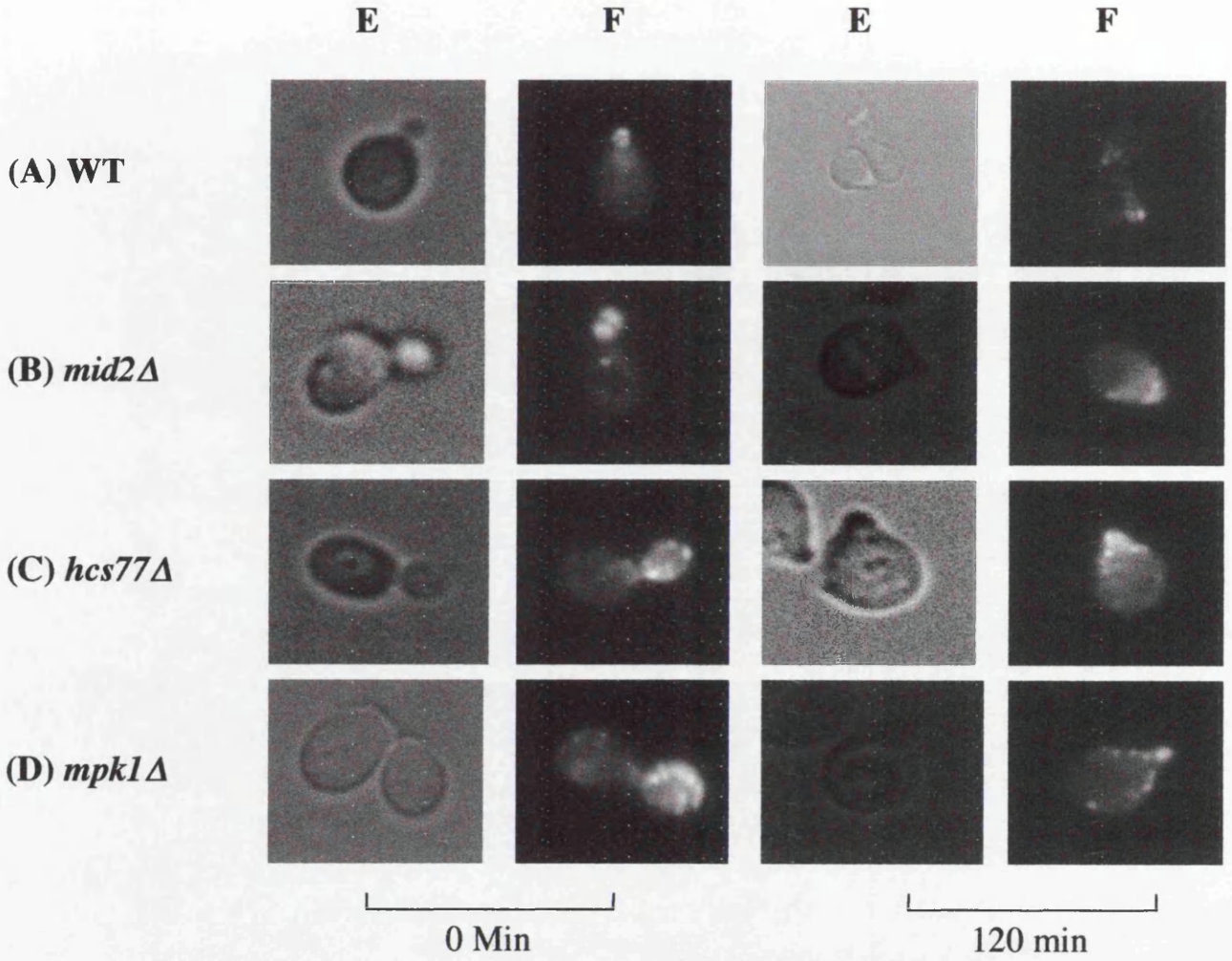

Figure 4.11. Pkc1p-MAPK pathway mutants display normal actin localisation when exposed to mating pheromone. *MAT* **a** (**A**) wild-type, (**B**) *mid2Δ*, (**C**) *hcs77Δ* and (**D**) *mpk1Δ* cells in the EG123 strain background, were grown to mid-logarithmic phase in pH 4.0 YPD at 25°C. The cell cultures were exposed to mating pheromone (α-factor; 12μM). Samples removed at time intervals were examined by fluorescent microscopy. Panel **E** represents the Nomarsky images and panel **F** represents Rhodamine Phaloidin stained cells. Note that live cells were in motion while images were taken and orientation of some corresponding images changed as a result.

To further assess the role of polarisation in death of Pkc1p-MAPK pathway mutants in mating pheromone, actin localisation was examined in *hcs77Δ* cells. *MAT* **a** wild-type and *MAT* **a** *hcs77Δ* cells in the EG123 strain background to mid-logarithmic phase in pH 4.0 selective media. These cells were then exposed to α-factor and samples were removed at time intervals. All cell samples were stained with rhodamine phaloidin and examined by fluorescent microscopy. In presence of mating pheromone, *hcs77Δ* cells displayed similar actin patch organisation as wild-type cells (Fig. 4.11A and C). This result indicates that *hcs77Δ* cells have no defect in polarisation in response to mating pheromone.

Since cells deficient in both cell surface sensors Hcs77p and Mid2p have no actin polarisation defect, then it is possible that cells deficient for *MPK1* also have no defect in actin polarisation in response to mating pheromone. To test this possibility *MAT* **a** wild-type and *MAT* **a** *mpk1Δ* cells in the EG123 strain background to mid-logarithmic phase in pH 4.0 selective media. These cells were then exposed to α-factor and samples were removed at time intervals. All cell samples were stained with rhodamine phaloidin and examined by fluorescent microscopy. The *mpk1Δ* cells showed similar actin patch localisation in presence of mating pheromone as wild-type cells (Fig. 4.11A and D). This result shows that *mpk1Δ* cells have no defect associated with actin polarisation when exposed to mating pheromone. These results are consistent with the Pkc1p-MAPK pathway mutants having no defects in actin polarisation during mating.

4.2.13. Pheromone-induced death in Pkc1p-MAPK pathway mutants is by cell lysis

One characteristic phenotype of the Pkc1p-MAPK pathway mutants is cell lysis at high temperatures. Since death in α-factor is associated with a defective Pkc1p-MAPK pathway, then death in mating pheromone may be the result of cell lysis. To test this possibility *MAT* **a** wild-type and *MAT* **a** *mid2Δ* cells in the EG123 strain background were grown to mid-logarithmic phase in pH 4.0 YPD. These cell cultures were then exposed to α-factor. Samples were removed at time intervals and were stained with propidium iodide (PI). Cells, which undergo lysis, allow the dye to leak into the cells. Cellular DNA become intercalated with propidium iodide and fluoresces yellow under the fluorescent microscope. After five hours of exposure to α-factor *mid2Δ* cells showed 95% lysis in

comparison to 13% lysis of wild-type cells (Fig. 4.12A). This result shows that *mid2Δ* cells die in α-factor because of cell lysis. The result further support evidence that mating pheromone-induced death of *mid2Δ* cells is a result of a defect in cellular integrity.

Since *mid2Δ* cells die in α-factor because of cell lysis, then cells which are deficient for the other cell surface sensor Hcs77p, may also die in α-factor because of cell lysis. To examine this possibility, *MAT* **a** wild-type and *MAT* **a** *hcs77Δ* cells in the EG123 strain background were grown to mid-logarithmic phase in pH 4.0 YPD. These cell cultures were then exposed to α-factor. Samples were removed at time intervals, stained with propidium iodide (PI) and were examined by fluorescent microscopy. After five hours of

exposure to α-factor, there was up to 60% cell lysis of *hcs77Δ* cells in comparison to 15% lysis of wild-type cells (Fig. 4.12B). This result shows that α-factor-induced death in *hcs77Δ* cells is because of cell lysis associated with defects in cellular integrity.

As cells which are deficient for each of the two proposed cell surface sensors, die in α-factor because of cell lysis then this suggests that the other mutants of the Pkc1p-MAPK pathway may also die in α-factor as a result of cell lysis. To test this possibility, *MAT* **a** wild-type and *MAT* **a** *mpk1Δ* cells in the EG123 strain background were grown to mid-logarithmic phase. These cell cultures were exposed to α-factor, and samples were removed at time intervals. All samples were stained with propidium iodide (PI) and were examined by fluorescent microscopy. Up to 95% of *mpk1Δ* cells had lysed after five hours of exposure to mating pheromone, in comparison to 15% lysis of wild-type cells (Fig. 4.12C). These results show that mutants of the Pkc1p-MAPK pathway die in α-factor because of cell lysis. Furthermore, these results indicate that survival of cells in mating pheromone requires a fully functional Pkc1p-MAPK pathway to maintain cellular integrity.

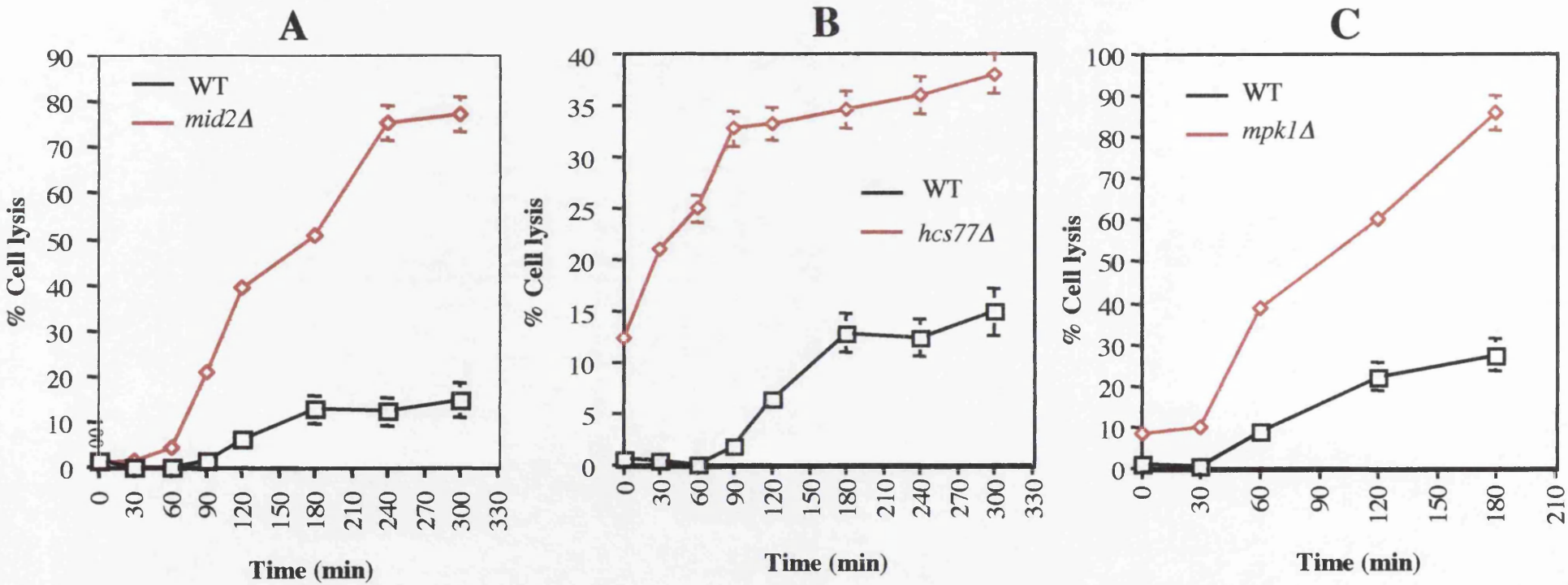

Figure 4.12. Pkc1p-MAPK pathway mutants die in mating pheromone as a result of cell lysis. *MAT* **a** wild-type, (**A**) *mid2Δ*, (**B**) *hcs77Δ* and (**C**) *mpk1Δ* cells in the EG123 strain background were grown to mid-logarithmic phase in pH 4.0 YPD. These cell cultures were exposed to mating pheromone (α-factor; 12μM). Samples removed at time intervals were stained with propidium iodide and were examined by microscopy. In the data presented n=3 and error is +/-SEM.

4.2.14. Osmotic stabilisation does not suppress the death of Pkc1p-MAPK pathway mutants in α-factor

Osmotic stabilisation suppresses the high temperature cell lysis defect of Pkc1p-MAPK pathway mutants (Irie *et al.* 1993; Lee *et al.* 1993; Paravicini *et al.* 1992). Vegetative growth defects of the Pkc1p-MAPK pathway mutants are suppressed in high osmolarity. It is possible that osmotic stabilisation can also suppress the α-factor-induced death of Pkc1p-MAPK pathway mutants. To test this possibility, *MAT* **a** wild-type and *MAT* **a** *mid2Δ* cells in the EG123 and S288C strain backgrounds were grown to mid-logarithmic phase in pH 4.0 10% sorbitol YPD. These cell cultures were exposed to α-factor and samples were removed at time intervals. All samples were stained with methylene blue, and their viability was assessed by microscopy. High osmolarity does not suppress the death of *mid2Δ* cells in α-factor (Fig. 4.13A). After five hours exposure to mating pheromone, the percentage of viable *mid2Δ* cells remaining in sorbitol is similar to that of non-osmotically stabilised cells (Fig. 4.1). These results suggest that the defect associated with *mid2Δ* death in α-factor is not one, which is remedial by osmotic stabilisation.

Cell lysis of *hcs77Δ* cells at high temperatures is remedied in presence of high osmolarity (Gray *et al.* 1997). Death of *hcs77Δ* cells in α-factor may therefore be osmotically remedial. To examine this possibility, mid-logarithmic phase *MAT* **a** wild-type and *MAT* **a** *hcs77Δ* cells in the EG123 strain background, cultured in 10% sorbitol pH 4.0 YPD, were exposed to α-factor. Samples were removed at time intervals, stained with methylene blue and their viability was assessed by microscopy. Presence of sorbitol only partially suppressed the death of *hcs77Δ* cells. After five hours exposure to mating pheromone, up to 50% of cells had died (Fig. 4.13B). This is in comparison to 60% death in mating pheromone, when cells were not osmotically stabilised (Fig. 4.5A). This result indicates that osmotic stabilisation of *hcs77Δ* cells does not substantially improves their survival in mating pheromone

Similar to other mutants of the Pkc1-MAPK pathway, cells deficient for *MPK1* display a cell lysis phenotype at high temperatures, which is osmotically remedial (Lee *et al.* 1993). It is possible that α-factor-induced death of *mpk1Δ* cells is also remedied by osmotic

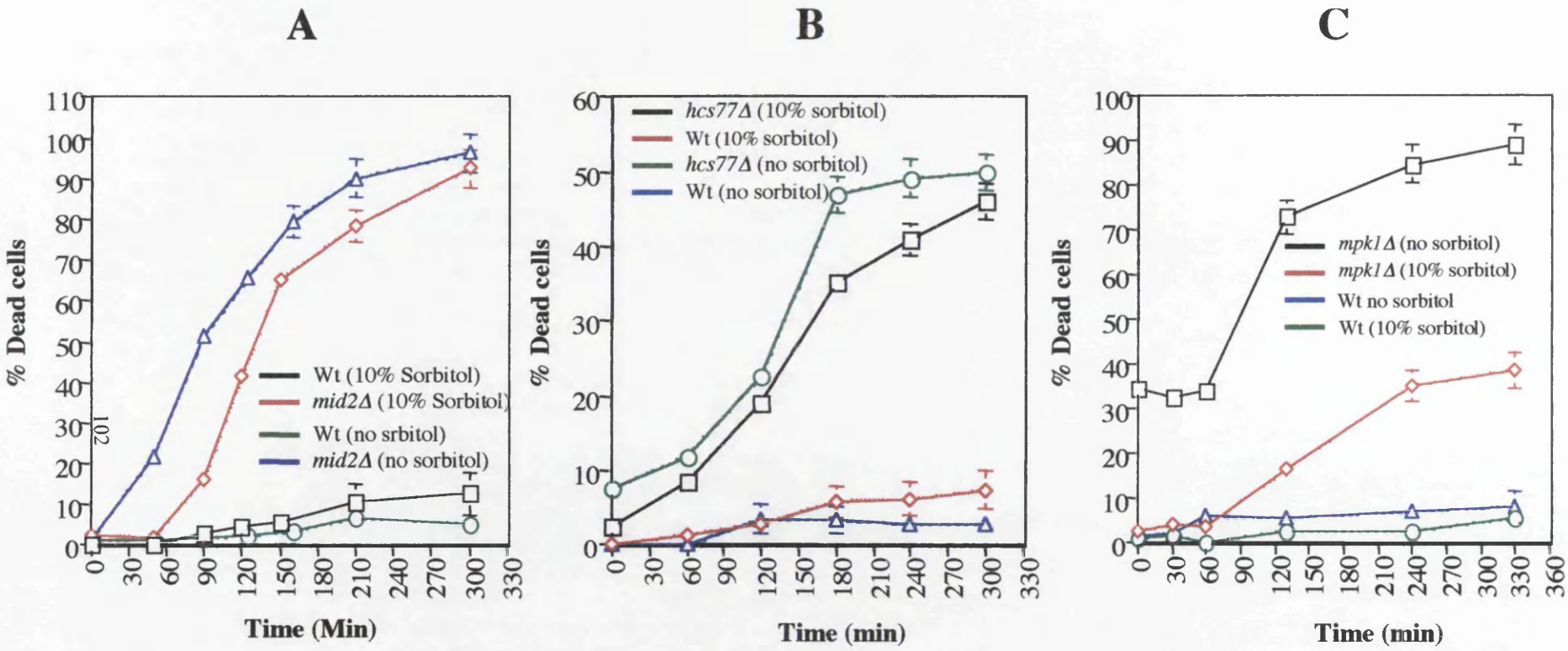

Figure 4.13. The death of Pkc1p-MAPK pathway mutants in mating pheromone is not osmotically remedial. *MAT* **a** wild-type, (**A**) *mid2Δ*, (**B**) *hcs77Δ* and (**C**) *mpk1Δ* cells in the EG123 strain background were grown to mid-logarithmic phase in pH 4.0 (+/-) 10% sorbitol YPD. These cell cultures were exposed to mating pheromone (α-factor; 12μM). Samples removed at time intervals were stained with methylene blue and their viability was examined by microscopy. In the data presented n=3 and error is +/-SEM.

stabilisation. To examine this possibility, *MAT* **a** wild-type and *MAT* **a** *mpk1Δ* cells in the EG123 strain background were grown to mid-logarithmic phase in 10% sorbitol pH 4.0 YPD. These cell cultures were then exposed to mating pheromone. Samples were removed at time intervals, stained with methylene blue and their viability was assessed by microscopy. Sorbitol partially suppressed the death of *mpk1Δ* cells in α-factor. In sorbitol, up to 40% of *mpk1Δ* cells died in presence of mating pheromone (Fig. 4.13C). This is in comparison to 90% dead *mpk1Δ* cells in absence of sorbitol (Fig. 4.2A). However, before addition of α-factor, osmotically stabilised *mpk1Δ* cells displayed improved initial survival. These results support the observations made by Errede *et al.* (1995), showing that sorbitol suppressed the death of *mpk1Δ* cells in mating pheromone. However, as before, osmotic stabilisation is not sufficient to fully suppress the pheromone-induced death. This result shows that the cell wall defects in Pkc1p-MAPK pathway mutants when exposed to α-factor, is not a defect which can be fully suppressed by high osmolarity.

4.2.15. Pkc1p pathway is not inhibited by high osmolarity in pheromone-treated cells

Pkc1p-MAPK pathway is known to be activated when cells are exposed to hypo-osmotic shock (Davenport *et al.* 1995). Philips and Herskowitz (1997) have proposed that this activated state of Pkc1p, during periods of high internal glycerol, ensures that the cell maintains its integrity before fusion during the mating response. Their proposal suggests that before cell fusion, mating partners have to have reached a state of osmotic balance. Thereby Pkc1p is inactivated to allow cell fusion (Philips and Herskowitz 1997). Therefore, a function of the Pkc1p pathway in response to mating pheromone is regulation of cell fusion, in response to osmotic balance. One would therefore expect that addition of 1M sorbitol to α-factor treated cells would turn off Pkc1p, compromising the cell integrity pathway and consequently cells would lyse. To test this hypothesis, mid-logarithmic phase *MAT* **a** wild-type cells were exposed to α-factor for two hours, until all cells had formed shmoos. After which two aliquots of α-factor treated cells were removed. To one aliquot, sorbitol was added to the concentration of 1M. Samples were removed at time intervals from both aliquots. All samples were stained with propidium iodide, and were examined by microscopy. Addition of sorbitol to α-factor treated cells did not lead to cell lysis (Fig. 4.14). This result indicates that osmotic balance does not down regulate Pkc1p.

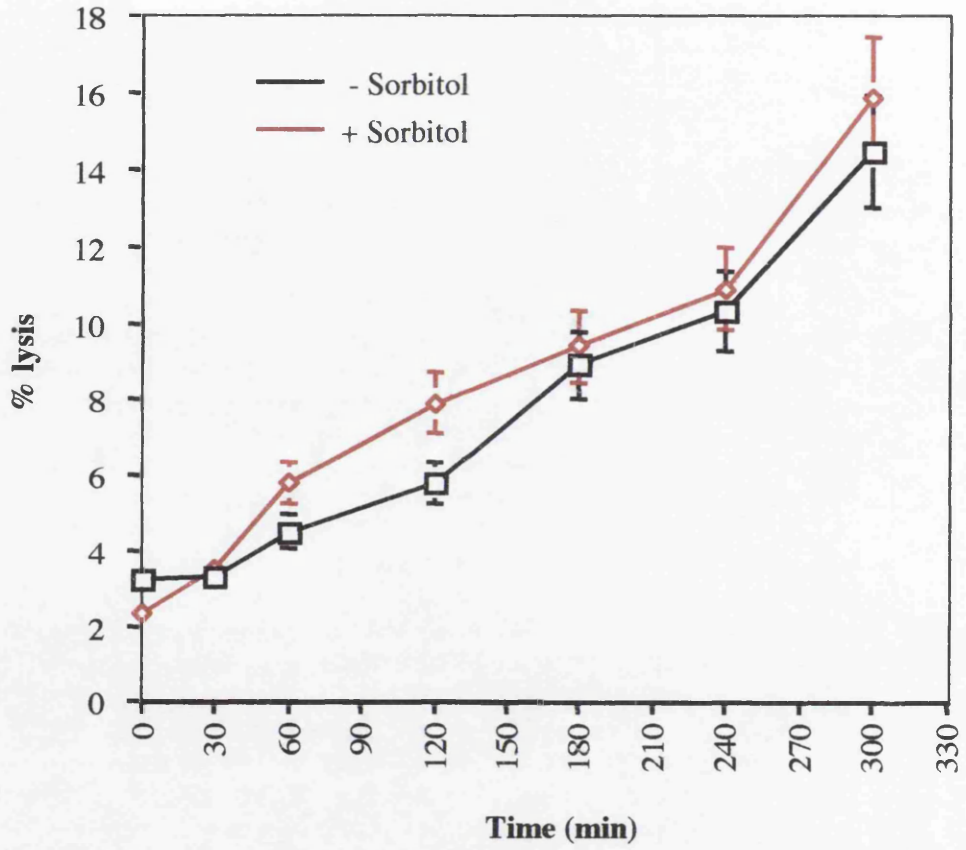

Figure 4.14. The Pkc1p-MAPK pathway does not regulate cell-cell fusion. Mid-logarithmic phase *MAT* **a** wild-type cells were exposed to mating pheromone for two hours (α-factor; 12μM). Two aliquots of the cell culture were removed; to one aliquot 1M sorbitol was added. The viability of samples removed at time intervals from both cell culture aliquots was assessed by fluorescent microscopy of propidium iodide stained cells. In the data presented n=3 and error is +/-SEM.

Cells treated with mating pheromone induce Mpk1p activation (Zarzov *et al.* 1996). If Pkc1p pathway was inactivated in high osmolarity, then one would expect that osmotically stabilised cells treated with α-factor, would have inactive Mpk1p (Kamada *et al.* 1995). Osmotically-stabilised cells show activation of *MPK1* transcription in response to mating pheromone (Fig. 4.4B). This result shows that Mpk1p can be activated in presence of osmotic stabilisers by α-factor treatment. Therefore, osmotic stabilisation does not down regulate Pkc1p during mating.

4.3. Discussion

Mid2p is an upstream regulator of Pkc1p-MAPK pathway, the null mutant of which displays a pheromone-induced death (Ono *et al.* 1994). In this chapter, death of *mid2Δ* cells in α-factor was confirmed in our strain backgrounds. This pheromone-induced death in *mid2Δ* cells is partially suppressed by over-expression of *HCS77*, suggesting that Hcs77p also serves a function, similar to Mid2p in response to pheromone, consistent with results obtained by Rajavel *et al.* (1999). Expression of *BCK1-20* also suppressed the pheromone-induced death of *mid2Δ* cells, providing supporting evidence that the Pkc1p-MAPK pathway has an important role in maintaining viability of cells during α-factor exposure. In addition, cells deficient for the cell surface sensor Mid2p, are defective for full activation of Mpk1p in response to mating pheromone, supporting observations made by Ketela *et al.* (1999). Therefore, death of *mid2Δ* cells is at least partially associated with defective Pkc1p-MAPK pathway. This defect in response to mating pheromone is independent of the calcium-signalling pathway. Cells deficient for *MPK1* also show an α-factor-induced death, in support of Errede *et al.* (1995), indicating that the whole of the Pkc1p-MAPK pathway is required for the α-factor response, to maintain cell integrity.

Hcs77p is also an upstream regulator of the Pkc1p-MAPK pathway, which has been proposed to just have a role in vegetative growth (Rajavel *et al.* 1999). However, *hcs77Δ* cells show a pheromone-induced death, though not as severe as that displayed by *mid2Δ* cells. This result is independently confirmed by results from Stirling and Stark (2000). This *hcs77Δ* phenotype is partially suppressed by over-expression of *MID2*, further emphasising the shared function that Hcs77p and Mid2p have in response to mating pheromone. Furthermore, over-expression of *PKC1* in *hcs77Δ* cells partially suppresses its phenotype in mating pheromone, indicating that death of *hcs77Δ* cells in pheromone is at least partially because of a defective Pkc1p-MAPK pathway. Cells deficient for the cell surface sensor Hcs77p, are defective for Mpk1p activation in α-factor, suggesting that *hcs77Δ* cells die in mating pheromone because of a defective cellular integrity. Cells deficient for both cell surface sensors Mid2p and Hcs77p, cannot activate Mpk1p in response to mating pheromone, showing that these two sensors have an essential and redundant function in the cell integrity pathway during α-factor exposure.

Since these mutants of the Pkc1p-MAPK pathway die as shmoos, it is possible that polarisation of the cells intensifies their cell surface defects leading to cell death. However, prevention of polarisation in Pkc1p-MAPK pathway mutants does not suppress the death of these mutants in α-factor. These results suggest that cell polarisation is not the key event leading to cell death in mating pheromone, and that other factors are contributing to this pheromone-induced death.

Philip *et al.* (2001) have shown that Mid2p and Hcs77p interact with Rom2p. As activators of Rho1p, these cell surface sensors may regulate actin cytoskeleton and cell wall biosynthesis through Pkc1p (Nonaka *et al.* 1995; Helliwell *et al.* 1998-b; Zhao *et al.* 1998). Furthermore, Marcoux *et al.* (1998), have shown that *MID2* over-expression suppresses the profilin deficient phenotype in yeast cells. Results in this chapter show that over-expression of *RHO1*, partially suppresses the *mid2Δ* phenotype in mating pheromone. These reports and results suggest a role for Mid2p and other Pkc1p-MAPK pathway components in actin re-organisation. However, actin patch localisation in Pkc1p-MAPK pathway mutants showed no underlying defect in actin organisation during the mating response. These results are consistent with reports that *mid2Δ* and *hcs77Δ* cells do not have a defect in actin organisation (Bettignies *et al.* 1999). The death of Pkc1p-MAPK pathway mutants in α-factor is therefore not because of defects associated with actin re-organisation.

Mid2p has been proposed to regulate chitin syntheis (Ketela *et al.* 1999). These authors suggest that *mid2Δ* cells exposed to mating pheromone have lower chitin content than wild-type cells. However, *mid2Δ* cells display similar chitin deposition to wild-type cells in response to pheromone. Furthermore, these authors measured chitin levels of *mid2Δ* cells, of which 70% of cell population would have been dead. The low chitin content that these authors observed may have been due to the majority of the cellular population being dead. However, chitin deposition does not provide a direct measurement of chitin synthesis and therefore further studies into chitin synthesis in *mid2Δ* cells should be carried out which account for the inviable population of cells. Furthermore, *hcs77Δ* and *mpk1Δ* cells do not display any defect in chitin deposition in response to pheromone. These results are consistent with reports that *mid2Δ* and *hcs77Δ* cells do not have a defect in chitin ring formation during vegetative growth (Bettignies *et al.* 1999). Together these

results indicate that the Pkc1p-MAPK pathway mutants die in α-factor because of cell wall defects that may not be associated with chitin deposition of the cell wall.

Cells deficient for components of the Pkc1p-MAPK pathway, display a lysis defect at high temperatures which is osmotically remedial (Kamada *et al.* 1995). Results in this chapter showed that Pkc1p-MAPK pathway mutants in α-factor, die as a result of cell lysis, and hence as a result of a defects in the cell integrity pathway. However, this pheromone-induced death, is not remedial by osmotic stabilisation (Rajavel *et al.* 1999). This latter result suggesting that Pkc1p-MAPK pathway mutants die in α-factor as a result of cell surface defects that cannot be remedied by high osmolarity. The Pkc1p-MAPK pathway therefore is essential during shmoo formation, as the pathway appears to be essential at all temperatures, this necessity for the cell integrity pathway is not osmotically remedial, and the pathway is not inactivated by osmotic stabilisation. One can infer from these results that there is a change in the context of the cell surface during shmoo formation. Furthermore, that the Pkc1p-MAPK pathway may respond to cell wall changes which are exacerbated by cell polarisation (see chapter 6 for detailed discussion).

Therefore, the role of Mid2p during the mating response is to activate the Pkc1p-MAPK pathway. This activation of the cell integrity pathway is required to maintain the viability of cells in α-factor. Mid2p and Hcs77p are both required for efficient Pkc1p-MAPK activity during the pheromone response, however, Mid2p appears to fulfill more functions during shmoo formation, as *mid2Δ* cells show a higher percentage of death in α-factor, than *hcs77Δ* cells. Activation of the Pkc1p-MAPK pathway *via* the two cell surface sensors is likely to be due to mechanical stress exerted by shmoo formation, rather than due to increased expression of the sensors. Unlike *MID2*, *HCS77* does not contain PRE elements in it 5' region and hence its expression is unlikely to be upregulated by pheromone. If increased expression of *MID2* was sufficient to activate the Pkc1p-MAPK pathway, then Hcs77p would not be required for activation of this pathway during the mating response.

It has been proposed that Pkc1p has a role to play in fusion of cells during mating (Philips *et al.* 1997). These authors propose that Pkc1p is inactivated when the two mating cells are in osmotic equilibrium, allowing cell wall degradation and hence fusion to occur. In

experiments carried out in this chapter, it was found that cells exposed to mating pheromone, which are osmotically stabilised, have an active Pkc1p-MAPK pathway, which maintains the cellular integrity. Therefore, high osmolarity does not turn off the Pkc1p pathway. Furthermore, osmotic stabilisation of wild-type cells in α-factor does not appear to lead to down regulation of the Pkc1p pathway, which would lead to a weak cell wall (and hence cell lysis). Therefore, factors other than high osmolarity may influence cell-cell fusion, which may include a mechanical signal triggered by cell-cell contact (see chapter 6 for detailed discussion).

"The three great essentials to achieve anything worthwhile are first, hard work; second, stick-to-itiveness; third, common sense."

Thomas A. Eddison

Chapter 5: Co-regulation of the Pkc1p-MAPK and the calcium siganalling pathways

5.1. Introduction

The calcineurin pathway of the budding yeast has been implicated in regulating events during pheromone-induced calcium influx, as calcineurin mutants and cells which are cultured in low calcium medium selectively die upon pheromone treatment (Cyert *et al.* 1991; Cyert and Thorner 1992; Iida *et al.* 1990; Ohsumi and Anraku 1985; Withee *et al.* 1997). Furthermore, calcineurin is activated by pheromone treatment, heat shock and by hypo-osmotic shock (Danielson *et al.* 1996; Hirata *et al.* 1995; Mendoza *et al.* 1994, 1996; Nakamura *et al.* 1993; Zhao *et al.* 1998). Mating pheromone treatment also leads to activation of the Pkc1p-MAPK pathway (Buehrer *et al.* 1997; Zarzov *et al.* 1996). In addition, heat and hypo-osmotic shocks lead to activation of Mpk1p (Davenport *et al.* 1995; Kamada *et al.* 1995). These observations are consistent with the calcineurin and Pkc1p-MAPK pathways playing a redundant role in regulation of cellular events associated with cell surface stress (Nakamura *et al.* 1996). In addition to co-regulation under some conditions, these two pathways share some common targets, for example: both pathways regulate the expression of *FKS2*, from distinct promoter elements, in response to heat shock (Zhao *et al.* 1998). These findings suggest roles for the Pkc1p-MAPK pathway and the calcineurin pathway in maintaining cellular integrity during cell surface stress. Moreover, the Pkc1p-MAPK and calcineurin pathways have been proposed to have a redundant role during vegetative growth, as a calcineurin null mutation is lethal in combination with *pkc1Δ* or *mpk1Δ* (Garrett-Engele *et al.* 1995, Nakamura *et al.* 1996).

The two cell surface sensors Mid2p and Hcs77p are upstream activators of Pkc1p-MAPK pathway when cells are heat shocked or treated with pheromone (Chapters 3 and 4; Gray *et al.* 1997; Ketela *et al.* 1999; Rajavel *et al.* 1999). The influx of calcium during periods of pheromone exposure has been associated with the putative stretch-activated cation channel Mid1p (Fischer *et al.* 1997; Iida *et al.* 1994; Kanzaki *et al.* 1999). Since mating pheromone exposure induces polarised growth involving remodeling of the cell wall,

calcium influx may respond to changes in the cell surface. In this chapter, the basis for co-regulation of the calcineurin and the Pkc1p-MAPK pathways is analysed.

5.2. Results

5.2.1. Cell surface stress activates the calcineurin pathway

Since both the Pkc1p-MAPK and calcineurin pathways are activated during heat shock, hypo-osmotic shock and pheromone exposure, one would expect that the calcineurin pathway, as in the case for the Pkc1p-MAPK pathway, also responds to other cell surface stresses. To test this hypothesis, *MAT* **a** wild-type EG123 and S288C cells were transformed with pBJ306 containing the CDRE-*lacZ* reporter construct (Jiang *et al.* 1999). This construct contains four copies of the calcineurin dependant responsive element (CDRE) from the promoter of the *FKS2* gene driving the expression of the *lacZ* reporter gene (Jiang *et al.* 1999). Expression of this reporter construct is completely dependent on calcineurin function (Jiang *et al.* 1999). The transformants were streaked onto selective media. The resulting colonies were grown to mid-logarithmic phase in selective media and were exposed to several different cell surface stresses (which are known to activate the Pkc1p-MAPK pathway), for 90 minutes. To keep the α-factor degrading enzyme Bar1p inactive, pH 4.0 media was used for α-factor treatment of cells.

The transformant cell cultures were exposed to α-factor, tunicamycin, chlorpromazine, calcofluor-white or zymolyase. Tunicamycin prevents glycosylation causing a secretion block, thereby activates the Pkc1p-MAPK pathway (Li *et al.* 2000). Chlorpromazine is a membrane-deforming drug that intercalates with the outer leaflet of the plasma membrane, leading to activation of the Pkc1p-MAPK pathway (Kamada *et al.* 1995). Calcofluor-white binds to and interferes with chitin chains in the cell wall and thereby activates the Pkc1p-MAPK pathway (Ketela *et al.* 1999). Finally, zymolyase is an enzyme mixture that digests the yeast cell wall and results in activation of the Pkc1p-MAPK pathway (De Nobel *et al.* 2000). The level of β-galactosidase activity in the transformants was measured and quantified using the Miller equation (section 2.22), before and after treatments with the above reagents.

Wild-type cells showed activation of the calcineurin reporter in response to all surface stresses tested (Fig. 5.1A and B). Furthermore, tunicamycin and chlorpromazine induced stronger activation of the calcineurin reporter construct. Furthermore, cells pre-incubated

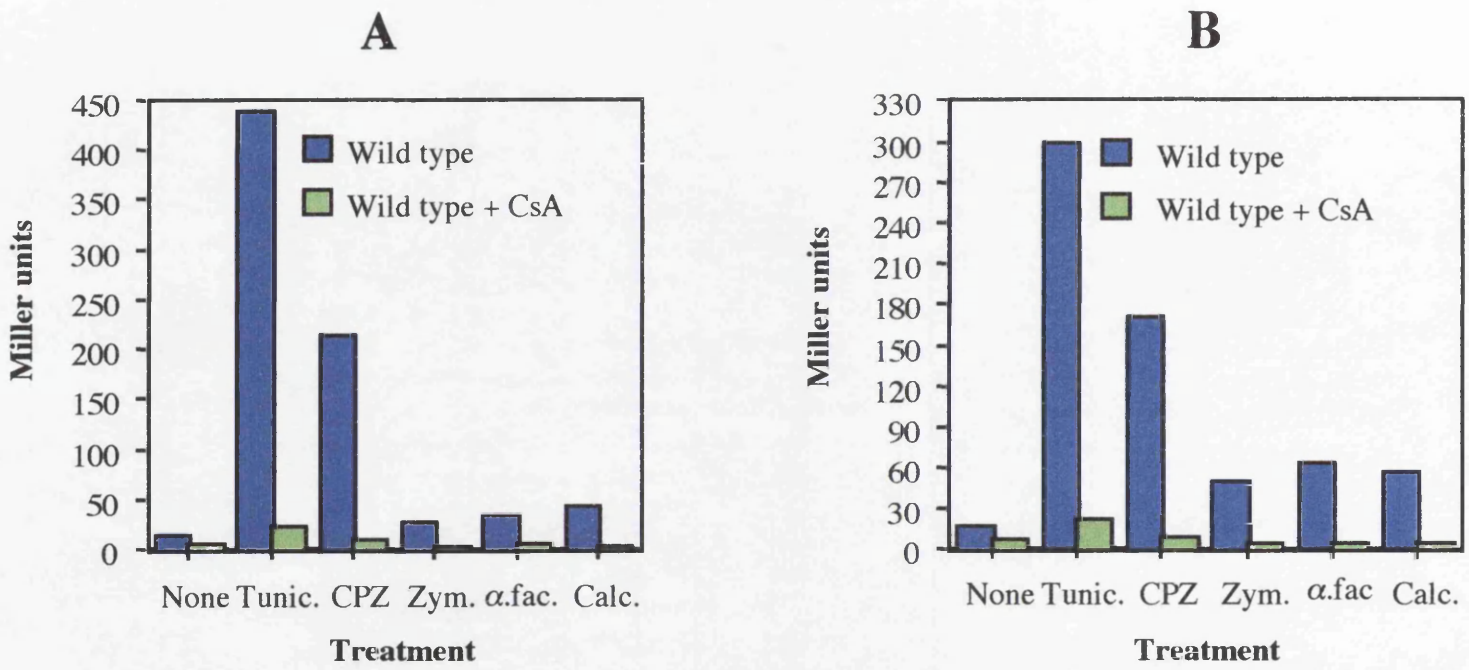

Figure 5.1. Cell surface stress activates calcineurin. *MAT* **a** wild-type cells in the (**A**) S288C and (**B**) EG123 strain background, transformed with pBJ306 containing the CDRE-*lacZ* reporter construct were grown to mid-logarithmic phase in Ura$^-$ selective media (pH 4.0 for α-factor) at 25°C. The cell cultures were incubated with or without cyclosporin-A (CsA - 100 μg/ml) for one hour, at 25°C. Cell cultures were incubated with tunicamycin (5 μg/ml), chlorpromazine (CPZ - 50 μg/ml), zymolyase (Zym - 2.5 U/ml), α-factor (α.fac - 12 μM) or calcofluor white (Calc - 100 μg/ml) for 90 minutes. Cell cultures were assayed for β-galactosidase activity and activity was quantified according to the Miller equation (section 2.22). The samples remained viable throughout the experiments (data not shown). The data presented are representatives of triplicate experiments.

in cyclosporin-A (a potent inhibitor of calcineurin) for one hour before all treatments displayed no reporter activation (Fig.5.1A and B). The majority of the cell population remained viable throughout these experiments (data not shown). These results show that treatments that directly or indirectly change the cell surface and that activate the Pkc1p-MAPK pathway, also activate the calcineurin reporter construct by a mechanism inhibited by cyclosporin-A. The calcineurin-signalling pathway, similar to the Pkc1p-MAPK pathway is therefore activated by cell surface stress.

5.2.2. Calcium influx is stimulated in response to cell surface stress

Ohsumi *et al.* (1985) and Iida *et al.* (1990) have shown that cells in mating pheromone show an increased rate of calcium influx. Calcineurin becomes activated in response to increase in cytosolic calcium and leads to Ca^{2+}/ calcineurin dependent gene expression (Mazur *et al.* 1995, Cunningham and Fink 1996). Here the influx of calcium in our wild-type cells during pheromone exposure is examined to verify these results. *MAT* **a** wild-type EG123 cells in mid-logarithmic phase and cultured at pH 4.0 YPD were exposed to α-factor for 90 minutes. $^{45}CaCl_2$ was added to the cell cultures that had been treated with α-factor and the control non-treated cells, as described in section 2.32. Samples were removed at time intervals and treated as described in materials and methods. Wild-type cells treated with α-factor showed stimulated calcium influx in comparison to untreated cells (Fig. 5.2A). These results confirm results obtained by Iida *et al.* (1990), showing that mating pheromone treatment leads to stimulated calcium influx and and hence verifies our methodology.

Since calcium influx is stimulated upon pheromone treatment, then it is possible that cell surface stress stimulates calcium influx. To examine this, $^{45}CaCl_2$ influx experiments were carried out as described in section 2.32, on wild-type cells in the EG123 background pre-incubated with either tunicamycin or chlorpromazine for one hour. It was found that tunicamycin and chlorpromazine treatments strongly stimulate calcium influx into wild-type cells, when compared to untreated cells (Fig. 5.2B and 5.2C). Furthermore, if 10% sorbitol was present in the growth medium, cells displayed calcium influx similar to non-sorbitol treated cells (Fig. 5.3A and 5.3B). One can infer from these results that cell

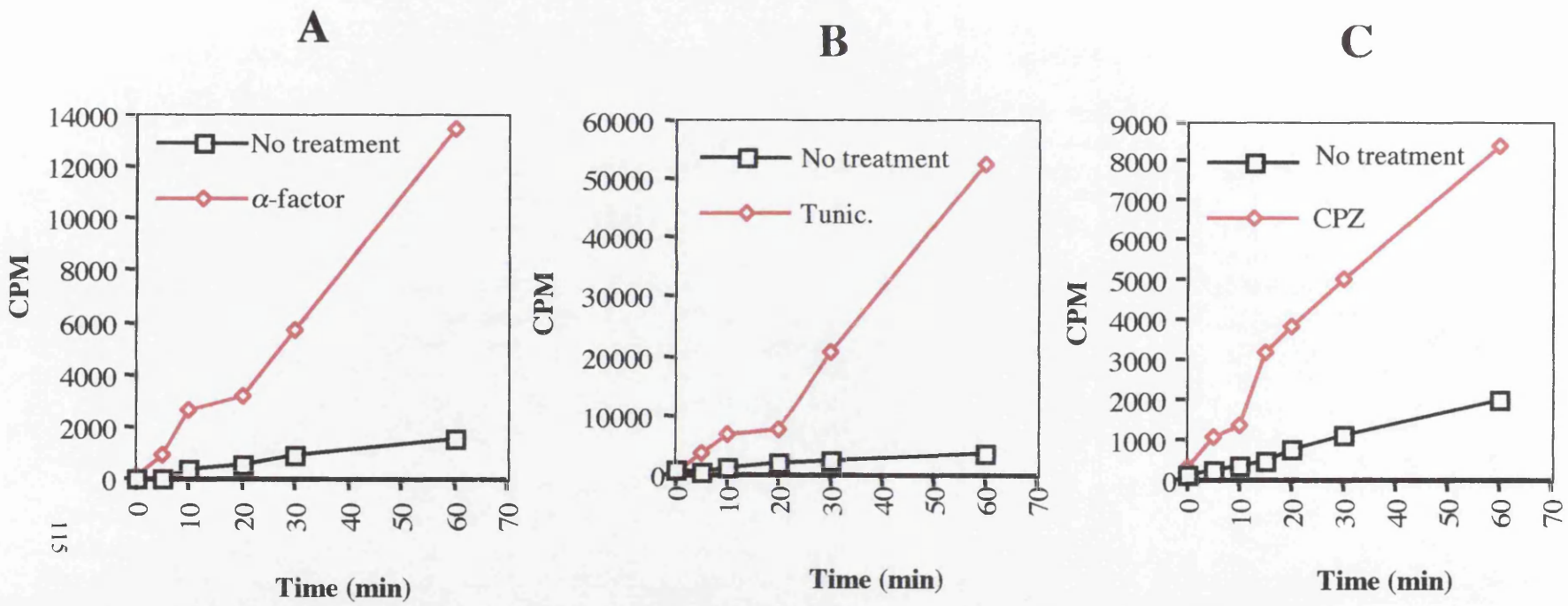

Figure 5.2. Cell surface stress stimulates calcium influx. *MAT* **a** wild-type cells in the EG123 strain background were grown to mid-logarithmic phase in YPD (pH 4.0 for α-factor treatment) at 25°C. These cell cultures were incubated with or without (**A**) α-factor (12 μM) for 90 minutes, (**B**) tunicamycin (Tunic - 5 μg/ml) for one hour or (**C**) chlorpromazine (CPZ - 50 μg/ml) for one hour. $^{45}CaCl_2$ was added to the cultures and samples were removed at time intervals. Calcium accumulation was monitored as described in section 2.32. The data presented are representatives of triplicate experiments.

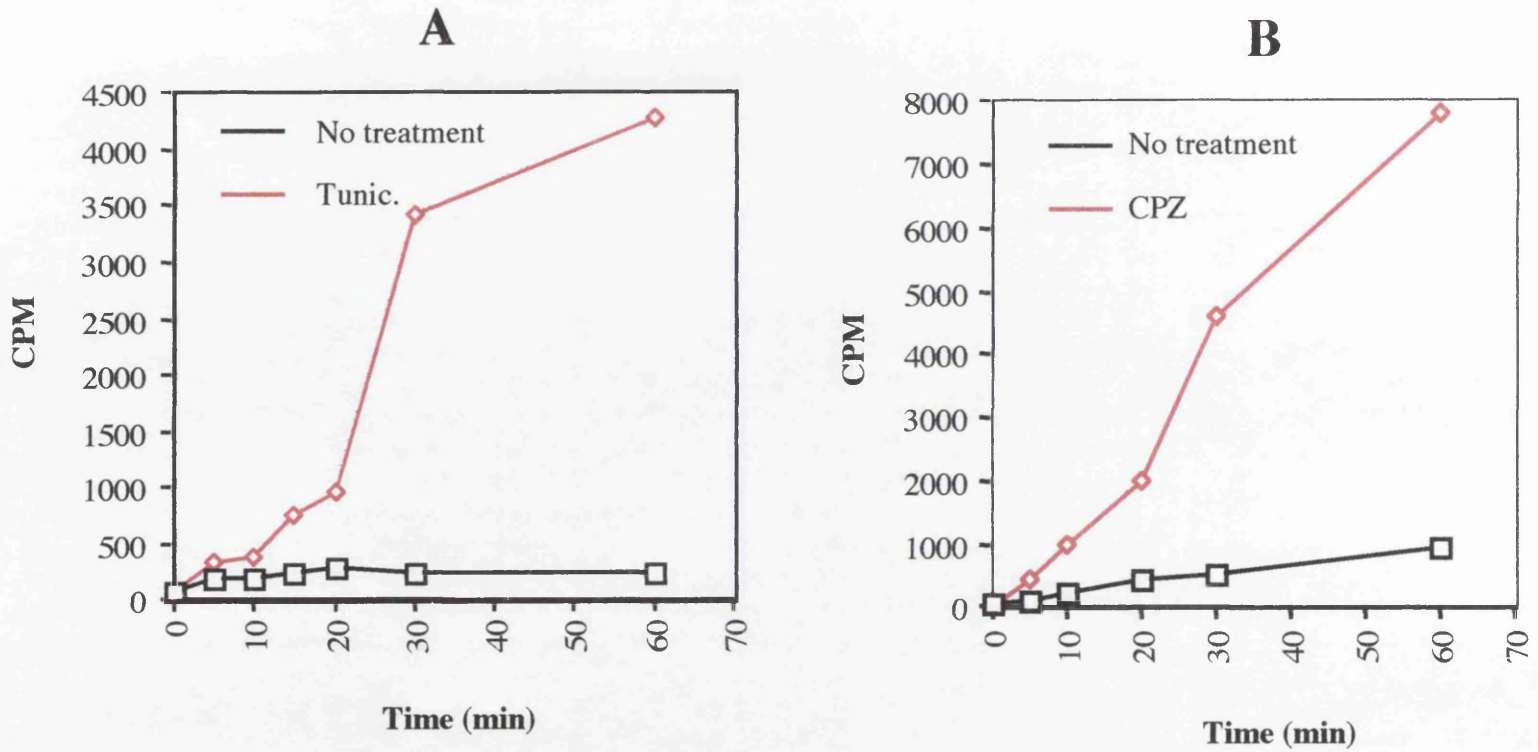

Figure 5.3. Calcium influx is stimulated by cell surface stress, even in the presence of high osmolarity. *MAT* **a** wild-type cells in the EG123 strain background were grown to mid-logarithmic phase in 10% sorbitol YPD at 25°C. These cell cultures were incubated with or without (**A**) tunicamycin (Tunic - 5 μg/ml) for one hour or (**B**) chlorpromazine (CPZ - 50 μg/ml) for one hour. $^{45}CaCl_2$ was added to the cultures and samples were removed at time intervals. Calcium accumulation was monitored as described in section 2.32. The data presented are representatives of triplicate experiments.

surface stresses stimulate calcium influx, independent of osmotic stabilisation, unlike the Pkc1p-MAPK pathway where osmotic stabilisation delays activation of the pathway.

5.2.3. Mid1p is required for calcium influx during cell surface stress

As shown in section 5.2.2, cell surface stress stimulates calcium influx in yeast cells. Mid1p has been characterised as a putative stretch-activated cation channel, which is required for efficient calcium influx during pheromone treatment (Iida *et al.* 1994, Kanzaki *et al.* 1999). Mid1p may also be required for calcium influx in response to other cell surface stresses that activate the calcineurin pathway. To test this possibility, calcium influx in *mid1Δ* cells was analysed upon tunicamycin treatment. Wild-type and *mid1Δ* cells were grown to mid-logarithmic phase in YPD and were treated with tunicamycin for one hour. $^{45}CaCl_2$ influx was monitored as described in section 2.32. *mid1Δ* cells treated with tunicamycin displayed a much reduced calcium influx in comparison to wild-type cells (Fig. 5.4). This result indicates that Mid1p is required for efficient stimulated calcium influx in response to cell surface stress.

5.2.4. Calcium influx in response to mating pheromone is dependent on cell polarisation

The Pkc1p-MAPK pathway becomes activated upon mating pheromone exposure (Zarzov *et al.* 1996). This activation of the Pkc1p-MAPK pathway is dependent upon cell polarisation (Zarzov *et al.* 1996). As shown above, cells exposed to mating pheromone display an induced calcium influx. Does the influx of calcium in mating pheromone require polarisation of the cells? *bni1Δ* cells fail to form shmoos upon pheromone treatment and α-factor treatment does not cause activation of the Pkc1p-MAPK pathway in *bni1Δ* mutants (Evangelista *et al.* 1997). Mid-logarithmic phase wild-type and *bni1Δ* cells in the W303 strain background in pH 4.0 YPD were exposed to α-factor for two hours. $^{45}CaCl_2$ influx was measured in these cell cultures as described in section 2.32. It was found that calcium influx is not stimulated in *bni1Δ* cells when exposed to cell surface stress in contrast to the congenic wild-type cells (Fig. 5.5). It can therefore be concluded that analogous to the Pkc1p-MAPK pathway, the calcium-signalling pathway is activated in response to cell polarisation during mating. The Pkc1p-MAPK and the

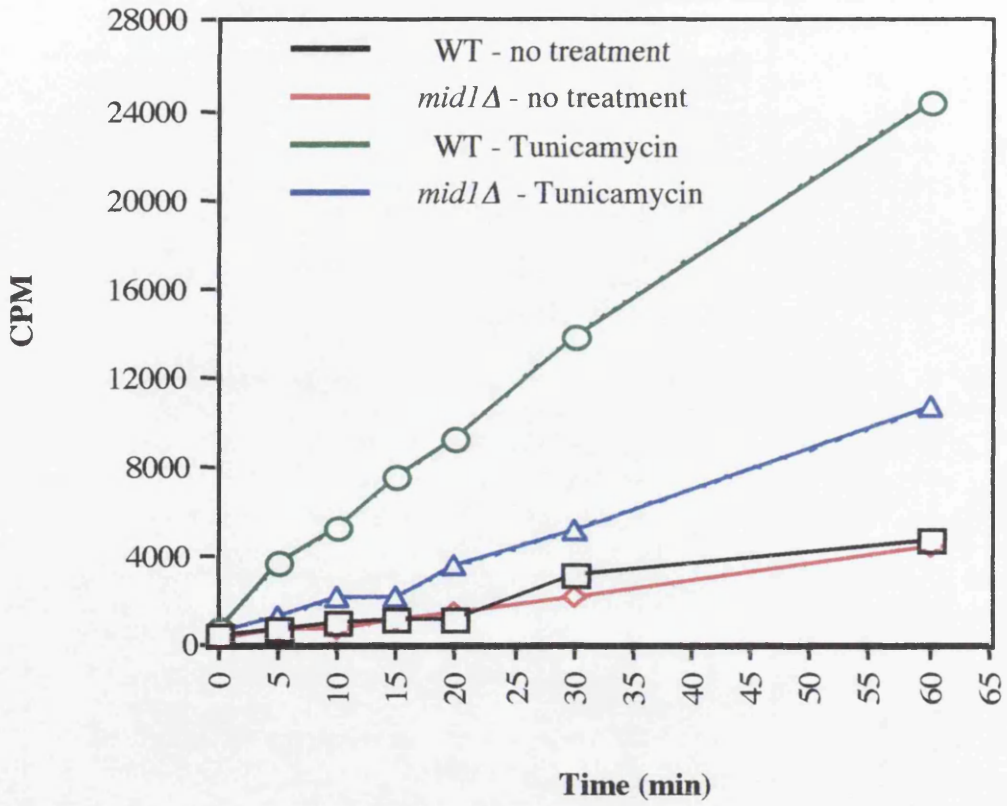

Figure 5.4. Mid1p is required for efficient calcium influx in response to cell surface stress. *MAT* **a** wild-type and *mid1Δ* cells in the EG123 strain background, were grown to mid-logarithmic phase in YPD. The cell cultures were incubated with tunicamycin (5 μg/ml) for one hour. $^{45}CaCl_2$ was added to the cultures and samples were removed at time intervals. Calcium accumulation was monitored as described in section 2.32. The data presented are representatives of triplicate experiments.

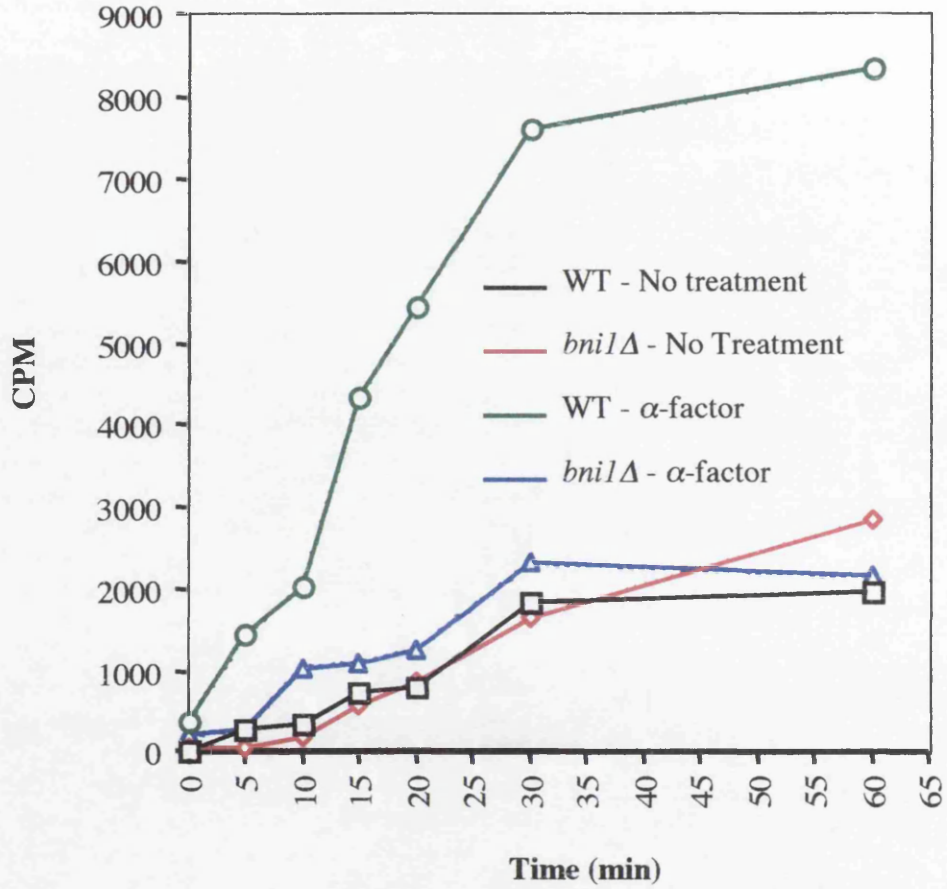

Figure 5.5. Influx of calcium in pheromone treated cells is dependent on polarisation. *MAT* **a** wild-type and *bni1Δ* cells in the W303 strain background were grown to mid-logarithmic cells in pH 4.0 YPD at 25°C. The cell cultures were exposed to α-factor (12 μM) for two hours. $^{45}CaCl_2$ was added to the cell cultures and samples were removed at time intervals. Calcium accumulation was monitored as described in section 2.32. The data presented are representatives of triplicate experiments.

calcium signalling pathways are thus co-regulated under a variety of conditions, all of which directly or indirectly implicate cell surface stress as the trigger.

5.2.5. The calcium-signalling pathway does not regulate the Pkc1p-MAPK pathway

The Pkc1p-MAPK pathway has been proposed to be activated in presence of high concentrations of calcium, suggesting that the Pkc1p-MAPK pathway functions downstream of the calcium-signalling pathway (Mizunuma *et al.* 1998). If the Pkc1p-MAPK pathway functions downstream of the calcium-signalling pathway, then one would expect that the calcium-signalling pathway is required to activate the Pkc1p-MAPK pathway in response to cell surface stress. To test this hypothesis dual phosphorylation (and hence activation) of Mpk1p after heat shock was examined by Western blot in cells that were grown in calcium-deficient media (Martin *et al.* 2000). Mid-logarithmic phase wild-type cells, grown in calcium-free or calcium-supplemented synthetic media at 25°C, were heat shocked for 15 minutes at 42°C. Mpk1p total protein was analysed by western blotting, with antibodies against Mpk1p. Mpk1p phosphorylation was analysed by Western blot using an antibody against the dually-phosphorylated form of Mpk1p. It was found that heat shock causes efficient phosphorylation of Mpk1p in the presence and absence of calcium in the medium (Fig. 5.6). One can infer from these results that calcium is not required for activation of Mpk1p in response to heat shock. The calcium siganlling pathway cannot therefore act upstream of the Pkc1p-MAPK pathway in response to cell surface stress. Indeed, loss of calcium signalling does not lead to a vegetative growth defect, as does loss of *PKC1*. Hence, calcium is unlikely to be involved in Pkc1p signalling.

5.2.6. Mid2p and Hcs77p are not the sensors for activation of calcineurin in response to cell surface stress

Results in chapters three and four showed that Mid2p and Hcs77p are required for activation of the Pkc1p-MAPK pathway in response to heat shock and pheromone treatment. Since the calcineurin-signalling pathway (like the Pkc1p-MAPK pathway), is activated when exposed to cell surface stress, it is possible that Hcs77p and Mid2p cell surface sensors are also required for activation of the calcineurin pathway. To test this

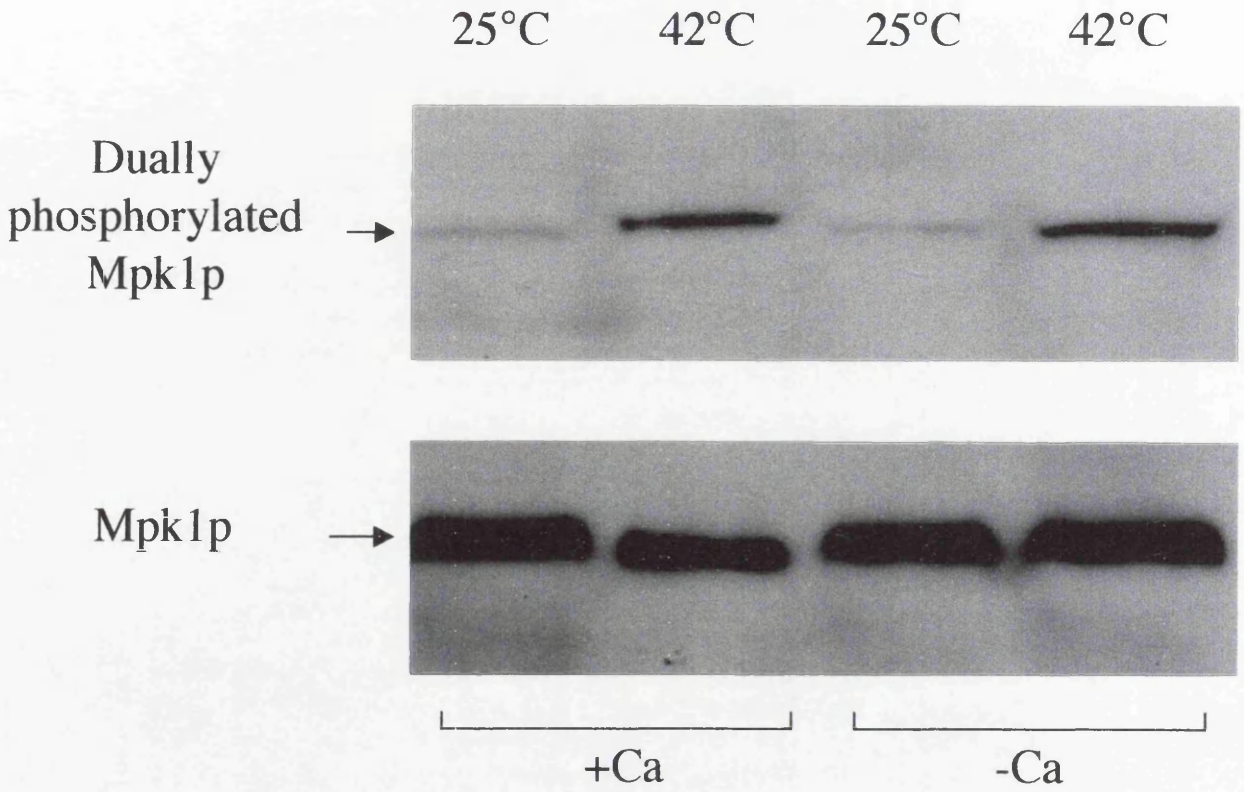

Figure 5.6. The Pkc1p-MAPK pathway is not regulated by the calcium-signalling pathway. Immunoblot analysis of Mpk1p phosphorylation (top panel) and total Mpk1p (bottom panel). *MAT* **a** wild-type cells in the EG123 strain background were grown to mid-logarithmic phase in Ca^- or Ca^+ synthetic media at 25°C. The cell cultures were heat shocked for 15 minutes at 42°C. Total protein was prepared from cell cultures and subjected to Bis-Tris-PAGE and western blotting. Mpk1p was detected by anti-Mpk1p antibodies. Activated Mpk1p was detected, by antibodies against the dually phosphorylated Mpk1p. The data presented are representatives of triplicate experiments.

possibility, *MAT* **a** *mid2Δ* cells in the EG123 and S288C strain backgrounds were transformed with pBJ306 containing the CDRE-*lacZ* reporter construct. All transformants were treated with cell surface stresses as described in section 5.2.1. When compared with wild-type cells, *mid2Δ* cells showed normal calcineurin reporter activation in response to cell surface stress (Fig. 5.7B). Furthermore, the activation of calcineurin reporter was inhibited by presence of cyclosporin-A (Fig. 5.7A). The majority of the cell population remained viable throughout the experiments, except for α-factor treated cells (data not shown). These results indicate that *mid2Δ* cells are not defective for activation of the calcineurin-signalling pathway in response to cell surface stress. Furthermore, these results show that Mid2p is not the sensor for activation of calcineurin in response to cell surface stress. The low calcineurin reporter activity observed in *mid2Δ* cells is due to the fact that these cells lose viability in presence of α-factor.

Hcs77p may be the sensor responsible for calcineurin activation in response to surface stress. To test this possibility, *MAT* **a** *hcs77Δ* EG123 cells were transformed with pBJ306 containing the CDRE-*lacZ* reporter construct. All transformants were streaked onto selective medium and the resulting colonies were exposed to cell surface stresses as described in section 5.2.1. The majority of the cell population remained viable throughout the experiments (data not shown). The *hcs77Δ* cells showed a similar fold induction of the calcineurin-reporter construct in response to cell surface stress as did wild-type cells (Fig. 5.8B). This calcineurin reporter activation was inhibited by presence of cyclosporin-A (Fig. 5.8A). These results show that *hcs77Δ* cells are not defective for activation of the calcineurin pathway in response to cell surface stress. Furthermore, these results indicate that the cell surface sensors required for activation of the Pkc1p-MAPK pathway are not required for activation of the calcineurin signalling pathway in response to cell surface stress.

Mid2p and Hcs77p share a redundant role in activation of the Pkc1p-MAPK pathway in response to cell surface stress (Chapters 3 and 4). It is possible that these two sensors, may also share a redundant role in activation of the calcineurin pathway in response to cell surface stress. However, deletion of both of these sensors is lethal to the cells and the *mid2Δhcs77Δ* cells are viable only in osmotically stabilised medium. Therefore, calcineurin-reporter activation in response to cell surface stress was analysed in the

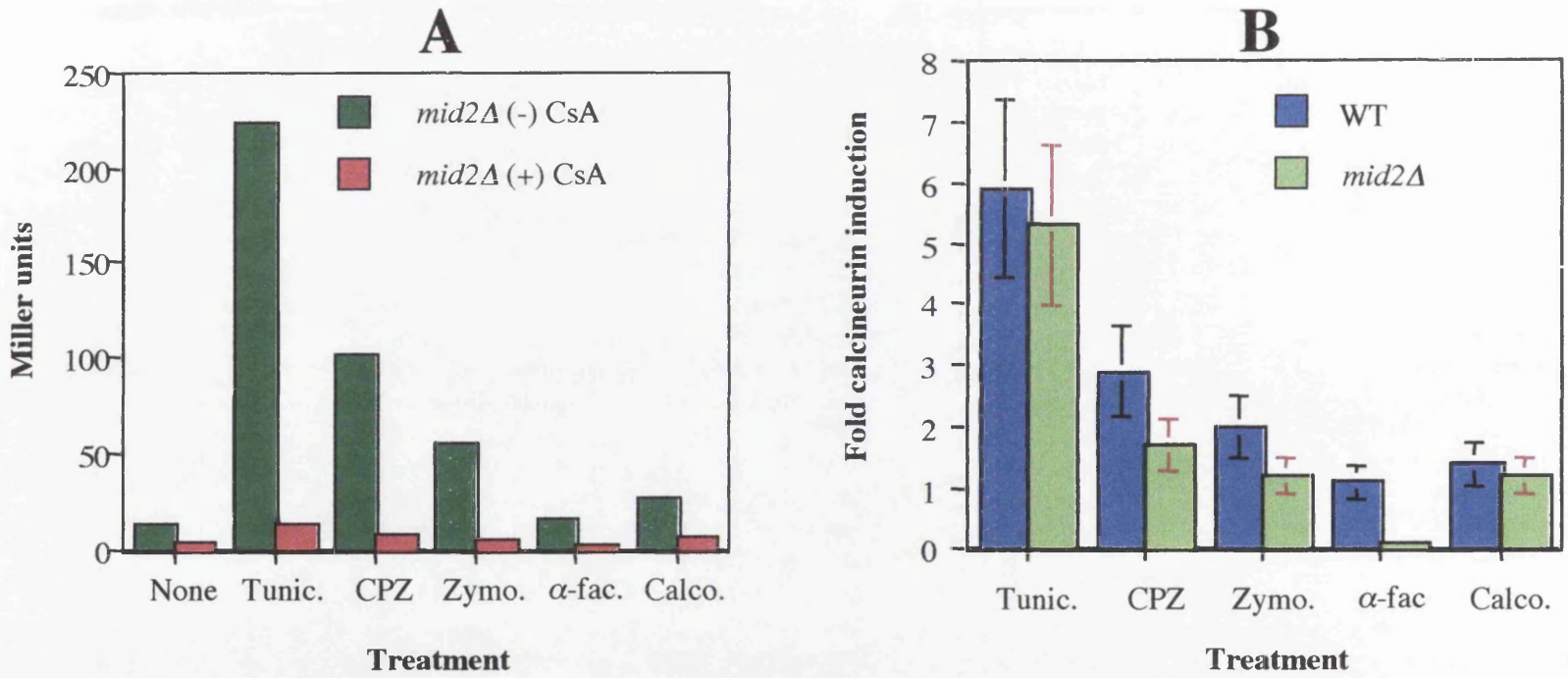

Figure 5.7. Mid2p is not required for activation of calcineurin in response to cell surface stress. *MAT* **a** wild-type and *mid2Δ* cells in the EG123 strain background, transformed with pBJ306 containing the CDRE-*lacZ* reporter construct, were grown to mid-logarithmic phase in Ura$^-$ selective media (pH 4.0 for α-factor treatment) at 25°C. The cell cultures were incubated with or without cyclosporin-A (100 μg/ml) for one hour, at 25°C. Cell cultures were incubated with tunicamycin (Tunic - 5 μg/ml), chlorpromazine (CPZ - 50 μg/ml), zymolyase (Zymo - 2.5 U/ml), α-factor (α-fac - 12 μM) or calcofluor white (Calc - 100 μg/ml) for 90 minutes. Cell cultures treated with cell surface stress were assayed for β-galactosidase activity and activity was quantified according to the Miller equation (**A**; section 2.22). Bar graph **B** shows fold activity of calcineurin in relation to basal activity. The samples were viable throughout the experiments, except for *mid2Δ* cells which display a pheromone induced death (data not shown). The CDRE activity of *mid2Δ* cells is therefore reduced as a direct result of lower viable number of cells present in mating pheromone. The data presented are representatives of triplicate experiments. N=3 and errors are +/- SEM.

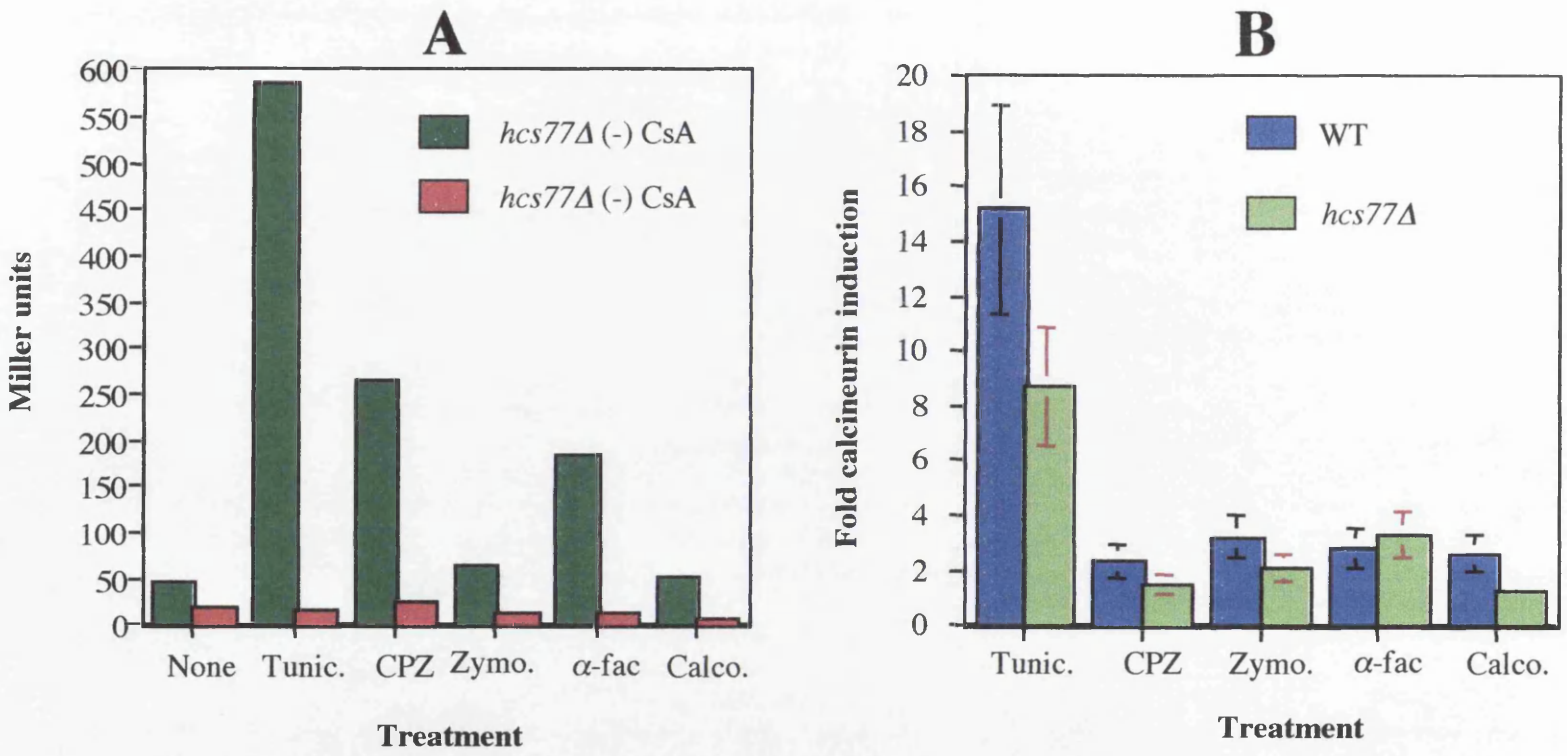

Figure 5.8. Hcs77p is not required for activation of calcineurin in response to cell surface stress. *MAT* **a** wild-type (WT) and *hcs77Δ* cells in the EG123 strain background transformed with pBJ306 containing the CDRE-*lacZ* reporter construct were grown to mid-logarithmic phase in Ura$^-$ selective media (pH 4.0 for α-factor treatment) at 25°C. The cell cultures were incubated with or without cyclosporin-A (CsA - 100 μg/ml) for one hour, at 25°C. Cell cultures were incubated with tunicamycin (Tunic - 5 μg/ml), chlorpromazine (CPZ - 50 μg/ml), zymolyase (Zym - 2.5 U/ml), α-factor (α-fac - 12 μM) or calcofluor white (Calc - 100 μg/ml) for 90 minutes. Cell cultures treated with surface stress were assayed for β-galactosidase activity and activity was quantified according to the Miller equation (**A**; section 2.22). Bar graph **B** shows fold activity of calcineurin in relation to basal activity. The cell samples remained viable throughout the experiments (data not shown). The data presented are representatives of triplicate experiments. N=3 and errors are +/- SEM.

presence of sorbitol. *MAT* **a** wild-type cells in the EG123 background transformed with pBJ306 containing the CDRE-*lacZ* reporter construct, were grown to mid-logarithmic phase in 10% sorbitol YPD and treated with cell surface stresses as described in section 5.2.1. The majority of the cell population remained viable throughout the experiments (data not shown). Osmotic stabilisation of the cells does not suppress calcineurin reporter activation in response to cell surface stress (Fig. 5.9). Furthermore, in presence of sorbitol the calcineurin reporter activation was still inhibited by cyclosporin-A (Fig. 5.9). Similarly, *mid2Δ* and *hcs77Δ* cells showed normal fold activation of calcineurin reporter construct in response to cell surface stress in presence of osmotic stabilisers (data not shown). These results show that cells, which are osmotically stabilised still activate the calcineurin pathway in response to cell surface stress.

Since the presence of sorbitol does not prevent activation of calcineurin during cell surface stress, then one can determine whether Hcs77p and Mid2p have a redundant role in activation of calcineurin in response to cell surface stress. Therefore, *MAT* **a** *mid2Δhcs77Δ* cells in the EG123 strain background were transformed with pBJ306 containing the CDRE-*lacZ* reporter construct. The cells were treated with various cell surface stresses as described in section 5.2.1. The majority of the cell population remained viable throughout the experiments except in the case of α-factor treated cells (data not shown). *mid2Δhcs77Δ* cells showed a similar degree of calcineurin reporter induction to wild-type cells in presence of cell surface stress (Fig. 5.10B). Calcineurin reporter induction was inhibited by the presence of cyclosporin-A (Fig. 5.10A). These results indicate that *mid2Δhcs77Δ* cells are not defective in activating calcineurin in response to cell surface stress, indicating that the two cell surface sensors are not required for the activation of calcineurin under such conditions. *mid2Δhcs77Δ* cells, similar to *mid2Δ* cells lose viability in presence of mating pheromone and therefore show a low calcineurin induction.

5.2.7. The *WSC* family of genes are not required for surface stress-induced calcium influx

WSC1/HCS77, *WSC2* and *WSC3*, are members of a family of putative cell surface sensors, proposed to play a role in maintenance of cell wall integrity (Verna *et al.* 1997). Since

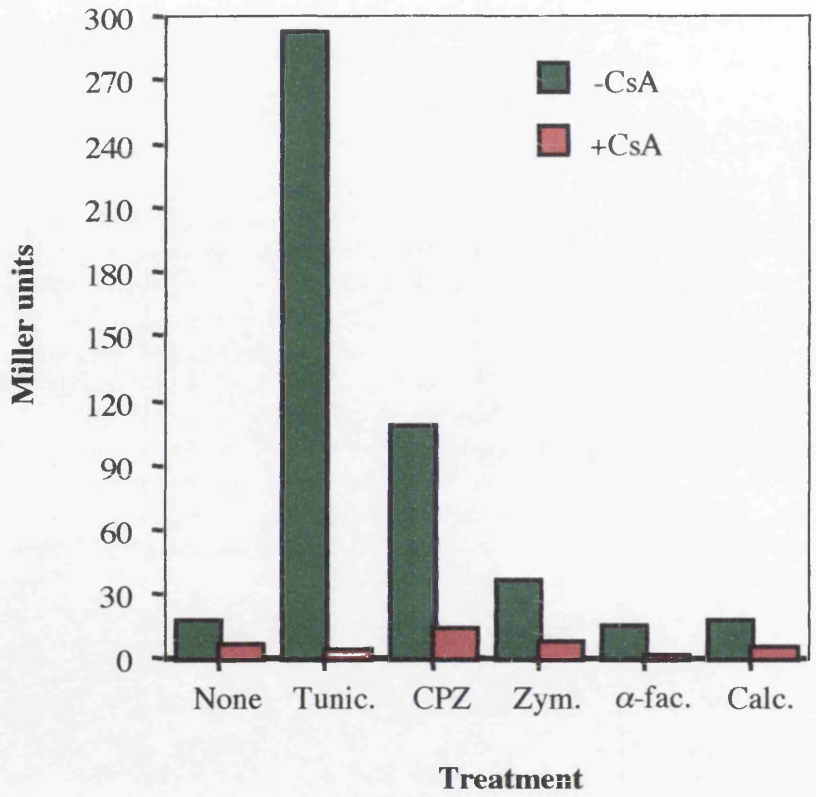

Figure 5.9. Calcineurin is activated in response to cell surface stress in presence of osmotic stabilisers. *MAT* **a** wild-type cells in the EG123 strain background transformed with pBJ306 containing the CDRE-*lacZ* reporter construct were grown to mid-logarithmic phase in 10% sorbitol Ura$^-$ selective media (pH 4.0 for α-factor treatment) at 25°C. The cell cultures were incubated with or without cyclosporin-A (100 μg/ml) for one hour, at 25°C. Cell cultures were incubated with tunicamycin (Tuni - 5 μg/ml), chlorpromazine (CPZ - 50 μg/ml), zymolyase (Zymo - 2.5 U/ml), α-factor (α-fac - 12 μM) or calcofluor white (Calc - 100 μg/ml) for 90 minutes. Cell cultures were assayed for β-galactosidase activity and activity was quantified according to the Miller equation (section 2.22). The majority of the cell population remained viable throughout the experiments (data not shown). The data presented are representatives of triplicate experiments. N=3 and errors are +/- SEM.

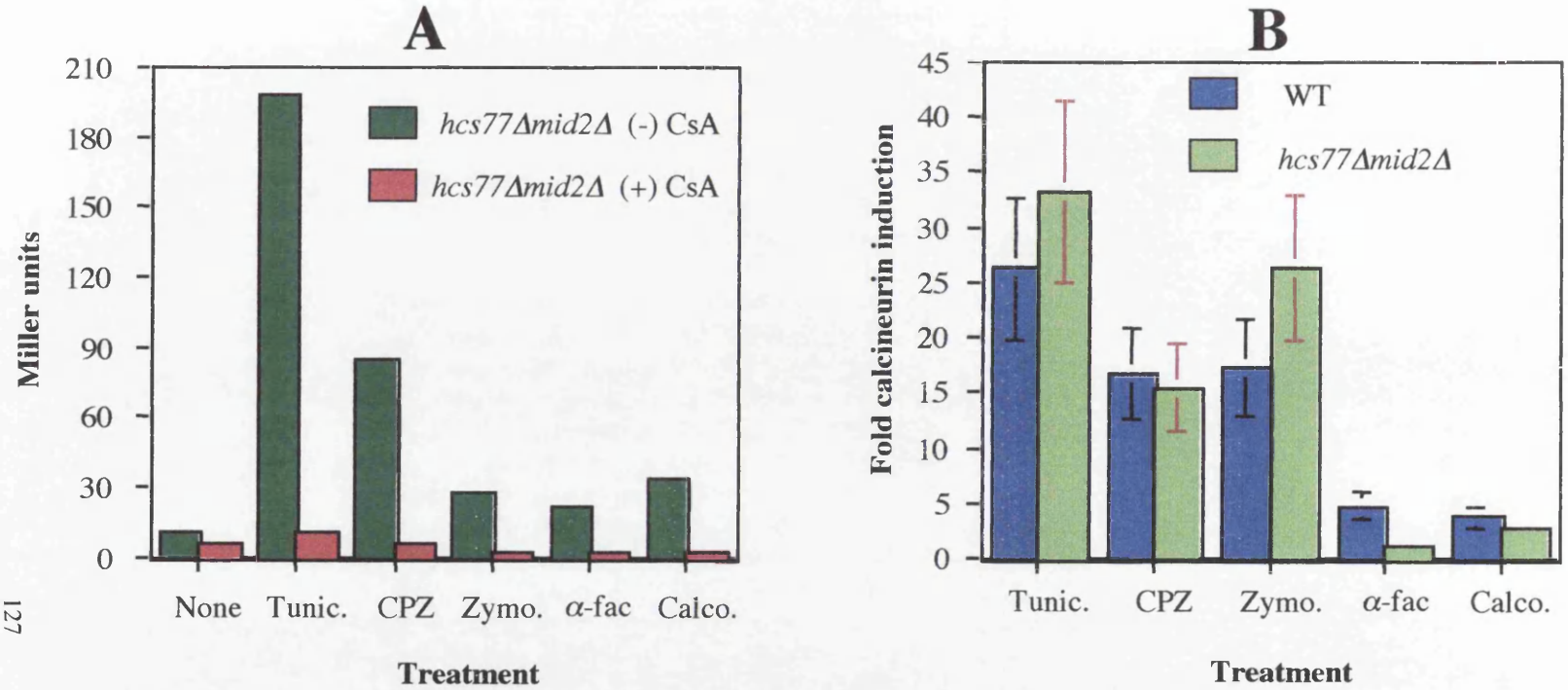

Figure 5.10. *mid2Δhcs77Δ* cells are not defective in activation of calcineurin in response to cell surface stress. *MAT* **a** wild-type and *mid2Δhcs77Δ* cells in the EG123 strain background transformed with pBJ306 containing the CDRE-*lacZ* reporter construct were grown to mid-logarithmic phase in 10% sorbitol Ura$^-$ selective media (pH 4.0 for α-factor treatment) at 25°C. The cell cultures were incubated with or without cyclosporin-A (CsA - 100 μg/ml) for one hour, at 25°C. Cell cultures were incubated with tunicamycin (Tunic. - 5 μg/ml), chlorpromazine (CPZ - 50 μg/ml), zymolyase (Zym. - 2.5 U/ml), α-factor (α-fac. - 12 μM) or calcofluor white (Calc. -100 μg/ml) for 90 minutes. Cell cultures treated with surface stress were assayed for β-galactosidase activity and activity was quantified according to the Miller equation (**A**; section 2.22). Bar graph **B** shows fold activity of calcineurin in relation to basal activity. The majority of the cell population remained viable throughout the experiments, except for mating pheromone treated mutants cells, which display a mating pheromone-induced death (data not shown). The low CDRE activity in mutant cells is therefore as a direct result of reduced number of viable cells. The data presented are representatives of triplicate experiments. N=3 and errors are +/- SEM.

Hcs77p and Mid2p are not the sensors for activation of calcineurin in response to cell surface stress, then the other *WSC* family members may fulfil this role. The *wsc* triple delete cells display low survival rate in heat shock and have been proposed to be required for normal response to heat shock (Verna *et al.* 1997). The *WSC* genes have also been found to be required for activation of the Pkc1p-MAPK cascade in heat shock (Gray *et al.* 1997; Verna *et al.* 1997). It is possible that these cell surface sensors, may also be required to activate the calcineurin pathway during cell surface stress by assisting in calcium influx. This hypothesis was tested, by assaying surface stress induced calcium influx in *wsc* triple deletion mutants during cell surface stress. The *wsc1Δwsc2Δwsc3Δ* cells in the SP1 strain background were grown to mid-logarithmic phase in 10% sorbitol YPD, and were treated with tunicamycin for one hour. Calcium influx was monitored as described in section 2.32. The *wsc1Δwsc2Δwsc3Δ* cells did not show reduced calcium influx in response to cell surface stress when compared to wild-type cells (Fig. 5.11). This result shows that calcium influx and hence calcineurin activation is not dependent on the *WSC* family of cell surface sensors.

5.2.8. The Pkc1p-MAPK pathway is not required for activation of calcineurin pathway in response to cell surface stress

Hcs77p and Mid2p are the only known cell surface sensors that are absolutely required for activation of the Pkc1p-MAPK pathway in response to cell surface stress (Chapters 3 and 4). Since Hcs77p and Mid2p are not required for activation of the calcineurin pathway, this indicates that the whole of the Pkc1p-MAPK pathway may not be required for calcineurin activation. To further test this possibility, activation of the calcineurin reporter was examined in *mpk1Δ* cells exposed to cell surface stress. *MAT* **a** *mpk1Δ* cells in the EG123 strain background were transformed with pBJ306 containing the CDRE-*lacZ* reporter construct. The transformed cells were treated to various cell surface stresses as described in section 5.2.1. The majority of the cell population remained viable throughout the experiments (data not shown). The *mpk1Δ* cells displayed a similar degree of calcineurin reporter induction in response to cell surface stress as that found in wild-type cells (Fig. 5.12B). Calcineurin-reporter activation was inhibited by the presence of cyclosporin-A (Fig. 5.12A). These results show that *mpk1Δ* cells are not defective in calcineurin activation in response to cell surface stress. It can be therefore concluded that

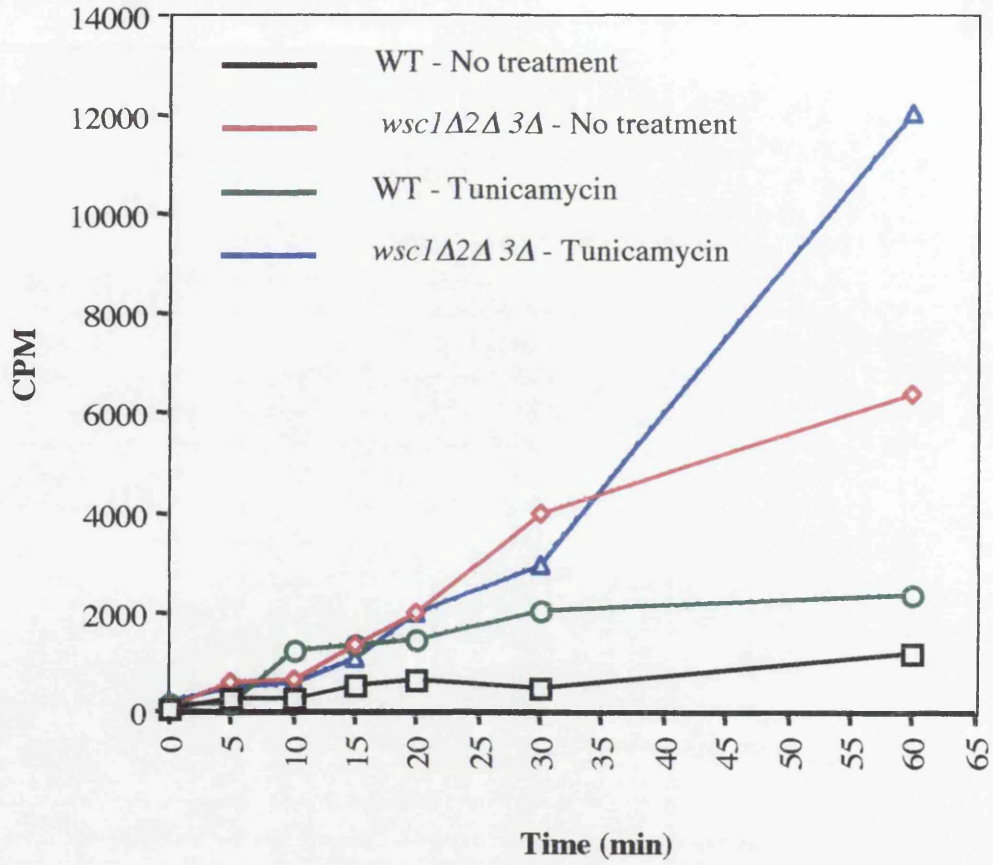

Figure 5.11. The Wsc family of cell surface sensors are not required for surface stress-induced calcium influx. *MAT* **a** wild-type and *MAT* **a** *wsc1Δwsc2Δwsc3Δ* cells in the SP1 strain background were grown to mid-logarithmic phase in 10% sorbitol YPD at 25°C. The cell cultures were incubated with or without tunicamycin (5 μg/ml) for one hour at 25°C. $^{45}CaCl_2$ was added to the cultures and calcium accumulation was monitored as described in section 2.32. The data presented are representatives of triplicate experiments.

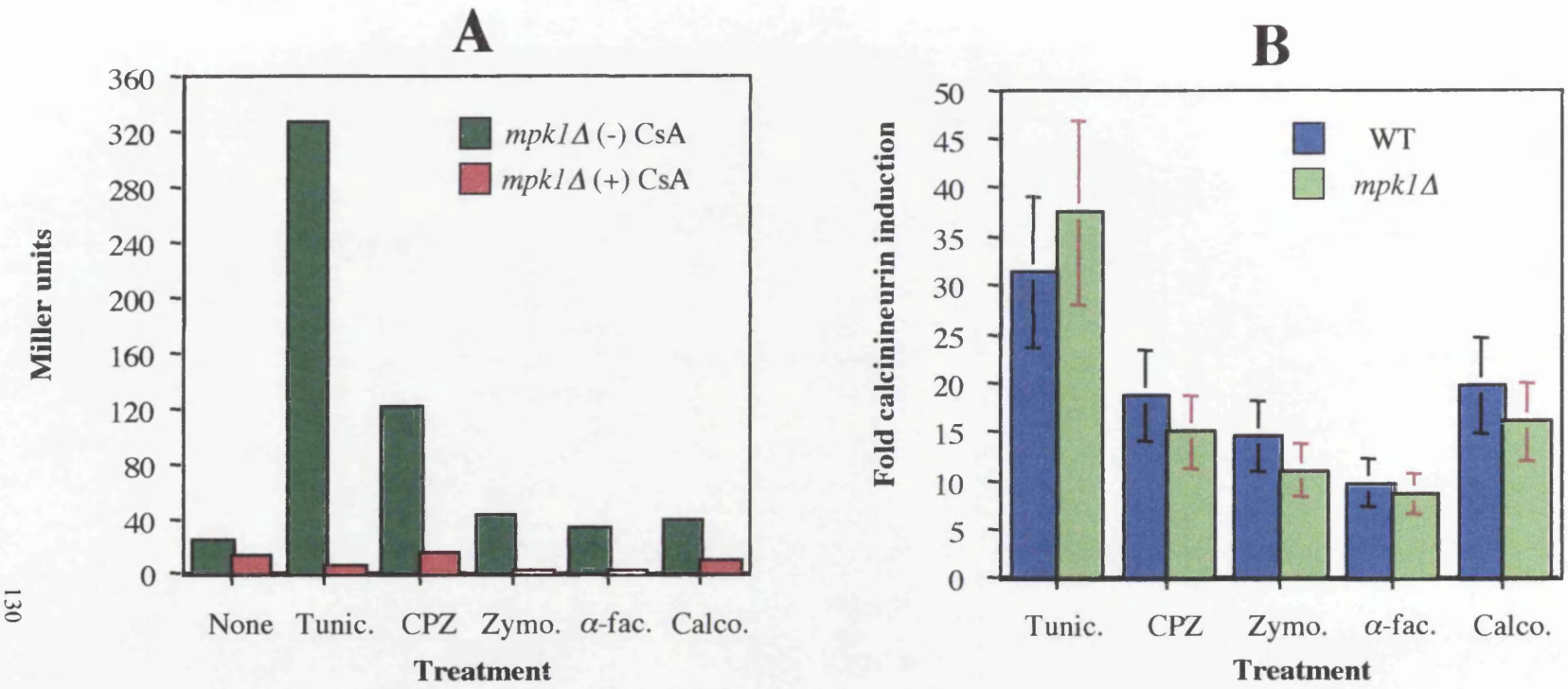

Figure 5.12. The Pkc1p-MAPK pathway is not required for activation of the calcineurin-signalling pathway. *MAT* **a** wild-type and *mpk1Δ* cells in the EG123 strain background transformed with pBJ306 containing the CDRE-*lacZ* reporter construct were grown to mid-logarithmic phase in 10% sorbitol Ura$^-$ selective media (pH 4.0 for α-factor treatment) at 25°C. The cell cultures were incubated with or without cyclosporin-A (CsA - 100 μg/ml) for one hour, at 25°C. Cell cultures were incubated with tunicamycin (Tunic - 5 μg/ml), chlorpromazine (CPZ - 50 μg/ml), zymolyase (Zymo - 2.5 U/ml), α-factor (α-fac - 12 μM) or calcofluor white (Calc - 100 μg/ml) for 90 minutes. Cell cultures treated with surface stress were assayed for β-galactosidase activity and activity was quantified according to the Miller equation (**A**; section 2.22). Bar graph **B** shows fold activity of calcineurin in relation to basal activity. The majority of the cell population remained viable throughout the experiments. The data presented are representatives of triplicate experiments. N=3 and errors are +/- SEM.

the Pkc1p-MAPK pathway does not regulate activation of the calcineurin-signalling pathway in response to cell surface stress.

5.2.9. *FKS2* expression is activated in response to cell surface stress

Glucan synthase is responsible for synthesis of β-1,3-glucan, which is an essential component of the cell wall (Douglas *et al.* 1994; Inoue *et al.* 1995; Mazur *et al.* 1995; Ram *et al.* 1995). Calcineurin activation leads to transcriptional activation of the glucan synthase subunit *FKS2* (Zhao *et al.* 1998). Since calcineurin is activated during cell surface stress in mutants of the Pkc1p-MAPK pathway, then does this activation also lead to transcriptional activation of *FKS2*? To assess this question, activation of *FKS2* transcription, in response to tunicamycin in wild-type, *mpk1Δ* and *mid2Δhcs77Δ* cells was examined. Mid-logarithmic phase wild-type, *mpk1Δ* and *mid2Δhcs77Δ* EG123 cells in 10% sorbitol YPD were exposed to tunicamycin for 0, 30, 60, 120 or 180 minutes. Total RNA was prepared from all samples, which was then analysed by northern blotting. The northern blots were probed for the *FKS2* or *ACT1* transcripts. In response to tunicamycin, *mpk1Δ* and *mid2Δhcs77Δ* cells displayed similar *FKS2* activation of transcription as that found for wild-type cells (Fig. 5.13A). Furthermore, this activation of transcription was inhibited in cells treated with cyclosporin-A (Fig. 5.13B). These results confirm that cells deficient in activation of the Pkc1p-MAPK pathway can activate *FKS2* in response to cell surface stress and are thus competent to activate the calcineurin pathway.

5.2.10. Pkc1p-MAPK pathway modulates basal calcineurin activity

Mutants of the Pkc1p-MAPK pathway, show normal calcineurin fold induction in response to cell surface stress (sections 5.2.6 and 5.2.8). However, in experiments carried out in sections 5.2.6 and 5.2.8, also showed that the basal calcineurin activity is different in mutants of the Pkc1p-MAPK pathway when compared to the congenic wild-type cells. Even though, *mid2Δ* and *hcs77Δ* cells displayed similar basal calcineurin-reporter activity to wild-type cells, *mid2Δhcs77Δ* cells showed a severely reduced calcineurin basal reporter activity (Fig. 5.14A, 5.14B and 5.15A). These results show that cells, which are mutants for both of the cell surface sensors, are defective for basal calcineurin activity.

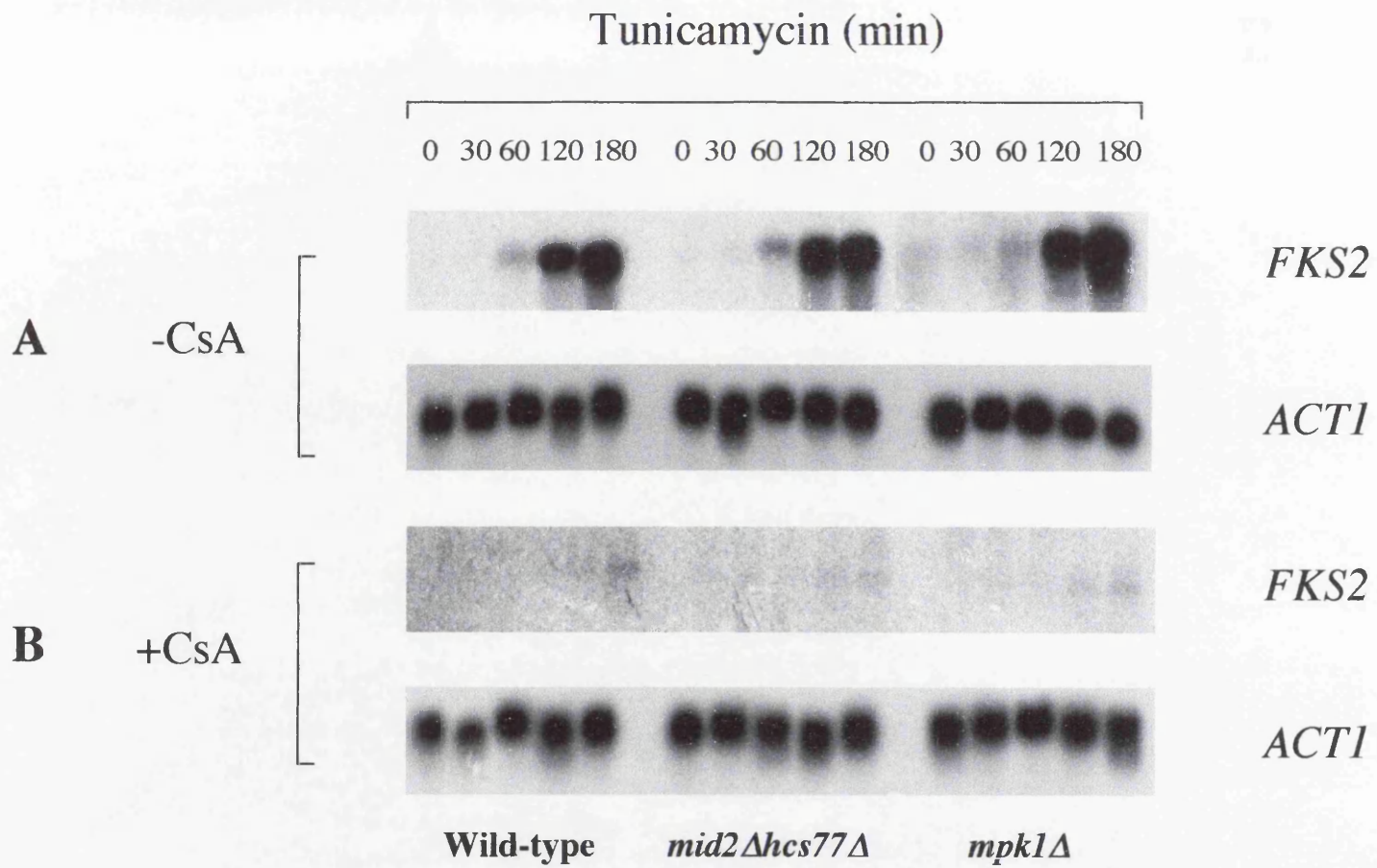

Figure 5.13. *FKS2* expression is activated in response to cell surface stress. Autoradiograph showing *FKS2* and *ACT1* expression levels in cell extracts. *MAT* **a** wild-type, *mid2Δhcs77Δ*, and *mpk1Δ* cells in the EG123 strain background were grown to mid-logarithmic phase in 10% sorbitol YPD at 25°C. The cell cultures were incubated (**B**) with or (**A**) without Cyclosporin-A (CsA) for one hour. The cell cultures were then incubated with tunicamycin (5 μg/ml) for three hours. Samples were removed at 0, 30, 60, 120 and 180 minutes. Total RNA was prepared from samples removed at time intervals. The resulting northern blots were probed with ^{32}P labeled *ACT1* (as loading control) or *FKS2* gene. The data presented are representatives of triplicate experiments.

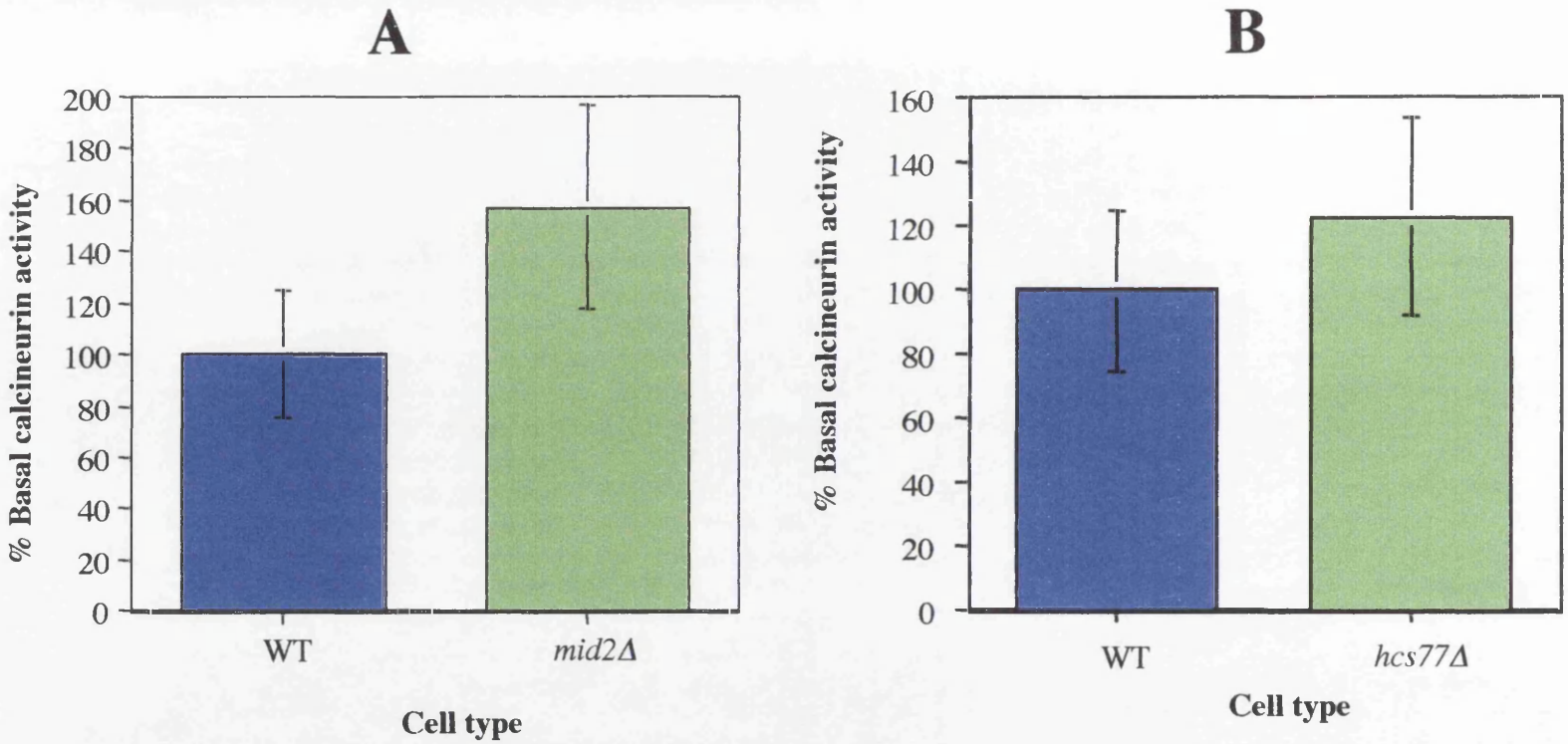

Figure 5.14. *mid2Δ* and *hcs77Δ* cells are not defective in basal calcineurin activity. *MAT* **a** wild-type (**A**) *mid2Δ* and (**B**) *hcs77Δ* cells in the EG123 strain background transformed with pBJ306 containing the CDRE-*lacZ* reporter construct were grown to mid-logarithmic phase in 10% sorbitol Ura$^-$ selective media at 25°C. Cell cultures were assayed for β-galactosidase activity and activity was quantified according to the Miller equation (section 2.22). The majority of the cell population remained viable throughout the experiments. The data presented are representatives of triplicate experiments. N=3 and errors are +/- SEM.

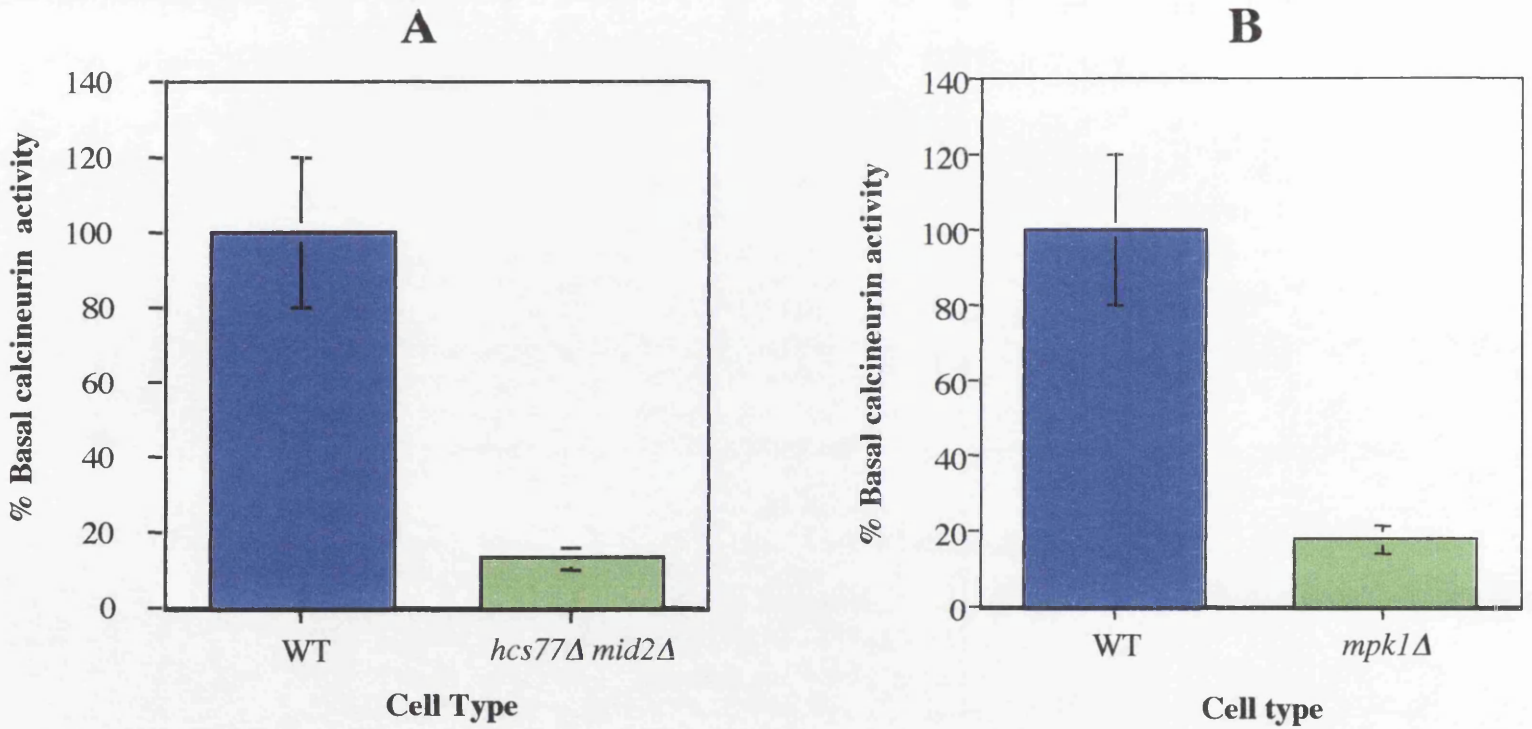

Figure 5.15. The Pkc1p-MAPK pathway modulates basal calcineurin activity. **(A)** *mid2Δhcs77Δ* and **(B)** *mpk1Δ* cells are defective in basal calcineurin activity. *MAT* **a** wild-type and mutant cells in the EG123 strain background transformed with pBJ306 containing the CDRE-*lacZ* reporter construct were grown to mid-logarithmic phase in 10% sorbitol Ura$^-$ selective media at 25°C. Cell cultures were assayed for β-galactosidase activity and activity was quantified according to the Miller equation (section 2.22). The majority of the cell population remained viabe throughout the experiments. The data presented are representatives of triplicate experiments. N=3 and errors are +/- SEM.

Therefore, Hcs77p and Mid2p have a redundant role in modulation of calcineurin basal activity.

Since Hcs77p and Mid2p have a redundant function in modulating calcineurin basal activity, then this suggests that the whole of the Pkc1p-MAPK pathway may be required for this basal modulation. The basal calcineurin-reporter activity of *mpk1Δ* cells, similar to *hcs77Δmid2Δ* cells, was greatly reduced in comparison to wild-type cells (Fig. 5.15B). This result shows that *mpk1Δ* cells are defective for the basal activity of calcineurin in vegetatively-growing cells. Together these results suggest that the whole of the Pkc1p-MAPK pathway is required for modulation of the basal calcineurin activity.

5.2.11. The Pkc1p-MAPK pathway does not regulate calcium influx

The Pkc1p-MAPK pathway may modulate basal calcineurin activity by regulation of calcium influx into the cells. Therefore, cells deficient for Pkc1p-MAPK activity would have lower basal calcium influx and hence reduced calcineurin basal activity. This hypothesis was tested by measuring influx of calcium in mid-logarithmic phase *mpk1Δ* and wild-type cells in the EG123 strain background pre-incubated with or without tunicamycin or chlorpromazine for one hour. 10% sorbitol was included in the YPD medium to maintain a high viable population of *mpk1Δ* cells at the beginning of each experiment. Assay of $^{45}CaCl_2$ influx was carried out as described in section 2.32. The *mpk1Δ* cells displayed similar initial calcium influx in comparison to wild-type cells (Fig. 5.16). Similar results were obtained for *mpk1Δ* cells in the W303 strain background (Data not shown). These results show that *mpk1Δ* cells are not defective for basal and stimulated influx of calcium during vegetative growth. Furthermore, these results show that the reduced basal calcineurin activity in *mpk1Δ* cells is not due to reduced calcium influx activity in these cells. However, *mpk1Δ* cells failed to accumulate calcium after the initial influx, whereas the wild-type cells continued influx and accumulation after initial uptake. These data suggest that *mpk1Δ* cells may show elevated for calcium efflux.

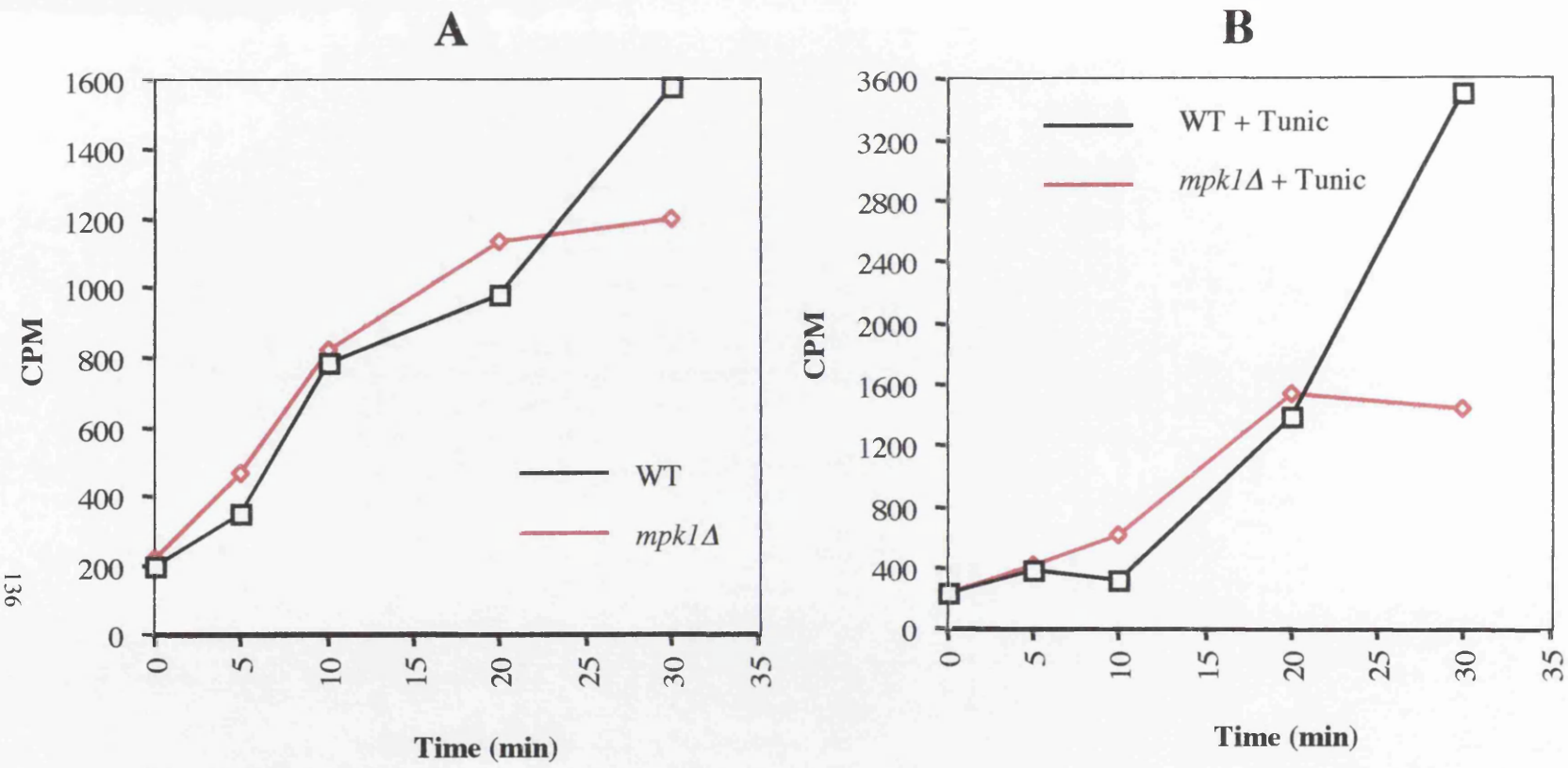

Figure 5.16. The Pkc1p-MAPK pathway does not regulate calcium influx. *MAT* **a** wild-type and *mpk1Δ* cells, in the EG123 strain background, were grown to mid-logarithmic phase in 10% sorbitol YPD. Cell cultures were incubated (**A**) without or with (**B**) tunicamycin (5 μg/ml) for one hour at 25°C. $^{45}CaCl_2$ was added to the cell cultures and samples were removed at time intervals. Calcium accumulation was assayed as described in section 2.32. The data presented are representatives of triplicate experiments.

5.2.12. The Pkc1p-MAPK pathway regulates calcium efflux

The inability to accumulate calcium by *mpk1Δ* cells could be due to a defect in regulation of calcium efflux. To test this hypothesis calcium efflux was assayed in *mpk1Δ* cells. Wild-type and *mpk1Δ* cells in the EG123 background were grown to mid-logarithmic phase in 10% sorbitol YPD containing $^{45}CaCl_2$. As before, sorbitol was maintained in the growth medium to maintain a high viable population of *mpk1Δ* cells at the beginning of each experiment. These cell cultures were then washed resuspended in 10% sorbitol YPD medium and were assayed for calcium efflux as described in section 2.33. The *mpk1Δ* cells displayed a much higher rate of calcium efflux than the congenic wild-type cells (Fig. 5.17A). This higher level of calcium efflux was also observed when EGTA was present in the washes of samples (Fig. 5.17B). This latter result eliminates the presence of non-specific binding of radio-labeled calcium to the cells after the washes.

These results show that *mpk1Δ* cells are defective in regulation of calcium efflux and therefore may not be able to sustain a basal level of calcium within the cells. Furthermore, these results indicate that the Pkc1p-MAPK pathway functions upstream of the calcium-signalling pathway, regulating calcium homeostasis.

5.2.13. Activation of Pkc1p pathway decreases calcium efflux in wild-type cells

Cells lacking components of the Pkc1p-MAPK pathway, show defects in calcium efflux. If internal calcium homeostasis is dependent on the Pkc1p-MAPK pathway, then one would expect that activation of this pathway would lead to decreased rate of calcium efflux. To test this possibility, wild-type cells were transformed with *Gal-PKC1* or *Gal-PKC1*-constitutively active plasmids (Roberts *et al.* 2000). The transformants were streaked onto selective plates, and the resulting colonies were grown to mid-logarithmic phase in selective media. $^{45}CaCl_2$ containing cell cultures were then incubated with galactose for three hours (to induce induction of the *PKC1* gene), washed and resuspended in galactose containing selective media. Efflux experiments were carried out as described in section 2.33. Cells over-expressing the constitutively active *PKC1* allele displayed a decrease in calcium efflux in comparison to cells over-expressing the wild-type form of *PKC1* (Fig. 5.17C). This result shows that activation of the Pkc1p pathway leads to

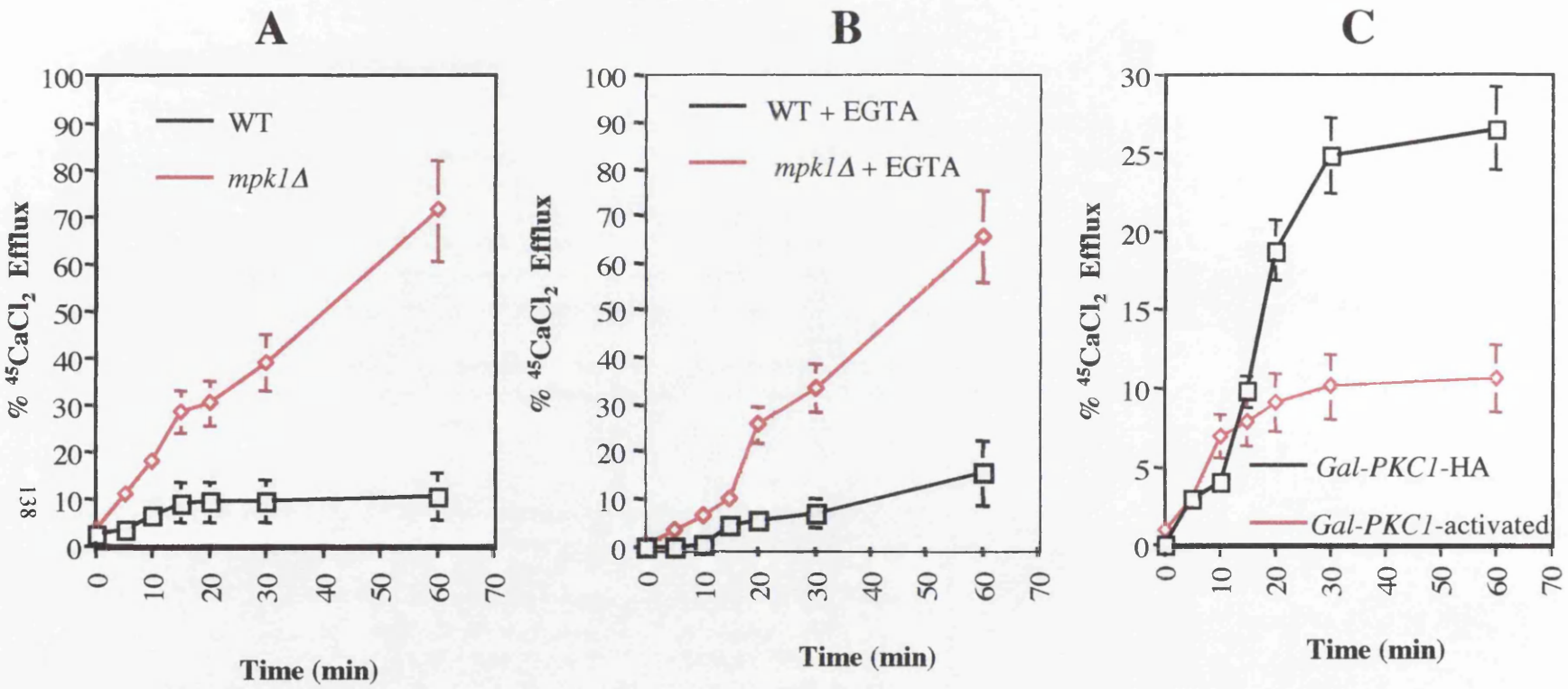

Figure 5.17. The Pkc1p-MAPK pathway regulates calcium efflux. *MAT* **a** wild-type and *mpk1Δ* cells in the EG123 strain background (**C**) transformed with *Gal-PKC1* or *Gal-PKC1* constitutively active plasmids, were grown to mid-logarithmic phase in 10% sorbitol (**A** and **B**) YPD or (**C**) Ura$^-$ selective media, containing $^{45}CaCl_2$. (**C**) Cell cultures were incubated with 2% galactose for three hours. Cell cultures were washed and resuspended in (**A**) 10% sorbitol YPD (**B**) 10% sorbitol YPDwith 10mM EGTA or (**C**) selective media with 2% galactose. Samples were removed at time intervals and assay of calcium efflux was carried out as described in section 2.33. The data presented are representatives of triplicate experiments. N=3 and errors are +/- SEM.

decreased calcium efflux. This result supports the proposal that the Pkc1p-MAPK pathway regulates functions upstream of the calcium-signalling pathway maintaining calcium homeostasis in yeast cells.

5.2.14. Mid1p is not required for calcium efflux

As a putative stretch-activated cation channel, Mid1p may also be involved in regulation of calcium efflux. As seen in section 5.2.12, *mpk1Δ* cells have a defective basal calcineurin activity that may be associated with high calcium efflux. If Mid1p regulates calcium efflux then it may be possible that it also has a low calcineurin activity. To test this possibility *mid1Δ* cells were transformed with the CDRE-*lacZ* reporter gene within pBJ306. The transformants were treated as described in section 5.2.1. The *mid1Δ* cells displayed similar calcineurin reporter induction in response to cell surface stress, as did wild-type cells (Fig. 5.18B). Calcineurin reporter activity was inhibited by presence of cylcosporin-A (data not shown). However, *mid1Δ* cells displayed a severely reduced basal calcineurin reporter activity in comparison to wild-type cells (Fig. 5.18A). These results show that *mid1Δ* cells have no defect in calcineurin activation during surface stress. However, Mid1p calcium channel activity is required for full basal calcineurin activity.

Since cells deficient for Mid1p channel activity have a reduced basal calcineurin activity, similar to that seen in *mpk1Δ* cells, it is possible that these cells also show elevated calcium efflux activity. If *mid1Δ* cells do display reduced efflux activity then that suggests that Mid1p may also function as a calcium exporter. To test this hypothesis, mid-logarithmic phase *mid1Δ* cells cultured in YPD $^{45}CaCl_2$ were assayed for calcium efflux as described in section 2.33. The *mid1Δ* cells displayed a similar calcium efflux pattern as wild-type cells (Fig. 5.19). This result shows that *mid1Δ* cells are not defective for calcium efflux. Furthermore, Mid1p does not regulate calcium efflux. It can be concluded therefore that Mid1p regulates basal calcineurin activity by regulation of calcium influx whereas the Pkc1p-MAPK pathway regulates basal calcineurin activity by inhibition of calcium efflux.

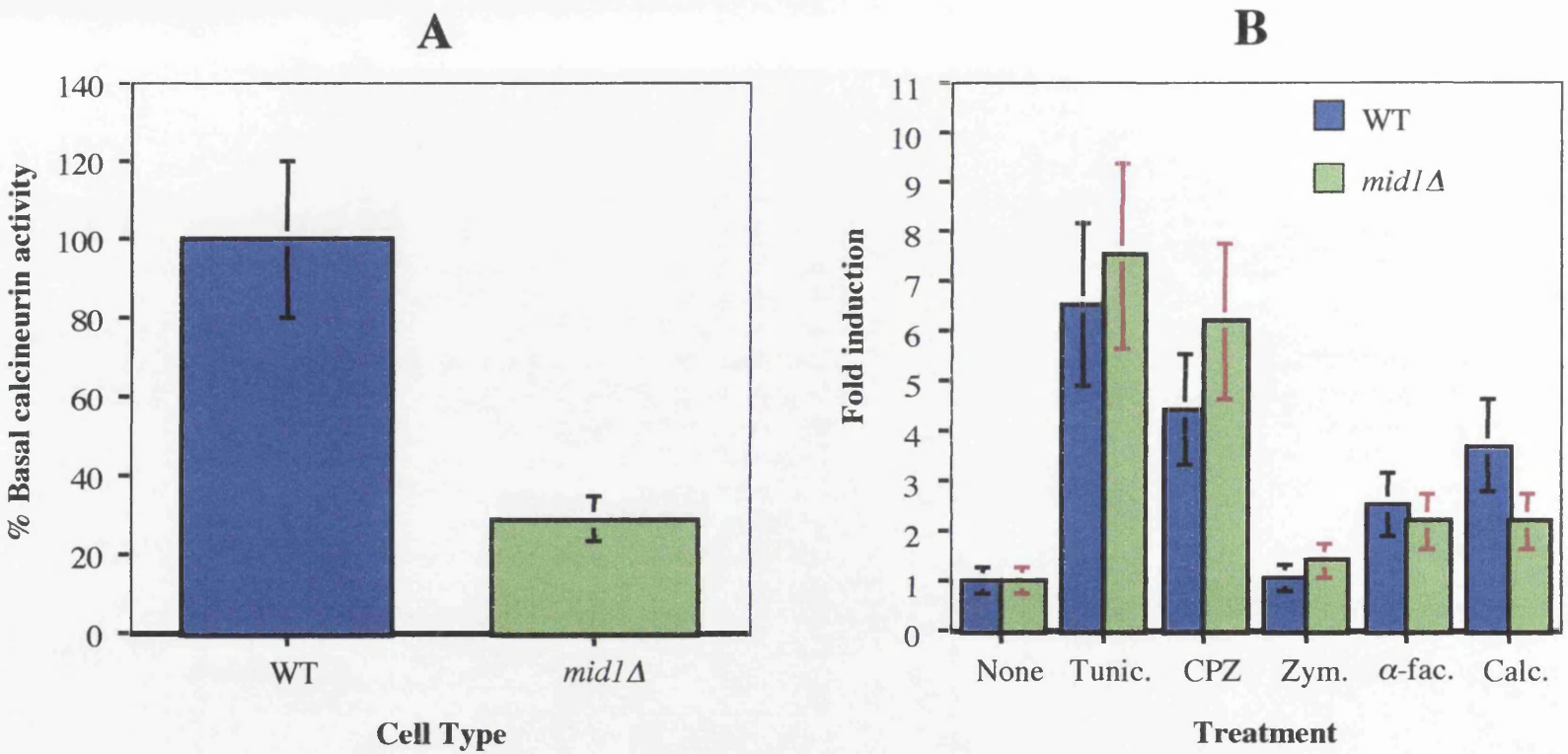

Figure 5.18. Mid1p regulates basal calcineurin activity in response to cell surface stress. *MAT* **a** wild-type and *mid1Δ* cells in the EG123 strain background transformed with pBJ306 containing the CDRE-*lacZ* reporter construct were grown to mid-logarithmic phase in Ura$^-$ selective media at 25°C. Media at pH 4.0 was used in α-factor treatments. (**B**) Cell cultures were incubated with tunicamycin (Tunic - 5 μg/ml), chlorpromazine (CPZ - 50 μg/ml), zymolyase (Zym - 2.5 U/ml), α-factor (α-fac - 12 μM), calcofluor white (Calc - 100 μg/ml) for 90 minutes. The cell cultures (**B**) treated or (**A**) not treated with cell surface stress were assayed for β-galactosidase activity and activity was quantified according to the Miller equation (section 2.22). The data presented are representatives of triplicate experiments. N=3 and errors are +/- SEM.

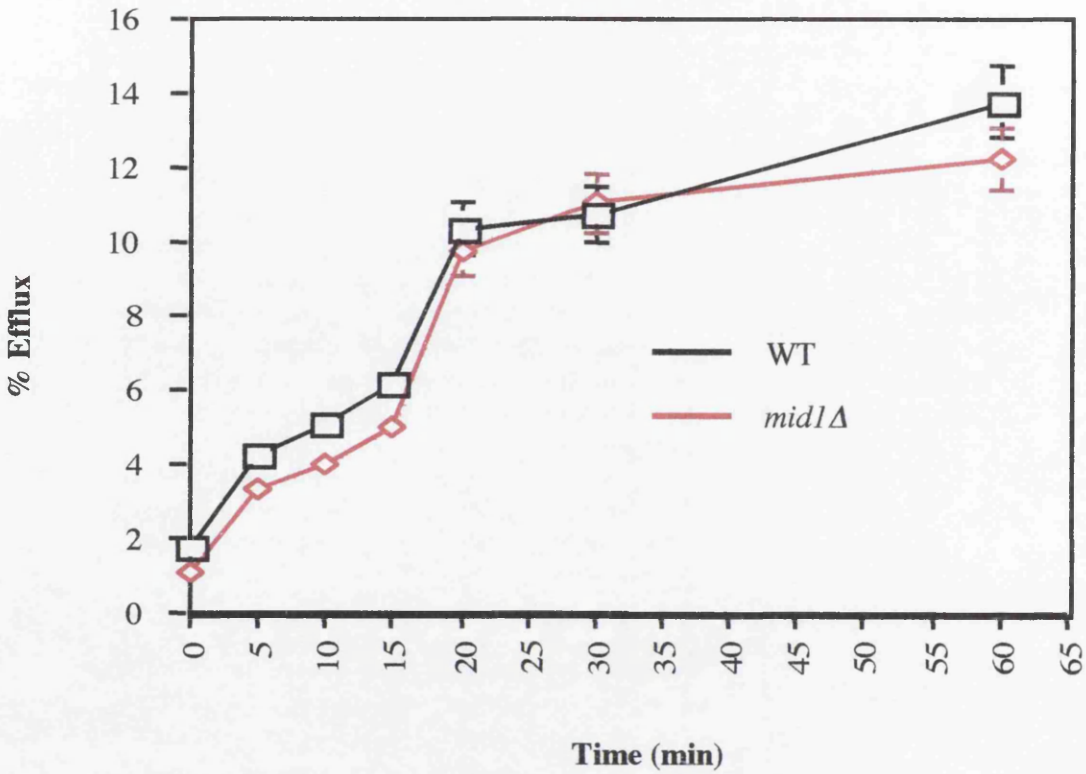

Figure 5.19. Mid1p is not required for calcium efflux. *MAT* **a** wild type and *mid1Δ* cells in the EG123 strain background, were grown to mid-logarithmic phase in $^{45}CaCl_2$ YPD. Cell cultures were washed and resuspended in YPD. Samples were removed at time intervals and assay of calcium efflux was carried out as described in section 2.33. The data presented are representatives of triplicate experiments. N=3 and errors are +/- SEM.

5.2.15. Calcineurin does not regulate calcium efflux

The Pkc1p-MAPK pathway modulates calcineurin basal activity. Calcineurin activity leads to transcriptional activation of *PMR1* and *PMC1* (Cunningham and Fink 1994-a,1996; Mendoza *et al.* 1994; Rudolph *et al.* 1994). Pmr1p and Pmc1p are ATPases, which are responsible for the majority of calcium exchange between the cellular stores and the cytoplasm. The Pkc1p-MAPK pathway also regulates calcium efflux from the cells. If the Pkc1p-MAPK pathway regulates calcium homeostasis, by regulating calcineurin activity, then one would expect that inhibition of calcineurin activity would lead to increased calcium efflux. To test this hypothesis, mid-logarithmic phase wild-type cells grown in $^{45}CaCl_2$ YPD, were incubated for one hour in presence or absence of cyclosporin-A. The cells were washed, while maintaining cyclosporin-A in the washes. Cells were resuspended in YPD with or without cyclosporin-A as appropriate and efflux of calcium was measured as described in section 2.33. It was found that calcium efflux in calcineurin-inhibited cells was similar to those cell not treated with cyclosporin-A (Fig. 5.20A). This result shows that calcineurin activity does not regulate calcium efflux. It can be concluded therefore, that the Pkc1p-MAPK pathway regulates calcium efflux independently of calcineurin and that the basal calcineurin activity is modulated as a result of the effect of the Pkc1p-MAPK pathway on calcium efflux.

To further analyse the role of calcineurin in calcium homeostasis, efflux of calcium in cells over-expressing constitutively active calcineurin was examined. Wild-type cells were transformed with *YEpCNA2* or *YEpCNA2Δ* (expressing a constitutively active *CNA*). Transformants were grown to mid-logarithmic phase in $^{45}CaCl_2$ selective media. The cells were washed and resuspended in selective media and calcium efflux was measured as described in section 2.33. It was found that constitutively active calcineurin does not affect the rate of calcium efflux when compared with cells over expressing the full-length calcineurin (Fig. 5.20B). This result further supports the previous results, showing that calcineurin does not regulate calcium efflux.

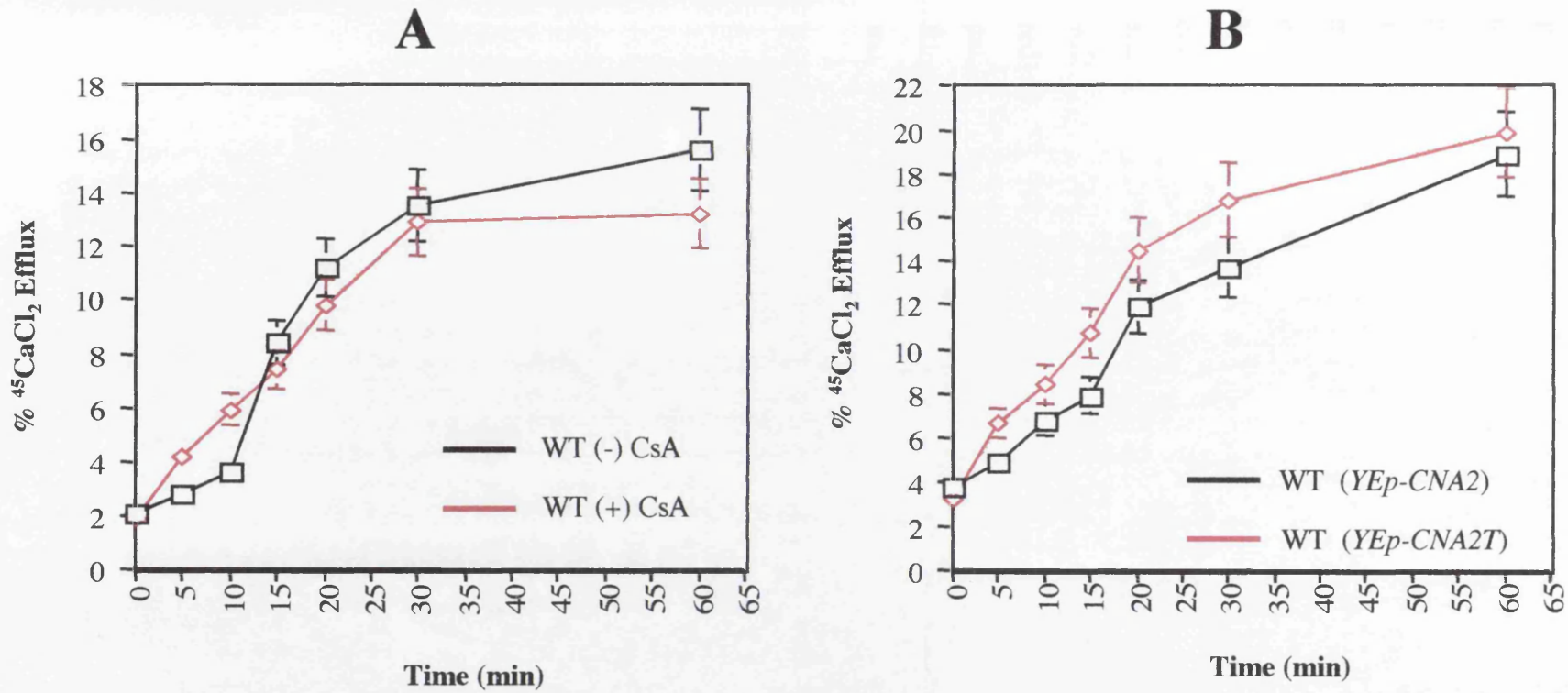

Figure 5.20. Calcineurin does not regulate calcium efflux. *MAT* **a** wild-type cells in the EG123 strain background, (**B**) transformed with *YEpCNA2* or *YEpCNA2Δ* were grown to mid-logarithmic phase in (**A**) YPD or (**B**) Ura⁻ selective media, containing $^{45}CaCl_2$ at 25°C. (**A**) Cell cultures were incubated with cyclosporin-A (100 μg/ml) for one hour. Cell cultures were washed and resuspended in (**A**) YPD or (**B**) Ura⁻ selective media. Samples were removed at time intervals and assay of calcium efflux was carried out as described in section 2.33. The data presented are representatives of triplicate experiments. N=3 and errors are +/- SEM.

5.2.16. Regulation of calcineurin by the Pkc1p-MAPK pathway does not depend on Rlm1p

The Pkc1p-MAPK pathway modulates calcineurin basal activity. The transcription factor Rlm1p is a target of the Pkc1p-MAPK pathway and becomes activated in response to phosphorylation by Mpk1p (Dodou *et al.* 1997; Watanabe *et al.* 1995; Watanabe *et al.* 1997). Most of the changes in gene expression, resulting from activation of Mpk1p, are mediated through Rlm1p (Jung and Levin 1999). Therefore, it is possible that regulation of calcineurin basal activity by the Pkc1p-MAPK pathway occurs at the transcriptional level through Rlm1p. To assess this possibility, the basal calcineurin activity in *rlm1Δ* cells transformed with pBJ306 containing the CDRE-*lacZ* reporter construct was analysed. The *rlm1Δ* cells displayed a basal calcineurin-reporter activity similar to that of wild-type cells (Fig. 5.21). More significantly, *rlm1Δ* cells did not display a severely reduced basal calcineurin activity (Fig. 5.21). This result suggests that the Pkc1p-MAPK pathway modulates basal calcineurin activity, independantly of transcriptional effects of Rlm1p. One can infer from this result that the Pkc1p-MAPK pathway may therefore modulate calcium efflux by a post transcriptional-mechanism.

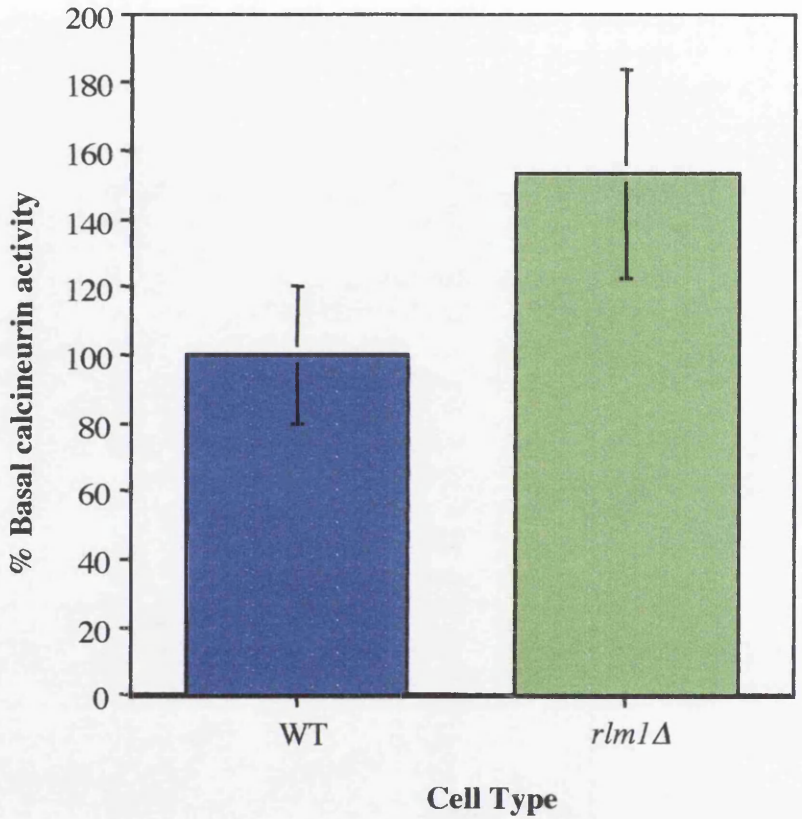

Figure 5.21. Pkc1p-MAPK pathway modulates basal calcineurin activity independently of Rlm1p. *MAT* **a** wild-type and *rlm1Δ* cells in the EG123 strain background transformed with pBJ306 containing the CDRE-*lacZ* reporter construct, were grown to mid-logarithmic phase in Ura$^-$ selective media at 25°C. Cell cultures, were assayed for β-galactosidase activity; and activity was quantified according to the Miller equation (section 2.22). The data presented are representatives of triplicate experiments. N=3 and errors are +/- SEM.

5.3. Discussion

The calcineurin-signalling pathway of the budding yeast is not required for vegetative growth under standard growth conditions (Cyert *et al.* 1991; Cyert and Thorner 1992; Kuno *et al.* 1991; Liu *et al.* 1991). However, the calcineurin-signalling pathway along with the Pkc1p-MAPK pathway is activated during times of pheromone exposure and heat shock (Buehrer *et al.* 1997; Kamada *et al.* 1995; Withee *et al.* 1997; Zarzov *et al.* 1996; Zhao *et al.* 1998). The Pkc1p-MAPK pathway is also activated when exposed to cell surface stress reagents such as zymolyase, calcofluor white, chlorpromazine or tunicamycin (De Nobel *et al.* 2000; Kamada *et al.* 1995; Ketela *et al.* 1999; Yun *et al.* 2000). Since cell surface change is the underlying cause leading to Pkc1p-MAPK pathway activation, then one would expect that the calcineurin pathway would also be activated in response to cell surface change. Evidence presented in section 5.2.1 shows that the calcineurin-signalling pathway is also activated in response to cell surface stress associated with drugs ranging from tunicamycinto calcofluor white. These data suggest that the calcineurin and Pkc1p-MAPK signalling pathways are co-regulated by cell surface stress to maintain cellular integrity.

The activation of the calcineurin pathway, in response to mating pheromone, is associated with calcium influx (Iida *et al.* 1990, Ohsumi and Anraku 1985). This pheromone-induced calcium influx has been associated with the putative stretch-activated calcium channel Mid1p and the calcium channel Cch1p (Fischer *et al.* 1997; Iida *et al.* 1994). Data presented in section 5.2.2 and 5.2.3, show that calcium influx is stimulated by cell surface change exerted by tunicamycin or chlorpromazine, and that this occurs at least in part through Mid1p channel activity. Data presented in section 5.2.4 provided evidence that cell surface change triggers calcium influx. Calcium influx was not stimulated in *bni1Δ* cells exposed to mating pheromone. This result not only provides evidence that calcium influx associated with polarisation requires the cell wall remodeling, but suggests that the calcineurin pathway responds to cell surface change, similar to the Pkc1p-MAPK pathway.

For activation of the Pkc1p-MAPK pathway, the two cell surface sensors Hcs77p and Mid2p share a redundant but essential function. These two cell surface sensors are

required for activation of the Pkc1p-MAPK pathway in response to cell surface stress. However, only the putative stretch-activated cation channel Mid1p can be implicated in activation of the calcineurin pathway in response to cell surface stress. It is plausible that since both the calcineurin and the Pkc1p-MAPK pathway respond to same cell surface stresses, that they also share the same cell surface sensors. However, these two cell surface sensors are dispensable for activation of calcineurin in response to cell surface stress (section 5.2.6). In addition, the *WSC* family are not the sensors for calcineurin activation in response to cell surface stress (section 5.2.7). Mutants of the *WSC* family even display higher calcium influx than wild-type cells in response to cell surface stress (section 5.2.7). Influx of calcium in these mutants may either be required to activate the calcineurin pathway for stabilisation of the cell surface or that the misshapen cell walls of these mutant cells allows higher passive influx of calcium. Together these results suggest that the Pkc1p-MAPK pathway is dispensable for activation of calcineurin in response to cell surface stress. The fact that *mpk1Δ* cells also display normal calcineurin activation in response to cell surface stress further supports these conclusions (section 5.2.8). Monitoring of *FKS2* expression in cells treated with tunicamycin, revealed supporting evidence that the Pkc1p-MAPK pathway is not required for activation of the calcineurin-signalling pathway (section 5.2.9).

Mizunuma *et al.* (1998) reported that high level of extracellular calcium can activate the Pkc1p-MAPK pathway, suggesting that the Pkc1p-MAPK pathway functions downstream of the calcium-signalling pathway. However, when cells grown in calcium deficient media, were heat shocked, Mpk1p was activated, similar to cells cultured in calcium containing media (section 5.2.5). These results indicate that the calcium-signalling pathway does not regulate the Pkc1p-MAPK pathway in response to cell surface stress. Furthermore, these results show that the Pkc1p-MAPK pathway does not function downstream of the calcium-signalling pathway, and suggests that these two pathways have independent surface sensing mechanisms.

The Pkc1p-MAPK pathway seems to play an important role in regulation of basal calcineurin activity. In *mid2Δhcs77Δ* and *mpk1Δ* cells the basal calcineurin activity is much reduced in comparison to wild-type cells (section 5.2.10). One possible model in which the Pkc1p-MAPK pathway may regulate calcineurin basal activity would involve

regulation of calcium influx. However, influx of calcium in *mpk1Δ* cells in presence and absence of cell surface stress induced by tunicamycin, was similar to that of wild-type cells (section 5.2.11). Despite displaying normal calcium influx, *mpk1Δ* cells were defective for accumulation of calcium, whereas wild-type cells showed continued net calcium influx and accumulation (section 5.2.11). The calcium accumulation defect of *mpk1Δ* cells may be associated with the high calcium efflux observed in these cells in comparison to the congenic wild-type cells (section 5.2.12). Similarly, calcium efflux was increased in wild-type cells over-expressing a constitutively active *PKC1* in comparison to wild-type cells over-expressing the *PKC1* gene (section 5.2.13). These results show that the Pkc1p-MAPK pathway regulates calcium homeostasis by regulation of calcium efflux from the cells. It can therefore be concluded that the calcium-signalling pathway functions downstream of the Pkc1p-MAPK pathway.

Calcineurin is involved in transcriptional regulation of the calcium store pumps, *PMR1* and *PMC1* (Cunningham and Fink 1994-a,1996; Mendoza *et al.* 1994; Rudolph *et al.* 1994). Calcineurin also inhibits the vacuolar H^+-ATPase Vcx1p, post-transcriptionally (Cunningham and Fink 1996). Therefore, calcineurin regulates calcium homeostasis at the transcriptional and post-transcriptional level. Regulation of calcium efflux by the Pkc1p-MAPK pathway however does not require calcineurin activity. When cells were inhibited for calcineurin activity calcium efflux was similar to that of cells not treated with the calcineurin inhibitor (section 5.2.15). In addition, calcium efflux was not influenced in cells expressing a constitutively active allele of calcineurin (section 5.2.15). The Therefore, the Pkc1p-MAPK pathway regulates calcium efflux independently of calcineurin. The *mpk1Δ* cells display reduced basal calcineurin activity, due presumably to reduced basal calcium accumulation, which in turn may result from the increased calcium efflux observed in these cells. This regulation of calcineurin activity at the basal level, by the Pkc1p-MAPK pathway is independent of the transcriptional activator Rlm1p, as *rlm1Δ* cells have normal basal calcineurin activity (section 5.2.16). Therefore, this result suggests that the regulation of basal calcium homeostasis occurs at the post-transcriptional level. Perhaps this regulation involves phosphorylation of calcium channels or pumps by Mpk1p.

"It is difficult to say what is impossible,
for the dream of yesterday
Is the hope of today
And reality of tomorrow."

Robert Goddard

Chapter 6: Final discussion

6.1. An alternative method for assaying Mpk1p activity in yeast

Jung and Levin (1999) have shown that Mpk1p activation positively regulates the transcription of the *MPK1* gene upon heat shock. Therefore, in principle, Mpk1p activity can be assayed by monitoring the expression of *MPK1*. This assay has been used hereinto monitor Mpk1p activity in response to various cell surface stresses that are known to induce Mpk1p activation. Furthermore, the results obtained by monitoring *MPK1* transcriptional activation in response to heat shock and pheromone, are mirrored by results obtained by other groups, using alternative methods for assaying Mpk1p activity (Ketela *et al.* 1999; Rajavel *et al.* 1999). These alternative methods for assaying Mpk1p activity include: the protein kinase assay which takes advantage of myelin basic protein (MBP) as a substrate, and using the anti-phospho-tyrosine antibody to detect the activated form of Mpk1p. Ketela *et al.* (1999) and Rajavel *et al.* (1999) have independently assayed Mpk1p activity in *mid2Δ* cells using the two alternative forms of Mpk1p assays and their results demonstrate similar activation properties as that reported herein. Therefore, monitoring *MPK1* transcriptional activation is an easy and alternative method for assaying Mpk1p activation.

6.2. Mid2p is an upstream activator of the Pkc1p-MAPK pathway

The Pkc1p-MAPK pathway of the budding yeast is involved in maintaining cellular integrity during vegetative growth and upon heat-shock. An upstream putative sensor Hcs77p has been identified that has been proposed to respond to membrane stretch associated with surface stress (Gray *et al.* 1997). Hcs77p is proposed to activate the Pkc1p-MAPK pathway *via* the GEF Rom2p, and is found to be required for GTP loading of Rho1p (Philip and Levin 2001). There are several lines of evidence, which show that Mid2p has a redundant role with Hcs77p as a putative upstream sensor of the Pkc1p-MAPK pathway. First, over-expression of *MID2* suppresses the temperature sensitive, growth defect of *hcs77Δ* cells (section 3.2.2; Rajavel *et al.* 1999). Second, 2μ *HCS77* suppresses the α-factor-induced death of *mid2Δ* cells (section 4.2.3; Rajavel *et al.* 1999). Third, *MID2* over-expression, like over-expression of *HCS77*, relieves the temperature

sensitivity phenotype of the *swi4* mutants (section 3.2.1). Fourth, *mid2Δ* and *hcs77Δ* are synthetic lethal: *mid2Δhcs77Δ* cells are inviable in the S288C strain background and viable only on osmotically stabilised medium in the EG123 strain background (section 3.2.5; Ketela *et al.* 1999; Rajavel *et al.* 1999). This phenotype of *mid2Δhcs77Δ* cells is more severe than *hcs77Δ* cells, which are only defective for growth at high temperatures (Gray *et al.* 1997). Fifth, the deduced amino acid sequence of Mid2p indicates that it is a type-I transmembrane protein, which is topologically similar to Hcs77p (Ono *et al.* 1994). Sixth, Mid2p localises to the plasma membrane (Ketela *et al.* 1999; Rajavel *et al.* 1999). Seventh, over-expression of *MID2* in *mpk1Δ*, *bck1Δ* and $pkc1^{ts}$ mutants does not suppress their temperature sensitive phenotype (section 3.2.3; Rajavel *et al.* 1999). Eighth, cells, that lack either Mid2p or Hcs77p, are compromised in Mpk1p activation in response to heat shock (section 3.2.7; Ketela *et al.* 1999; Rajavel *et al.* 1999). Finally, *mid2Δhcs77Δ* cells cannot activate Mpk1p in response to heat shock or pheromone treatment (sections 3.2.7 and 4.2.8).

Does Mid2p have similar vegetative functions as Hcs77p? Deletion of *MID2* leads to no apparent vegetative defects (section 3.2.4). Unlike *hcs77Δ* cells, which are defective for growth at 37°C, *mid2Δ* cells in the strain backgrounds S288C, EG123 and W303, are able to proliferate at all temperatures (section 3.2.4). These data are confirmed by recent findings, showing that Mid2p is not required for vegetative growth (Ketela *et al.* 1999; Rajavel *et al.* 1999). Therefore, even though Mid2p and Hcs77p have redundant functions during vegetative growth and in activation of Mpk1p, deletion of these sensors does not lead to similar vegetative phenotypes. The phenotypic difference, between *mid2Δ* and *hcs77Δ* cells, may be explained by the following model. Both of these cell surface sensors contribute to activation of Mpk1p in response to cell surface stress. To fulfill this role, Hcs77p and Mid2p are complexed with Rom2p and Rho1p, leading to activation of the cell integrity pathway (Philip *et al.* 2001). On the other hand, Hcs77p and not Mid2p, is required for actin depolarisation associated with stress. In its role in actin depolarisation Hcs77p can form a complex with Rom2p, Rho1p and Pkc1p, independently of the MAPK cascade (Delley and Hall 1999). Hence, Mid2p and Hcs77p have predominantly overlapping but also distinct functions that may exist in separate complexes within the cell. According to this model, during vegetative growth, Hcs77p regulates more functions than Mid2p in response to cell surface stress and hence cells deficient for its functions are

defective for growth at high temperatures. Mpk1p may therefore, not be the initial target of Pkc1p to support growth and cell integrity at high temperatures.

What role does Mid2p fulfill during the G_1/S transition? The cell integrity pathway is activated during the G_1/S transition by an unknown mechanism that may be dependent on Cdc28/Cln complex (Mazzoni *et al.* 1993). At the G_1/S transition, the cell undergoes the morphogenic changes, which lead to bud formation (Lew and Reed 1993, 1995). Hence, the Pkc1p pathway may be activated by cell surface stress incurred during bud growth. The cyclin-dependent kinase Cdc28p in complex with the G_1 cyclins Cln1p, Cln2p and Cln3p are required for bud formation. The activity of Cdc28p regulates the activation of Mpk1p during this period of polarised growth (Gustin *et al.* 1998; Marini *et al.* 1996; Zarzov *et al.* 1996). This may be due to a distinct role for Cdc28p in activating the pathway or may be a consequence of bud emergence, which is inititiated by Cdc28p activity. Since *MID2* over-expression relieves the temperature sensitivity phenotype of the *swi4Δ* cells, as does over-expression of *HCS77* or *PKC1* - it suggests a role for the cell integrity pathway during bud emergence (section 3.2.1; Gray *et al.* 1997). *MID2* over-expression also leads to activation of Skn7p (Ketela *et al.* 1999). Skn7p activity is sufficient for suppression of the *swi4^{ts}swi6Δ* growth defect to restore expression of *CLN1* and *CLN2* cyclins in these mutants (Bouquin *et al.* 1999). These data suggest that Mid2p in a separate complex from the Pkc1p pathway can interact with Rho1p and Skn7p leading to increased cyclin expression and cell cycle regulation at START. It is possible that the activation of the cell integrity pathway, during polarised growth occurs in response to changes in the cell surface resulting from bud formation, rather than as a direct response to the cell cycle machinery. There has been no direct link made between Cdc28p and Mpk1p, suggesting that Cdc28p activation may lead to cell wall changes associated with bud formation independently of the cell integrity pathway. This change in the cell surface may then lead to activation of Mpk1p and Skn7p *via* the cell surface stress sensors Mid2p and/or Hcs77p. The activation of Mpk1p during the G_1/S phase then leads to increase in cell wall gene expression, which is required for formation of the new bud (Igual *et al.* 1996).

Rho1p activation leads to other cellular events, which include regulation of actin cytoskeleton, and cell wall biosynthesis through Pkc1p (Nonaka *et al.* 1995, Helliwell *et*

al. 1998-b; Zhao *et al.* 1998). Rho1p binds directly to and activates *FKS1* (β-1,3-glucan synthase), and can hence control cell wall synthesis directly (Drgonova *et al.* 1996; Qadota *et al.* 1996). In regulation of the actin cytoskeleton, Rho1p interacts with Bni1p, which binds to profilin (Evangelista *et al.* 1997; Kohno *et al.* 1996). Profilin deficient cells display delocalised deposition of cell wall chitin, form buds at random sites and the actin cytoskeleton is drastically altered, the latter being remedied by over-expression of *MID2* (Marcoux *et al.* 1998). Therefore, Mid2p may have a role in regulation of the actin cytoskeleton, through activation of Rho1p. Delley and Hall (1999) have already described the role of Hcs77p in depolarisation of the actin cytoskeleton and Fks1p *via* Pkc1p, but find no role for Mid2p in this process. It is possible that these two putative cell surface sensors in response to cell surface stress control cell morphogenesis through Rho1p and independently or *via* Pkc1p.

How do Mid2p and Hcs77p sense cell surface stress? One possible mechanism, by which yeast cells sense surface stress, is through detection of mechanical changes in the cell wall. Kamada *et al.* (1995) have proposed that the Pkc1p pathway is activated by Rho1p, through mechano-sensors which sense an outward stretch of the plasma membrane. This outward stretch is proposed to be induced by turgor pressure, which then leads to Rho1p activation. Hcs77p and Mid2p perhaps function as the mechano-sensors to activate Rho1p and the Pkc1p pathway. Mid2p and Hcs77p show topological similarity to mammalian integrins, in that they possess a large extracellular domain, a single membrane spanning domain and a small cytoplasmic region (Alberts *et al.* 1994). Integrins mediate interactions between the cell and the environment, they interact with the actin cytoskeleton hence regulating shape, orientation and movement of cells. Integrins mediate these changes in the cell through RhoA (Alberts *et al.* 1994; Clarke and Brugge 1995). As sensors of cell surface stress, Hcs77p and Mid2p may mediate cell surface change to the different stress response pathways, analogous to mammalian integrins.

6.3. Mid2p and Hcs77p are upstream regulators of Pkc1p-MAPK pathway during the mating response

When yeast cells are exposed to mating pheromone, the Pkc1p-MAPK pathway is activated (Zarzov *et al.* 1996). There are several lines of evidence showing that Mid2p

and Hcs77p are upstream regulators of the Pkc1p-MAPK pathway during the mating response. First, *mid2Δ* cells display a pheromone-induced death (section 4.2.1; Ono *et al.* 1994). Second, this pheromone-induced death can be partially suppressed by over-expression of downstream components of the Pkc1p-MAPK pathway (section 4.2.3). In particular over-expression of *HCS77* in *mid2Δ* cells can compensate for lack of *MID2* during α-factor exposure (section 4.2.3; Ketela *et al.* 1999; Rajavel *et al.* 1999). Third, cells deficient for *HCS77* also display a pheromone-induced death, though this is not as severe as the death of *mid2Δ* cell in α-factor (section 4.2.5; Stirling and Stark 2000). Fourth, the death of *hcs77Δ* cells in α-factor, can be suppressed by over-expression of *MID2*, emphasising the overlapping role of Hcs77p and Mid2p during the mating response (section 4.2.6). Fifth, the pheromone-induced death of *hcs77Δ* cells can also be suppressed by over-expression of downstream components such as *PKC1* and *RHO1* (section 4.2.6). Sixth, Mpk1p activation assays show that both *mid2Δ* and *hcs77Δ* cells are defective for activation of Mpk1p in response to mating pheromone (section 4.2.4 and 4.2.7). Seventh, *mid2Δhcs77Δ* cells, cannot activate Mpk1p in response to mating pheromone, indicating that these two putative cell surface sensors have a redundant role in activation of Mpk1p in response to α-factor (section 4.2.8).

The data presented herein, supports a model in which the whole of the Pkc1p-MAPK pathway is required to maintain viability/integrity during mating pheromone exposure. Firstly, *mpk1Δ* cells die in presence of mating pheromone (section 4.2.2; Errede *et al.* 1995). Second, death of *mpk1Δ* cells, similar to death of *mid2Δ* and *hcs77Δ* cells is not osmotically remedial (section 4.2.14). Third, *mid2Δmpk1Δ* cells show similar death kinetics as *mid2Δ* cells, suggesting that the death of *mid2Δ* cells in α-factor may not be dependent on a defective parallel pathway (section 4.2.2). Fourth, *mid2Δ* and *hcs77Δ* cells are defective in α-factor-induced activation of Mpk1p. These results suggest that *mid2Δ* cells die in α-factor at least in part due to a defective Pkc1p-MAPK pathway.

These data together suggest a model in which the Pkc1p-MAPK pathway becomes activated when exposed to α-factor, through the two putative cell surface sensors Hcs77p and Mid2p. In this model, the Pkc1p-MAPK pathway is activated by these mechanosensitive sensors as a result of surface stress induced by shmoo formation and

also as a direct result of cell wall remodeling that occurs in preparation for cell-cell fusion. Therefore, analogous to their vegetative role, Hcs77p and Mid2p have a redundant role in activation of the Pkc1p-MAPK pathway, in response to mating pheromone. Buehrer *et al.* (1997) have suggested that the protein product of an unknown Ste12p-activated gene is required to activate Mpk1p in response to mating pheromone. However, activation of the Pkc1p-MAPK pathway *via* the two cell surface sensors is more likely to be due to mechanical stress exerted by shmoo formation, rather than due to increased expression of the sensors. Unlike *MID2*, *HCS77* does not contain PRE elements in it 5' region and hence its expression is unlikely to be upregulated by pheromone. If increased expression of *MID2* was sufficient to activate the Pkc1p-MAPK pathway, then Hcs77p would not be required for activation of this pathway during the mating response. Therefore, it is possible, that the production of such Ste12p dependent factor, may be required to initiate changes in the cell surface. In a model in which, cell surface change leads to activation of the Pkc1p-MAPK pathway during the mating response, transcriptional activation of one or more PRE-containing genes encoding cell wall remodeling proteins may be required to initiate this cell surface change.

6.4. The role of Pkc1p-MAPK pathway during mating

In this section, the data presented suggests a model in which the Pkc1p-MAPK pathway is required for maintaining cell integrity and viability, as a direct result of cell wall remodeling during shmoo formation.

Evidence presented herein suggests that the cause of mating pheromone-induced death in Pkc1p-MAPK pathway mutants is not a consequence of cell polarisation (shmoo formation). First, prevention of cell polarisation in *mid2Δ* cells by latrunculin-A, does not prevent pheromone-induced death of these cells (section 4.2.9). Second, *mid2Δbni1Δ* cells, which are also defective for shmoo formation, show similar kinetics of death as *mid2Δ* cells in α-factor (section 4.2.9; Evangelista *et al.* 1997). Third, treatment of *hcs77Δ* cells with latrunculin-A, does not prevent α-factor-induced death of these cells (section 4.2.9). Fourth, prevention of polarisation in *mpk1Δ* cells with latrunculin-A, does not prevent α-factor-induced death of these mutants (section 4.2.9). The pheromone-induced death of Pkc1p-MAPK pathway mutants may be the consequence of changes that

occur in the cell wall during events leading to polarisation which renders the survival of these mutants and not the polarisation event itself.

The following data show that the death of the cell integrity pathway mutants in α-factor is not because of chitin deposition defects. First, *mid2Δ* cells have no defect in chitin deposition when exposed to α-factor (section 4.2.11). Second, *hcs77Δ* cells display normal chitin deposition in response to mating pheromone (section 4.2.11). Third, analysis of chitin deposition in *mpk1Δ* cells by calcofluor staining shows that these mutants have no defect in chitin deposition in response to α-factor (section 4.2.11). These results are consistent with reports that *mid2Δ* and *hcs77Δ* cells do not have a defect in chitin ring formation during vegetative growth (Bettignies *et al.* 1999). These data suggest that defects associated with mutants of the Pkc1p-MAPK pathway, are not associated with chitin deposition. Recent reports by Ketela *et al.* (1999) has suggested that Mid2p is involved in chitin synthesis. These authors measured the chitin content of *mid2Δ* cells exposed to α-factor for two hours. However, more than half the *mid2Δ* population dies after two hours of pheromone exposure (section 4.2.1). Therefore, the chitin levels measured by these authors may not have represented the real level of chitin in *mid2Δ* cells.

There are several lines of evidence which suggest that the Pkc1p-MAPK pathway mutants are not defective for actin localisation during shmoo formation. First, actin localisation, detected by rhodamine phloidine staining shows that *mid2Δ* cells are not defective for actin patch localisation (section 4.2.12). Second, *hcs77Δ* cells show normal actin patch localisation (section 4.2.12). Third, rhodamine phaloidin staining of *mpk1Δ* cells reveals that these mutants display normal actin patch localisation (section 4.2.12). These results are consistent with recent publications indicating that *mid2Δ* and *hcs77Δ* cells do not have a defect in actin organisation (Bettignies *et al.* 1999).

Do Pkc1p-MAPK pathway mutants die in α-factor as a direct result of cell wall defects? Results in chapter four have provided evidence that mutants of the Pkc1p-MAPK pathway die in presence of α-factor as a result of cell lysis, that may be associate with weak cell walls. First, *mid2Δ* cells display cell lysis, that correlates with cell death in presence of

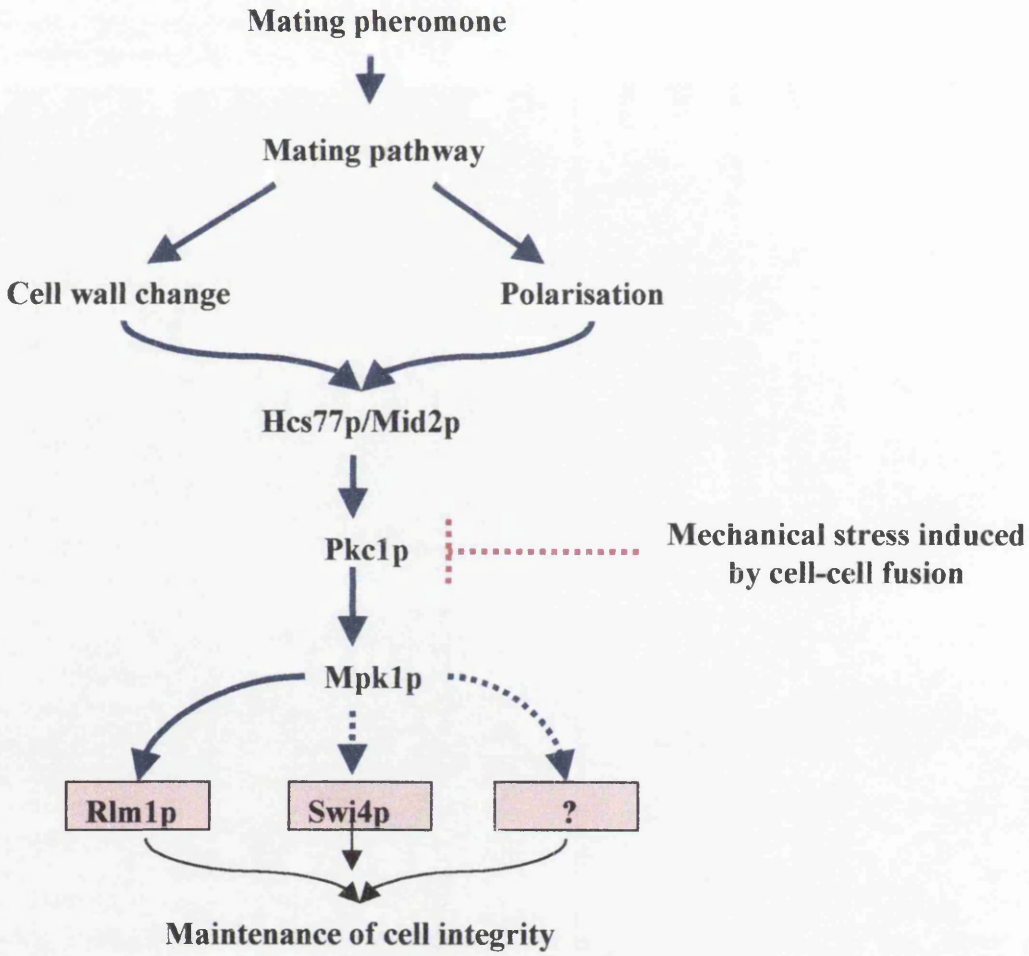

Figure 6.1. Proposed model for the role of Pkc1p-MAPK pathway during the mating pheromone response. Solid arrows indicate activation, dashed arrows and lines are speculative and lines with bars indicate inhibition. See text for details.

mating pheromone (section 4.2.13). Second, *hcs77Δ* cells also die in α-factor as a result of cell lysis (section 4.2.13). Third, propidium iodide staining reveals that *mpk1Δ* cells die in α-factor as a result of cell lysis (section 4.2.13). As described previously this cell wall weakness may not be associated with a defect in chitin deposition. This cell lysis phenotype of Pkc1p-MAPK pathway mutants in α-factor is similar to the vegetative phenotype of some of these mutants, at high temperatures (section 4.2.13). Unlike the vegetative phenotype of Pkc1p-MAPK pathway mutants, this α-factor-induced cell lysis is not remedial by high osmolarity (section 4.2.14). Taken together, these results suggest a model in which the Pkc1p-MAPK pathway responds to and actively maintains cell integrity during shmoo formation. This model suggests that there is a change in the context during pheromone exposure, partly caused by the pheromone response pathway and partly induced by remodeling of the cell wall. This possibility is favoured, because one can envisage the pheromone response pathway causing change in the cell wall in preparation for cell-cell fusion. The Pkc1p-MAPK pathway would be required to maintain the viability of this unique state of the cell.

The data presented herein show that the Pkc1p-MAPK pathway does not regulate cell-cell fusion. Philips and Herskowitz (1997) have proposed that Pkc1p regulates cell-cell fusion. Before cell-cell fusion can occur, intervening cell wall material must be removed. However, removal of cell wall material prematurely can lead to cell lysis. One possibility raised by the work of Philips and Herskowitz (1997) is that secretion of glycerol at the shmoo tip leads to hyper-osmotic state between two mating partners in close proximity. This high osmolarity condition may then inactivate the Pkc1p pathway in the mating partners, allowing dissolution of the cell walls and cell-cell fusion. The data presented herein suggests that the Pkc1p-MAPK pathway does not regulate cell-cell fusion by this mechanism. First, cells treated with α-factor and then exposed to higher osmolarity do not lyse (section 4.2.15). According to the Philips and Herskowitz (1997) model, one would have expected polarised cells to lyse after osmotic stabilisation, as the cell integrity pathway would be inactive, leading to weak cell walls. Second, contrary to expectation Mpk1p remains active in mating pheromone-treated cells when osmotically stabilised (section 4.2.15; Kamada *et al.* 1995). Although the secretion of glycerol is important for efficient cell-cell fusion, our data suggests that the Pkc1p pathway is not involved in this glycerol-dependent mechanism. Hyper-activation of the Pkc1p pathway leads to arrest as

prezygotes. This result may reflect a role for the Pkc1p pathway in cell-cell fusion or it may be an artifact of hyper-activated Pkc1p leading to cell wall physically incapable of undergoing cell-cell fusion. It remains a possibility that the Pkc1p pathway regulates cell-cell fusion by being inactivated by direct cell-cell contact. This possibility can be addressed by monitoring Pkc1p activity on a single cell basis during cell-cell fusion and in mutants that arrest as prezygotes, such as *fus1Δfus2Δ* cells.

6.5. The Ca^{2+} / calcineurin pathway responds to cell surface stress

Analogous to the Pkc1p-MAPK pathway, the calcineurin-signalling pathway in yeast is active when treated with α-factor, or heat- / hypo-osmotically shocked (Beeler *et al.* 1997; Buehrer *et al.* 1997; Davenport *et al.* 1995; Withee *et al.* 1997; Zhao *et al.* 1998). The Pkc1p-MAPK pathway is also activated in cells treated with the membrane deforming drug chlorpromazine, tunicamycin treatment resulting in secretion blocks, addition of the cell wall degrading enzymes zymolyase and calcofluor-white treatment which interferes with the make up of the cell wall (Buehrer *et al.* 1997; Davenport *et al.* 1995; De Nobel *et al.* 2000; Kamada *et al.* 1995; Ketela *et al.* 1999; Yun *et al.* 2000). Evidence presented herein shows that the calcineurin pathway is also activated in response to these treatments (section 5.2.1). Therefore, the calcineurin pathway appears to monitor cell surface stress consistent with its known role in regulating cell wall synthesis by regulating the expression of some cell wall genes (Zhao *et al.* 1998). Furthermore, this role of calcineurin is consistent with the observation that Mid1p, an upstream activator of calcineurin pathway, is a mechanosensitive Ca^{2+} channel. Cells appear to respond to cell surface stresses, by two signalling pathways (Pkc1p and calcineurin) involved in reinforcing the cell surface. Therefore, the Pkc1p-MAPK and the calcineurin pathway are co-regulated in response to cell surface stress.

6.6. Mid1p is a putative channel for Ca^{2+} influx during cell surface stress

Calcineurin activation usually results from increased influx of extracellular calcium (Withee *et al.* 1997). In response to mating pheromone for example, cells require influx of calcium for viability, presumably *via* activation of calcineurin. Calcineurin mutants lose viability in response to α-factor (Iida *et al.* 1990; Withee *et al.* 1997). Evidence

presented herein shows that that Mid1p functions as a putative Ca^{2+} channel allowing Ca^{2+} influx as a result of cell surface change during cell surface stress. First, in exposure to cell surface damage associated with chlorpromazine and tunicamycin, cells show stimulated calcium influx (section 5.2.2). Hence, activation of calcineurin in response to cell surface stress appears to require the influx of calcium, similar to that found during α-factor treatment of cells. In response to mating pheromone, calcium influx occurs by the putative calcium channels Mid1p and Cch1p (which is similar to the α-subunit of the mammalian voltage gated calcium channels; Fischer *et al.* 1997; Iida *et al.* 1994). No *bona fide* calcium channel has yet been identified in yeast and therefore Mid1p and Cch1p are the only possible candidates for this function. Second, Mid1p and Cch1 appear to have a redundant role in allowing calcium influx, and have been therefore proposed to form components of one calcium channel (Fischer *et al.* 1997). Third, Mid1p has been shown to function as a stretch-activated cation channel that is permeable to calcium in Chinese hamster ovary (CHO) cells (Kanzaki *et al.* 1999). Expression of Mid1p in CHO cells confers sensitivity to mechanical stress that results in increase in both calcium conductance and the concentration of free cytosolic calcium. Fourth, *bni1Δ* cells (which cannot form shmoos) show no stimulated Ca^{2+} uptake in response to α-factor treatment (section 5.2.4; Evangelista *et al.* 1997). The final line of evidence is that influx of calcium in response to tunicamcyin occurs *via* Mid1p (section 5.2.3). However, Mid1p is not responsible for all of the increased calcium influx during cell surface stress or α-factor treatment (section 5.2.3). Therefore, another calcium channel may be contributing to this induced calcium influx.

6.7. The calcineurin pathway has an independent surface sensing mechanism from that of the Pkc1p-MAPK pathway

Evidence presented herein indicates that the cell surface sensors for the calcineurin pathway are distinct from those of the Pkc1p-MAPK pathway. First, *mid2Δ* cells are not defective for calcineurin activation in response to cell surface stress (section 5.2.6). Second, *hcs77Δ* cells display normal calcineurin activation when exposed to cell surface stress (section 5.2.6). Third, *mid2Δhcs77Δ* cells exposed to cell surface stress are able to activate calcineurin in response to cell surface stress, similar to the congenic wild-type cells (section 5.2.6). Fourth, *wsc1Δwsc2Δwsc3Δ* cells are not defective for stress induced

calcium influx (section 5.2.7). Finally, calcium influx is stimulated by cell surface damage (sections 5.2.3 and 5.2.4). However, evidence presented herein shows that activation of the Pkc1p-MAPK pathway in response to cell surface stress does not require Ca^{2+} influx. In the absence of extra-cellular calcium, cells can still activate Mpk1p in response to heat shock (section 5.2.5). Therefore calcium influx is not required for heat activation of the Pkc1p-MAPK pathway. There has been suggestions that high extra-cellular calcium activates the Pkc1p-MAPK pathway (Mizunuma *et al.* 1998). This report by Mizunuma *et al.* (1998) suggests that the activation of the Pkc1p-MAPK pathway in response to cell surface stress would require influx of calcium. The results discussed above and in section 5.2.5 indicates that the calcium-signalling pathway does not operate upstream of the Pkc1p-MAPK pathway. In fact, cells defective for both the calcineurin and the Pkc1p-MAPK signalling pathways are inviable, indicating that these two pathways are partially redundant (Garrett-Engele *et al.* 1995; Nakamura *et al.* 1996). Furthermore, these results indicate that the cell responds to cell surface stress by two independent mechanisms which involve: activation of the cell integrity pathway *via* integrin-like surface proteins and activation of the calcineurin pathway *via* calcium influx though mechano-sensitive cation channels.

Results presented in chapter four, show that the entire Pkc1p-MAPK pathway is dispensable for the activation of the calcineurin pathway. Cells lacking *MPK1* show similar calcineurin fold activation as wild-type cells when exposed to cell surface stress (section 5.2.8). Cells lacking both cell surface sensors Mid2p and Hcs77p are not defective for activation of the calcineurin pathway in response to cell surface stress (section 5.2.6). Furthermore, Northern analysis of *FKS2* expression in *mid2Δhcs77Δ* and *mpk1Δ* cells supports a mechanism by which transcriptional regulation of cell wall associated genes can occur independently of the Pkc1p-MAPK pathway during stress response (section 5.2.9).

6.8. The Pkc1p-MAPK modulates the calcineurin-signalling pathway

In spite of separate sensing mechanisms, data presented in section 5.2.10 indicates that the Pkc1p-MAPK pathway modulates calcineurin activity. First, *mid2Δhcs77Δ* cells have a severely reduced basal calcineurin activity in comparison to the congenic wild-type cells

(section 5.2.10). Second, the basal calcineurin activity in *mpk1Δ* cells is severely compromised (section 5.2.10). These data suggest a model in which the two pathways are co-regulated to "fine tune" the response of the cell to a surface stress. In this model the Pkc1p-MAPK pathway does not affect the inducibility of the calcineurin pathway but rather sets the basal activity of calcineurin signalling. Thus, it can be envisaged that cell surface stress activates the Pkc1p-MAPK pathway and calcium-signalling pathway by separate mechanisms, but activation of the Pkc1p-MAPK pathway reinforces the calcium-signalling pathway. This interconnection may ensure an efficient response to cell surface stress. Furthermore, these data suggest that the calcium-signalling pathway is functioning downstream of the Pkc1p-MAPK pathway signalling.

6.9. The Pkc1p-MAPK pathway is a regulator of calcium homeostasis

Calcineurin activation requires influx of extracellular calcium. Data presented herein indicate that the Pkc1p-MAPK pathway modulates calcineurin activity by regulating Ca^{2+} homeostasis by inhibiting calcium efflux from the cell. First, *mpk1Δ* cells are not defective for initial calcium influx (section 5.2.11). Second, *mpk1Δ* cells are defective for calcium accumulation (section 5.2.11). After the initial influx of calcium into cells, *mpk1Δ* cells fail to accumulate calcium to the same extent as wild-type cells (section 5.2.11). Third, this failure to accumulate calcium correlates with high calcium efflux in *mpk1Δ* cells (section 5.2.12). Fourth, cells expressing the hyper-activated form of *PKC1* display reduced Ca^{2+} efflux (section 5.2.13). By regulating calcium efflux, the Pkc1p-MAPK pathway can regulate the level of calcineurin activity in response to incoming calcium. Therefore, activation of the Pkc1p-MAPK pathway leads to and is sufficient for reduction in activity of the calcium efflux machinery. Furthermore, the Pkc1p-MAPK pathway regulates calcium homeostasis without affecting calcium influx.

The following data indicate that the Pkc1p-MAPK pathway regulates calcium homeostasis independently of the calcineurin-signalling pathway. First, Ca^{2+} efflux is normal in cell treated with cyclosporin-A (section 5.2.15). Second, hyper-activation of the calcineurin-signalling pathway does not change the Ca^{2+} efflux kinetics of cells when compared to cells expressing full-length calcineurin (section 5.2.15). Therefore, high calcium efflux in *mpk1Δ* cells is the cause of reduced basal calcineurin activity rather than as a result of it.

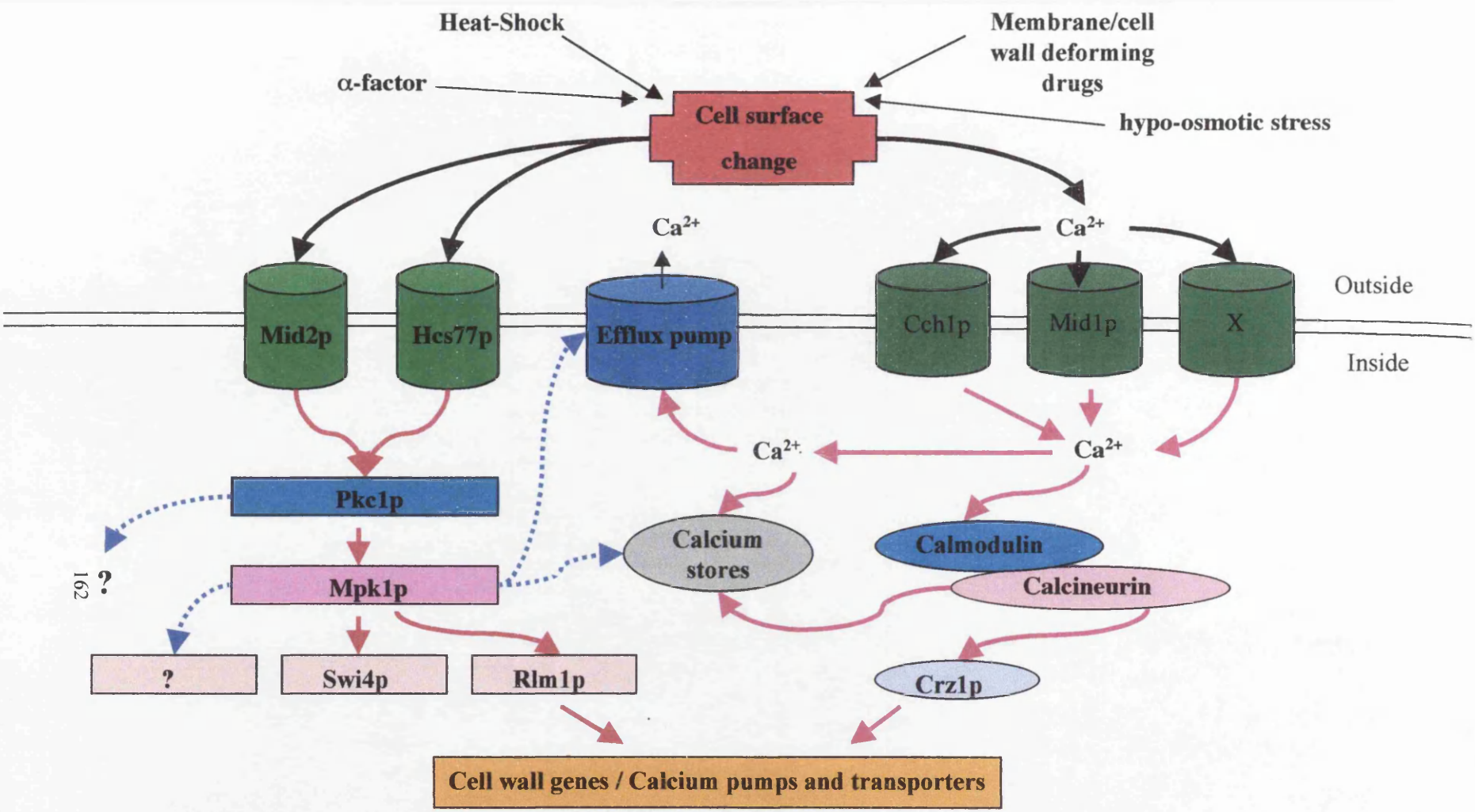

Figure 6.2. The co-regulation of the Pkc1p-MAPK pathway and the calcineurin pathway in response to cell surface stress. Solid arrows indicate activation and dashed arrows are speculative, indicating also that other components may be involved. See text for details.

The calcineurin pathway is known to regulate calcium homeostasis transcriptionally by monitoring expression of *PMC1* and *PMR1*, but also post-transcriptionally by monitoring activity of Vcx1p (Cunningham and Fink 1996; Matheos *et al.* 1997; Stathopoulos and Cyert 1997). The Pkc1p-MAPK pathway appears not to control these calcium pumps at the transcriptional level, as observed by Northern analysis (Data not shown). It seems however, that the Pkc1p-MAPK pathway exerts its effect on calcium homeostasis, post-transcriptionally, as *rlm1Δ* cells in which most of the Pkc1p-MAPK pathway dependent gene expression is abolished, display no defect in basal calcineurin activity (section 5.2.16). Our data suggest a model in which the Pkc1p-MAPK pathway functions upstream of the calcium signalling pathway, and may be involved in phosphorylating and thereby activating an efflux factor, leading to its inhibition. This efflux factor is not Mid1p, as *mid1Δ* cells show normal Ca^{2+} efflux from the cells (section 5.2.14). The Pkc1p-MAPK pathway may also be involved in regulation of the Ca^{2+} stores, thereby influencing accumulation of Ca^{2+} within cells. However, depletion of cytoplasmic calcium in *mpk1Δ* cells may itself lead to calcium efflux from calcium stores to replenish the cytoplasmic concentration of calcium. In this model, the Pkc1p-MAPK pathway may only be regulating a calcium efflux factor. This regulation of calcium homeostasis by Mpk1p, does not appear to be as a direct result of cell wall defects, as an activated allele of *PKC1*, reduces calcium efflux in wild-type cells in comparsion to cells expressing the full length *PKC1* (section 5.2.13). Indeed calcium influx remains unchanged in cells expressing the activated allele of *PKC1*, indicating that efflux of calcium in *mpk1Δ* cells is a direct result of calcium efflux regulation rather than a cell wall defect (data not shown).

There already exists a Ca^{2+} hemeostasis regulation mechanism in mammalian cells, involving the PKC and the ERK pathway. In mammalian cells, PKC regulates the activity of many channels, including Ca^{2+} and K^{+} channels (Ma *et al.* 2001). The ERK pathway in human platelets is proposed to regulate store mediated calcium entry (SMCE), independently of PKC (Rosado and Sage 2001). SMCE is a mechanism for Ca^{2+} influx, in response to depletion of Ca^{2+} stores. This regulation is thought to occur by a physical and reversible coupling of the endoplasmic-reticulum with the plasma membrane. Other studies have shown that activation of PKC stimulates Ca^{2+} entry in platelets, without evoking the release of Ca^{2+} from intracellular stores (Rosado and Sage 2000). However, no study thus far has revealed a connection between Ca^{2+} efflux, the protein kinase C and

the MAPK cascades. Our understanding of regulation of calcium efflux in yeast, by the Pkc1p-MAPK pathway, is hindered by the fact that no efflux factor has yet been identified in yeast. However, Ca^{2+} exchangers and pumps have been identified in the plasma membranes of mammalian cells, responsible for efflux of Ca^{2+} (Carafoli *et al.* 1999). Further studies into Ca^{2+} homeostasis in yeast may identify similar factors in yeast, which would lead to discovery of more direct interactions between it and the cell integrity pathway.

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