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Cell Fusion During Mating in Saccharomyces cerevisiae

by

Jennifer Philips

DISSERTATION

Submitted in partial satisfaction of the requirements for the degree of

DOCTOR OF PHILOSOPHY

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of the

UNIVERSITY OF CALIFORNIA

San Francisco

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CELL FUSION DURING MATING IN *SACCHAROMYCES CEREVISIAE*

JENNIFER PHILIPS

ABSTRACT

Successful zygote formation during yeast mating requires cell fusion of the haploid mating partners. To ensure that cells do not lyse while mating, the cell wall is degraded only after cell-cell contact and only in the region of contact. The signals that control cell wall degradation and the machinery that mediates this process are poorly understood. To understand how cell fusion is regulated, mutants defective in cell fusion were identified. Two mutants were defective in the *FPS1* gene, which codes for a glycerol facilitator (Luyten et al., 1995). Additional analyses indicated that their defect results from inability to regulate osmotic balance, suggesting that cells monitor their osmotic state before committing to breaking down their cell wall. We also observed that protein kinase C, previously recognized for its role in osmotic regulation (Davenport et al., 1995), negatively regulates cell fusion, further linking osmosensing pathways to regulation of cell fusion. We propose that high intracellular osmolarity activates Pkc1p, which mediates a checkpoint that inhibits cell wall breakdown.

To identify additional genes involved in cell fusion, we looked for genes whose overexpression relieved the defect in cell fusion caused by hyperactive protein kinase C. This strategy led to the identification of two highly similar, novel genes, *KEL1* and *KEL2*, that encode proteins composed of two domains,

one containing six kelch repeats, a motif initially described in the *Drosophila* protein Kelch (Xue and Cooley, 1993), and the other predicted to form coiled coils. Both Kel1p and Kel2p localize to the site where cell fusion occurs during mating and to regions of polarized growth during vegetative growth. Co-immunoprecipitation and two-hybrid analyses indicate that Kel1p and Kel2p physically interact. Despite this association, only *kel1Δ* mutants exhibit a phenotype, being defective in cell morphology and cell fusion. We suggest that during mating, Pkc1p monitors the osmotic and morphologic integrity of the cell. If cells are not osmotically stable, as in *fps1Δ* mutants, or exhibit disrupted morphology, as in *kel1Δ* mutants, Pkc1p inhibits cell fusion.

CELL FUSION DURING MATING IN *SACCHAROMYCES CEREVISIAE*

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CHAPTER ONE

INTRODUCTION

Cell fusion occurs during several biological events such as muscle development when myoblasts fuse to form a syncytial myotube and in sexual reproduction when gametes fuse. In the yeast *Saccharomyces cerevisiae*, two haploid cells fuse during mating to produce a diploid zygote. Intercellular fusion requires the successful completion of a series of events, beginning with cell-cell recognition and culminating in fusion of plasma membranes and intracellular organelles. For the plasma membranes to make contact, extracellular material separating the interacting cells must be eliminated. In particular, the zona pellucida surrounding the egg, extracellular matrix material separating myoblasts, and cell wall material separating haploid yeast must be removed. Cell-cell contact is important in triggering these events, but the molecular details are poorly understood. This thesis describes the progress I have made in understanding the control of cell fusion in yeast. Through the identification and characterization of genes involved in this process, we are beginning to understand some of the signals which regulate cell fusion. The hope is that understanding cell fusion in yeast will provide insights into cell fusion in other systems and, more generally, into how cells respond to cell-cell contact.

The mating pathway in *Saccharomyces cerevisiae* is initiated when haploid cells (**a** and α cells) respond to the peptide pheromone secreted from the opposite cell type and culminates in the formation of an **a**/α diploid zygote (Fig. 1-1). The pheromones cause cells to arrest the cell cycle in G1, induce transcription of genes required for mating, and undergo a mophological alteration called shmoo formation (for review, see Bardwell et al., 1994; Herskowitz, 1995). A good deal of work, discussed in more detail below, has been done to understand the responses to pheromone. Considerably less is known about the events that occur after cell-cell contact, including cell wall

Figure 1-1. Steps in Mating in *Saccharomyces cerevisiae*

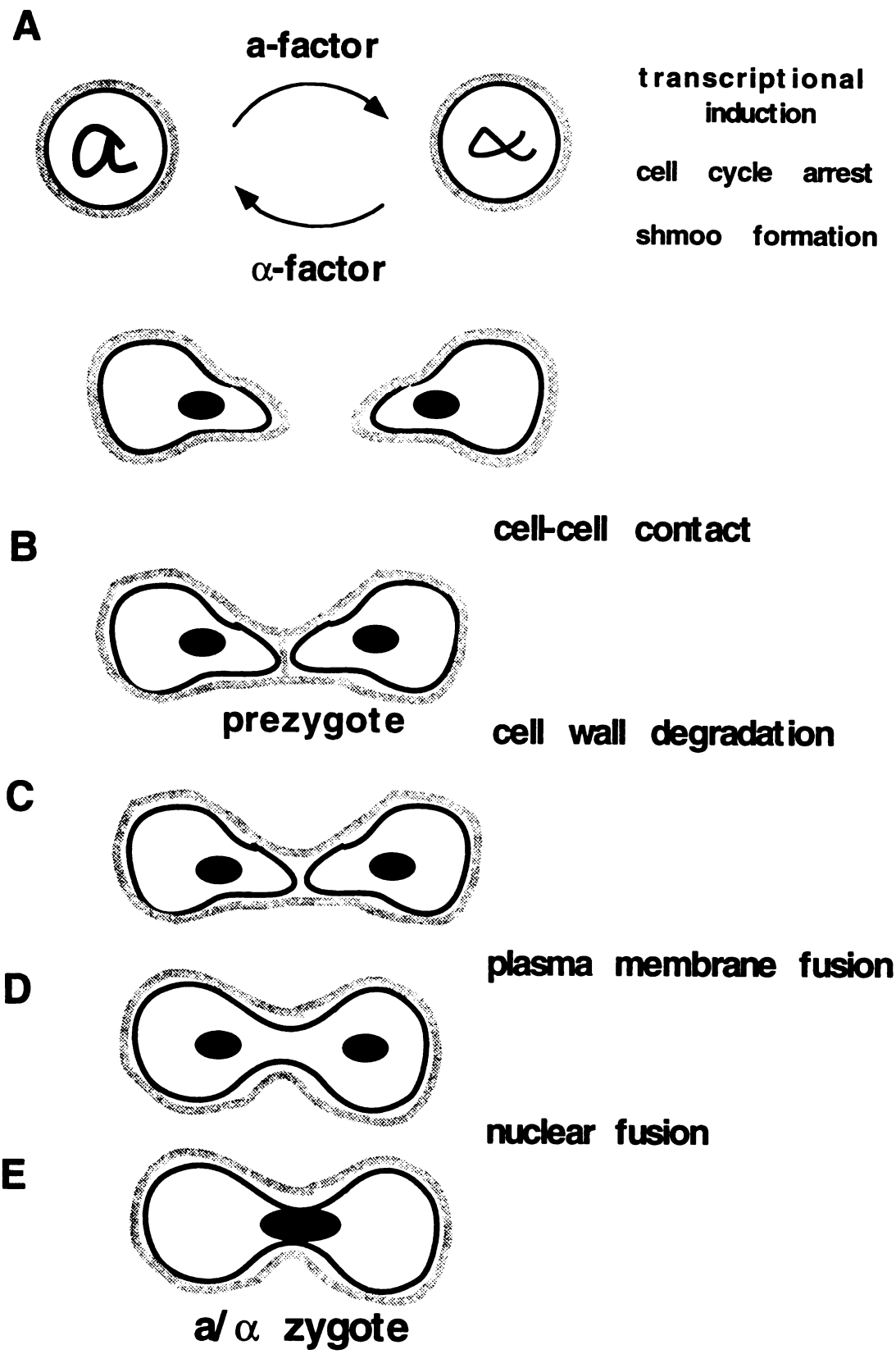
A. Cells respond to peptide pheromones by inducing a morphological response called shmoo formation, cell cycle arrest, and transcriptional activation.

B. Cells grow towards each other until they make contact. A structure in which cells are adhered and the intervening cell wall is intact is termed a prezygote. The cell wall is indicated in gray; the plasma membrane is in black.

C. Cell wall degradation allows the plasma membranes to come into direct contact.

D. Plasma membrane fusion results in a heterokaryon.

E. Fusion of intracellular organelles produces an **a**/α diploid zygote.


A
a-factor
a
α
α-factor
transcriptional induction
cell cycle arrest
shmoo formation
B
cell-cell contact
prezygote
cell wall degradation
C
plasma membrane fusion
D
nuclear fusion
E
a/α zygote

degradation, plasma membrane fusion, and fusion of intracellular organelles. The focus of this thesis is how cell contact triggers cell wall degradation.

During conjugation, cells use a pheromone gradient to grow towards their mating partner until they come into contact (Jackson and Hartwell, 1990a; 1990b; Dorer et al., 1995; Segall, 1993). The agglutinin proteins mediate adhesion between partners which is reversible by sonication (Lipke and Kurjan, 1992). The cell walls then become irreversibly adhered, at which point the mating partners can be separated from each other only if the cell wall is removed (Trueheart et al., 1987). Once cells make contact with each other, the cell wall is removed beginning in the center of the region of contact and preceeding towards the edges (Osumi et al., 1974; Gammie et al., submitted). Since the cell wall is not degraded with pheromone treatment, some signal must be generated when cells make contact that initiates cell wall remodeling. It is important that the cell wall be removed in a highly regulated manner; if it is degraded in the wrong place or at the wrong time, then cells will lyse. Mutants defective in cell fusion accumulate dumbbell-shaped structures called prezygotes in which cells are adhered, but the intervening cell wall remains, presenting a barrier to plasma membrane fusion, cytoplasmic mixing, and nuclear fusion (Trueheart et al., 1987; McCaffrey et al., 1987).

The molecular events involved in triggering cell wall degradation in response to cell contact are poorly understood. In principle, all the machinery required for cell wall degradation could be extracellular and could be activated by cell-cell contact. For example, extracellular enzymes in the cell wall of an **a** cell could be activated by processing enzymes in the wall of an α cell. Another possibility is that cell-cell contact generates an intracellular signal that activates the cell fusion machinery. The intracellular signal could

regulate cell wall degradation by altering the balance of synthetic and degradative activities, modulating their synthesis, activity, or localization. This thesis describes the progress we have made in understanding the signals that regulate cell fusion. From such work, it is clear that intracellular signalling molecules regulate the cell fusion machinery; however, exactly what constitutes the machinery remains largely obscure.

The remainder of the Introduction is divided into three parts. The first section discusses cell fusion during mammalian fertilization, muscle development, and *Chylamydomonas* mating, focusing on events which may be analogous to cell fusion in yeast. In these examples, the molecular details connecting cell-cell contact, signalling, and cell fusion are emerging but scanty. In most cases, potential signalling molecules have been identified and polarized secretion seems to play a role, but how the two are related in unclear. Next, I return to a discussion of the mating pathway and cell fusion in *Saccharomyces cerevisiae*. Finally, I consider the pathways involved in maintaining cell integrity in yeast.

Mammailan Fertilization

To fertilize an egg, mammalian sperm bind to the zona pellucida (ZP), an extracellular matrix surrounding the egg (for review, see Wassarman, 1995; Snell and White, 1996), which plays an important role in cell-cell recognition, preparing cells for fusion, and preventing polyspermy. Two secretory events, the acrosome reaction and cortical granule exocytosis, modify the zona pellucida during fertilization. Sperm binding to the zona pellucida triggers the acrosome reaction, an exocytotic event which allows the sperm to digest and penetrate the ZP (Fig. 1-2). The acrosome reaction also modifies the sperm cell surface by replacing the original plasma membrane with the inner acrosomal membrane which is thought to be important for fusion

Figure 1-2. Steps in Mammalian Fertilzation

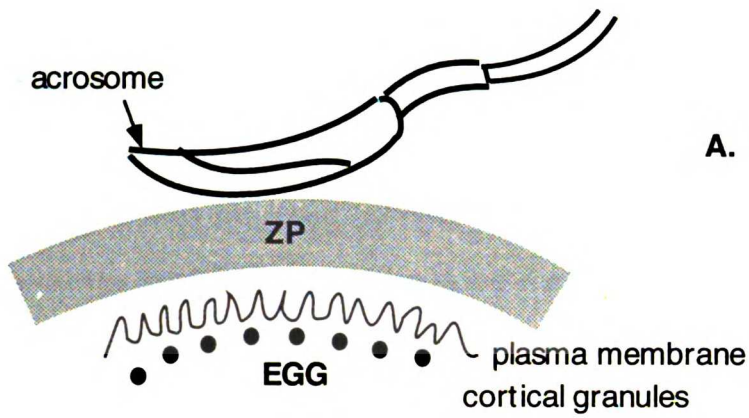
A. Sperm bind to the zona pellucida (ZP).

B. The acrosome reaction allows sperm to penetrate the ZP.

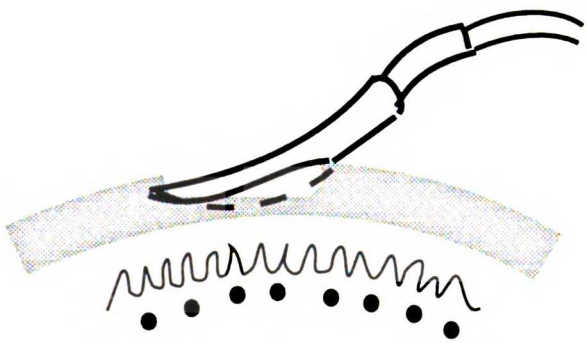
C. Plasma membranes of sperm and egg bind.

D. Plasma membrane fusion occurs, triggering cortical granule exocytosis which modifies the ZP.

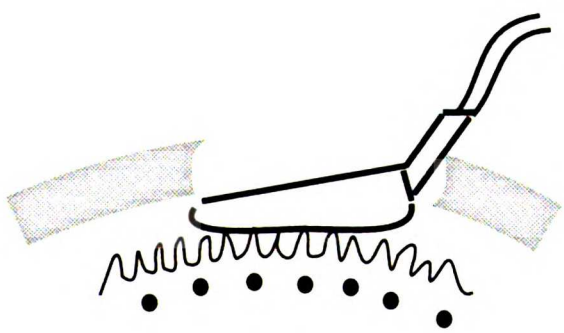
This figure was derived from Snell and White (1996).



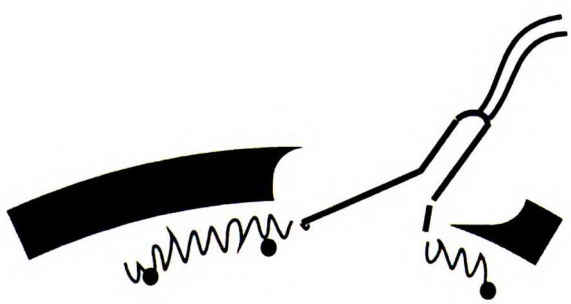
A. ZP binding



B. acrosome reaction ZP penetration



C. plasma membrane binding



D. plasma membrane fusion cortical granule exocytosis

competence (Yanagimachi, 1994). Once sperm contact the egg, the equatorial region of the sperm head fuses with actin-rich regions of the egg plasma membrane that contain microvilli. Fertilin, a disintigrin family member, may mediate plasma membrane binding and/or fusion (see below). Plasma membrane fusion is followed by release of cortical granules from the egg. The contents of the cortical granules remove the ligand on the zona pellucida that mediates sperm binding, thereby making it refractory to further sperm binding.

The ZP and the secretory events that modify it are essential in coordinating fertilization, but how sperm binding triggers the acrosome reaction is poorly understood. Sperm bind to ZP3, a major glycoprotein constituent of the zona pellucida. Several candidate ZP3 receptors on mouse sperm have been identified, including β-1,4-galactosyltransferase (GalTase) (Miller et al., 1992; Gong et al., 1995), p95 (Leyton and Saling, 1989), and sp56 (Bleil and Wassarman, 1990), which are thought to recognize oligosaccharide side chains on ZP3. Transgenic studies in mice may help to clarify the relative importance and role in fertilization of putative receptors. For instance, such analysis has shown that mice lacking ZP3 are sterile (Liu et al., 1996). Males lacking GalTase are not sterile, but their sperm fail to undergo the acrosome reaction in response to ZP3 (Lu et al., 1997). Conversely, mice overexpressing GalTase are hypersensitive to the ZP3-induced acrosome reaction (Youakim et al., 1994).

Potentially important signalling molecules involved in mediating the acrosome reaction include calcium, diacylglycerol, protein kinases, and heterotrimeric G proteins. Sperm binding to the ZP is accompanied by a rise in intracellular calcium and diacylglycerol (Florman, 1994; Roldan and Harrison, 1992), and calcium channel agonists induce acrosomal exocytosis

(Roldan et al., 1994). One of the potential ZP3 receptors, p95, is tyrosine phosphorylated upon ZP3 binding, suggesting a role for protein kinases (Leyton and Saling, 1995). Finally, if sperm are treated with ZP3 or antibodies that cluster GalTase, then the acrosome reaction is triggered in a pertussis-toxin sensitive manner, suggesting the involvement of a heterotrimeric G protein (Gong et al., 1995). How these signalling molecules regulate the machinery involved in acrosomal exocytosis is unclear.

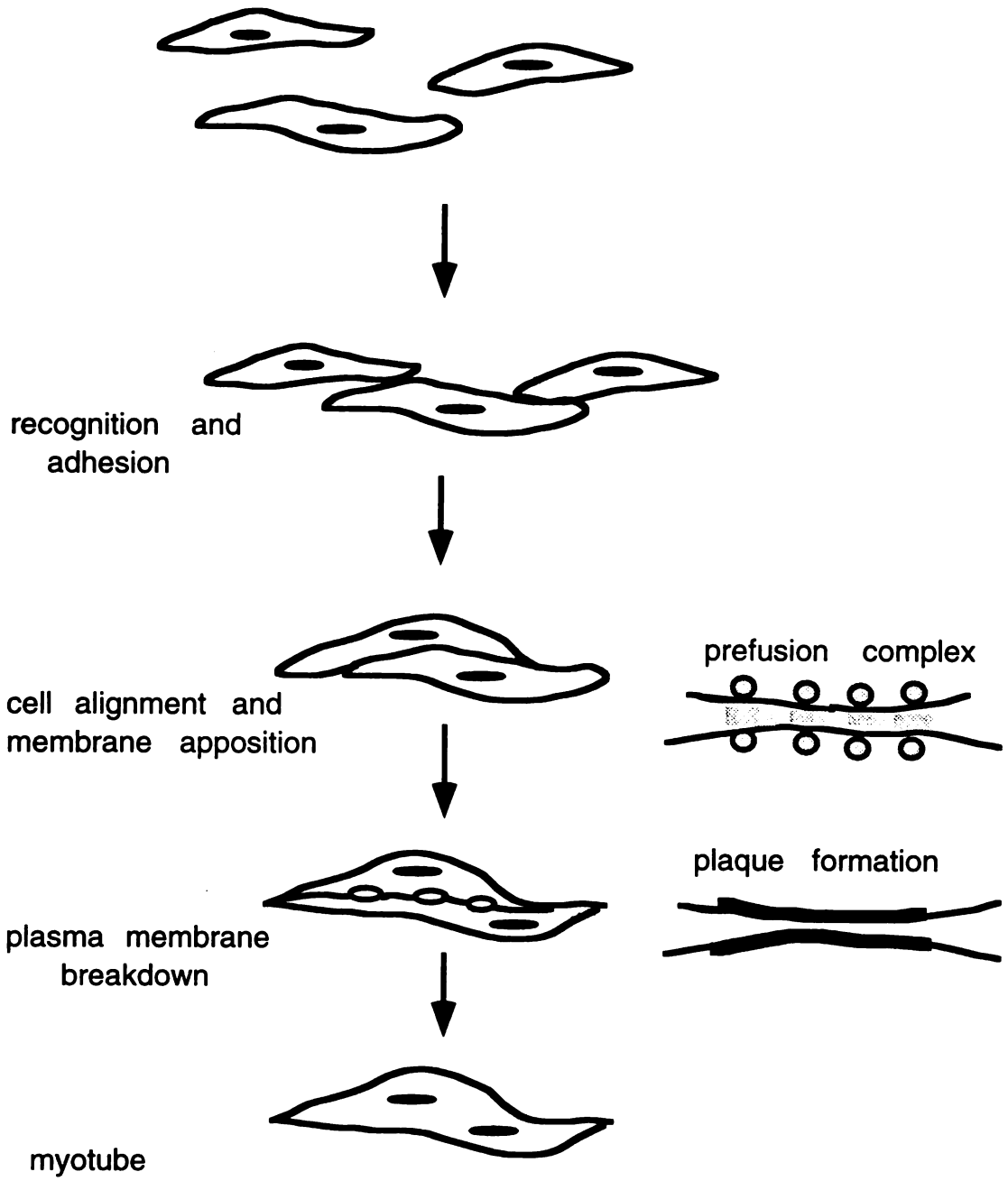
Myoblast Fusion

During muscle development, myoblast fuse to form a syncytial myotube (for review, see Wakelam, 1985). Myoblast fusion requires a cell-cell recognition event since myoblasts fuse in a stereotyped pattern to produce the proper muscle architecture (Fig. 1-3). The process is initiated when myoblasts aggregate and then elongate and align along their long axes. Although myoblasts are not surrounded by a structure similar to the zona pellucida or yeast cell wall, extracellular matrix material has to be removed to allow myoblasts to come into direct contact. In addition, fusion of the plasma membranes may require clearing proteins from the cell surface, permitting close apposition of the fusing membranes. Degradation of extracellular proteins could be accomplished, at least in part, by the metalloendoprotease m-calpain, which may be secreted by fusing myoblasts (Brustis et al., 1994). Additional evidence that secretion is important in myoblast fusion comes from an electron microcopic analysis in *Drosophila* that describes ultrastructural changes during myoblast fusion (Doberstein et al., 1997). Vesicles from adjacent myoblasts align at the plasma membrane in a structure termed the prefusion complex, which is composed of paired vesicles (one vesicle in each cell) and associated electron-dense extracellular material, which may play a role in aligning the vesicles. Electron-dense plaques are

Figure 1-3. Steps in Myoblast Fusion

Myoblasts recognize, adhere, and align with each other. At the ultrastructural level, prefusion complexes and plaque formation are observed. Plasma membranes break down to form a myotube.

This figure was derived from Doberstein et al. (1997).


recognition and adhesion
cell alignment and membrane apposition
prefusion complex
plaque formation
plasma membrane breakdown
myotube

observed which may result from fusion of the paired vesicles with the plasma membrane. Plasma membrane fusion events occur in particular regions of the cell surface, with plasma membrane outside the fusion area remaining intact. How the fusion machinery is localized or locally activated is not known.

Myoblasts fuse in cell culture (Holtzer et al., 1958), making the fusion event accessible to pharmocological manipulation and biochemical analysis. Such studies have identified a variety of agents that disrupt cell fusion and a number of signalling molecules implicated in fusion (reviewed by Knudsen, 1992). For example, a calcium influx is observed prior to fusion and is required for fusion but not for earlier events such as alignment. Nitric oxide synthase (NOS) and cGMP levels rise in fusing myoblasts, and experimental manipulation of NOS activity alters cell fusion (Lee et al., 1994). Although various manipulations alter myoblast fusion *in vitro*, the relative importance of the implicated molecules *in vivo* is unclear. In addition, the tissue culture system has been limited in identifying novel molecules involved in fusion. Analysis of myoblast fusion in *Drosophila* will be useful to identify novel genes and determine which factors are important *in vivo*.

Plasma Membrane Fusion

During cell-cell fusion and viral-cell fusion, the exocytoplasmic leaflets of membrane bilayers are joined. In contrast, during intracellular fusion events, the cytoplasmic leaflets, which differ in compositon and physical properties from the outer leaflets, are joined. Thus, ideas about plasma membrane fusion during intercellular fusion events are derived largely from lessons from the viral model (for review, see Houvila et al., 1996). In the case of influenza virus, the hemagglutinin (HA) protein mediates plasma membrane binding and fusion (for review, see White, 1992). HA, a type I

integral membrane protein which exists as a trimer, undergoes a conformation change as the pH decreases in the endosome, which exposes a fusion domain, a hydrophobic stretch of amino acids which tends to form an amphipathic α helix and β sheet. After contact of the fusion domain with the target membrane, the HA trimers are thought to aggregate, forming a fusion pore. During sperm-egg fusion, the glycoprotein fertilin (also called PH-30) has been proposed to function similarly to the HA peptide during viral fusion (Blobel et al., 1992). Fertilin, the founding member of the ADAM family (containing a disintigrin and metalloprotease domain) of proteins, is a dimer; the fertilin-β subunit includes a disintegrin domain which is thought to bind to an egg integrin. The fertilin-α subunit includes a twenty-two amino acid region resembling the HA fusion domain. The idea is that fertilin-β binds to an egg integrin, causing a conformational change which exposes the fusion domain in fertilin-α, which, alone or in conjunction with other factors, mediates plasma membrane fusion. Consistent with this hypothesis, antibodies against fertilin disrupt fusion (Primakoff et al., 1987), and synthetic peptides derived from the proposed fertillin fusion domain are sufficient to mediate liposome fusion (Muga et al., 1994). Meltrin-α, a fertilin-related protein, is implicated in myoblast fusion (Yagami-Hiromasa et al., 1995), raising the possibility that additional cell-cell fusion events are mediated by ADAM family members. However, meltrin-α expression is not limited to fusogenic cells (Harris et al., 1997), suggesting that it does not dictate plasma membrane fusion. Furthermore, the *S. cerevisiae* genome apparently does not contain any ADAM family members, suggesting that additional fusion proteins exist (Huovila et al., 1996).

Cell Fusion in *Chlamydomonas*

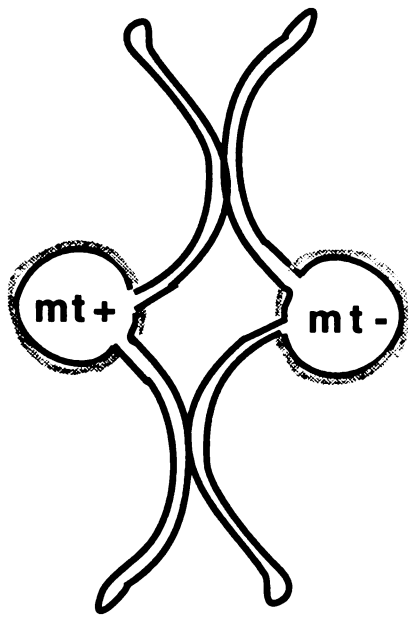
When deprived of nitrogen, vegetative cells of the biflagellated alga *Chlamydomonas reinhardtii* differentiate into gametes that are competent to undergo fertilization (for review, see Snell, 1993). Cells of opposite mating type (mt^+ and mt^-) adhere through agglutinin molecules located on their flagellar surface (Adair et al., 1982; 1983; Goodenough et al., 1985), whose expression is induced during gametic differentiation (Fig. 1-4). Flagellar adhesion aligns the two gametes and brings them into close apposition in preparation for cell fusion. Flagellar adhesion activates adenylate cyclase (Zhang and Snell, 1994), causing a rise in cyclic AMP which initiates a series of events important for fertilization (Pijst et al., 1984; Pasquale and Goodenough, 1987). These events include recruitment of agglutinins from the cell body to the flagella to maintain adhesiveness during fertilization (Hunnicutt et al., 1990). *Chlamydomonas* gametes are separated by a cellulose-deficient, glycoprotein-rich cell wall that must be shed to allow plasma membrane fusion. During gametic differentiation, production of lysin, a metalloprotease that degrades the cell wall, is increased (Kinoshita et al., 1992). Prior to flagellar agglutination, lysin is found in the periplasm in an inactive form (Matsuda et al., 1987; Buchanan et al., 1989). Lysin is converted into an active form in response to flagellar agglutination by a serine protease which is released from the cell body into the periplasm, presumably through a secretory event (Snell et al., 1989). Thus, in *Chlamydomonas*, cell wall degradation is initiated by cell-cell contact (flagellar agglutination) which activates intracellular signalling pathways that regulate secretion of a serine protease. Once the cell wall is degraded, plasma membrane fusion occurs at specialized sites on the cell. Flagellar adhesion causes the formation of an actin-rich fertilization tubule on mt^+ gametes which interacts with mt^-

Figure 1-4. Steps in Mating in *Chlamydomonas reinhardtii*

In response to nitrogen limitation, vegetative *Chlamydomonas* cells differentiate into fusion-competent gametes. Cell of opposite mating type (mt^+ and mt^-) adhere through agglutinin molecules on the flagellar surface. Flagellar adhesion stimulates an increase in cAMP which is important in the following responses: secretion of a serine protease which is involved in degrading the cell wall, recruitment of agglutinins from the cell body to flagella to maintain adhesiveness, and formation of an actin-rich mating projection on mt^+ gametes which is the site of plasma membrane fusion.

Nitrogen Limitation

differentiation,
increased agglutinin
and lysin expression

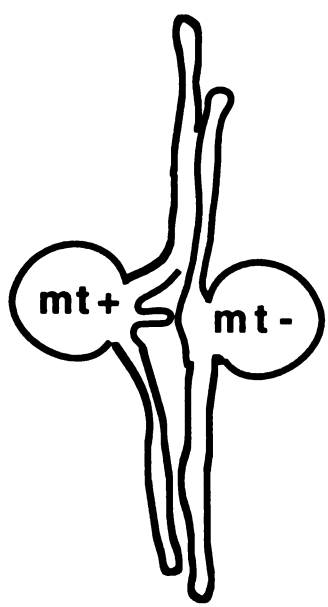


flagellar adhesion

cAMP

agglutinins recruited from
cell body to flagella

secretion of serine protease



cell wall shed

formation of mt^+
actin-rich
mating projection

plasma membrane fusion

mating structures (not actin rich) which serve as the site of plasma membrane fusion. Fertilization tubules from mt^+ gametes have been purified, providing a starting point for a biochemical approach to identify molecules involved in plasma membrane fusion (Wilson et al., 1997). As in mammalian fertilization and myoblast fusion, essentially nothing is known about how the signalling molecules, in this case adenylate cyclase and protein kinases, cause the biological responses, including release of the serine protease, formation of actin-rich mating structures, and recruitment of agglutinins to the flagella.

The Mating Pathway in *Saccharomyces cerevisiae*

This description of the mating pathway in yeast is not meant to be an exhaustive review but to highlight aspects of the mating pathway that are important in thinking about cell fusion. First is a summary of factors involved in cell fusion (for an additional review, see Marsh and Rose, 1997). Second, I review the proteins involved in pheromone production and signal transduction because some of these proteins are also required for cell fusion (see Table 1-1 and Appendix 1). Finally, because it seems likely that the fusion machinery has to be properly localized within the cell, particular attention is focused on the establishment of polarized growth and the organization of the cytoskeleton that occurs during mating. An additional reason to pay particular attention to these events is that the cell fusion machinery may act upon proteins involved in polarity, secretion, or morphogenesis to effect cell fusion.

Cell Fusion in Yeast

A summary of the proteins involved in cell fusion is provided in Table 1-1. Except for Pkc1p, loss of these proteins causes a defect in cell fusion evidenced by an accumulation of prezygotes during mating. There are a couple points worth noting. First, there are no mutants that completely

eliminate the ability of cells to fuse; all the mutants that exist exhibit reduced but still measurable cell fusion, suggesting that there are multiple, redundant pathways controlling cell fusion. Another possibility is that the key factors have not been identified, perhaps because they are also essential for growth or an earlier step in mating. Second, Pkc1p is the only known negative regulator of cell fusion (Philips and Herskowitz, 1997).

The proteins involved in cell fusion can be roughly divided into three categories. First, Fus1p and Fus2p are highly induced by pheromone and are specifically required for cell fusion (Trueheart et al., 1987; McCaffrey et al., 1987; Elion et al., 1995). The second category is comprised of proteins which also function in pheromone communication in addition to their role in cell fusion. Axl1p, Ste6p, Ram1p, Kex2p, and Ste13p are required for pheromone production. Fus3p is a MAP kinase involved in signal transduction in response to pheromone (Elion et al., 1990). Members of the last group -- Fps1p, Spa2p, Pea2p, Bni1p, Kel1p, Rvs161p,Tpm1p, Pkc1p and Chs5p -- have additional roles during vegetative growth, such as glycerol transport, bud-site selection, endocytosis, cell-wall construction, and morphogenesis. For proteins in the third category, caution should be exercised in interpreting thier role in cell fusion based upon their functions during vegetative growth. For instance, during vegetative growth, Rvs161p is involved in the actin cytoskeleton, endocytosis, and recovery from nutrient limitation, but at least the first two functions are genetically separable from its role in cell fusion, which seems to be to stabilize Fus2p (Brizzio et al., submitted). It is presently unclear into which category Fig1p and Fig2p belong. They are both highly induced by pheromone, suggesting that they should be categorized with Fus1p and Fus2p (Erdman et al., 1998). However, it has not been reported whether mutants lacking Fig1p and Fig2p exhibit defects in pheromone production or

TABLE 1-1

PROTEINS INVOLVED IN CELL FUSION

NAME	FUNCTIONS	HOMOLOGY/ DOMAINS	LOCALIZATION	PHEROMONE INDUCED?	REFERENCES
Fus1p		SH3; transmembrane	shmoo tip (plasma membrane)	yes	Trueheart et al, 1987; McCaffrey et al., 1987
Fus2p		coiled coil	shmoo tip (intracellular structure)	yes	Trueheart et al., 1987; Elion et al., 1995
Fus3p	signal transduction	MAPK	?	yes	Elion et al., 1990
Axl1p	a-factor processing; bud site selection	insulin-processing enzymes	?	?	Brizzio et al., 1996; Dorer et al., 1997; Appendix 1

NAME	FUNCTIONS	HOMOLOGY/ DOMAINS	LOCALIZATION	PHEROMONE INDUCED?	REFERENCES
Ste6p	a-factor export	ABC-cassette transporter	Golgi	yes	Elia and Marsh, 1996; Appendix 1
Ram1p	a-factor prenylation	prenyl transferase	?	?	Brizzio et al., 1996
Kex2p	α-factor processing	KR-endopeptidase	Golgi	?	Brizzio et al., 1996
Ste13p	α-factor processing	dipeptidyl-aminopeptidase	Golgi	?	Brizzio et al., 1996
Fps1p	glycerol transport	MIP family of transporters	?	no (Philips, unpublished)	Philips and Herskowitz, 1997

NAME	FUNCTIONS	HOMOLOGY/ DOMAINS	LOCALIZATION	PHEROMONE INDUCED?	REFERENCES
Spa2p	morphology; bud site selection	coiled coil	shmoo tip	no	Dorer et al., 1997
Pea2p	morphology; bud site selection	coiled coil	shmoo tip	?	Dorer et al., 1997
Bni1p	morphology; bud site selection	formin	shmoo tip	no	Dorer et al., 1997
Kel1p	morphology	kelch; coiled coil	shmoo tip	no	Chapter 3
Rvs161p	endocytosis; morphology; survival in nutrient limitation; Fus2p stability	amphiphysin	shmoo tip	yes	Brizzio et al., submitted

NAME	FUNCTIONS	HOMOLOGY/ DOMAINS	LOCALIZATION	PHEROMONE INDUCED?	REFERENCES
Tpm1p	vesicle transport	tropomyosin	actin cables	?	Liu and Bretscher, 1992
Chs5p	chitin synthase III activity; morphology	neurofilaments	shmoo tip (intracellular structure)	no	Santoz et al., 1997
Pkc1p	cell integrity	protein kinase C	?	?	Philips and Herskowitz, 1997
Fig1p	morphology	four transmembrane domains	*	yes	Erdman et al., 1998
Fig2p	morphology; agglutination	GPI-anchor	*	yes	Erdman et al., 1998

The table lists proteins that are required for cell fusion. Additional functions are listed in column two.

For many of these proteins, numerous studies have contributed to their characterization. We reference those papers that describe their role in cell fusion.

*The full-length proteins have not been localized.

signal transduction. Second, *fig1Δ* and *fig2Δ* mutants display a variety of morphological defects during mating, so it is unclear whether their primary function is cell fusion.

Fus1p and Fus2p are described in more detail in the following section. The other proteins involved in cell fusion will be mentioned throughout the discussion of the mating pathway where appropriate. The Pkc1p pathway is the subject of the last section of the Introduction and Chapters 2 and 3. Fps1p and Kel1p are the subject of Chapters 2 and 3, respectively.

Fus1p and Fus2p Are Specifically Required for Cell Fusion

Fus1p and Fus2p were described over ten years ago (Trueheart et al., 1987; McCaffrey et al., 1997). More than fifteen proteins involved in cell fusion have been identified since, yet Fus1p and Fus2p remain unique (although further characterization may show that Fig1p or Fig2p is similar). Their synthesis is highly induced by pheromone, and their major function seems to be cell fusion (Trueheart et al., 1987; McCaffrey et al., 1987; Elion et al., 1995). They are localized in the right place and expressed at the right time to play a direct role in cell fusion, but exactly what they do is unclear. Fus1p is a transmembrane protein with an intracellular SH3 domain that has been shown to bind actin by two-hybrid analysis (Trueheart et al., 1987; Trueheart et al., 1989; Amberg et al., 1995). Fus1p localizes to a sharp rim on pointed projections (Trueheart et al., 1987), whereas Fus2p localizes to punctate structures within shmoo tips (Elion et al., 1995). Fus2p has no homology to known proteins, but structural predictions suggest that it is likely to form coiled coils (Elion et al., 1995). Genetic data suggest that Fus1p and Fus2p function in parallel pathways. First, overexpression of each can partially suppress loss of the other. Second, loss of both results in a fusion defect that is much more severe than seen in either single mutant alone (Trueheart et

al., 1987). The defect in cell fusion of a *fus1Δ* or *fus2Δ* mutants is greatly enhanced when they mate with a *fus1Δ* or *fus2Δ* mutant compared to mating with a wild-type strain, suggesting that at least some activities required for cell fusion can be provided by either partner.

Pheromones Mediate Cell-Cell Communication

The pheromones are used by cells to select a mating partner in a process called courtship (Jackson and Hartwell, 1990a; 1990b). Cells chose the partner that secretes the highest level of pheromone, even if that partner is not fertile, suggesting that partner selection is a distinct step from zygote formation (Jackson and Hartwell, 1990a; 1990b). To court a partner successfully, cells have to produce and respond to pheromone (Jackson, and Hartwell, 1990a; 1990b). Transcription of the structural genes for **a**-factor is induced by α-factor, and transcription of the structural genes for α-factor is induced by **a**-factor (Strazdis and MacKay, 1983; Achstetter, 1989). Components involved in pheromone maturation and export, such as Ste13p (Achstetter, 1989) and Ste6p (Wilson and Herskowitz, 1984), are also induced by pheromone (see below). Thus, if both partners are able to produce and respond to pheromone, there is a mutually reinforcing conversation.

Proteins involved in pheromone production in **a** cells (Axl1p, Ste6p, and Ram1p) and in α cells (Kex2p and Ste13p) are also involved in cell fusion (for a discussion of their role in cell fusion, see Chapter 4 and Appendix 1). A different set of machinery is used for production of **a**-factor than α-factor. Redundant genes, *MFα1* and *MFα2*, encode α-factor precursors, which undergo maturation and export through the secretory pathway (Julius et al., 1984). The α-factor precursor is processed by the peptidases Kex1p, Kex2p, and Ste13p to the mature tridecapeptide (Fuller et al., 1988). **a**-factor, a dodecapeptide, is encoded by *MFA1* and *MFA2* (Michaelis and Herskowitz,

1988). Pro-**a**-factor undergoes carboxy-terminal modifications and two amino-terminal proteolytic processing events (Fig. 1-5). A carboxy-terminal CAAX motif targets **a**-factor for farnesylation by Ram1p/Ste16p and Ram2p, two subunits of the prenyl transferase (He, et al., 1991). Additional carboxy-terminal processing is accomplished by Ste14p, a carboxy-terminal methyltransferase (Marr et al., 1990), and Rce1p and Afc1p, which cleave the three carboxy-terminal amino acids (Boyartchuk et al., 1997). Ste24p mediates the first amino-terminal proteolytic processing event (Fujimura-Kamada et al., 1997); Axl1p and Ste23p have homology to insulin-degrading enzymes and are required for an additional endoproteolytic cleavage (Adames et al., 1995). Unlike α-factor, **a**-factor is not exported through the secretory pathway; rather Ste6p, a member of the ABC-transporter family of proteins, exports mature **a**-factor (Michaelis, 1993; Kuchler et al., 1989).

Signal Transduction in Response to Pheromones

The peptide pheromones bind to seven-transmembrane spanning receptors -- **a**-factor binds to the **a**-factor receptor (Ste3p) on α cells; α-factor binds the α-factor receptor (Ste2p) on **a** cells. Receptor activation induces three intracellular responses: arrest of cell division in the G1 phase of the cell cycle, induction of genes required for mating, and a morphological response called shmoo formation (for reviews see Bardwell, 1994; Herskowitz, 1995). How the signal transduction pathway mediates cell cycle arrest and transcriptional induction is well-understood and diagrammed in Fig. 1-6.

Upon ligand binding, receptors activate a heterotrimeric G protein, composed of Gα (Gpa1p), Gβ (Ste4p), and Gγ (Ste18p). Ligand binding is thought to cause Gα to exhange GDP for GTP, resulting in dissociation of Gα from Gβγ. Release of Gα allows Gβγ to transmit the signal (Nomoto et al., 1990; Whiteway et al., 1990). It is unclear how the signal is tranduced from

Figure 1-5. Steps in a-factor Biogenesis

The **a**-factor precursor contains an amino-terminal extension, the mature portion (in black), and a CAAX motif. The precursor undergoes several carboxy-terminal modifications, including farnesylation, removal of the three carboxy-terminal amino acids, and carboxymethylation. Amino-terminal proteolytic processing occurs in two steps, the first mediated by Ste24p and the second by Axl1p and Ste23p. Ste6p transports mature **a**-factor from the cell. Proteins in bold are required for efficient cell fusion.

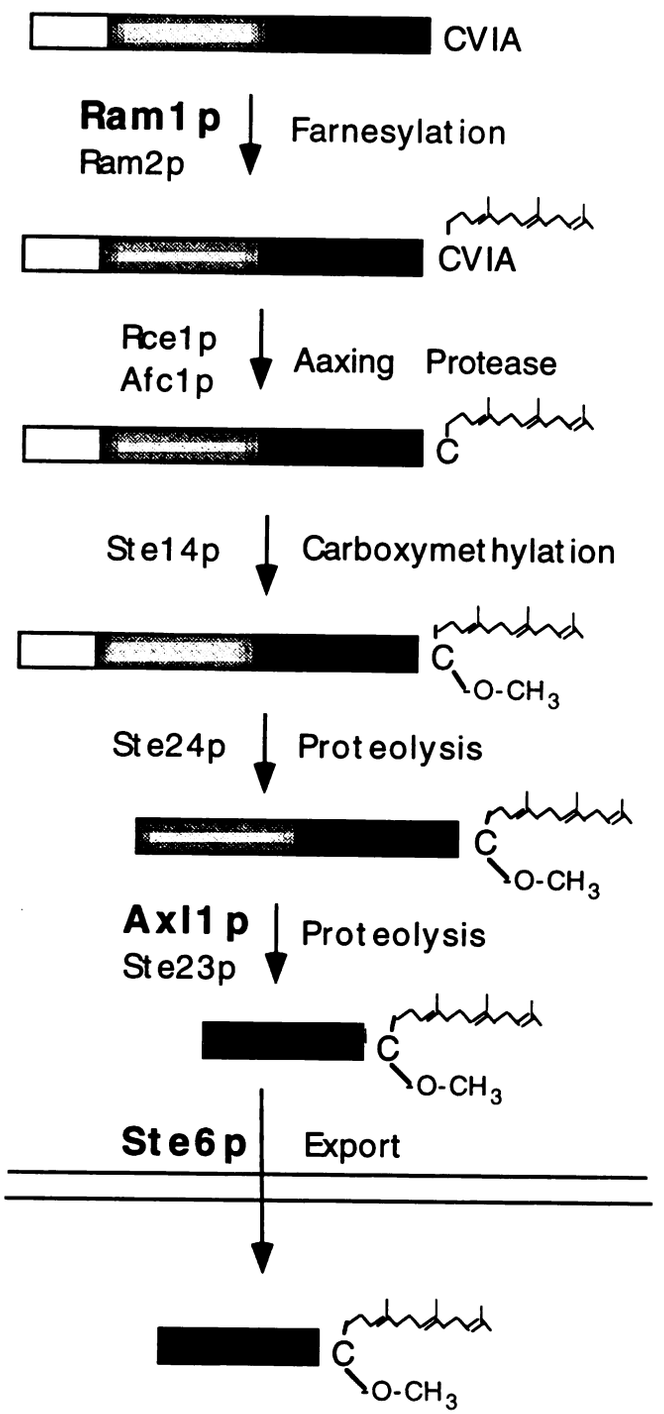
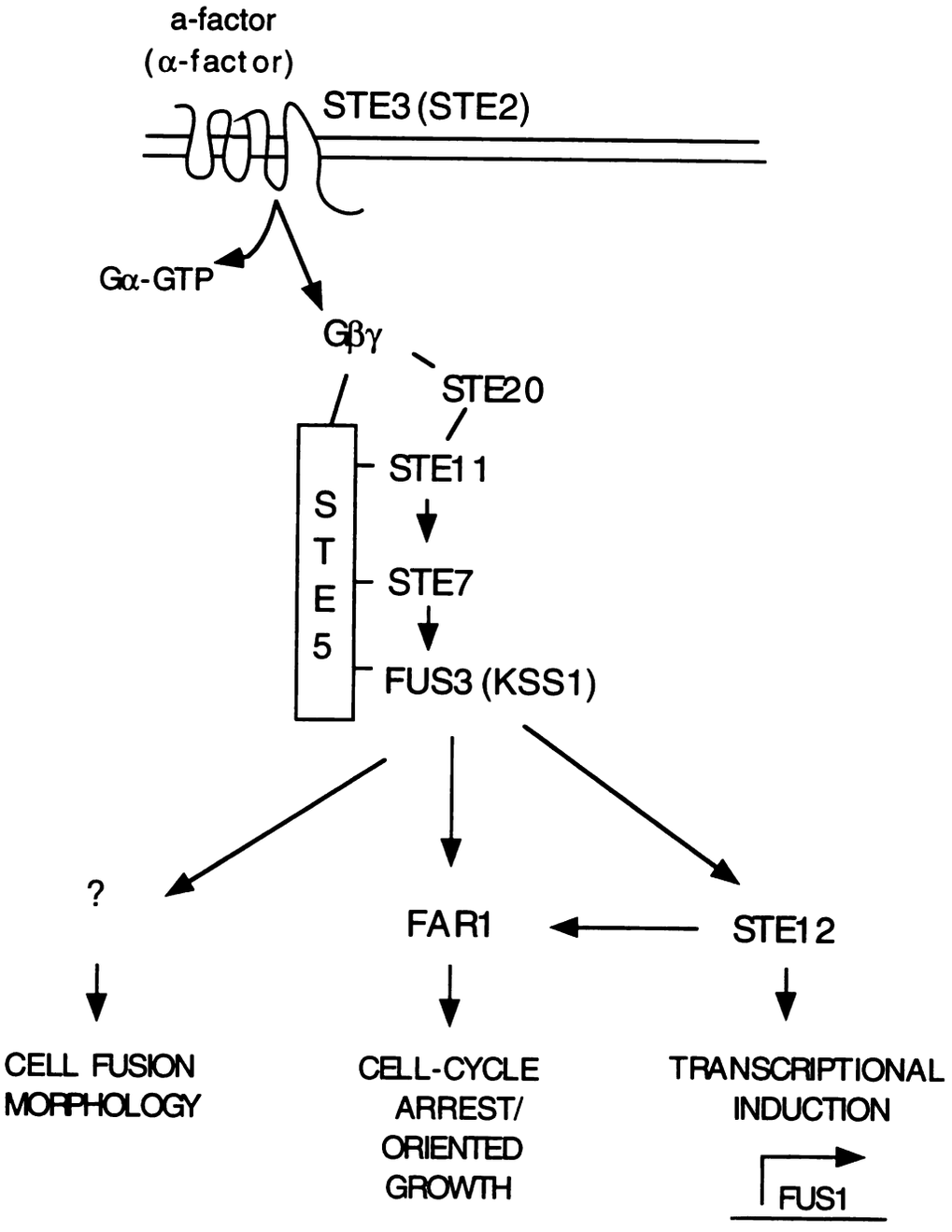

CVIA
Ram1p
Ram2p
Farnesylation
CVIA
Rce1p
Afc1p
Aaxing Protease
C
Ste14p
Carboxymethylation
C
O-CH3
Ste24p
Proteolysis
C
O-CH3
Axl1p
Ste23p
Proteolysis
C
O-CH3
Ste6p
Export
C
O-CH3

Figure 1-6. Signal Transduction in Response to Pheromones

α-factor and **a**-factor bind to cell-surface receptors, Ste2p and Ste3p, respectively, which activate a heterotrimeric G protein. Release of Gβγ activates a MAP kinase cascade composed of Ste11p, Ste7p, and Fus3p/Kss1p. Ste20p and Ste5p are required to transduce the signal from Gβg to Ste11p. Fus3p (and Kss1p) activate Ste12p, which induces transcription of genes required for mating. Fus3p acts through Far1p to arrest cell division in G1. Far1p also plays a role in orientation of growth in response to a pheromone gradient. Fus3p has a role in cell fusion and morphogenesis, but the relevant targets are unknown.


a-factor
(α-factor)
STE3 (STE2)
Gα-GTP
Gβγ
STE20
STE11
STE7
FUS3 (KSS1)
STE5
?
FAR1
STE12
CELL FUSION
MORPHOLOGY
CELL-CYCLE
ARREST/
ORIENTED
GROWTH
TRANSCRIPTIONAL
INDUCTION
FUS1

Gβγ to the downstream MAP kinase pathway composed of Ste11p (a MEK kinase), Ste7p (a MAPK kinase), and Fus3p (a MAP kinase). Both Ste20p and Ste5p are required for signal transduction and can bind to Gβ, making them potential effectors (Whiteway et al., 1995; Leeuw et al., 1998). Ste5p also binds the components of the MAP kinase pathway and is thought to function as a scaffold, perhaps spatially restricting the signalling components or preventing inappropriate cross-talk between various MAP kinase pathways (for review, see Elion, 1995). One possibility is that by binding both Gβ and Ste11p, Ste5p brings Ste11p into close proximity of Ste20p which also binds Gβ. Ste20p is a PAK-family kinase that can directly phosphorylate Ste11p (Leberer et al., 1992) and may thereby activate it. The signal would then be propagated by the MAP kinase module. Ste11p directly phosphorylates and activates Ste7p (Neiman and Herskowitz, 1994). Similarly, Ste7p phosporylates and activates Fus3p (Errede et al., 1993).

Fus3p is required for cell cycle arrest, transcriptional induction, and cell fusion. It targets Far1p and Ste12p (Elion et al., 1993), which mediate cell cycle arrest and transcriptional induction, respectively. The target involved in cell fusion is not known. Far1p causes cell cycle arrest by inhibiting the activity of Cdc28p-Cln2p kinase (Peter et al., 1993). Ste12p is a transcription factor that induces expression of genes required for mating by binding to a DNA element called the pheromone response element (PRE; Dolan et al., 1989; Errede and Ammerer, 1989; Song et al., 1991). Several genes required for cell fusion, including *FUS1*, *FUS2*, *FIG1*, *FIG2*, and *RVS161*, are induced by pheromone (Trueheart et al., 1987; McCaffrey et al., 1987; Brizzio et al., submitted; Erdman et al., 1998). In the absence of Fus3p, transcriptional induction still occurs because another MAP kinase, Kss1p, can activate Ste12p, suggesting that the defect in cell fusion of *fus3* mutants is not due to a failure in gene induction.

Fus3p may also regulate morphogenesis separately from its role in gene induction. *fus3-1* mutants are reported to form elongated projections whereas *fus3*-2 mutants are defective in projection formation, although they both induce *FUS1* expression (Elion et al., 1990).

Morphological Response to Pheromone

Exogenously supplied pheromone causes a morphological alteration called shmoo formation in which cells change from a round shape to a pear shape. This morphological change is accompanied by rearrangement of the cytoskeleton, reorganization of intracellular organelles, and alterations in the cell wall. The yeast actin cytoskeleton is composed of cortical actin patches associated with the cell surface and cytoplasmic cables aligned along the axis of cell growth (Mulholland et al., 1994). The polarized distribution of actin is regulated in a cell-cycle dependent manner during vegetative growth (Adams and Pringle, 1984; Kilmartin and Adams, 1984). During mating, cells arrest in G1, a phase of the cell cycle during which the actin cytoskeleton is largely unpolarized. However, upon pheromone treatment, the actin cytoskeleton is organized towards the shmoo tip (Hasek et al., 1987; Gehrung and Snyder, 1990; Ford and Pringle, 1986; Read et al., 1992). The polarized distribution of actin is thought to localize cell surface growth by directing secretion (Field and Schekman, 1980; Adams and Pringle, 1984; Novick and Botstein, 1985). Consistent with this idea, vesicles localize and accumulate in the shmoo tip (Gammie et al., submitted; Baba et al., 1989). Additionally, mutants defective in polarized delivery of vesicles, such as actin and tropomyosin mutants, are also defective in shmoo formation (Read et al., 1992; Liu and Bretscher, 1992). Since actin is required for polarization, an event which occurs prior to cell fusion, it is unclear whether actin also plays a direct role in cell fusion. Extra-nuclear microtubules also reorganize in response to pheromone, extending

from the spindle pole body to the shmoo tip. Microtubules are important for nuclear fusion (Meluh and Rose, 1990; Berlin et al., 1990; Rose and Fink, 1987) but are dispensible for shmoo formation and cell fusion (Hasek et al., 1987; Read et al., 1992).

Polarized secretion may be important for cell surface modifications that occur with pheromone treatment. The yeast cell wall is composed primarily of mannoproteins and three fibrous polysaccharides, β-1,3-glucan, β-1,6-glucan, and chitin (for a review of the cell wall, see Cabib et al., 1993). Alterations in the cell wall after pheromone treatment are evidenced by modified electron microscopic appearance, increased sensitivity to glucanase, and altered staining with fluorescently-labeled concanavalin A (conA), which labels mannoproteins, and calcofluor, which labels chitin (Lipke et al., 1976; Tkacz and MacKay et al., 1979; Schekman and Brawley, 1979; Baba et al., 1989). Localized deposition of chitin and mannoproteins occurs in the shmoo tips (Sheckman and Brawley, 1979: Tkacz and MacKay, 1979). The significance of these cell wall modifications is not clear, but they might be important in morphogenesis or cell fusion. Consistent with such a possibility, mutants defective in Chs3p and Chs5p, proteins required for deposition of chitin in the shmoo tip, exhibit defects in shmoo morphology. *chs5Δ* mutants also exhibit a defect in cell fusion, which is not due simply to the lack of chitin in the shmoo tip, because *chs3Δ* mutants, which are more defective than *chs5Δ* mutants in chitin deposition, do not exhibit a defect in cell fusion (Santoz et al, 1997).

Polarized secretion may also be important to localize the fusion machinery to the site of future cell contact. Several proteins which are important in cell fusion, including Fus1p, Fus2p, Bni1p, Spa2p, Pea2p, Kel1p, and Rvs161p, localize in a polarized fashion towards the shmoo tip (Trueheart et al., 1987;

Elion et al., 1995; Evangelista et al., 1997; Snyder et al., 1991; Valtz and Herskowitz, 1996; Philips and Herskowitz, submitted; Brizzio et al., submitted). Mutants lacking tropomyosin are defective in organizing actin and secretion to the shmoo tip and also exhibit a defect in cell fusion, suggesting that cell fusion depends upon localized secretion (Liu and Bretscher, 1992). However, additional mechanisms must exist for localizing or maintaining the localization of some fusion components because some proteins, such as Spa2p, remain localized in the absence of actin (Ayscough et al., 1997; Snyner et al., 1991).

A number of proteins important in morphogenesis during shmooing seem to be largely dispensible for morphology during budding. A subset of these, including Pea2p, Spa2p, Chs5p, and Bni1p, are also required for cell fusion (Dorer et al., 1997; Santos et al., 1997). *spa2Δ*, *pea2Δ*, *bni1Δ*, and *chs5Δ* mutants fail to form tight mating projections, instead forming oval or peanut-shaped shmoos (Gerhung and Snyder, 1990; Valtz and Herskowitz, 1996, Evangelista et al., 1997). It is unclear whether the defect in cell fusion in these mutants is a consequence of the shmoo morphology defect. Perhaps the inability to form tight mating projections results in diffusely localized cell fusion machinery. Another possibility is that these proteins are part of the fusion machinery or are directly involved in organizing it.

Polarity Establishment Genes Direct Cell Polarity

Polarized growth requires, first, that cells chose a site to polarize towards and, second, that they organize growth towards that site. Organization of actin towards the chosen site during shmoo formation depends upon a set of factors called the polarity establishment proteins, including Cdc24p, Cdc42p, and Bem1p, which are also required to organize actin towards the bud site during vegetative growth (see Chenevert, 1994; Drubin and Nelson, 1996).

Cdc42p is a Rho-family GTPase that localizes to regions of polarized growth (Ziman et al., 1993). Cdc24p is a guanine-nucleotide exchange factor that is thought to activate Cdc42p by promoting exchange of GDP for GTP (Ziman and Johnson, 1994; Zheng et al., 1994). In the absence of Cdc42p or Cdc24p, cells fail to grow in a polarized manner and instead increase in size isotropically (Adams and Pringle, 1984; Adams et al., 1990; Sloat et al., 1981; Sloat and Pringle, 1978). Bem1p, an SH3-domain containing protein, localizes to regions of polarized growth during mating and budding (Chenevert et al., 1992; Chenevert, 1993) and interacts directly with Cdc24p (Peterson et al., 1994; Zheng et al., 1995). Bem1p is also found in a complex with actin (Leeuw et al., 1995). At high temperature, *bem1Δ* mutants exhibit a similar defect in polarized actin organization seen in the absence of Cdc42p or Cdc24p activity. Exactly how these proteins organize and polarize the cytoskeleton is unclear. Suggested effectors of Cdc42p (based upon supposed or demonstrated binding) are numerous, including a WASP-related molecule, Las17p/Bee1p (Li, 1997), the formins Bni1p and Bnr1p (Evangelista et al., 1997; Imamura et al., 1997; Kohno et al., 1996), the PAK kinases Ste20p, Cla4p, and Skm1p (Cvrckova et al., 1995; Peter et al., 1996; Leberer et al., 1997; Martin et al., 1997), and Gic1p and Gic2p (Brown et al., 1997; Chen et al., 1997). Based upon the phenotype of mutants lacking these proteins, none alone can account for the role of Cdc42p in polarized growth, suggesting that Cdc42p functions through multiple effectors.

Choosing the Site of Polarized Growth During Mating

Cells are able to polarize growth towards their mating partner by detecting a pheromone gradient. If presented with two potential mating partners, a pheromone-producing partner and a pheromoneless partner, a cell will mate with the pheromone-producing partner (a behavior termed mating partner

discrimination; Jackson and Hartwell, 1990b). If exogenous pheromone is added, then cells fails to discriminate, mating randomly with either partner (Dorer et al., 1995). The implication is that exogenous pheromone disrupts a gradient of pheromone used for partner selection. Segall (1993) showed directly that cells orient growth in response to a pheromone gradient. Because yeast cells choose a mating partner among several possible partners, the site for cell fusion is probably not predetermined on the surface of the cell, but becomes localized towards the chosen mating partner.

A picture is emerging of how that site is specified. One idea is that positional information provided by the pheromone gradient creates an internal landmark recognized by polarity establishment proteins that direct growth to this site (Chenevert, 1994). Because mutants defective in the G protein and the receptor are defective in mating partner discrimination, it was suggested that they are likely candidates for the landmark (Jackson et al., 1991; Schrick et al., 1997). Molecular data substantiate this hypothesis. Gβ binds to the polarity establishment protein Cdc24p (Zhao et al., 1995; Nern and Arkowitz, 1998), and mutations in Cdc24p that disrupt this interaction cause a defect in orientation (Nern and Arkowitz, 1998). Orientation is probably not as simple as Cdc24p binding to Gβ, because mutants defective in Far1p also fail to orient (Valtz et al., 1995; Dorer et al., 1995). Far1p binds to Bem1p and Gβ, and these interactions are functionally important for Far1p to carry out its role in orientation (M. Peter, personal communication). These data suggest that Far1p may also recruit polarity establishment genes to the landmark.

Relationship between Orientation and Fusion

During mating or shmooing, if cells are unable to use the positional information of the gradient, they are still able to direct growth towards a point

on the cell surface. Specifically, mutants defective in *FAR1* or *CDC24* that fail to orient still form a projection (Valtz et al., 1995; Dorer et al., 1995; Nern and Arkowitz, 1998). Additionally, if treated with a uniform level of pheromone rather than a gradient, cells polarize and form a shmoo. Instead of determining the site of the projection based upon an extracellular signal, the site is chosen based upon an internal landmark, the bud site (Madden and Snyder, 1992; Valtz et al., 1995; Nern and Arkowitz, 1998). The term "default mating" is used to describe the situation where mating cells polarize towards the bud site, as opposed to normal, chemotropic mating where cells polarize towards a pheromone gradient (Dorer et al., 1995; 1997). Strains which are defective in orientation, either due to mutation or exogenously added pheromone, mate with a lower efficiency than strains that can orient. The idea is that if by chance they orient towards a partner which is orienting towards them, then they can mate.

Mutants have been identified that are especially defective in default mating but only mildly defective when they position the shmoo site based upon the extracellular information provided by the pheromone gradient (Dorer et al., 1997). Suprisingly, the genes so identified (*AXL1, FUS1, FUS2, FUS3, BNI1, RVS161, PEA2,* and *SPA2*) are all required for cell fusion during normal mating. These results suggest that if cells are unable to use a gradient to determine the site where they will make contact and fuse with their partner, they exhibit a more stringent requirement for components involved in cell fusion. This stringent requirement seems to apply to a variety of cell fusion genes that would be expected to perform distinct functions. Genes required for pheromone production (*AXL1*), those specifically required for fusion (*FUS1* and *FUS2*), and those involved in morphogenesis (*BNI1, PEA2, SPA2*) are all represented. However, default mating does not heighten the

requirement for all cell fusion genes because *CHS5* is required for cell fusion but not for default mating (Dorer et al., 1997).

There is no molecular understanding for the greater dependence on cell fusion proteins during default mating compared to chemotropic mating. There are several possible explanations. When growing in a gradient, cells improve their orientation as they grow towards the gradient (Segall, 1993). Mutants that are defective in determining the initial site of growth may also be defective in the fine corrections. Fine-tuning of oriented growth between two partners may be important in properly localizing the fusion machinery. Another possibility is that there is something structurally different between a shmoo that is organized towards a bud site compared to one that is organized in response to a mating-determined landmark. Consistent with such a possibility, shmoos with altered morphology are observed when *fig1Δ*, *fig2Δ*, and *fig4Δ* mutants mate in liquid, but not when polarizing in isotropic pheromone (Erdman et al., 1998). The recruitment of polarity establishment factors to a site on the cell surface determined by an extracellular cue depends upon different factors than recruitment to the bud site (for example Far1p versus Bud1p). Perhaps if the projection is organized towards the bud site, some fusion components fail to localize or some bud proteins interfere with fusion.

The Cell Integrity Pathway

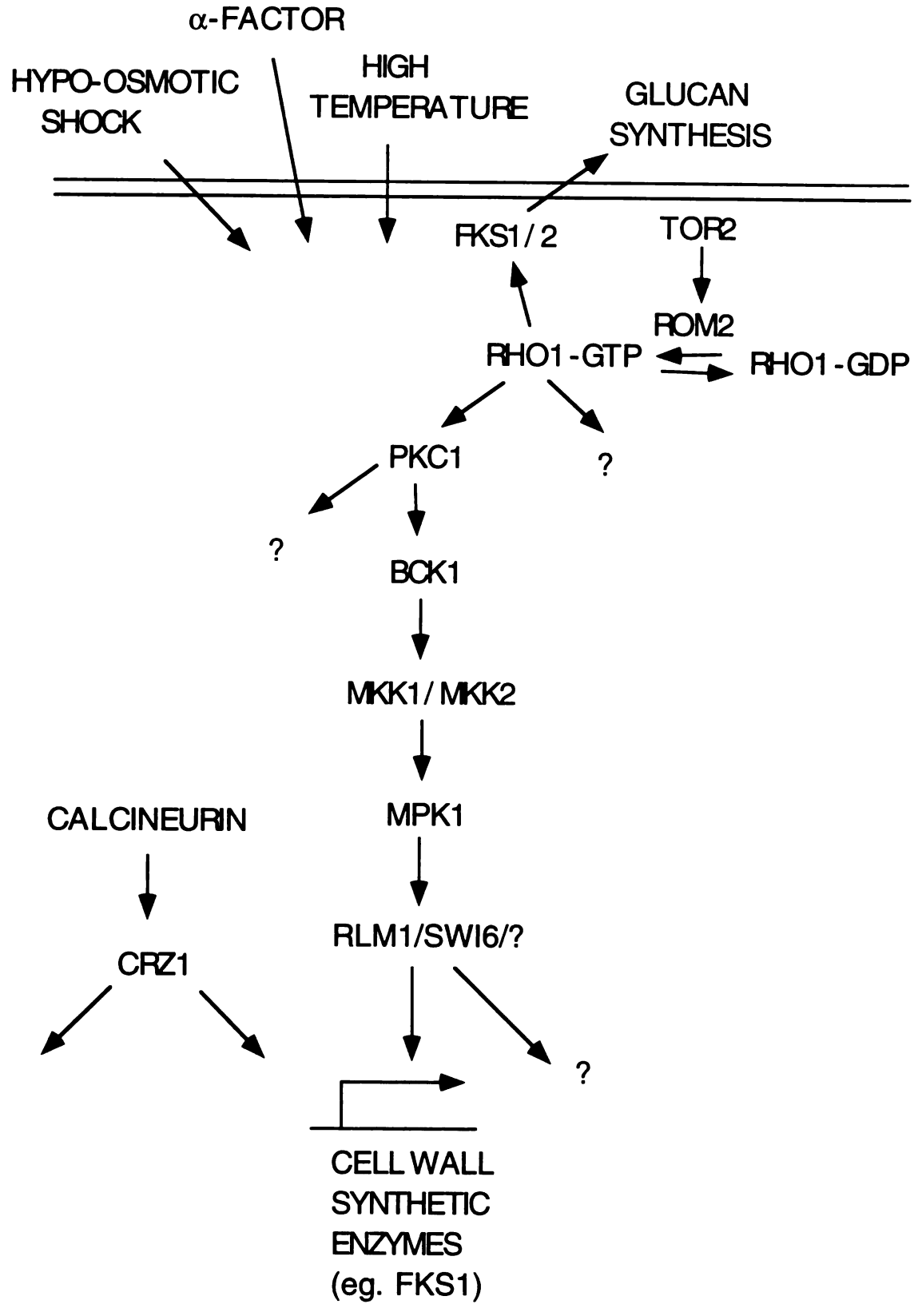
The yeast cell wall, composed primarily of mannoproteins and glucan polymers (β-1,3-glucan, β-1,6-glucan, and chitin) is important in providing structural integrity to the cell (for review, see Cabib et al., 1997); in its absence, cells lyse unless osmotically supported. The cell wall is not a static structure, but instead is constantly altered by changing activity of synthases and degradative enzymes. Cell fusion could be regulated by altering the relative

balance between synthetic and degradative activities. Alterations in these activities could be achieved through a novel pathway that regulates cell wall remodelling during fusion or could be controlled by modulating pathways known to regulate cell integrity. The activity of intracellular enzymes that regulate cell wall biosynthesis could be modulated, or secretion of cell wall modifying enzymes could be regulated.

The protein kinase C pathway is involved in maintaining cell integrity (for review see Banuett, 1998). Pkc1p of *S.cerevisiae* is related to the α, β, and γ isoforms of mammalian protein kinase C, composed of potential phorbal ester- and calcium-binding domains, a pseudosubstrate domain, and a catalytic domain (Levin et al., 1990). Mutants which are defective in *PKC1* exhibit altered cell wall composition and are inviable unless grown in the presence of an osmotic stabilizing agent, such as 1M sorbitol (Shimizu et al., 1994; Roemer et al., 1994; Levin and Bartlett-Heubusch, 1992; Paravicini et al., 1992). Pkc1p is thought to regulate a MAP kinase module composed of the MEK kinase, Bck1p/Slk1p (Lee and Levin, 1992; Costigan et al., 1992), two redundant MEKs, Mkk1p and Mkk2p (Irie et al., 1993), and the MAP kinase Mpk1p/Slt2p (Mazzoni et al., 1993; Lee et al., 1993; Torres et al., 1991; Fig. 1-7). Mpk1p is activated in response to conditions that threaten cell integrity such as hypo-osmotic shock, high temperature, and treatment with membrane-intercalating drugs such as chlorpromazine (Davenport et al., 1995; Kamada et al., 1995). Mutants defect in the Pkc1p pathway are synthetically lethal with mutants defective in cell wall construction, consistent with a role for Pkc1p in cell integrity (Roemer et al., 1994; Garrett-Engele et al., 1995). Because *pkc1Δ* mutants require osmotic support at all temperatures whereas mutants defective in the MAP kinase module require osmotic support only at high

Figure 1-7. The Cell Integrity Pathways

Cells respond to hypo-osmotic shock, pheromone, and high temperature by activating the Pkc1p pathway. Pkc1p is activated by the small GTP-binding protein Rho1p and acts upstream of a MAP kinase module, composed of Bck1p, Mkk1p/Mkk2p, and Mpk1p, to regulate transcription and perhaps other targets. Rho1p is regulated by Rom2p, a guanine-nucleotide exchange factor, which is controlled by the phosphatidylinositol 3-kinase homolog Tor2p. In addition to activating Pkc1p, Rho1p activates Fks1p and Fks2p, glucan synthases. Rlm1p and Swi6p are transcription factors that may respond to Pkc1p activity. Calcineurin, via the transcription factor Crz1p, regulates an overlapping set of genes.


α-FACTOR
HYPO-OSMOTIC SHOCK
HIGH TEMPERATURE
GLUCAN SYNTHESIS
FKS1/2
TOR2
ROM2
RHO1-GTP
RHO1-GDP
PKC1
?
?
BCK1
MKK1/MKK2
MPK1
CALCINEURIN
RLM1/SWI6/?
CRZ1
?
CELL WALL SYNTHETIC ENZYMES (eg. FKS1)

temperatures, Pkc1p is thought to have targets in addition to the MAP kinase module. These additional targets remain obscure.

Pkc1p regulates cell integrity through the MAP kinase module, at least in part, by regulating transcription of genes involved in cell wall construction. For example, transcription of *FKS1*, which encodes a subunit of the β-1,3-glucan synthase (Douglas et al., 1994), *KRE6*, involved in β-1,6-glucan synthesis (Roemer et al., 1994), and *CDS2*, which encodes chitin synthase III (Bulawa, 1993), is reduced in *pkc1* or *mpk1Δ* mutants (Igual et al., 1996). Additionally, Bgl2p, a β-glucanase, is overproduced in *pkc1* mutants (Shimizu et al., 1994), suggesting that Pkc1p regulates cell integrity both by negatively regulating degradative enzymes and positively regulating synthetic enzymes. The transcriptional regulation by the MAP kinase branch of the Pkc1p pathway may be controlled in part by the transcription factors Rlm1p and Swi6p (Watanabe et al., 1997; Dodou and Treisman, 1997; Madden et al., 1997).

Genetic and biochemical data indicate that Pkc1p functions downstream of Rho1p, a small GTPase (Nonaka et al., 1995; Kamada et al., 1996), which is regulated by the phosphatidylinositol 3-kinase homolog Tor2p via the exchange factor Rom2p (Schmidt et al., 1997). Strains containing certain temperature-sensitive alleles of *rho1* exhibit a cell lysis phenotype similar to *pkc1Δ* mutants (Kamada et al., 1996). Activation of Pkc1p suppresses some *rho1* alleles but not others, and *rho1* mutants display more severe growth defects than *pkc1* mutants, suggesting that Rho1p has functions in addition to activating Pkc1p (Nonaka et al., 1995; Kamada et al., 1996). Consistent with this idea, Rho1p regulates cell integrity in at least two ways -- by directly activating Pkc1p (Kamada et al., 1996), which presumably activates transcription of cell wall components, and by directly activating the β-1,3-

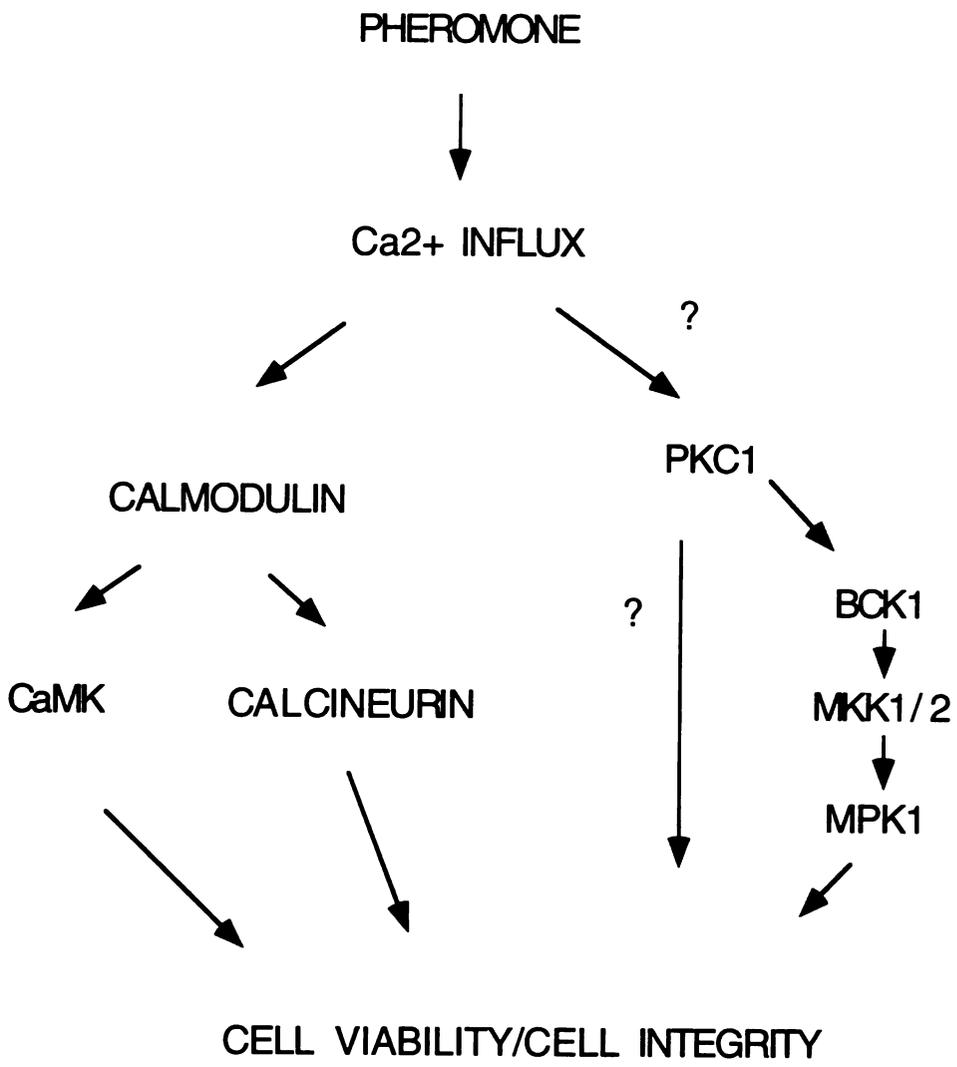
glucan synthase (Drgonova et al., 1996; Qadota et al., 1996). Additional upstream regulators of the Pkc1p pathway have been identified and are reviewed in Banuett (1998).

The Ca^{2+}/calmodulin-dependent protein phosphatase 2B (calcineurin) is involved in both ion homeostasis and cell integrity and is suggested to serve a distinct, yet overlapping function with the Pkc1p pathway (Garrett-Engele et al., 1995; Nakamura et al., 1996). Calcineurin is composed of a catalytic subunit, encoded by the functionally redundant *CNA1* and *CNA2* genes (Cyert et al., 1991; Liu et al., 1991), and a regulatory subunit, encoded by *CNB1* (Cyert and Thorner, 1992; Kuno et al., 1991). Mutants defective in calcineurin activity are synthetically lethal with *pkc1*, *bck1*, and *mpk1* mutations (Garrett-Engele et al., 1995; Nakamura et al., 1996). Additionally, a constitutively activated version of calcineurin partially suppress the growth defect of *mpk1* and *pkc1* mutants (Garrett-Engele et al., 1995), and overexpression of *MPK1* suppresses some of the phenotypes of calcineurin mutants (Nakamura et al., 1996). The calcineurin and Pkc1p pathways may partially overlap in controlling expression of genes important in cell wall biosynthesis (Fig. 1-8). For example, two genes, *FKS1* and *FKS2*, which both encode the catalytic subunit of the β-1,3-glucan synthase, are regulated by Pkc1p and calcineurin (Eng et al., 1994; Douglas et al., 1994; Mazur et al., 1995; Igual et al., 1996).

The Pkc1p and calcineurin pathway may function downstream of a calcium signal to maintain cell integrity during pheromone treatment. At the time of projection formation, pheromone-treated cells experience a rise is intracellular calcium and activation of Mpk1p, both of which are required for for cells to remain viable (Iida et al., 1990; Zarzov et al., 1996; Errede et al., 1995; Buehrer and Errede, 1997). If wild-type cells are treated with pheromone in calcium-free media, they die within 2-4 hours (Iida et al., 1990). At

Figure 1-8. Maintenance of Cell Viability in the Presence of Pheromone

Pheromone treatment causes an influx of calcium which is required to maintain viability with prolonged pheromone treatment. At least part of the requirement for Ca^{2+} may be to activate calcineurin and CaMK (Ca^{2+}-calmodulin-dependent kinase). The Pkc1p pathway is required to maintain cell integrity with prolonged pheromone treatment, but it is not known whether Pkc1p activation depends upon the Ca^{2+} influx.


PHEROMONE
Ca2+ INFLUX
?
CALMODULIN
PKC1
?
BCK1
CaMK
CALCINEURIN
MKK1/2
MPK1
CELL VIABILITY/CELL INTEGRITY

temperatures that are permissive for vegetative growth, *mpk1Δ* mutants lyse in pheromone, suggesting that Mpk1p is important in maintaining cell integrity under such conditions (Errede et al., 1995). Mutants defective in calcineurin activity also lose viability in the presence of pheromone (Cyert et al., 1991; Cyert and Thorner, 1992;). Whether the calcium influx is important in activating the Pkc1p pathway is unclear. However, part of the requirement for the calcium influx seems to be activation of calmodulin, a calcium-binding protein, which in turn activates calcineurin and the calcium-calmodulin-dependent protein kinase (CaMK; Moser et al., 1996). In the case of *mpk1* mutants, the loss of viability upon pheromone treatment is suppressed by osmotic stabilizing agents, suggesting that the defect is due to cell lysis (Errede et al., 1995). Although it is not clear whether the loss of viability exhibited by calcineurin mutants is due to cell lysis (Withee et al., 1997), it seems likely that calcineurin also has a role in maintaining cell wall integrity during pheromone treatment because pheromone-induced transcription of *FKS2* depends upon calcineurin (Mazur et al., 1995). In addition, overexpression of *MPK1* partially restores viability to calcineurin-defective mutants in pheromone (Nakamura et al., 1996), consistent with the idea that both Pkc1p and calcineurin regulate cell wall integrity during pheromone treatment.

In conclusion, the Pkc1p pathway is required to maintain cell integrity particularly under conditions such as hypo-osmotic shock and pheromone treatment. The calcineurin pathway may contribute to cell integrity by regulating an overlapping set of genes involved in cell wall construction. The Pkc1p pathway, calcineurin pathway, and calcium influx are also required to maintain cell viability, and perhaps integrity, during pheromone

CHAPTER TWO

OSMOTIC BALANCE REGULATES CELL FUSION DURING MATING IN *SACCHAROMYCES CEREVISIAE*

A version of Chapter 2 originally appeared as Philips, J. and I. Herskowitz. 1997. *J. Cell Biol.* 138:961-974. It is reproduced with copyright permission of The Rockefeller University Press.

ABSTRACT

Successful zygote formation during yeast mating requires cell fusion of the two haploid mating partners. To ensure that cells do not lyse as they remodel their cell wall, the fusion event is both temporally and spatially regulated: the cell wall is degraded only after cell-cell contact and only in the region of cell-cell contact. To understand how cell fusion is regulated, we identified mutants defective in cell fusion based upon their defect in mating to a *fus1 fus2* strain (Chenevert et al., 1994). Two of these cell fusion mutants are defective in the *FPS1* gene, which codes for a glycerol facilitator (Luyten et al., 1995). To determine whether inability to maintain osmotic balance accounts for the defect in cell fusion in these mutants, we analyzed the behavior of an *fps1Δ* mutant with reduced intracellular glycerol levels due to a defect in the *GPD1* (glycerol-3-phosphate dehydrogenase) gene (Albertyn et al., 1994b): deletion of *GPD1* partially suppressed the cell fusion defect of *fps1* mutants. In contrast, overexpression of *GPD1* exacerbated the defect. The fusion defect could also be partially suppressed by 1 M sorbitol. These observations indicate that the fusion defect of *fps1* mutants results from inability to regulate osmotic balance and provide evidence that the osmotic state of the cell can regulate fusion. We have also observed that mutants expressing hyperactive protein kinase C exhibit a cell fusion defect similar to that of *fps1* mutants. We propose that Pkc1p regulates cell fusion in response to osmotic disequilibrium. Unlike *fps1* mutants, *fus1* and *fus2* mutants are not influenced by expression of *GPD1* or by 1 M sorbitol. Their fusion defect is thus unlikely to result from altered osmotic balance.

INTRODUCTION

The joining of two cells occurs during certain specialized cell-cell interactions such as sperm-egg fusion during fertilization, myoblast fusion during myotube formation, and gamete fusion during yeast mating. Intercellular fusion requires successful completion of a number of different events, the molecular details of which are poorly understood. The interacting cells must first recognize and adhere to each other. Extracellular material separating the interacting cells must then be removed. The zona pellucida surrounding the egg, extracellular matrix components separating myoblasts, and cell wall material separating haploid yeast cells must be removed in order to place the plasma membranes of the interacting cells into apposition. Finally, the plasma membranes of the two cells fuse, forming a single heterokaryon which can then undergo fusion of intracellular organelles.

The mating pathway of *Saccharomyces cerevisiae* culminates in the fusion of two haploid cells of opposite mating type (**a** and α) into an **a**/α diploid zygote. The events leading up to cell-cell contact are well characterized. Haploid cells secrete peptide pheromones (**a**-factor by **a** cells and α-factor by α cells) which are important for intercellular recognition and preparing cells for fusion. These pheromones activate a G protein-coupled receptor on the surface of the opposite mating partner, which in turn activates a MAP kinase cascade, inducing a morphological response (shmoo formation), cell cycle arrest, and transcriptional induction (for reviews see Kurjan, 1992; Sprague and Thorner, 1992; Herskowitz, 1995). The mating pheromones prepare cells to fuse by inducing expression and localization of fusion components. In particular, synthesis of Fus1p and Fus2p, proteins

required for cell fusion, is induced by pheromone (Trueheart et al., 1987; McCaffrey et al., 1987; Elion et al., 1995). These proteins are localized to the region of future cell contact (Trueheart et al., 1987; Elion et al., 1995). Cells polarize the actin cytoskeleton and secretory apparatus towards their selected mating partner by detecting a pheromone gradient (Jackson and Hartwell, 1990; Madden and Snyder, 1992; Segall, 1993). As a result, new membrane and cell wall material is deposited at the site of future cell contact (Field and Schekman, 1980; Adams and Pringle, 1984; Novick and Botstein, 1985; Hasek et al., 1987; Read et al., 1992), which may be important for localized cell wall modifications (Lipke et al., 1976; Tkacz and MacKay, 1979; Schekman and Brawley, 1979; Baba et al., 1989) and targeting of the fusion machinery.

Although pheromones activate cells for fusion, cell wall degradation does not begin until the mating partners contact each other. Initially, cell surface agglutinins mediate attachment of the mating partners (Lipke and Kurjan, 1992) which is reversible by sonication. The cell walls then become irreversibly attached. Once cell-cell contact occurs, a thinning of the cell wall is observed which begins in the center of the region of cell contact and proceeds towards the edges (Osumi et al., 1974). Cell wall degradation and remodeling normally occur quickly, so that few cells in a population of mating cells are adhered but not fused (Trueheart et al., 1987). In mutants defective in cell fusion, zygote formation is blocked after the cells have adhered but before the intervening wall has been degraded, producing a dumbbell-shaped structure called a prezygote. The persistence of the cell wall in these mutants creates a physical barrier between mating partners, preventing cytoplasmic mixing and nuclear fusion (Trueheart et al., 1987; McCaffrey et al., 1987).

Products of the *FUS1-FUS3*, *FUS5-FUS8*, and *CEF1* genes are required for cell fusion (Trueheart et al., 1987; McCaffrey et al., 1987; Elion et al., 1995; Elion et al., 1990; Kurihara et al., 1994; Elia and Marsh, 1996). Fus1p is a transmembrane protein with an intracellular SH3 domain (Trueheart et al., 1987; Trueheart and Fink, 1989). Fus2p has no similarity to known proteins (Elion et al., 1995). Despite their lack of homology, Fus1p and Fus2p have overlapping functions, as overexpression of one can partially suppress loss of the other. Absence of both proteins results in a synthetic fusion defect. In addition, the fusion defect is greatly enhanced when both mating partners are mutant, suggesting that at least some activities required for cell fusion can be provided by either partner (Trueheart et al., 1987). Unlike *FUS1* and *FUS2*, which are specifically required for cell fusion, the other *FUS* genes have additional functions during mating. For instance, *FUS3*, whose role in cell fusion is unknown, encodes a MAP kinase that functions in the pheromone response pathway (Elion et al., 1990). *FUS5*, *FUS8*, and *CEF1* genes correspond to *AXL1*, *RAM1*, and *STE6*, respectively. These genes were previously identified for their role in **a**-factor production (Adames et al., 1995; Powers et al., 1986; Kuchler et al., 1989), suggesting that high levels of pheromone may play a role in cell fusion (Elia and Marsh, 1996; Brizzio et al., 1996). It is also possible that these proteins, for example, Ste6p, the **a**-factor transporter, are required for cell fusion independently of their role in pheromone production or secretion (Elia and Marsh, 1996). Whether the products of these genes play direct roles in cell fusion or are involved in regulating fusion is unclear.

Cell fusion requires that cell contact be sensed and that the cell surface be remodeled in response to this contact. To prevent cell lysis and maintain cell integrity, cell wall degradation must be highly regulated, occurring only after cell-cell contact and only in the region of cell-cell contact. We show that the

glycerol facilitator, Fps1p, is required for cell fusion. Our studies on Fps1p provide evidence that the osmotic state of the cell regulates cell fusion. We present additional studies that suggest protein kinase C, previously recognized for its role in osmotic regulation (Davenport et al., 1995), negatively regulates cell fusion, further linking osmosensing pathways to regulation of cell fusion.

MATERIALS AND METHODS

Yeast Strains and Media

Yeast strains are described in Appendix 4; plasmids are described in Appendix 5. Standard yeast growth conditions and genetic manipulations are described in Rose et al. (1990). Cells were grown at 30°C in YEPD medium unless otherwise noted. DNA manipulations were performed as described in Sambrook et al. (1989).

Yeast Plasmids and Transformations

YEp*GPD1* is a 2μ *URA3* plasmid (derived from YEplac195) containing the *GPD1* gene, as described in Albertyn et al. (1994b) (kindly provided by S. Hohmann, Goteborg University). pJP67 (YCp50-DS1) is a YCp50-derived plasmid containing the *PKC1-R398P* allele as described in Nonaka et al. (1995) (kindly provided by Y. Takai, Osaka University). The 4.3 kb SphI fragment containing *PKC1-R398P* from pJP67 was cloned into BamHI and SalI sites of pRS306 to generate pJP72. This plasmid was used to integrate *PKC1-R398P* at its genomic locus, generating strain JP317. Plasmids containing *PKC1* under control of the *GAL1* promoter are pDL242 (p*GAL1*[*PKC1-R398A*]), pDL293 (p*GAL1*[*PKC1::HA*]), and pDL295 (p*GAL1*[*PKC1-K853R::HA*]) as described in Watanabe et al. (1994). They were kindly provided by D. Levin (Johns Hopkins University). pJW192 codes for a RAS2-GFP fusion protein under control of the *GPD* promoter on a 2μ *TRP1* marked plasmid (kindly provided by J. Whistler, University of California, Berkeley). Yeast transformations were performed by the lithium acetate method (Ito et al., 1983).

Strain Construction

The *GPD1* gene was deleted from strains using pUC*gpd1Δ::TRP1*, a construct designed to replace *GPD1* with *TRP1* as described in Albertyn et al. (1994b) (kindly provided by S. Hohmann, Goteborg University). Strains were confirmed to be *gpd1Δ* by their sensitivity to high osmolarity media and by PCR analysis. *fus1Δ* strains were constructed using pJP2, which contains a substitution of the *FUS1* ORF by *TRP1*. This plasmid was generated by cloning the 1.9 kb PstI-KpnI fragment containing *FUS1* into the PstI-KpnI sites of pBluescript KS+. The *FUS1* ORF was removed by cloning a ClaI-HincII fragment containing *TRP1* into the AccI-HincII sites of *FUS1*. *fus2Δ* strains were generated using pKOFUS2, a plasmid in which the 1.6 kb HindIII fragment containing *FUS2* was replaced by the 1.1 kb HindIII fragment containing *URA3*. *fus1Δ* and *fus2Δ* strains were confirmed by their defective mating and by PCR analysis. *fps1Δ* deletion strains were generated using either pJP31 or pJP52. pJP31 replaces *FPS1* with *LEU2;* pJP52 replaces *FPS1* with *URA3* (Fig. 2-2*b*). The BglII-HindIII fragment containing *FPS1* was cloned into the BamHI-HindIII sites of pUC18. To generate pJP31, *LEU2* was removed from pUC18-LEU2 (Herskowitz collection), in which *LEU2* is cloned into the SalI site of pUC18. A PstI-XbaI fragment, containing *LEU2*, was cloned into the NsiI-AccI site of *FPS1*. pJP52 was constructed from a plasmid in which a 111-nucleotide fragment was inserted at the stop codon of *FPS1*, generating a BamHI site 70 nucleotides 3' to the stop codon. This plasmid was cut with BamHI and NsiI and a BamHI-NsiI fragment containing *URA3* was inserted. The *URA3* fragment was obtained from pSM32 (Herskowitz collection), a pUC18 plasmid containing *URA3*. *fps1Δ* strains were confirmed by defective mating and by PCR analysis.

Mating Assays

Quantitative mating was as described in Valtz et al. (1995) except that 6 x 10^6 cells of each mating partner were mixed. Mating assays scored microscopically were performed by mixing equal numbers of log phase **a** and α cells (6 x 10^6), collecting cells on 0.45 µm filters (Millipore Corp., Bedford, MA), and incubating on YEPD plates for approximately 4 h at 30°C. Cells were resuspended in 5 ml 70% ethanol by vortexing, washed, and resuspended in 50% glycerol + 1 µg/ml 4,6-diamidino-2-phenylindole (DAPI; Sigma, St. Louis, MO). Samples were sonicated and viewed with a Zeiss Axioskop microscope at 100X. Percentage prezygotes was defined as prezygotes/(prezygotes + zygotes). At least 100 partnered cells (zygotes + prezygotes) were counted per sample. Numbers represent the average of at least three experiments unless otherwise noted. Assays in which one partner contained the RAS2-GFP plasmid were performed in essentially the same manner, except that strains were grown in SD-TRP to select for the plasmid. In this case, cells were resuspended from the filter into 5 ml YEPD by vortexing, sonicated, and viewed with an Olympus BX50 microscope at 100X. Mating assays scored on plates were performed by spreading a lawn of 9 x 10^6 log phase *fus1 fus2* mating tester cells on a YEPD plate. Patches of cells grown on YEPD (or on minimal media to select for plasmids) were replica plated to the lawn. After incubation at 30°C for 3.5-4.5 h, plates were replica plated to media selective for growth of diploids. For cells expressing *PKC1* alleles under control of the *GAL1* promoter (pDL242, pDL293, pDL295), patches were grown on raffinose - URA plates to select for the plasmid but maintain the *GAL1* promoter inactive. They were replica plated to a lawn of a *MATα fus1 fus2* strain at 30°C, on either YEPD or YEPGalactose. Cells were allowed to mate for 3.75 h and then replica plated to select for diploids in the presence of glucose.

Cloning of *FPS1*

FPS1 was cloned by complementation of the mating defect of the M8 mutant (Chenevert et al., 1994). Five plasmids which rescued the mating defect were isolated from a 2μ YEp24-derived library (Carlson and Botstein, 1982) from 22,000 transformants screened. All contained a common 6.5 kb overlapping DNA segment. An EcoRI-SalI fragment was subcloned into pRS316, a CEN-ARS vector, and deletion analysis was performed to identify the complementing ORF (Fig. 2-2*c*). A plasmid containing a 2.2 kb XhoI-HindIII fragment (pJP27) fully restored mating to the M8 and M11 mutants. The 2.4 kb BglII-HindIII segment containing *FPS1* was cloned into pRS306, a *URA3* marked, integrating vector to form pJP30, which was able to restore mating and fusion when integrated at the *FPS1* genomic locus. A wild-type strain was transformed with the integrating plasmid, marking the *FPS1* locus with *URA3* (JP150), and then crossed to M8 and M11 mutants, which were *ura3*. Twelve of 13 complete tetrads from the M8 cross showed 2:2 segregation of Ura3 $^{+}$:Ura3^{-} and Mating^{+}:Mating^{-} phenotypes. One tetrad showed 1:3 segregation of Ura3^{+}:Ura3^{-} and 2:2 segregation of Mating^{+}:Mating^{-}, presumably due to gene conversion at *URA3*. Ten complete tetrads from the M11 cross showed 2:2 segregation of Ura3 and mating phenotypes. In all spores, the Ura3^{+} phenotype cosegregated with mating proficiency, indicating that the mutations responsible for the mating defects of M8 and M11 are linked to *FPS1*.

Analysis of *fps1Δ mpk1Δ* Double Mutants

Segregants obtained by crossing an *fps1Δ* strain with an *mpk1Δ* strain were allowed to germinate on YEPD containing 1 M sorbitol at 25^{0}C. Cells were passaged on YEPD (except the *mpk1Δ fps1Δ* double mutant, which was grown

on YEPD + 1 M sorbitol), streaked for single colonies on either YEPD or YEPD + 1 M sorbitol, and grown for two days at 25°C.

Assaying of Intracellular Glycerol

Intracellular glycerol was assayed enzymatically using a glycerol determination kit (Boehringer Mannheim, Indianapolis, IN) essentially as described (Albertyn et al., 1994a). Cells were grown to mid-log phase in YEPD unless selecting for plasmids, in which case they were grown in SD-URA. From each culture, three 10 ml aliquots were separately filtered onto 0.45 μm filters (Millipore Corp., Bedford, MA), washed quickly with 5 ml ice-cold YEPD, and resuspended in 2 ml 0.5 M Tris-HCl pH 7.5 by vortexing. Samples were heated to 95°C for 10 min, and cell debris removed by centrifugation. Aliquots were pooled, and glycerol determinations were performed as per manufacturer's specifications. Protein concentrations were determined using the Bio-Rad Protein Assay (Hercules, CA). Glycerol concentrations were normalized to total protein. Fold increase in glycerol concentrations was determined by normalizing to the level of intracellular glycerol in wild-type cells. Data represent the average of at least three experiments.

RESULTS

Identification of Mutants Defective in Cell Fusion

Mutants defective in cell fusion were identified based upon their defect in mating to a *fus1 fus2* mutant (Chenevert et al., 1994). *fus1 fus2* double mutants are mildly compromised in mating to a wild-type strain but are severely defective in mating to *fus1* or *fus2* strains (Trueheart et al., 1987; Elion et al., 1995). Hence, a screen to find mutants defective in mating to a *fus1 fus2* strain should identify mutants defective in *FUS1*, *FUS2*, and other genes required for fusion. To determine if any of the mutants were defective in *FUS1* or *FUS2*, plasmids containing these genes were tested for their ability to restore mating to the mutants, which identified one mutant as defective in *FUS2* (Chenevert et al., 1994).

To identify additional cell fusion mutants, mutants exhibiting a mating defect but normal shmoo morphology (Class 4 mutants in Chenevert et al., 1994) were examined microscopically to ascertain if prezygotes accumulated when the mutants were mated to a wild-type strain. Prezygotes were scored as structures in which the nuclei of mating partners remained unfused, as evidenced by two distinct DAPI staining structures, and in which a septum was visible between adherent mating partners. Nine of thirteen mutants exhibited increased frequency of prezygotes, indicative of a fusion defect (data not shown). The mutants were of two classes: five were **a**-cell specific and four were non-cell-type specific, exhibiting mating defects as both **a** and α cells (see Appendix 1). Two mutants of the latter class, designated M8 and M11 (B6 and J10, respectively, in Chenevert et al., 1994), were further characterized and are described here.

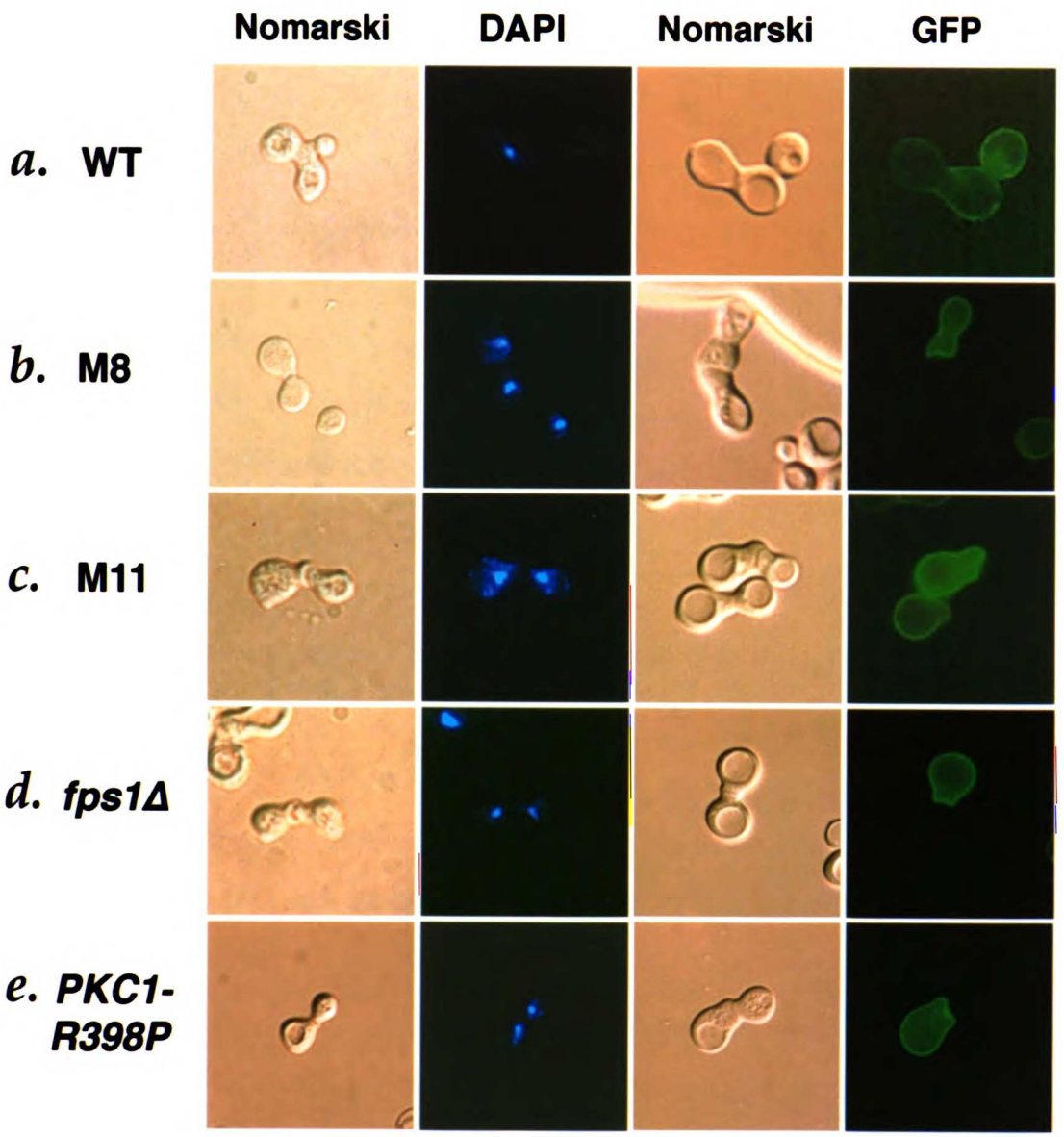
M8 and M11 exhibit normal pheromone signalling and events leading up to cell-cell contact. They produce and respond to pheromone normally, as assayed by cell cycle arrest and shmoo formation (Chenevert et al., 1994; J. Philips, unpublished observations). We observed that in matings between M8 or M11 mutants and a wild-type partner, the percentage of partnered cells ([prezgotes+zygotes]/total cells) was normal, suggesting that the defect in mating is not due to inability of the partners to find or adhere to each other. Fig. 1 illustrates the aberrant morphological structures that accumulated in mating mixes in which one partner is M8 or M11. More than 30% of the partnered cells were prezygotes (Figs. 2-1*b* and*c*). In contrast, in mating reactions between wild-type partners (Fig. 2-1*a*), fewer than one percent of the partnered cells were prezygotes. Further evidence that cell fusion was blocked prior to plasma membrane fusion was obtained by mating cells to a partner that produces a RAS2-GFP fusion protein (kindly provided by J. Whistler, University of California, Berkeley), which localizes green fluorescence around the periphery of the cell (J. Whistler and J. Rine, personal communication). In mating reactions containing wild-type cells, zygotes showed the green fluorescent signal throughout the entire zygote (Fig. 2-1*a*). In contrast, when mutants were mated to the wild-type partner containing RAS2-GFP, prezygotes were found in which the green fluorescent signal remained restricted to one cell, indicating a failure of plasma membrane fusion and cytoplasmic mixing (Figs. 2-1*b* and *c*). We conclude that M8 and M11 mutants are defective in cell fusion but are normal for earlier events of mating.

M8 and M11 are Defective in the *FPS1* Gene

To clone the gene defective in the M8 mutant, the strain was transformed with a high-copy YEp24-derived library, and 22,000 transformants were screened for ability to mate with a *fus1 fus2* strain. Five plasmids were

Figure 2-1. Morphological phenotype of cell fusion mutants.

(a) Wild-type (IH3160), (b) M8 (IH3179), (c) M11 (IH3182), (d) *fps1Δ* (JP147), and (e)*PKC1-R398P* (JP317) strains were mated on filters to wild-type strain IH3186 (two left columns) or to wild-type strain JP333 carrying the RAS2-GPF fusion plasmid (two right columns) as described in Materials and Methods. Nuclei were visualized by DAPI staining (second column). RAS2-GFP was visualized by fluorescence microscopy (fourth column).


Nomarski
DAPI
Nomarski
GFP
a. WT
b. M8
c. M11
d. fps1Δ
e. PKC1-R398P

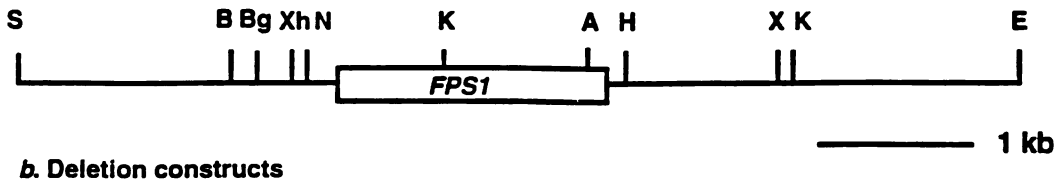
identified which restored mating. All contained a common 6.5 kb overlapping DNA segment. This segment was subcloned into pRS316, a CEN-ARS vector, and further subcloning was performed to identify the minimal fragment capable of complementation (Fig. 2-2*c*). A 2.2 kb XhoI-HindIII fragment (pJP27) restored mating to the M8 and M11 mutants. This fragment contains the *FPS1* ORF which fully restored mating and cell fusion to M8 and M11 when integrated at its genomic locus (Fig. 2-3 and data not shown). The mutation responsible for the mating defect of M8 and M11 was demonstrated to be allelic to *FPS1* by following the segregation of M8 and M11 in crosses where the *FPS1* locus was marked (see Materials and Methods). The plasmid complementation and segregation analyses indicate that the mating defect of M8 and M11 strains is due to mutations in *FPS1*, which are designated *fps1-1* and *fps1-2*, respectively.

Mating Defect of *fps1Δ* Mutants

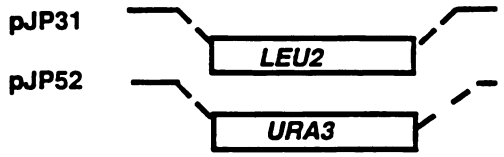
Analysis of strains deleted for *FPS1* confirmed that Fps1p is required for cell fusion. *FPS1* was deleted from the genome by replacing the *FPS1* coding sequence with either *LEU2* or *URA3* (Fig. 2-2*b*; see Materials and Methods). *fps1Δ* strains, like *fps1-1* and *fps1-2* mutants, were defective in mating to *fus1 fus2* strains (Fig. 2-4) and accumulated prezygotes that were morphologically indistinguishable from those observed in mating reactions with *fps1-1* and *fps1-2* mutants (Fig. 2-1*d*). In mating mixes of *fps1Δ* or *fps1-2* mutants to a wild-type partner, 30-45% of partnered cells were prezygotes. To determine if the *fps1-1* allele was quantitatively similar to the *fps1Δ* allele, *FPS1* was deleted in two different *fps1-1* strains. These *fps1Δ* deletion strains behaved like the *fps1-1* parent strains with respect to the percentage of prezygotes accumulated (data not shown). Thus, *fps1-1* and *fps1-2* appear to be null alleles of *FPS1*. *FPS1* deletion mutants in the EG123 strain background have a

Figure 2-2. Restriction map (a), disruption constructs (b), and deletion analysis (c) of *FPS1* region. The ability of each plasmid to complement the mating defect of the M8 and M11 mutants is indicated on the right: +, complementation; -, no complementation. pJP are plasmids carrying the indicated segments. Restriction enzymes: S, SalI; B, BamHI; Bg, BglII; Xh, XhoI; N, NsiI; K, KpnI; H, HindIII; X, XbaI; E, EcoRI. Additional information is given in the Materials and Methods and in the text.

a. Restriction map of *FPS1* region



b. Deletion constructs



c. Deletion analysis of subclones

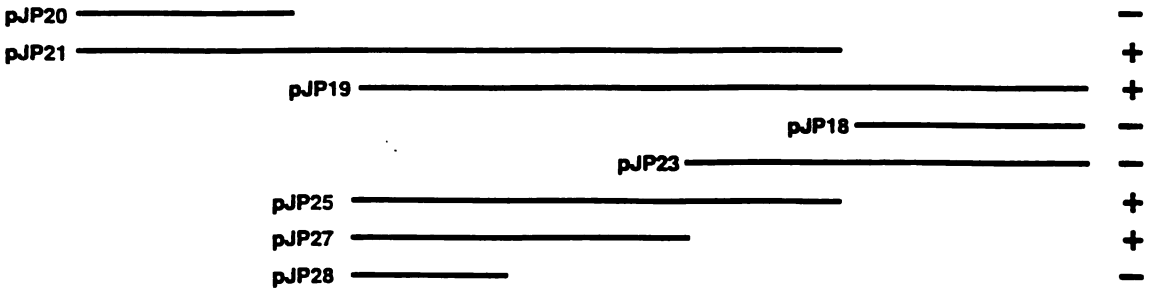
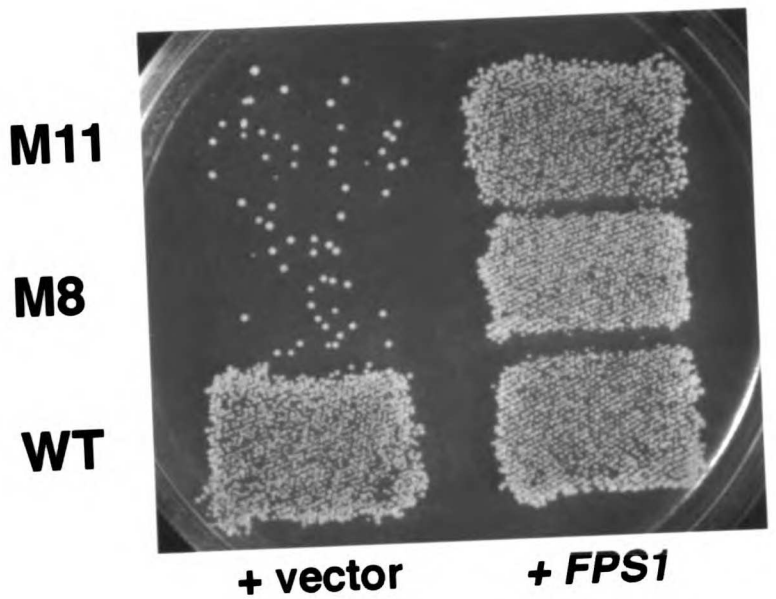


Figure 2-3. The *FPS1* gene complements the mating defect of M8 and M11. Right column: WT (JP150), M8 (JP154), and M11 (JP158), all carrying *FPS1* plasmid pJP30 at the *FPS1* genomic locus; left column: WT (JP153), M8 (JP157), and M11 (JP161), all carrying the vector alone (pRS306). Strains were mated to a *MATα fus1 fus2* strain (IH2351) as described in Materials and Methods.


M11
M8
WT
+ vector
+ FPS1

defect in cell fusion (data not shown).

antitative mating assays demonstrated that, like *fus1 fus2* strains, *fps1Δ* were mildly defective in mating, exhibiting a two- to three-fold se in diploid formation (Table 2-1). Unlike *fus1 fus2* mutants, which an enhanced defect when both mating partners are mutant (Trueheart 987; McCaffrey et al., 1987; Elion et al., 1995), *fps1Δ* mutants did not gnificantly worse or accumulate more prezygotes when mated to an artner than to a wild-type partner. In fact, when *fps1Δ* mutants are to wild-type cells, they yielded 61 +/- 17 % or 32 +/- 5 % prezygotes, ling upon the mating type of the mutant, whereas matings between *1Δ* mutants yield 18 +/- 9 % prezygotes. Unlike *fus1* and *fus2* mutants, between *fps1Δ* mutants may exhibit some suppression of the fusion In contrast, *fps1Δ* mutants mated to *fus1 fus2* mutants at a much fficiency compared to wild-type, a decrease of approximately 20-fold 2-1).

ol Accumulation in *fps1Δ* Mutants Is Correlated with Defective Cell

1p is a member of the major intrinsic protein family (MIP), an onarily conserved family of channel proteins that transport small les (reviewed by Reizer et al., 1993). It is most similar to the *E.coli* l facilitator, GlpF, and has been proposed to function as a glycerol rter in yeast (Luyten et al., 1995). Consistent with its role as a glycerol rter, *fps1Δ* mutants contain approximately two-fold more intracellular l than wild-type strains, suggesting that they are defective in glycerol Luyten et al., 1995). We observed a similar increase in intracellular l in our *fps1Δ* mutant (Table 2-2).

Table 2-1

Quantitative Mating Defect of *fps1Δ* Mutants

	a strain	α strain	mating efficiency (%)
1.	WT (IH3196)	WT (IH3190)	100
2.	*fps1Δ* (JP147)	WT (IH3190)	56
3.	WT (IH3196)	*fps1Δ* (JP226)	35
4.	*fps1Δ* (JP147)	*fps1Δ* (JP226)	40
5.	WT (IH3196)	WT (IH2350)	100
6.	WT (IH3196)	*fus1 fus2* (IH2351)	20
7.	*fps1Δ* (JP147)	*fus1 fus2* (IH2351)	<1

Mating efficiencies were calculated as described in Materials and Methods. Mating efficiencies were normalized to the isogenic wild-type mating. For lines 2-4, the values were normalized to the efficiency of IH3196 x IH3190 matings (line 1). For lines 6 and 7, the values were normalized to the efficiency of IH3196 x IH2350 mating (line 5).

Table 2-2

Intracellular Glycerol Concentrations in *fps1Δ* Strains

	Fold Change in Intracellular Glycerol
WT	1.0
fps1Δ	2.1 +/- 0.2
gpd1Δ	1.3 +/- 0.3
fps1Δ gpd1Δ	1.1 +/- 0.2
WT + vector	1.0
WT + 2μ *GPD1*	2.2 +/- 0.3
fps1Δ + vector	2.4 +/- 0.5
fps1Δ + 2μ *GPD1*	4.2 +/- 0.9

* Strains were WT (IH3196), *fps1Δ* (JP147), *gpd1Δ* (JP168), and *fps1Δ gpd1Δ* (JP165). Fold change in intracellular glycerol was determined by normalizing to the glycerol level of the WT strain (n=5). Strains containing plasmids were WT + vector (JP400), WT + YEp*GPD1*(JP199), *fps1Δ* + vector (JP401) and *fps1Δ* + YEp*GPD1*(JP200). Fold change in intracellular glycerol was determined by normalizing to WT + vector (n=3). Glycerol concentrations were determined as described in Materials and Methods. Values are the means +/- SD.

To ascertain whether the defect in cell fusion of *fps1Δ* strains results from elevated levels of intracellular glycerol, we determined whether reducing intracellular glycerol levels restored cell fusion. We reduced intracellular glycerol levels by deleting the *GPD1* gene, which encodes the NADH-dependent glycerol-3-phosphate dehydrogenase (GPDH) required for the first step of glycerol biosynthesis (Gancedo et al., 1968). Although yeast cells have a second NADH-dependent GPDH (Gpd2p), Gpd1p is responsible for approximately 95% of the NADH-dependent GPDH activity in the cell (Albertyn et al., 1994b). We found that the level of intracellular glycerol in the *fps1Δ GPD1* mutant was 2.1-fold higher than in the wild-type strain (*FPS1 GPD1*) and was only 1.1-fold higher than wild-type strains in the *fps1Δ gpd1Δ* mutant (Table 2-2).

We next determined whether this decrease in intracellular glycerol level in *fps1Δ gpd1Δ* mutants suppressed the mating defect. We found that deletion of *GPD1* suppressed the mating defect of *fps1Δ* strains, whereas deletion of *GPD1* in *FPS1* strains had no effect on mating (Fig. 2-4). To determine if *gpd1Δ* suppressed the cell fusion defect of *fps1Δ* strains, we microscopically assayed the double mutant for accumulation of prezygotes: deletion of *GPD1* in an *fps1Δ* mutant decreased the number of prezygotes from 42% to 14% (Table 2-3). Again, we saw little effect of the *GPD1* deletion in an *FPS1* strain. Thus, deletion of *GPD1* partially restored both intracellular glycerol levels and mating to a *fps1Δ* mutant.

If increased intracellular glycerol is responsible for the cell fusion defect of *fps1Δ* mutants, we hypothesized that this defect would be exacerbated by further increasing intracellular glycerol levels. Overexpression of *GPD1* in *fps1Δ* strains raises intracellular glycerol levels (Luyten et al., 1995). We observed that overexpression of *GPD1* in *fps1Δ* mutant strain JP200 led to a

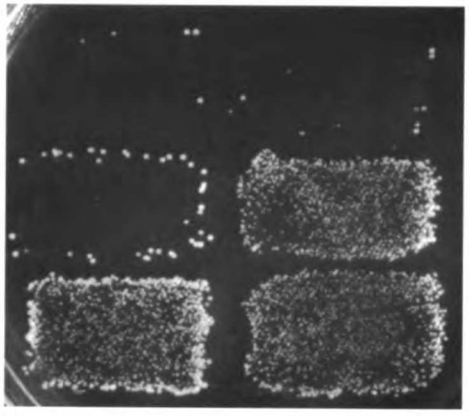
2-4. Suppression of mating defect of *fps1Δ* by *gpd1Δ* and 1 M sorbitol

ches of *MAT**a*** strains, WT (IH3160), *fps1Δ* (JP147), *fus1Δ* (JP52) (left
n) and *gpd1Δ* (JP168), *fps1Δ gpd1Δ* (JP165), and *fus1Δ gpd1Δ* (JP285)
column), were mated to a *MATα fus1 fus2* strain (IH2351) as described
erials and Methods.

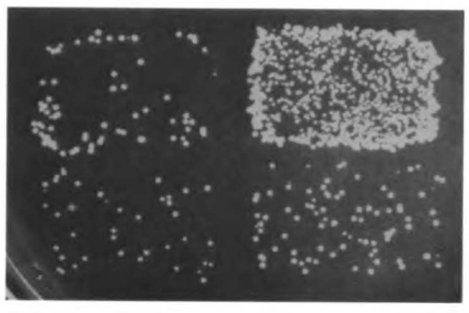
ches of *MATα* strains, *fps1Δ* (JP226) and *fus2Δ* (JP257) (left column) and
gpd1Δ (JP233) and *fus2Δ gpd1Δ* (JP287) (right column), were mated to a
fus1 fus2 strain (IH2353) as described in Materials and Methods.

ches of *MAT**a*** strains, WT (IH3196) and *fps1Δ* (JP147), were mated on
(left panel) or YEPD containing 1M sorbitol (right panel) to a *MATα*
s2 strain (IH2351).

a


a fus1Δ
a fps1Δ
a WT
a fus1Δ gpd1Δ
a fps1Δ gpd1Δ
a gpdΔ1

b


α fps1Δ
α fus2Δ
α fps1Δ gpd1Δ
α fus2Δ gpd1Δ

c

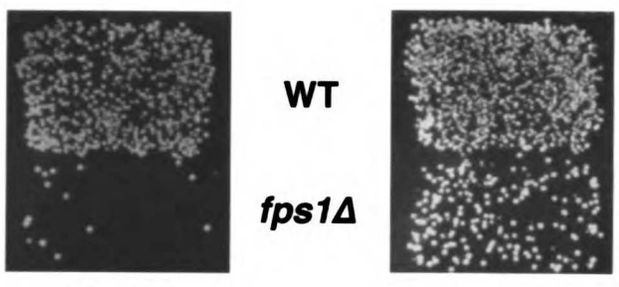

WT
fps1Δ
YEPD
1M SORBITOL

Table 2-3

Effect of Altering *GPD1* Expression on the Defect in Cell Fusion of *fps1Δ* Mutants

	% prezygotes in matings to*	
genotype	α *GPD1*	α *gpd1Δ*
a WT	0.75 +/- 0.55	0.75 +/- 0.55
a *gpd1Δ*	1.50 +/- 0.75	1.75 +/- 0.55
a *fps1Δ*	42.3 +/- 6.7	55.5 +/- 4.6
a *fps1Δ gpd1Δ*	14.0 +/- 2.5	26.0 +/- 7.2

	α WT+ vector	α WT+ 2μ *GPD1*
a WT + vector	2	<1
a WT + 2μ *GPD1*	5	<1
a *fps1Δ* + vector	20	7
a *fps1Δ* + 2μ *GPD1*	84	33

* % prezygotes represents the number of prezygotes/(zygotes+prezygotes). In lines 1-4, α strains were WT (IH3186) and *gpd1Δ* (JP236). **a** strains were WT (IH3196), *gpdΔ* (JP168), *fps1Δ* (JP147), and *fps1Δ gpd1Δ* (JP165). At least 100 partnered cells were counted for each experiment. Values are the means of four experiments +/- SD. In lines 5-8, the α strain was IH3190. **a** strains were WT + vector (JP400), WT +YEp*GPD1*(JP199), *fps1Δ* + vector (JP401), and *fps1Δ* + YEp*GPD1*(JP200). More than one hundred patnered cells were counted per sample. Data are from one representative experiment.

1.8-fold increase in glycerol levels in comparison with the *fps1Δ* strain carrying a control plasmid (JP401) (Table 2-2, lines 7 and 8). We observed that the glycerol-overproducing strain JP200 exhibited an enhanced defect in cell fusion, producing 84% prezygotes in comparison with strain JP401, which yielded 20% prezygotes when mated to a wild-type strain (Table 2-3). No significant increase in prezygotes was observed in the *FPS1* strain carrying the *GPD1* plasmid. We also examined the ability of these strains to mate with partners that overexpress *GPD1*. Once again, we observed that mating with JP200 yielded nearly 5-fold more prezygotes than JP401: 33% versus 7% (Table 2-3, lines 7 and 8, second column). Overexpression of *GPD1* increased the levels of intracellular glycerol and exacerbated the defect in cell fusion of *fps1Δ* mutants. We conclude that the defect in cell fusion in *fps1Δ* mutants correlates with increased levels of intracellular glycerol.

In contrast to what was observed when *GPD1* was overexpressed in the *fps1Δ* mutant, we found that when *GPD1* was overexpressed in the mating partner, there was a decrease in prezygotes formed, from 20% to 7% for the *fps1Δ* strain carrying the control plasmid (Table 2-3, line 7) and from 84% to 33% for the *fps1Δ* strain carrying the *GPD1* plasmid (Table 2-3, line 8). One explanation for this improvement in fusion is that overexpression of *GPD1* in the mating partner led to increased extracellular glycerol, which restored osmotic balance to the *fps1Δ* mutant. Consistent with the idea that glycerol produced by the mating partner may osmotically stabilize the *fps1Δ* mutant, we found that deletion of *gpd1Δ* in the partner somewhat exacerbated the defect of the *fps1Δ* mutant. *fps1Δ* mutants mated to *GPD1* strains produced 42% prezygotes which increased to 55% when mated to a *gpd1Δ* partner (Table 2-3, line 3). Additionally, *fps1Δ gpd1Δ* mutants mated to *GPD1* strains

produced 14% prezygotes which increased to 26% when mated to a *gpd1Δ* partner (Table 2-3, line 4).

High Osmolarity Partially Supresses the Cell Fusion Defect of *fps1Δ* Mutants

The correlation between increased intracellular glycerol levels and a defect in cell fusion suggests at least two possible causes of the cell fusion defect. Intracellular glycerol itself may inhibit cell fusion. Another possibility is that an imbalance between intracellular and extracellular solute levels inhibits fusion. To distinguish between these possibilities, we determined whether restoration of osmotic balance by 1 M sorbitol could suppress the cell fusion defect of *fps1Δ* mutants. If elevated intracellular glycerol *per se* inhibits cell fusion, then 1 M sorbitol should either have no effect or may exacerbate the cell fusion defect of *fps1Δ* mutants, since 1 M sorbitol induces *GPD1* expression (Hirayama et al., 1995). If the mating defect is due to osmotic imbalance, then mating in the presence of 1 M sorbitol may suppress the defect. We observed the latter: the mating defect of *fps1Δ* mutants was partially alleviated by 1 M sorbitol (Fig. 4c). Because this mating assay involves mating to a *fus1 fus2* strain, it was possible that the improvement in mating resulted from an effect of the 1 M sorbitol on the *fus1 fus2* partner rather than on the *fps1Δ* partner. To address this issue, we examined the effect of 1M sorbitol on *fps1Δ* mutants mated to a wild-type strain. We found that the fusion defect was suppressed in the presence of 1 M sorbitol; the number of prezygotes declined from 37% to 10% (Table 2-4). Moreover, the presence of 1M sorbitol did not improve mating by *fus1 fus2* mutants (data not shown) or result in decreased levels of prezygotes (Table 2-4). We conclude that the mating defect of *fps1Δ* mutants can be suppressed by restoring osmotic balance to these cells.

Table 2-4

1M Sorbitol Partially Suppresses the Cell Fusion Defect of *fps1Δ* Strains

genotypes	% prezygotes* in matings on YEPD unsupplemented	+1M Sorbitol
a WT	<1	<1
a *fps1Δ*	37 +/- 4	10 +/- 2
a *fus1Δ*	12 +/- 3	14 +/- 2
α *fus2Δ*	7 +/- 1	15 +/- 2
α WT (JP2350)	2	2
α *fus1 fus2*	65	93

* % prezygotes represents the number of prezygotes/(zygotes+prezygotes). Cells were mated either on YEPD or YEPD supplemented with 1M sorbitol. **a** strains were WT (IH3196), *fps1Δ* (JP147), and *fus1Δ* (JP52); they were mated to an α WT strain (IH3186). α strains were WT (IH2350), *fus2Δ* (JP257), and *fus1 fus2* (IH2351); they were mated to an **a** WT strain (IH3196). For lines 1-4, values are the mean of three experiments +/- SD; for lines 5 and 6, values are the average of two experiments.

Additional evidence that the cell fusion defect of *fps1Δ* mutants is not due to an absolute increase in glycerol but rather to a difference between intracellular and extracellular glycerol comes from comparing the behavior of wild-type cells overexpressing *GPD1* and *fps1Δ* mutants. Both of these strains contained approximately two-fold more intracellular glycerol than wild-type strains containing vector alone (Table 2-2; lines 6 and 7), yet the *fps1Δ* mutant exhibited a more severe cell fusion defect (Table 2-3, lines 6 and 7). One explanation for this difference is that the wild-type cells, which contain a higher level of glycerol due to overexpression of *GPD1*, can efficiently release this glycerol, so that extracellular glycerol levels also increase. The *fps1Δ* mutant, which releases glycerol inefficiently, has higher intracellular glycerol and decreased extracellular glycerol (Luyten et al., 1995). These data suggest that the osmotic imbalance, rather than the absolute levels of intracellular glycerol, accounts for the cell fusion defect of *fps1Δ* mutants.

Activated Alleles of *PKC1* Inhibit Cell Fusion

The protein kinase C pathway is induced by conditions in which intracellular solute is higher than extracellular solute (Davenport et al., 1995). Because *fps1Δ* mutants have higher intracellular glycerol concentrations than wild-type cells, we wondered whether the cell fusion defect of *fps1Δ* mutants was influenced or mediated by the *PKC1* pathway. If activation of the *PKC1* pathway is responsible for the defect in cell fusion of *fps1Δ* mutants, then activation of Pkc1p in an otherwise wild-type background should give a similar defect. We therefore examined the effect of expressing an activated allele of *PKC1* on mating and cell fusion.

A CEN-ARS plasmid containing such an allele of *PKC1* (*PKC1-R398P*) was introduced into a wild-type strain (IH3196) to generate strain JP300. *PKC1-R398P* alters the pseudosubstrate binding site of Pkc1p, creating a dominant,

activated allele (Nonaka et al., 1995). Expression of this allele under control of its own promoter had no detectable effect on cell viability (data not shown). The activated allele did, however, result in a mating defect similar to that of the *fps1Δ* mutant (Fig. 2-5*a*). Assays of cell cycle arrest and shmoo formation indicated that JP300 responded normally to pheromone (data not shown). Furthermore, JP300 produced pheromone normally, as assayed by halo formation. When JP300 was mated to a wild-type strain, prezygotes accumulated. JP300 exhibited normal partnership, indicating that its defect did not result from inability to respond or adhere to its partner, but rather to a defect in cell fusion (Table 2-5, lines 1 and 2; Fig. 2-1*e*). We conclude that activation of the *PKC1* pathway is sufficient to cause a defect in cell fusion. We were able to activate the *PKC1* pathway at the time of mating, by expressing an activated allele of *PKC1* (*PKC1-R398A*) under control of the *GAL1* promoter (Watanabe et al., 1994). Expression of this activated allele when cells were mating resulted in a mating defect, whereas no significant mating defect was seen when cells expressed wild-type *PKC1* or *PKC1-K853R*, an allele mutated in the kinase domain (Fig. 2-5*b*). Hence, a constitutively active allele of Pkc1p expressed during mating causes a mating defect.

Pkc1p Does Not Act Through Fps1p To Inhibit Cell Fusion

Activated alleles of Pkc1p and loss of function of *FPS1* caused quantitatively very similar cell fusion defects (Table 2-5), raising the possibility that Pkc1p negatively regulates cell fusion by inhibiting Fps1p. If the only target of Pkc1p for inhibiting cell fusion were Fps1p, then the activated allele of Pkc1p should not exacerbate the defect in fusion of an *fps1Δ* strain. However, we found that the *PKC1-R398P* allele increased the defect in cell fusion of the *fps1Δ* strain (Table 2-5, lines 3 and 4). In addition, if Pkc1p negatively regulates Fps1p, then deletion of *GPD1* should suppress the *PKC1-*

Figure 2-5. Inhibition of mating by activated *PKC1* alleles.

a. Patches of *MATa* strains, WT (IH3196), *gpd1Δ* (JP168), and *fps1Δ* (JP163), containing YCp50 (left column) or YCp50-*PKC1-R398P* (pJP67) (right column), were mated to a *MATα fus1 fus2* strain (IH2351).

b. A wild-type strain (IH3196) containing plasmids pDL295 (p*GAL1*[*PKC1-K853R::HA*]), pDL242 (p*GAL1*[*PKC1-R398A]*), or pDL293 (p*GAL1*[*PKC1::HA*]), were mated to a *MATα fus1 fus2* strain (IH2351) on either YEPD (left column) or YEP Galactose (right column) as described in Materials and Methods.

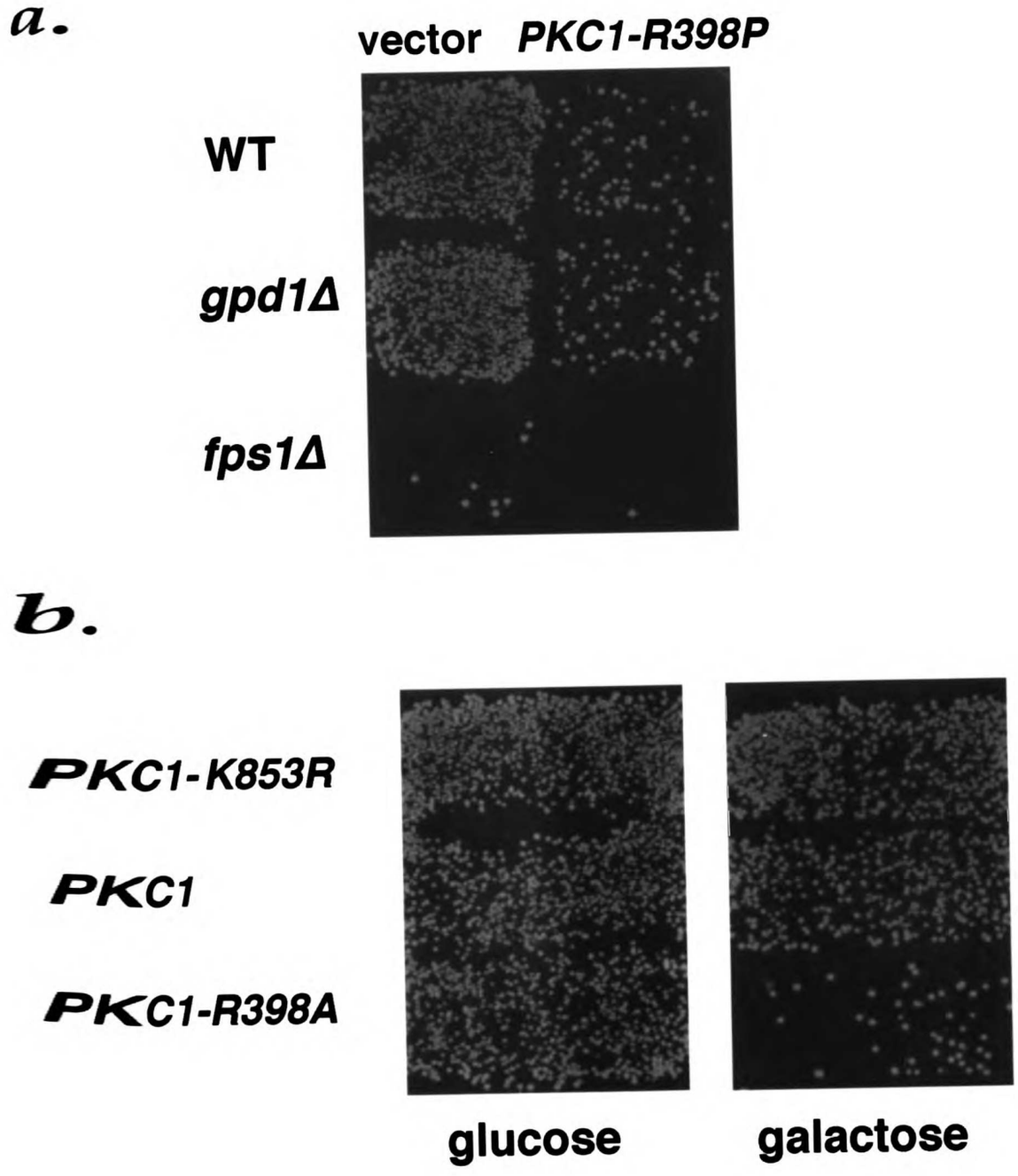

a.
vector
PKC1-R398P
WT
gpd1Δ
fps1Δ
b.
PKC1-K853R
PKC1
PKC1-R398A
glucose
galactose

Table 2-5

The *PKC1-R398P* Allele Causes a Defect in Cell Fusion

genotype	plasmid	% prezygotes*	% partnership
a WT	vector	2.0 +/- 0.7	41.6 +/- 1.6
a WT	*PKC1-R398P*	16.3 +/- 1.1	46.3 +/- 3.9
a *fps1Δ*	vector	26.3 +/- 6.4	40.0 +/- 2.5
a *fps1Δ*	*PKC1-R398P*	82.6 +/- 4.9	44.0 +/- 0.7
a *gpd1Δ*	vector	2.0 +/- 0.7	40.7 +/- 7.9
a *gpd1Δ*	*PKC1-R398P*	18.3 +/- 1.5	41.0 +/- 1.9

* % prezygotes represents the number of prezygotes/(zygotes+prezygotes).

% partnership represents the number of (zygotes + prezygotes)/total cells.

a strains were WT (IH3196), *fps1Δ* (JP163), and *gpd1Δ* (JP168). The strains were transformed with either YCp50 containing *PKC1-R398P* (pJP67) or YCp50 alone and were mated to a wild-type α strain (IH3186). More than one hundered partnered cells were counted per experiment to determine % prezygotes. More than two hundred total cells were counted per experiment to determine % partnership. Values are the mean of three experiments +/- SD.

R398P allele, as it partially suppressed an *fps1Δ* mutant (Fig. 2-4; Table 2-3). Deletion of *GPD1* did not, however, suppress the *PKC1-R398P* allele, suggesting that Pkc1p acts downstream or parallel to glycerol accumulation (Table 2-5, lines 2 and 6; Figs. 2-6 and 2-8).

***fps1Δ mpk1Δ* Double Mutants Require Osmotic Support for Viability**

One explanation for the defect in *fps1Δ* mutants is that increased activity of the *PKC1* pathway accounts, at least in part, for the defect in cell fusion. Additional evidence that this pathway is active came from analysis of a mutant defective in both *FPS1* and *MPK1*, which codes for the MAP kinase regulated by Pkc1p (Lee et al., 1993: Torres et al., 1991). Although both *fps1Δ* and *mpk1Δ* mutants grow normally at 25°C (Van Aelst et al., 1994; Lee et al., 1993), we were unable to obtain double mutant segregants when spores were germinated at 25°C on YEPD in eight tetrads analyzed. The *fps1Δ mpk1Δ* double mutant was obtained, however, when spores were germinated in the presence of 1 M sorbitol: 14 double mutants were obtained from 18 tetrads; all were unable to grow on YEPD lacking sorbitol. The remaining 58 spores, none of which were double mutants, grew normally on unsupplemented YEPD at 25°C (Fig. 2-6). The inviability of the *fps1Δ mpk1Δ* strain demonstrates that the *fps1Δ* mutant, in the absence of osmotic stabilizing agents, requires *MPK1* for viability and thus that Mpk1p is active in *fps1Δ* mutants.

Mutations in *FUS1* and *FUS2* Are Not Suppressed by Altering Osmotic Conditions

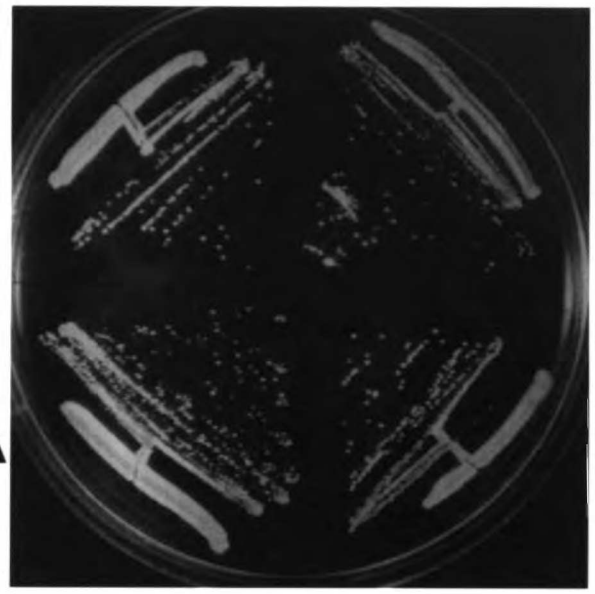
We have carried out a variety of analyses to determine whether the cell fusion defect of *fus1* and *fus2* mutants has a similar basis to that of *fps1* mutants. First, we determined whether deletion of *GPD1* restored mating and cell fusion to *fus1* or *fus2* mutants. Although deletion of *GPD1*

Figure 2-6. Synthetic lethality of *fps1Δ mpk1Δ*.

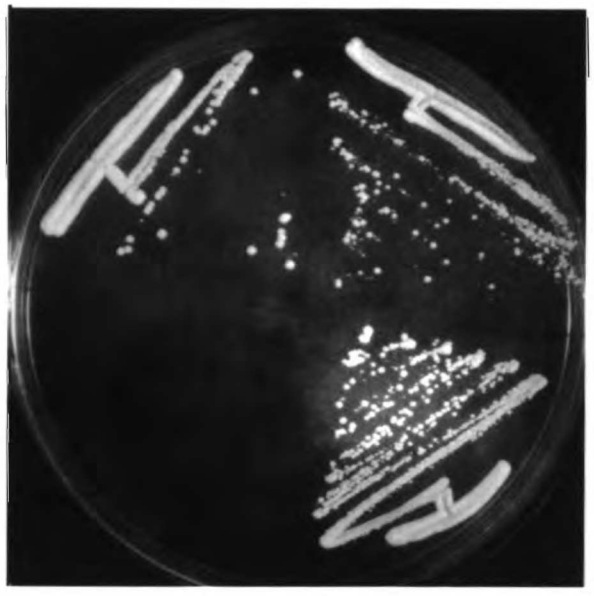
a. *mpk1Δ* (JP326), *fps1Δ* (JP325), *mpk1Δ fps1Δ* (JP327) and wild-type strains were grown on YEPD + 1 M sorbitol at 25^{0}C for two days.

b. *mpk1Δ* (JP326), *fps1Δ* (JP325), *mpk1Δ fps1Δ* (JP327) and wild-type strains were grown on YEPD 25^{0}C for two days.

a.



b.



improved mating in *fps1Δ* mutants, it did not improve matings of *fus1* and *fus2* mutants (Fig. 2-4). Similarly, microscopic examination of mating mixes did not reveal a significant change in prezygote accumulation in *fus1 gpd1* or *fus2 gpd1* double mutants compared with *fus1 GPD1* or *fus2 GPD1* strains. Moreover, overexpression of *GPD1* did not exacerbate the cell fusion defect of *fus1* mutants (data not shown). We conclude that the cell fusion defect of *fus1* and *fus2* strains is unlikely to be due to an accumulation of intracellular glycerol. Additional evidence that the defect in cell fusion in *fus1* and *fus2* mutants differs from that in *fps1Δ* mutants came from analyzing the ability of mutants to mate in the presence of 1 M sorbitol. Accumulation of prezygotes was not detectably altered for *fus1Δ* mutants by mating in the presence of 1 M sorbitol, whereas *fus2Δ* and *fus1 fus2* strains showed an exacerbation of the fusion defect, a result opposite to that seen with *fps1Δ* mutants (Table 2-5). Thus, *fus1* and *fus2* mutants differ from *fps1Δ* mutants in their genetic interactions with *GPD1* and in their response to 1 M sorbitol. We conclude that it is unlikely that the cell fusion defect associated with mutations in *FUS1* and *FUS2* is due to an inability to maintain osmotic stability.

DISCUSSION

During conjugation, haploid cells of opposite mating type reorganize their cell walls to allow cell fusion. The signals that control cell wall degradation and membrane fusion and the machinery that mediates these processes are not known. We have found that the *FPS1* gene, which codes for a glycerol transporter, is essential for efficient cell fusion. We identified *fps1* mutants based on their defect in mating to an enfeebled (*fus1 fus2*) mating partner and showed that they are specifically defective in cell fusion: they accumulated prezygotes during mating but were normal for pheromone signalling. The fusion defect of *fps1* mutants correlates with their increased level of intracellular glycerol relative to wild-type cells: the defect was partially suppressed by reducing intracellular glycerol and exacerbated by further increasing intracellular glycerol levels. The defect appears to result from osmotic imbalance rather than a high level of glycerol *per se* since extracellular 1 M sorbitol partially relieved the fusion defect. We propose that during mating, cells monitor their osmotic state before committing to breaking down and remodeling their cell wall. In particular, under conditions of osmotic imbalance, such as in strains lacking Fps1p, cells interrupt cell wall breakdown. The *fps1* mutant thus reveals a checkpoint for cell wall breakdown during mating.

Because *fps1* mutants accumulate a high level of intracellular glycerol, we reasoned that they experience a situation analogous to that of wild-type cells exposed to hypotonic conditions, which would lead to activation of the *PKC1* pathway (Davenport et al., 1995; Kamada et al., 1995). We therefore anticipated that constitutive activation of the *PKC1* pathway, for example, due to alteration of Pkc1p itself, would cause a fusion defect similar to that of *fps1*

nts. This prediction was borne out. Additional analyses of the
itutively activated *PKC1* mutant indicate that Pkc1p functions
nstream of Fps1p and glycerol accumulation. We suggest that activation
kc1p couples sensation of hypo-osmotic conditions to inhibition of cell
n. Other genes required for cell fusion, in particular, *FUS1* and *FUS2*, do
ppear to participate in this osmolarity checkpoint, as mutants defective
ese genes are not influenced by osmolarity.

otic Balance Governs Cell Fusion

During vegetative growth, *fps1* mutants accumulate approximately
fold more intracellular glycerol than wild-type cells, which does not
e any apparent growth defect (Van Aelst et al., 1991; J. Philips,
blished observations). This increase in intracellular glycerol causes a
t in cell fusion during mating. In principle, Fps1p, which contains six
ive membrane-spanning domains (Van Aelst et al., 1991), might play a
n mating that is distinct from its role in transport. Our analyses,
ever, indicate that its role in mating is a consequence of its function in
rol transport and that in its absence, the process of cell wall breakdown is
ited. Our observations indicate that glycerol functions as an osmolyte
ng the mating process rather than being directly involved, for example,
gnalling between the mating partners. This conclusion comes from our
vations that glycerol *per se* is not required during mating since the defect
1 mutants can be suppressed by extracellular sorbitol or by deleting
l, which reduces intracellular glycerol levels. We thus favor the
thesis that mating cells of yeast are exceptionally sensitive to osmotic
uilibrium prior to undergoing the potentially lethal morphogenetic
ges required for cell fusion. Cells lacking *FPS1* sense that they are not
tically balanced and respond by blocking cell wall breakdown. In other

words, cells possess a checkpoint that ensures that they do not degrade their cell wall under hypo-osmotic conditions.

It is unclear whether the the osmotic imbalance perceived by the *fps1* mutant results from a difference between the mutant and its environment or a disparity between mating partners. If the osmolarity between mating partners were monitored, we might expect that an **a** *fps1* and α *fps1* mutant mated to each other would form zygotes at normal frequency. Although the number of prezygotes does not return to wild-type levels when *fps1Δ* mutants are mated to *fps1Δ* mutants, the reduction in the number of prezygotes compared to *fps1Δ* mutants mated to wild-type may be significant (Table II), indicating that a difference in osmolarity between mating partners may be monitored.

The *PKC1* Pathway May Mediate the Cell Fusion Checkpoint

Support for the hypothesis that osmosensing pathways regulate cell fusion was obtained by analyses of the *PKC1* pathway. This pathway is required for maintenance of cell integrity and cell wall construction (reviewed by Errede and Levin, 1993) and is induced when cells are subjected to conditions, such as hypo-osmotic shock, that threaten cell integrity (Davenport et al., 1995; Kamada et al., 1995). We found that activation of Pkc1p using a constitutively activated allele of *PKC1* led to a fusion defect essentially identical to that of *fps1* mutants: *PKC1-R398P* and *fps1* strains accumulated prezygotes that were morphologically indistinguishable from each other.

One possibility is that Pkc1p blocks cell fusion by inhibiting *FPS1*. Two experiment suggest that this is not the case. First, the *PKC1-R398P* mutation has a more severe phenotype in an *fps1Δ* mutant than in a wild-type strain. Additionally, cell fusion is not restored to the *PKC1-R398P* mutant by

reducing intracellular glycerol levels due to deletion of *GPD1*. These data, in conjunction with the previously demonstrated role of Pkc1p in responding to hypo-osmotic shock, lead us to propose that Pkc1p lies downstream of Fps1p and Gpd1p and functions as a part of a checkpoint to monitor osmotic balance during mating (Fig. 2-7).

If activation of the *PKC1* pathway is responsible for the defect in cell fusion of *fps1Δ* mutants, then Pkc1p should be active in *fps1Δ* mutants and deletion of *pkc1* should suppress the mating defect. Because 1 M sorbitol suppresses the mating defect of *fps1Δ* mutants, and *pkc1Δ* mutants require 1 M sorbitol for viability (Levin and Bartlett-Heubusch, 1992; Paravicini et al., 1992), it is impossible to ask whether *pkc1Δ* suppresses the mating defect. However, evidence that the *PKC1* pathway is active in *fps1* mutants comes from the analysis of mutants defective in both Fps1p and Mpk1p, the MAP kinase in the *PKC1* pathway (Lee et al., 1993; Torres et al., 1991). We have observed that *fps1Δ mpk1Δ* mutants are inviable unless supported osmotically, indicating that Mpk1p is active in the *fps1* mutants. The *FPS1 mpk1* strains are inviable only at high temperature (37°C) (Lee et al., 1993), whereas the *fps1 mpk1* strains are also inviable at low temperatures (25°C). One explanation for the inviability of the *fps1 mpk1* double mutant is that *fps1* mutants depend upon Mpk1 activity in order to respond to the osmotic imbalance caused by their inefficient release of glycerol.

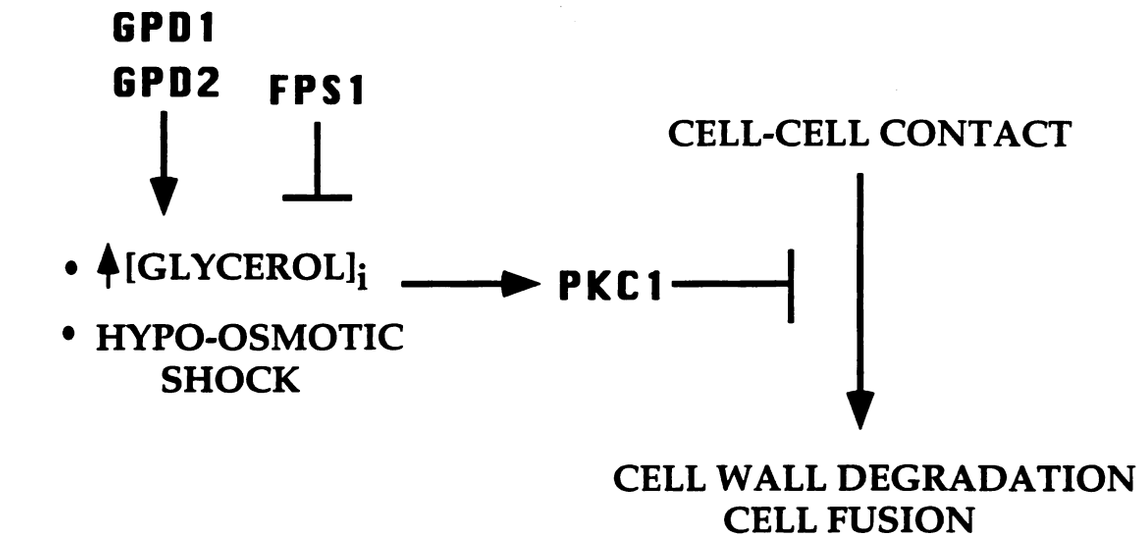
At least two different models could explain the role of *PKC1* in cell fusion. According to one view, Pkc1p negatively regulates cell fusion as part of a checkpoint to ensure that cells are not osmotically vulnerable prior to fusion (Fig. 2-7*a*). In this case, Pkc1p is utilized only under conditions, such as hypo-osmotic shock, that make cell fusion particularly dangerous to cell integrity. According to a second view, Pkc1p constitutively inhibits cell fusion, so that

Figure 2-7. Regulation of cell fusion by osmosensing pathways.

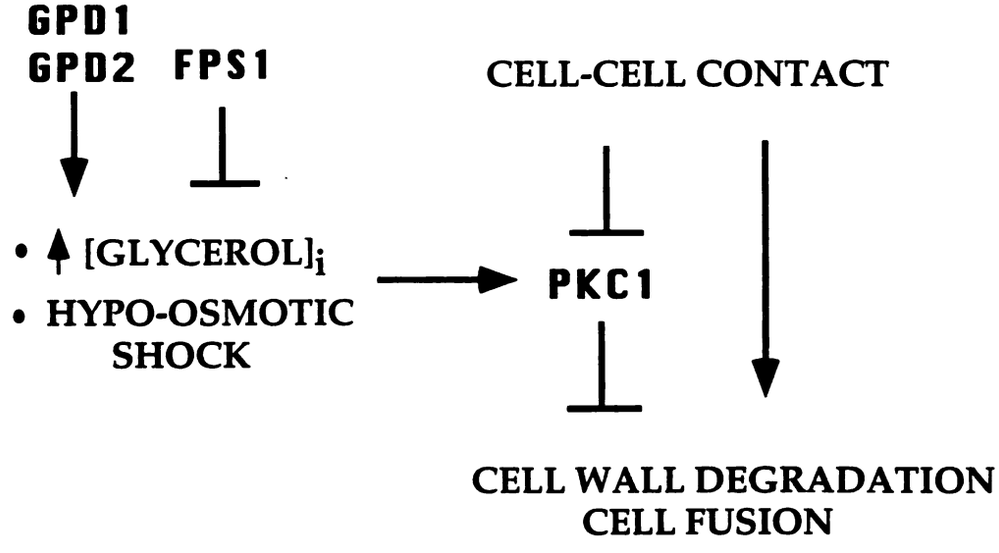
a. Increased intracellular glycerol levels due to deletion of *FPS1* and exacerbated by overexpression of *GPD1* activate the protein kinase C pathway, which responds to hypo-osmotic shock. Pkc1p is part of a checkpoint which inhibits cell fusion if cells are hypo-osmotically shocked.

b. Pkc1p is a negative regulator of cell fusion which is turned off or overridden in order for cells to fuse. When cells are osmotically stressed, because of deletion of *fps1Δ* and exacerbated by increased expression of *GPD1*, Pkc1p is activated, preventing cell fusion.

a.


GPD1
GPD2
FPS1
CELL-CELL CONTACT
• ↑[GLYCEROL]i
• HYPO-OSMOTIC SHOCK
PKC1
CELL WALL DEGRADATION
CELL FUSION

b.


GPD1
GPD2
FPS1
CELL-CELL CONTACT
• ↑ [GLYCEROL]i
• HYPO-OSMOTIC SHOCK
PKC1
CELL WALL DEGRADATION
CELL FUSION

cell fusion occurs only when Pkc1p is antagonized (Fig. 2-7*b*). According to this model, Pkc1p is a key regulator of cell fusion, inhibiting fusion until a signal turns it off. In this case, the cell fusion defect of *fps1* mutants results from activation of the osmotic sensing pathway, which prevents the Pkc1p-dependent inhibition from being relieved during cell fusion.

Previous work has shown that Mpk1p is activated in response to mating pheromone (Errede et al., 1995; Zarzov et al., 1996), presumably due to activation of Pkc1p, and is necessary for viability of yeast cells under these conditions (Errede et al., 1995). Our observations indicate, in contrast, that activation of Pkc1p inhibits a late step in mating. To explain the apparently paradoxical actions of the *PKC1* pathway in mating, we suggest, as in model two above, that Pkc1p is a negative regulator of cell fusion (Fig. 2-7*b*) which, during normal mating, is activated and then subsequently inhibited. We propose that Pkc1p is first activated in response to pheromone and thereby inhibits cell wall degradation during initial stages of pheromone response and projection formation. When mating partners come in contact with each other, they generate a mechanical force as the cell walls become irreversibly adhered to each other, which generates a signal to turn off the *PKC1* pathway. We suggest that the signal to turn off the *PKC1* pathway is a mechanical signal. It has previously been suggested that the *PKC1* pathway responds to membrane stretch due to various stimuli, such as low external osmolarity, high temperature, and drug-induced membrane stretch (Kamada et al., 1995).

Proteins Required for Cell Fusion: Regulators and Machinery

Genes required for cell fusion have been identified by a number of different strategies (Trueheart et al., 1987; McCaffrey et al., 1987; Elion et al., 1995; Elion et al., 1990; Kurihara et al., 1994; Elia and Marsh, 1996). The studies presented here allow us to make a distinction between proteins that regulate

of the process of fusion (such as Pkc1p or, more indirectly, Fps1p) and potential participants in fusion itself (such as Fus1p and Fus2p). This distinction comes from the following observations. Fps1p is not directly required for cell fusion: its requirement can be relieved by deleting *GPD1* or by mating in the presence of high osmolarity. Rather, Fps1p is required to maintain osmotic balance and thereby avoid tripping a checkpoint that inhibits cell fusion. We have shown here that Pkc1p also governs fusion: cell expressing activated forms are defective in fusion presumably because they activate the cell-fusion checkpoint. It is not clear whether Fus1p and Fus2p are regulators of fusion or play more direct roles in this process or whether they are targets for the inhibition mediated by the *PKC1* pathway. The fusion defect of *fus1* and *fus2* mutants is not influenced by deletion of *GPD1* or osmotic stabilizers, and thus it is unlikely that these mutants exhibit a fusion defect by evoking the osmotic checkpoint.

Our findings on fusion during yeast mating may have implications for other examples of cell fusion. Maintenance of cell integrity is essential for all cell fusion processes. We therefore expect that processes such as sperm-egg and myoblast fusion may also be regulated by osmolarity. It would be striking if protein kinase C were involved in monitoring osmotic balance during sperm-egg or myoblast fusion. Eyster and McFarland (1995) have, in fact, reported that endogenous modulators of protein kinase C can regulate myogenesis. Perhaps activated versions of protein kinase C could block sperm-egg or myoblast fusion as they block fusion between mating partners in yeast. The targets of the inhibition triggered by the osmolarity-induced checkpoint in yeast remain to be determined. We are seeking to identify such targets by genetic strategies. These proteins might be directly involved in cell

fusion during mating and illuminate mechanisms relevant to other cell fusion events.

CHAPTER THREE

IDENTIFICATION OF Kel1p, A KELCH DOMAIN-CONTAINING PROTEIN, INVOLVED IN CELL FUSION AND MORPHOLOGY IN *SACCHAROMYCES CEREVISIAE*

ABSTRACT

We showed previously that protein kinase C, which is required to maintain cell integrity, negatively regulates cell fusion (Philips, J. and I. Herskowitz. 1997. *J. Cell Biol.* 138:961-974). To identify additional genes involved in cell fusion, we looked for genes whose overexpression relieved the defect caused by activated alleles of Pkc1p. This strategy led to the identification of a novel gene, *KEL1*, which encodes a protein composed of two domains, one containing six kelch repeats, a motif initially described in the *Drosophila* protein Kelch (Xue, F. and L. Cooley. 1993. *Cell.* 72:681-693), and another domain predicted to form coiled coils. *KEL2*, which corresponds to ORF YGR238c, encodes a protein highly similar to Kel1p. Like *KEL1*, overexpression of *KEL2* suppressed the mating defect associated with activated Pkc1p. Consistent with a role for Kel1p in cell fusion, *kel1Δ* mutants exhibit a defect in cell fusion which is exacerbated by activated alleles of Pkc1p or loss of *FUS1*. Kel1p is also required for proper morphology during vegetative growth. Both Kel1p and Kel2p localize to the site where cell fusion occurs during mating and to regions of polarized growth during vegetative growth. Co-immunoprecipitation and two-hybrid analyses indicate that Kel1p and Kel2p physically interact. This interaction likely occurs *in vivo* since localization of Kel2p depends upon *KEL1*. Despite this association, *kel2Δ* mutants are not impaired in cell fusion or morphology. We conclude that Kel1p has a role in cell morphogenesis and cell fusion and may act upstream or parallel to Pkc1p.

INTRODUCTION

Cell fusion occurs during a variety of biological processes -- for example, in sperm-egg fusion during fertilization, in myoblast fusion during myotube formation, and in microorganisms such as yeast during mating. In yeast, this process is initiated when haploid cells respond to the peptide pheromone secreted by cells of the opposite mating type (**a** cells responding to α-factor and α cells to **a**-factor). The pheromones bind a seven-transmembrane receptor, stimulating a signal transduction cascade which results in cell cycle arrest, transcriptional induction of genes required for mating and cell fusion (Trueheart et al., 1987; McCaffrey et al., 1987; Elion et al., 1995), and a morphological response called shmoo formation (for reviews see Bardwell et al., 1994; Herskowitz, 1995). Cells polarize their actin cytoskeleton towards their mating partner by detecting a pheromone gradient (Jackson and Hartwell, 1990; Madden and Snyder, 1992; Segall, 1993), resulting in the polarized deposition of proteins to the region of future cell contact (Trueheart et al., 1987; Elion et al., 1995).

Once the mating partners come into contact, a series of events, poorly understood at the molecular level, must be successfully executed to produce a diploid zygote. First, the cell wall must be removed in the region separating the two cells. Since inappropriate removal of cell wall material could result in lysis, correct spatial and temporal regulation of this event is critical for maintenance of cell integrity. Hence, degradation of the cell wall does not occur until a cell comes into contact with its mating partner. The cell wall is then degraded beginning in the center of the region of contact and proceeding towards the edges (Osumi et al., 1974; Gammie et al., submitted). Cell wall

degradation normally occurs quickly, so few cells are observed that have adhered but failed to fuse (Trueheart et al., 1987). Once cell wall material is removed, plasma membranes fuse, and then intracellular organelles such as nuclei and mitochondria fuse to produce an **a**/α diploid zygote.

Our previous work demonstrated that Pkc1p and Fps1p regulate cell fusion (Philips and Herskowitz, 1997). Mutants defective in the *FPS1* gene, predicted to encode a glycerol transporter (Luyten et al., 1995), accumulate intracellular glycerol and exhibit a defect in cell fusion (Luyten et al., 1995; Philips and Herskowitz, 1997). The fusion defect can be alleviated if intracellular glycerol concentration is restored to wild-type levels or if 1 M sorbitol is provided extracellularly to osmotically stabilize the cells, suggesting that osmotic imbalance is the cause of the defect in cell fusion. Since the Pkc1p pathway responds to conditions that threaten cell integrity such as hypo-osmotic shock (Davenport et al., 1995; Kamada et al., 1995), we proposed that Pkc1p inhibits cell fusion if cells are not osmotically balanced, a situation that makes cell fusion particularly dangerous (Philips and Herskowitz, 1997). Supporting the idea that Pkc1p negatively regulates cell fusion, cells expressing an activated allele of *PKC1 (PKC1-R398P)* exhibit a defect in cell fusion (Philips and Herskowitz, 1997). *PKC1-R398P* alters the pseudosubstrate binding site of Pkc1p, creating a dominant, activated allele (Nonaka et al., 1995). Pkc1p is proposed to function upstream of a MAP kinase module composed of the MEKK, Bck1p/Slk1p (Costigan et al., 1992; Lee and Levin, 1992), two redundant MEKs, Mkk1p and Mkk2p (Irie et al., 1993), and the MAPK, Mpk1p/Slt2p (Torres et al., 1991; Mazzoni et al., 1993; reviewed by Errede and Levin, 1993). Consistent with the idea that the Pkc1p pathway may inhibit cell wall degradation during mating, the pathway is activated in response to pheromone (Errede et al., 1995; Zarzov et al., 1996; Buehrer and Errede, 1997).

Furthermore, mutants defective in *MPK1* lyse in response to pheromone (Errede et al., 1995). These observations implicate Pkc1p in the regulation of cell fusion, but the nature of the fusion machinery and how it is controlled is unclear.

Numerous mutants have been identified that are defective in cell fusion (for review, see Marsh and Rose, 1997). When such mutants mate, they adhere but fail to fuse, producing dumbbell-shaped structures called prezygotes. In prezygotes, the cell wall persists between mating partners, presenting a barrier to plasma membrane fusion, cytoplasmic mixing, and nuclear fusion. Proteins required for cell fusion fall into three classes. The first class is comprised of Fus1p and Fus2p whose synthesis is highly induced by pheromone and seems to be specifically required for fusion (Trueheart et al., 1987; McCaffrey et al., 1987; Elion et al., 1995). *fus1 fus2* double mutants are mildly compromised in mating to a wild-type strain but are severely defective in mating to *fus1* or *fus2* strains (Trueheart et al., 1987). The other two classes include proteins that have functions during mating or vegetative growth in addition to their role in cell fusion. Axl1p, Ram1p, and Ste6p affect cell fusion in **a** but not α cells (Elia and Marsh, 1996; Brizzio et al., 1996; Dorer et al., 1997). They were originally identified for their role in **a**-factor production and mating (Adames et al., 1995; Powers et al., 1986; Kuchler et al., 1989) and later shown to affect cell fusion. The third class of proteins is required in both cell types for fusion and is also needed for processes in addition to cell fusion and mating. This class include Rvs161p, Fps1p, Spa2p, Pea2p, Bni1p, and Chs5p (Brizzio et al., submitted; Philips and Herskowitz, 1997; Dorer et al., 1997; Santos et al., 1997). These proteins regulate morphogenesis and cell integrity, which may be modulated during mating to bring about cell fusion.

Here we describe a novel protein, Kel1p, which is a member of this final class of fusion proteins. We identified Kel1p because its overexpression suppressed the mating defect associated with an activated allele of Pkc1p. Kel1p localizes to regions of polarized growth during mating and vegetative growth, and mutants lacking Kel1p exhibit defects in cell fusion and morphology.

MATERIALS AND METHODS

Yeast Strains and Media

Yeast strains are described in Appendix 4. Plasmids are described in Appendix 5. Standard yeast growth conditions and genetic manipulations are described in Rose et al. (1990). Cells were grown at 30°C in YEPD medium unless otherwise noted. DNA manipulations were performed as in Sambrook et al. (1989).

Yeast Plasmids and Transformations

Plasmids are listed in Table I. pJP72 is a pRS306-derived plasmid containing the *PKC1-R398P* allele as described in Nonaka et al. (1995) (Philips and Herskowitz, 1997). This plasmid was used to integrate *PKC1-R398P* at its genomic locus, generating strain JP317. Control strain JP338 was constructed by integrating pRS306 at *ura3*. Yeast transformations were performed by the lithium acetate method (Ito et al., 1983).

Cloning of *KEL1* and *KEL2*

KEL1 was cloned by suppression of the mating defect of JP317. A plasmid (pPHS11) containing an approximately 6 kb insert was isolated from a 2μ library (Nasmyth; see Fig. 1B). A HindIII-HindIII fragment from pPHS11 was subcloned into the HindIII site of YEp351 to generate pJP83. An SphI-SphI fragment from pPHS11 was subcloned into the SphI site of YEp351 to generate pJP81. A BamHI-BamHI fragment from pPHS11 was subcloned into the BamHI site of YEp351 to generate pJP84. pJP81, which contains YHR158c, was indistinguishable from pPHS11 in its ability to suppress the mating defect of JP317 (data not shown). To confirm that the ORF responsible for the suppression was YHR158c, YHR158c was disrupted by digesting pJP81 with

SpeI, treating with *E. coli* DNA polymerase I Klenow fragment and deoxynucleoside triphosphates, and ligating, to generate pJP82. pJP82 failed to suppress the mating defect exhibited by JP317. Additionally, a construct which contains approximately half of the YHR158c sequence (pJP84) partially suppressed the mating defect JP317, whereas a construct which removes the first 89 nucleotides of YHR158c (pJP83) failed to suppress (Fig. 1B).

Oligonucleotides OJP27 (5'-ACC TCT TGT AAC TAC TAC ATA CG) and OJP28 (5'-TCT TCT TGA CCT GAA CAT TGG) were used to amplify *KEL2* from yeast genomic DNA by PCR. The amplified product was cut with PstI and cloned into the PstI site of YEp351 to generate pJP92.

Strain Construction

The *KEL1* gene was deleted from yeast strains using pJP94, a construct that replaces *KEL1* with *hisG-URA3-hisG* (Alani et al., 1987). This plasmid was constructed by ligating the SphI-SphI fragment containing *KEL1* into the SphI site of pGEM11(F^+). BglII sites were introduced at the start position and 31 nucleotides upstream of the stop codon of *KEL1* by PCR. The BglII-BamHI fragment from pNKY51 (Alani et al., 1987) was ligated into the BglII sites to replace the *KEL1* ORF with *hisG-URA3-hisG*. *kel1Δ* strains were verified by PCR analysis.

kel2Δ deletion strains were generated using either pJP138 or pJP113. pJP113 replaces *KEL2* with *LEU2;* pJP138 replaces *KEL2* with *hisG-URA3-hisG* (Alani et al., 1987). These plasmids were generated by ligating a PstI-PstI fragment containing *KEL2* into the PstI site of pBluescript. BglII sites were introduced 11 nucleotides upstream of the start site and 7 nucleotides downstream of the stop codon by PCR. A BamHI fragment containing *LEU2* was ligated into the BglII sites to generate pJP113. A BglII-BamHI fragment containing *hisG-*

URA3-hisG from pNKY51 was inserted into the BglII sites to generate pJP138. *kel2Δ* strains were confirmed by PCR analysis.

fus1Δ strains were constructed using pJP2 (Philips and Herskowitz, 1997) and confirmed by defective mating and PCR analysis.

Microscopic and Plate Mating Assays

Mating assays scored microscopically were performed as described in Philips and Herskowitz (1997). Prezygotes were defined as structures in which the nuclei of mating partners remained unfused, as evidenced by two distinct DAPI-staining structures, and in which a septum was visible between adherent mating partners. Percentage prezygotes was defined as prezygotes/(prezygotes + zygotes). At least 100 partnered cells (zygotes + prezygotes) were counted per sample. Numbers represent averages of at least three experiments. Mating assays scored on plates were performed as described in Philips and Herskowitz (1997).

Construction of tagged Kel1p and Kel2p

GFP (S65T, F64L) (Cormack, et al., 1996) was fused to the carboxy terminus of both Kel1p and Kel2p. BglII sites were introduced just prior to the stop codon of *KEL1* and *KEL2* by oligonucleotide-mediated mutagenesis (Kunkel et al., 1987) using oligonucleotides OJP43 (5'-CAT TAC GCA TAT TGT CTT TTA AGA TCT ATC GCT GTC AGC ATC) for *KEL1* and OJP46 (5'-AAC TAT ATA CTC TCG AAC AAA GAT CTT AGT CAT TGG AAG ACG) for *KEL2*. A SalI-SacII fragment of *KEL1* which contained the introduced BglII site was cloned into the SalI-SacII sites of pJP81 to generate pJP127 (YEp351-*KEL1*). GFP was obtained on a BglII-BamHI fragment from pEBO413 (a gift from E. O'Shea, UCSF) and ligated into the BglII site of pJP127 to generate pJP129 (YEp351-*KEL1-GFP*). To construct an integrating construct, the SphI-SphI fragment containing *KEL1-GFP* from pJP129 was inserted into the SphI site of YIp5 to

generate pJP139 (YIp5-*KEL1-GFP*). The SphI-SphI fragment from pJP127 was cloned into the SphI site of YIp5 to generate pJP143 (YIp5-*KEL1*). pJP139 and pJP143 were used to integrate *KEL1-GFP* and untagged *KEL1* at the *KEL1* locus by digesting with XbaI prior to transformation. When integrated at the *URA3* locus, Kel1p-GFP complemented the mating defect of *kel1Δ* mutants (data not shown). To construct an HA-tagged version of Kel1p, an oligonucleotide containing two copies of the HA epitope was inserted into pJP127 to generate pJP202 (YEp351-*KEL1-HA*). The SphI-SphI fragment from pJP202 containing *KEL1-HA* was cloned into the SphI site of YCplac111 to generate pJP209 (YCplac111-*KEL1-HA*). The SphI-SphI fragment from pJP127 containing the untagged version of *KEL1* was ligated into the SphI site of YCplac111 to generate pJP207 (YCplac111-*KEL1*). For *KEL2*, the BglII-BamHI fragment from pEBO413 containing *GFP* was inserted into the BglII site generated in *KEL2* by oligonucleotide-mediated mutagenesis (Kunkel et al., 1987). This fusion construct, expressed from the 2μ plasmid YEp351, was designated pJP126. To construct an HA-tagged version of Kel2p, an oligonucleotide containing two copies of the HA epitope was inserted into the BglII site to create pJP131. pJP123 encodes Kel2p on YEp351 lacking an epitope tag.

Microscopy

Localization of Kel1p was analyzed in strains harboring pJP139 or pJP143 grown in SD-Ura. Localization of Kel2p was determined using strains harboring pJP126 or pJP123 grown in SD-Leu. Cells were grown to mid-log phase, sonicated, pelleted in a microcentrifuge, and resuspended in Fluoromount G (Southern Biotechnology Associates, Inc.; Birmingham, AL). Samples were viewed with a Olympus BX50 microscope at 100x (Olympus Corp., Lake Success, NY).

Morphology of wild-type (IH3196), *kel1Δ* (JP363), *kel2Δ* (JP371), and *kel1Δ kel2Δ* (JP385) strains was determined by growing cells to mid-log phase in synthetic complete medium. IH3196 harboring YEp351 or pJP81 was grown to mid-log phase in SD-Ura. IH3196 harboring pRS426 or pJP160 was grown to mid-log phase in SGal-Ura. Cells were sonicated, pelleted in a microcentrifuge, and resuspended in 50% glycerol prior to viewing with an Axioskop microscope at 100x (Carl Zeiss, Inc., Thornwood, NY).

Northern Analysis

RNA preparations and sample analysis were performed as described previously (Cross and Tinkelenberg, 1991). The probes used were DNA restriction fragments that were gel-purified and labeled by random-prime labeling using a Prime-it kit (Stratagene). The fragments used were a 1.7 kb PstI-HincII fragment containing coding sequence for *FUS1*, a 1.7 kb HindIII-BamHI fragment containing coding sequence for YHR158c, and a 0.8 kb HpaI-SalI fragment for *TCM1* (Schultz and Friesen, 1983).

Coimmunoprecipitation Analysis

Yeast cell extracts were prepared by growing cells overnight in SD-Ura Leu media. Cells were inoculated into YEPD and allowed to grow for approximately two generations to an OD_{600} of approximately 0.4. 100-ml cultures were harvested, resuspended in 250 µl ice cold lysis buffer (50 mM Tris-HCl pH8.0, 1% sodium deoxycholate, 1% Triton-X100, 0.1% SDS, 50 mM NaF, 80 mM βglycerophosphate, 1 mM Na_3VO_4, and a cocktail of protease inhibitors (Boehringer)) and mixed with an equal volume of glass beads. After vortexing vigorously ten times with 10 s pulses, samples were centrifuged at 14,000 rpm for 15 min in a microfuge at 4°C. The supernatants were removed and lysis buffer added to a final volume of 0.5 ml. Samples were centrifuged again for 15 min at 4°C. 50 µl supernatant was removed for

detection of Kel1p-GFP in the extract (data not shown). Immunoprecipitations were performed on the remaining sample at 4°C for 2 h by incubating the remaining supernatant with 25 µl of a 50% slurry of protein G-sepharose bound to anti-HA antibodies (12CA5) with rocking. Precipitates were washed four times with 0.5 ml cold lysis buffer, resuspended in 50 µl loading buffer, and boiled for five min before resolving by 7.5% SDS-PAGE. Immunoblot analysis was performed with anti-HA (HA11, Babco) or anti-GFP antibodies as described below.

Immunoblot Analysis

Extracts which were not used for coimmunoprecipitations were prepared as follows. Cells were grown to an OD_{600} of approximately 0.3. 15 ml of culture was pelleted in a microfuge and incubated on ice for 5 min. Pellets were resuspended in 150 µl ice-cold solution 1 (1.85 N NaOH, 7.4% β-mercaptoethanol) and incubated on ice for 10 min. 150 µl ice-cold 50% TCA was added. Cells were incubated on ice for 10 min, followed by centrifugation for 2 min at 4°C. Pellets were washed with 1 ml ice-cold acetone. Samples were centrifuged for 2 min at 4°C. The pellet was resuspended in sample buffer, boiled for 5 min, and resolved by 7.5% SDS-PAGE.

SDS-PAGE was followed by electroblotting onto nitrocellulose filters using the Minigel system (Bio-Rad Labs., Hercules, CA). Blots were incubated in TBST (TBS with 0.1% Triton X-100) with 10% nonfat dry milk for approximately 1 h. Blots were incubated with primary antibody (mAb HA11 diluted 1:10,000 (Babco); mAb anti-GFP (C163; a gift from P. O'Farrell, UCSF) diluted 1:2) in TBST supplemented with 2% nonfat dry milk at 4°C overnight. Blots were washed three times for 5 min in TBST and incubated with peroxidase-linked secondary antibody (Bio-Rad Labs., Hercules CA) diluted 1:2,000 in TBST with 2% nonfat dry milk for 1 h. Blots were washed twice for

5 min in TBS supplemented with 0.3% Triton X-100 followed by three washes in TBST. Blots were developed using an enhanced chemiluminescence detection kit (Amersham Corp., Arlington Heights, IL).

Two-Hybrid Analysis

Two-hybrid assays were performed as described (Gyuris et al., 1993). Yeast strain EGY48 containing the *LexAop-LacZ* reporter plasmid pSH18-34 was cotransformed with pEG202-based plasmids expressing LexA DNA-binding domain fusions and pJG4-5-based plasmids containing transcriptional activation domain fusions (Gyuris et al., 1993). β-galactosidase activities were measured essentially as described by Stern et al. (1984). Cultures were grown overnight in S raffinose-His Ura Trp. Expression of Kel1p-AD was induced with galactose for approximately 1 h. Data are expressed in Miller units $(OD_{420} \times 1000)/(OD_{600} \times \text{min} \times \text{ml})$ as determined from at least three independent transformants, tested in duplicate.

Analysis of *mpk1Δ kel1Δ* and *mpk1Δ kel2Δ* Strains

An *mpk1Δ* strain (IH3077) was crossed with two independent *kel1Δ* strains (JP445A and JP445B) generated by gene replacement or two independent *kel2Δ* strains (JP446A and JP446B) generated by gene replacement. Segregants were allowed to germinate on YEPD containing 1 M sorbitol at 30°C. Cells were streaked for single colonies on either YEPD or YEPD + 1 M sorbitol and grown for 2 d at 34.5°C.

RESULTS

Overexpression of *KEL1* and *KEL2* Suppresses *PKC1-R398P*

Cells expressing an activated allele of *PKC1* (*PKC1-R398P)* exhibit impaired mating due to a defect in cell fusion (Philips and Herskowitz, 1997). Expression of this allele under control of its own promoter resulted in a mating defect that was easily detected when cells were mated to a *fus1 fus2* mutant strain, which is also partially defective in cell fusion (Fig. 3-1A). To identify additional genes important for cell fusion, we performed a high copy suppressor screen for improved mating of cells expressing *PKC1-R398P*. Strain JP317 (*PKC1-R398P*) was transformed with a high-copy library, and approximately 4500 transformants were screened by replica plating for mating with a *fus1 fus2* mutant strain. Four plasmids, which contained distinct inserts, were identified that restored mating when retransformed. One of them, designated pPHS11, is described here. The others are described in Appendix 2.

Sequencing revealed that pPHS11 contained two full-length ORFs, YHR158c and *REC104*, and part of YHR159w and YHR156c. Subcloning demonstrated that YHR158c was responsible for suppression (see Materials and Methods and Fig. 3-1B). Fig. 1A shows that a 2μ plasmid containing YHR158c (pJP81) suppressed the mating defect of JP317. To ascertain whether overexpression of YHR158c suppressed the fusion defect of JP317, we examined the matings microscopically. We found that pJP81 reduced the number of prezygotes that accumulated in a strain expressing *PKC1-R398P* by 50%. Northern blot analysis indicated that the YHR158c transcript was present in **a**, α, and **a**/α diploid cells, consistent with it having a function

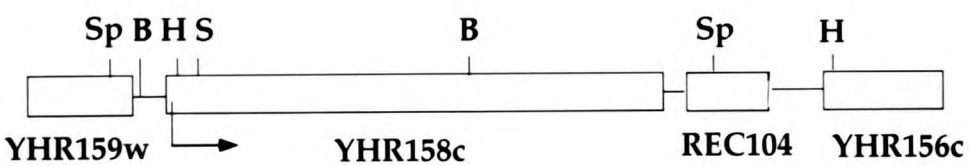
Fig. 3-1. Identification of *KEL1* and *KEL2* as suppressors of *PKC1-R398P*.

A. High copy *KEL1* and *KEL2* suppress the mating defect associated with *PKC1-R398P*. JP317 (*MAT**a** PKC1-R398P*) carrying 2μ plasmids containing *KEL1* (pJP81), *KEL2* (pJP92), or vector (YEp351) were mated to a *MATα fus1 fus2* strain (IH2351) as described in Material and Methods. The wild-type control (JP338) harbored vector YEp351.

B. Restriction map and subcloning analysis of pPHS11. pPHS11 is the original plasmid obtained from the high copy suppressor screen. pJP81-84 are YEp351-derived plasmids carrying the indicated segments. pJP82 was generated by digesting pJP81 with SpeI, filling in with Klenow (designated by *), and religating to destroy the reading frame of YHR158c. The ability of each plasmid to suppress the mating defect of JP317 is indicated on the right: +, suppression; -, no suppression; +/-, partial suppression. Restriction enzymes: S, SpeI; B, BamHI; H, HindIII; Sp, SphI. Additional information is provided in Materials and Methods and in the text.

A

B



		Suppressing Activity
pPHS11	———	+
pJP83	H ——— H	–
pJP81	Sp ——— Sp	+
pJP84	B ——— B	+/-
pJP82	Sp —*— Sp	–

during vegetative growth (see below), and was not induced by pheromone (Fig. 3-2).

YHR158c is predicted to encode an 1164 amino acid protein. Analysis of the sequence revealed six internal repeats in the amino-terminal half of the protein, followed by a domain predicted to form coiled-coils in the carboxy-terminal half of the protein (Fig. 3-3A). The repeats of approximately 50 amino acids each belong to the kelch family (Xue and Cooley, 1993; Bork and Doolittle, 1994), named after the *Drosophila* protein in which these repeats were first identified. The proteins most similar to YHR158c are a protein predicted in the *S. cerevisiae* database (YGR238c), Tea1p of *Schizosaccharomyces pombe* (Mata and Nurse, 1997), and a protein predicted by the *S. pombe* sequencing project (accession number Z98603). All four proteins have the same domain structure: six kelch repeats in the amino-terminal half of the protein followed by a predicted coiled-coil domain in the carboxy terminus. Because YHR158c and YGR238c contain kelch repeats, we have named the two genes *KEL1* and *KEL2*, respectively. The two *S. cerevisiae* genes are located in duplicated regions of the *S. cerevisiae* genome (chromosomal block 29), between *SPO12* and *SOL3* for *KEL1* and between YGR230w and *SOL4* for *KEL2* (Wolfe and Shields, 1998). Kel1p and Kel2p are 62% identical in the region containing kelch repeats and 44% identical over their entire length. An alignment of the kelch repeats in Kel1p, Kel2p, and Tea1p is shown in Fig. 3-3B. Tea1p is slightly more similar to the two *S. cerevisiae* proteins than it is to the second *S. pombe* protein (see dendrogram in Fig. 3-3C).

When expressed from a 2μ plasmid, Kel2p, like Kel1p, suppressed the mating defect associated with activated Pkc1p (Fig. 3-1A); however, the suppression was weaker and more variable from experiment to experiment

Fig. 3-2. Northern analysis of YHR158c and *FUS1* mRNA. mRNA was prepared as described in Materials and Methods from ***a/α*** wild-type (JP54), ***MATα*** wild-type (IH3197), ***a*** wild-type (IH3196), ***a*** *YHR158cΔ* (JP358), and ***a*** *fus1Δ* (JP52) strains after cells were treated with α-factor for 20 minutes (+, treated; -, untreated). Northern blots were hybridized with a probe to YHR158c (top panel) or *FUS1* (third panel). Blots were stripped and hybridized with a probe to *TCM1* to serve as a loading control.

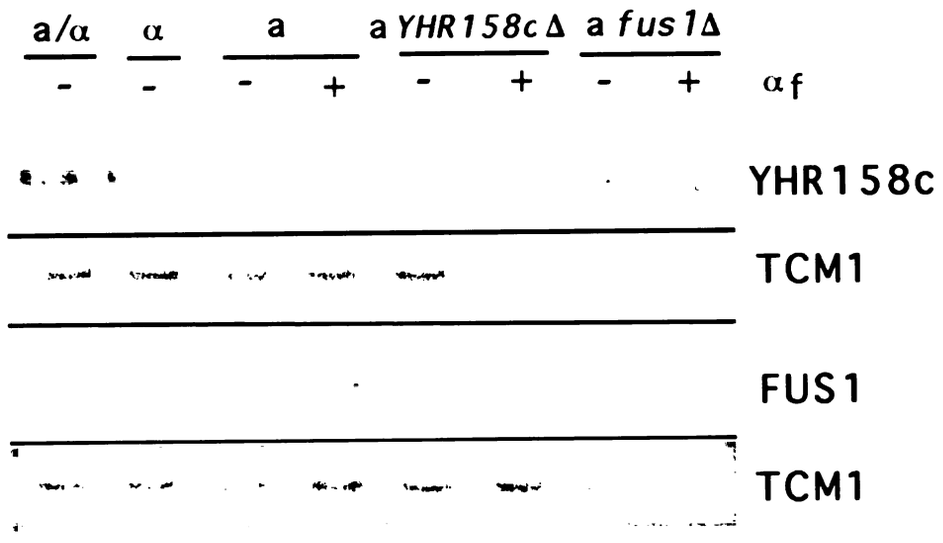

a/α
α
a
a YHR158cΔ
a fus1Δ
- - - + - + - +
αf
YHR158c
TCM1
FUS1
TCM1

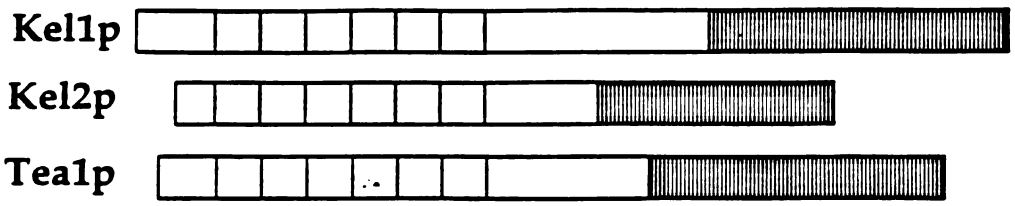
Fig. 3-3. Relationship of Kel1p, Kel2p, and Tea1p.

A. Domain structure of Kel1p, Kel2p, and Tea1p. Grey boxes indicate kelch repeats. Striped boxes indicate predicted coiled-coil domains.

B. Alignment of the kelch repeats in Kel1p, Kel2p, and Tea1p. Consensus residues are indicated in bold (residues present in at least three of the six repeats). Consensus sequence is listed underneath repeats in bold. # indicates presence of amino acids excluded from the alignment.

C. Dendrogram indicating the relationship between Kel1p, Kel2p, Tea1p, and the protein predicted by the *S. pombe* sequencing project (Z98603). Dendrogram was generated using Pileup and Cluster analysis.

A



B

Kel1p

```
VW NRIKLQNSPF PRYR HVASAYVTDKNQ      IYVIGGLHDQSVYGDT WILTAFDNATRFST
TT IDISEAT    PPPR VGHAAVLCGNA       FVVFGGDTH KVNKEGLMDDDIYLLNINSY
KW TVPAPVGPR  PLGR YGHKISIIATTQMKTK LYVFGGQFD DTYFNDLAVYDLSSFRRPDS
HW EFLKPRTFT  PPPI TNFTMISYDSK       LWVFGGDTL QGLVNDVFMYDPAIN
DW FIIDTTGEK  PPPV QEHATVVYNDL       MCVVGGKDEHDAYLNSVYFLNLKSR
KW FKLPVFTAGI PQGR SGHSLTLLKNDK      ILIMGGDKFDYARVEEYDLHTSDIDMQRGT
 W            PPPR  GH                VFGGD     Y N     D
```

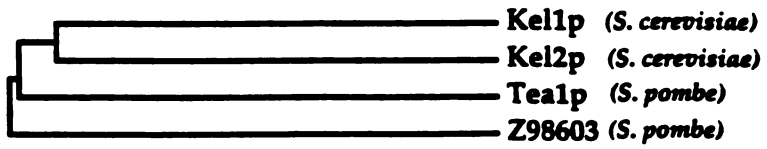
Kel2p

```
IW NRVKLKNSPF PRYR  HSSSFIVTNDNR     IFVTGGLHDQSVYGDV WQIAANADGTSFTS
KR IDIDQNT    PPPR VGHASTICGNA       YVVFGGDTH KLNKNGLLDDDLYLFNINSY
KW TIPQPIGRR  PLGR YGHKISIIASNPMQTK LYLFGGQVD ETYFNDLVVFDLSSFRRPNS
HW EFLEPVGDL  PPPL TNHTMVAYDNK       LWVFGGETP KTISNDTYRYDPAQS
EW SKVKTTGEK  PPPI QEHASVVYKHL       MCVLGGKDTHNAYSNDVYFLNLLSL
KW YKLPRMKEGI PQER SGHSLTLMKNEK      LLIMGGDKTDYASPNIHDLQTSETDQGEGT
KW       G    PPPR  GH S I  N       L VFGG      Y ND    DL
```

Tea1p

```
PW SKLTVRGSSN VLPR  YSHASHLYAEGGQE  IYIFGGVASDSQPKNDLWVLNLATSQ
FT SLRSLGET   PSPR  LGHASILIGNA      FIVFGGLTNHDVA#NSLYLLNTSSL
VW QKANASGAR  PSGR  YGHTISCLGSK      ICLFGGRLL DYYFNDLVCFDLNNLNTSDS
RW ELASVVNDP  PPAR  AGHVAFTFSDK      LYIFGGTDG ANFFND
LW CYHPKQSAWS#PNPR  AGHAASVVEGI      LYVFGGRASDGTFLNDLYAFRLSSK
HW YKLSDLPFT  PSPR  SSHTLSCSGLT      LVLIGGKQGKGAS#NVYMLDTSRFRLGSVPTT
 W  K S       PSPR   GHA S  G        LY FGG         NDL
```

C



than seen with Kel1p. The inability of Kel2p to suppress as well as Kel1p may be due to lower protein levels. Western blot analysis of both HA-tagged and GFP-tagged versions indicated that Kel1p was more abundant than Kel2p (Fig. 3-4D and data not shown).

Kel1p and Kel2p Localize to Regions of Polarized Growth

During mating, cells polarize the actin cytoskeleton and secretory apparatus towards their selected mating partner (Field and Schekman, 1980; Ford and Pringle, 1986; Hasek et al., 1987; Madden and Snyder 1992; Read et al., 1992; Segall, 1993). Many proteins required for cell fusion, including Fus1p, Fus2p and Spa2p, localize to the region of future cell contact (Trueheart et al., 1987; Elion et al., 1995; Gehrung and Snyder, 1990). To determine whether Kel1p is similarly localized, a fusion protein was constructed in which GFP was joined to the carboxy terminus of Kel1p (see Materials and Methods). Cells were treated with α-factor, and the localization of Kel1p-GFP was examined by fluorescence microscopy. Kel1p-GFP expressed from 2μ, CEN-ARS, or integrating plasmids localized to shmoo tips (Fig. 3-4A, panel a and data not shown). Focusing through sections of cells indicated that Kel1p-GFP was localized to the periphery of the shmoo tip. When overexpressed, Kel1p-GFP suppressed the fusion defect associated with *PKC1-R398P*. In this case, Kel1p-GFP remained localized to the shmoo tip, but the green fluorescent signal was more intense and more broadly localized than when the fusion construct was integrated (data not shown). As with pheromone treatment, Kel1p-GFP localized to regions of polarized growth in vegetative cells (Fig.3- 4A, panel b). Kel1p-GFP could be seen as a single spot in unbudded cells. In cells with small- or medium-sized buds, Kel1p localized to the bud tip. In large-budded cells, Kel1p-GFP was often localized to the neck region separating the mother and daughter cell.

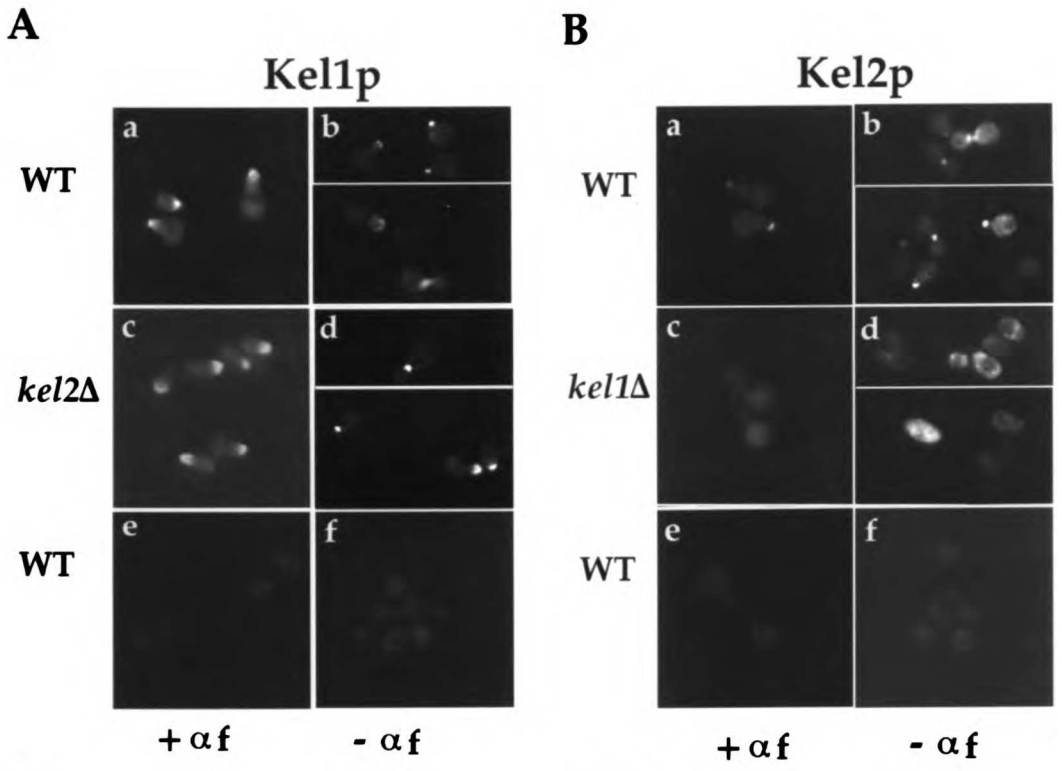
Fig. 3-4. Localization of Kel1p and Kel2p.

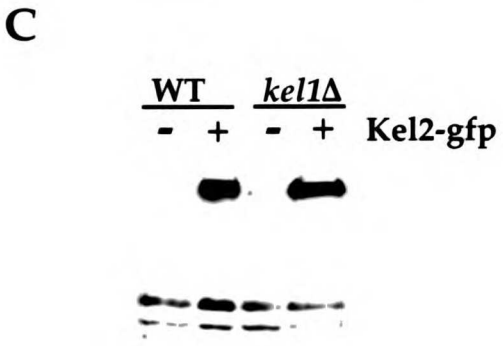
A. An integrating plasmid containing Kel1p-GFP (pJP139) was used to localize Kel1p in wild-type cells (IH3196) (a and b) and in *kel2Δ* (JP370) cells (c and d). No signal was observed in wild-type cells expressing an untagged control plasmid (pJP143) (e and f). Cells in panels a, c, and e were treated with 25 μg/ml α-factor for approximately two hours. Panels b, d, and f are composites from two different photographs.

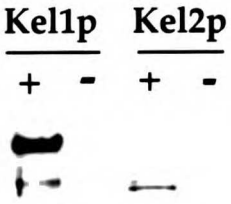
B. YEp351 containing Kel2p-GFP (pJP126) was used to localize Kel2p in wild-type cells (a and b) and in *kel1Δ* cells (JP363) (c and d). No signal was observed in wild-type cells expressing an untagged, control plasmid (pJP123) (e and f). Cells in panels a, c, and e were treated with 25 μg/ml α-factor for approximately two hours. Panels b, d, and f are composites from two different photographs.

C. Kel2p is present in *kel1Δ* strains. Wild-type (IH3196) and *kel1Δ* (JP363) strains harboring plasmids encoding Kel2p-GFP (pJP126) or untagged control plasmid (pJP123) were analyzed by Western blot as described in Materials and Methods. Monoclonal antibodies recognizing GFP (C163) were a generous gift from P. O'Farrell.

D. Kel1p is more highly expressed than Kel2p. JP317 was transformed with YEp351-derived plasmids containing Kel1p-HA (pJP202), untagged Kel1p (pJP127), Kel2p-HA (pJP131), or untagged Kel2p (pJP123). + indicates HA-tagged versions; - indicates untagged versions. Extracts were prepared as described in Materials and Methods. Lanes were equally loaded, and western blot analysis was performed with monoclonal antibodies recognizing the HA tag (HA11, Babco).


A
Kel1p
WT
kel2Δ
WT
a
b
c
d
e
f
+αf
-αf
B
Kel2p
WT
kel1Δ
WT
a
b
c
d
e
f
+αf
-αf


C
WT
kel1Δ
- + - +
Kel2-gfp


D
Kel1p
Kel2p
+ - + -

To determine if Kel2p could function similarly to Kel1p, we examined its subcellular localization. GFP was fused to the carboxy terminus of Kel2p. When expressed from a 2μ plasmid, but not when expressed from a CEN-ARS plasmid, we were able to detect Kel2p-GFP signal. Like Kel1p-GFP, Kel2p-GFP localized to the shmoo tip in pheromone-treated cells (Fig. 3-4B, panel a). Also, like Kel1p, Kel2p-GFP localized to regions of polarized growth during vegetative growth. Kel2p-GFP was found as a single spot in unbudded cells. In cells with small- or medium-sized buds, Kel2p-GFP localized to the bud tip. In large-budded cells, Kel2p-GFP was detected at the bud tip or at the neck between mother and bud (Fig. 3-4B, panel b). We conclude that Kel1p and Kel2p localize to regions of polarized growth both during mating and budding.

Localization of Kel2p Depends Upon *KEL1*

Because Kel1p and Kel2p exhibit a similar pattern of localization, we determined whether their localization depends upon each other. In *kel2Δ* strains, localization of Kel1p was indistinguishable from that in wild-type strains (Fig. 3-4A, panels c and d). However, Kel2p failed to localize in the absence of Kel1p both with and without pheromone treatment (Fig. 3-4B, panels c and d). To determine whether Kel2p was present in the *kel1Δ* strain, we examined Kel2p-GFP by immunoblot and observed that the protein was present at equivalent levels in wild-type and *kel1Δ* strains (Fig. 3-4C). We conclude that Kel1p is required to localize Kel2p to regions of polarized growth and that Kel2p is dispensable for localization of Kel1p. Despite the considerable homology between Kel1p and Kel2p, Kel1p apparently interacts with a determinant at the site of polarized growth that Kel2p fails to interact with in the absence of Kel1p.

Kel1p and Kel2p Are Present in a Complex

Since the localization of Kel2p was dependent upon Kel1p, we wondered whether the two proteins physically interact. To address this question, coimmunoprecipitation and two-hybrid analyses were performed. An epitope-tagged version of Kel2p (Kel2p-HA) was constructed in which a double hemagglutinin (HA) tag was fused to the carboxy terminus of Kel2p as described in Materials and Methods. Kel2p-HA was expressed in a strain which harbored a GFP-tagged version of Kel1p or an untagged version. In cells expressing Kel2p-HA, we were able to immunoprecipitate Kel2p with anti-HA antibodies (Fig. 3-5A, lower panel, lanes 1 and 2). If the cells co-expressed Kel1p-GFP, Kel1p co-immunoprecipitated with Kel2p-HA, as detected by immunoblot with anti-GFP antibodies (Fig. 3-5A, upper panel, lane 2). To determine whether Kel1p-GFP was specifically co-immunoprecipitated by Kel2p-HA, immunoprecipitations were performed from cells expressing the untagged version of Kel2p. We were unable to immunoprecipitate Kel1p-GFP with anti-HA antibodies in such extracts (Fig. 3-5A, upper panel, lane 3).

Similar experiments were performed to determine whether Kel1p can associate with itself. An HA-tagged version of Kel1p was expressed in a cell which also harbored an integrated copy of a GFP-tagged version of Kel1p. Kel1p-HA was immunoprecipitated using antibodies which recognize the HA tag. Kel1p-GFP co-immunoprecipitated as determined by western blotting (Fig. 3-5A, upper panel, lane 5). In control experiments, we were unable to immunoprecipitate Kel1p-GFP if an untagged version of Kel1p, rather than an HA-tagged version, was co-expressed (Fig. 3-5A, upper panel, lane 6). These data indicate that, in addition to forming a complex with Kel2p, Kel1p forms a complex with itself.

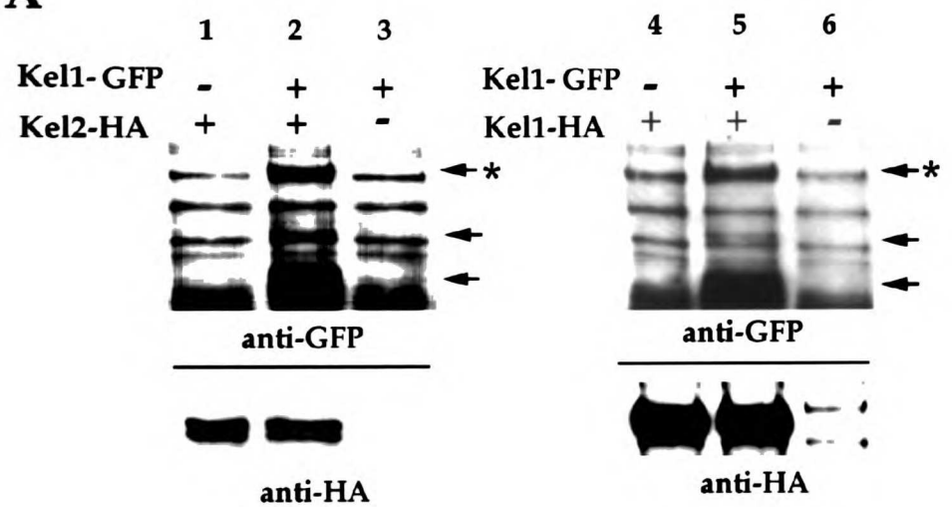
Fig. 3-5. Kel1p and Kel2p Physically Interact

A. Coimmunoprecipitation of Kel2p and Kel1p. HA-tagged Kel1p or Kel2p was expressed in cells (JP363) which coexpressed Kel1p-GFP. HA-tagged proteins were immunoprecipitated as described in Materials and Methods, and the ability to coimmunoprecipitate Kel1p-GFP was assessed. In lanes 1-3, cells expressed Kel2p-HA (pJP131), indicated by +, or untagged Kel2p (pJP123) indicated by -. In lanes 4-6, cells expressed Kel1p-HA (pJP209), indicated by +, or untagged Kel1p (pJP207), indicated by -. Immunoprecipitated HA-tagged proteins were detected in the lower blots using antibodies which recognize the HA epitope (HA11, Babco). Equal amounts of Kel2p-HA were immunoprecipitated in lanes 1 and 2, whereas no band was detected in extracts prepared from strains expressing the untagged control (lane 3). Equal amounts of Kel1p-HA were immunoprecipitated in lanes 4 and 5, whereas no band was detected in extracts prepared from strains expressing the untagged control (lane 6). Cells co-expressed Kel1p-GFP (pJP139), indicated by +, or untagged Kel1p (pJP143), indicated by -. Monoclonal antibodies recognizing GFP were used to detect co-immunoprecipitated Kel1p-GFP in the upper blots. Arrowhead with an asterisk indicates full-length Kel1p-GFP. Other arrowheads indicate breakdown products of Kel1p-GFP which also coimmunoprecipitate.

B. Two-hybrid analysis of Kel1p and Kel2p. Cells expressed Kel2p or Bicoid fused to the lexA DNA-binding domain (DBD; pJP158 and pRFHM1, respectively) and Kel1p fused to a transcriptional activation domain (AD; pJP167) or vector. Ability to activate transcription from a reporter construct was determined as described in Materials and Methods. Miller Units were

determined as described in Materials and Methods. Values are means +/- standard deviation.

A



B

DBD	AD	Miller Units
Kel2p	Kel1p	527 +/- 46
Kel2p	vector	4.0 +/- 0.4
Bicoid	Kel1p	4.9 +/- 0.4

Additional evidence that Kel1p and Kel2p physically interact was obtained by two-hybrid analysis. Full-length Kel2p was fused to the lexA DNA-binding domain (Kel2-DBD) to generate pJP158 (see Materials and Methods). On its own, this fusion protein did not activate transcription from a reporter construct in which lexA operator sites lie upstream of the *lacZ* gene (pSH18-34; Gyuris et al., 1993). Full-length Kel1p was fused to a transcriptional activation domain (Kel1-AD) (see Materials and Methods) and expressed under control of the *GAL1,10* promoter (pJP167). When Kel1-AD and Kel2-DBD were co-expressed, β-galactosidase activity increased more than one hundred-fold compared to cells that expressed either Kel1-AD or Kel2-DBD alone (Fig. 3-5B). When cells were grown on glucose to repress Kel1-AD, β-galactosidase was not produced (data not shown). When co-expressed with a Bicoid-lexA DNA-binding domain fusion (pRFHM1; Gyuris et al., 1993), Kel1-AD did not activate transcription of *lacZ*, indicating that interaction with the Kel2-DBD fusion is specific. To test whether Kel2p interacts with itself, Kel2-DBD and Kel2-AD constructs were examined in the two-hybrid system. When expression of Kel2-AD was induced by galactose for one hour or less, no interaction was detectable. Longer inductions resulted in an interaction that was approximately 10% that seen for Kel1p-AD and Kel2-DBD (data not shown). Based upon the co-immunoprecipitation and two-hybrid analyses, we conclude that Kel1p physically interacts with Kel2p and itself. Our data indicate, moreover, that the Kel1p-Kel2p interaction is significant *in vivo*, as the localization of Kel2p depends upon Kel1p.

***kel1Δ* Mutants Are Defective in Cell Fusion**

To determine whether *KEL1* has a role in cell fusion, we examined the phenotype of a strain in which *KEL1* was deleted. In a diploid strain, one copy of the *KEL1* open reading frame was replaced with the *URA3* gene (see

Materials and Methods). Examination of haploid segregants indicated that deletion of *KEL1* had no effect on growth rate, budding pattern (in *kel1Δ* haploids and in **a**/α *kel1Δ/kel1Δ* diploids), or sensitivity to high and low temperatures. *kel1Δ* mutants did exhibit a mating defect (Fig. 3-6A). This defect was not due to inability to produce or respond to pheromone normally, as determined by halo assays and shmoo formation (Fig. 3-4B, panel c, and data not shown). Additionally, *kel1Δ* mutants signalled in response to pheromone, as evidenced by normal transcriptional induction of *FUS1* (Fig. 3-2). *kel1Δ* mutants did, however, exhibit a defect in cell fusion, displaying a four- to five-fold increase in the number of prezygotes compared to wild-type cells (Table 3-1, lines one and two; Table 3-2, lines one and two). The defect was not exacerbated by mating to *kel1Δ* cells (data not shown).

fus1Δ and *fus2Δ* mutants display only mild defects in cell fusion; *fus1Δ fus2Δ* strains exhibit a more severe defect (Trueheart et al., 1987). We therefore determined whether the *kel1Δ fus1Δ* double mutant had a more severe defect in cell fusion than either single mutant. In matings between a *kel1Δ fus1Δ* double mutant and a wild-type mating partner, approximately 54% of the partnered cells were prezygotes, a defect significantly worse than seen with *kel1Δ* or *fus1Δ* single mutants (9.4% and 22.4%, respectively; Table 3-1). These results indicate that loss of *KEL1* results in a defect in cell fusion that is exacerbated by loss of *FUS1*. Loss of *KEL1* exacerbated the defect in cell fusion of **a** *fus1Δ* and α *fus1Δ* cells, indicating that *KEL1* is required in both cell types for efficient cell fusion (Table 3-1 and data not shown).

kel2Δ Mutants Do Not Exhibit a Defect in Cell Fusion

To determine whether *KEL2* was also required for cell fusion, we constructed a strain in which *KEL2* was deleted and analyzed its phenotype. One copy of the *KEL2* open reading frame was replaced with the *LEU2* gene

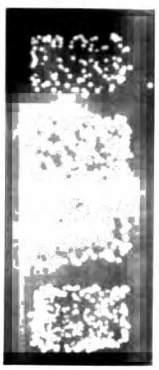
Fig. 3-6. Kel1p and Kel2p are Involved in Mating and Morphology

A. *kel1Δ* mutants are defective in mating. *kel1Δ* strain JP363 was transformed with a CEN-ARS plasmid containing *KEL1* (pJP207), a 2μ plasmid expressing Kel2p (pJP92), or vector (YCplac111). The wild-type control (IH3196) was transformed with vector (YCplac111). Strains were mated to an α *fus1 fus2* strain (IH2351) as described in Materials and Methods.

B. *kel1Δ* and *kel1Δ kel2Δ* mutants exhibit altered morphology. Morphology of isogenic (a) wild-type (IH3196), (b) *kel1Δ* (JP363), (c) *kel2Δ* (JP371), and (d) *kel1Δ kel2Δ* (JP385) strains.

C. Morphological phenotype exhibited by cells overexpressing *KEL1* or *KEL2*. Morphology exhibited by a wild-type strain (IH3196) transformed with (a) vector (YEp351), (b) 2μ *KEL1* (pJP81), (c) vector (pRS426) or (d) p*GAL KEL2* (pJP160) grown in SD-Ura (a and b) or S galactose-Ura (c and d).

A



kel1Δ + vector

kel1Δ + p*KEL1*

kel1Δ + 2μ *KEL2*

WT + vector

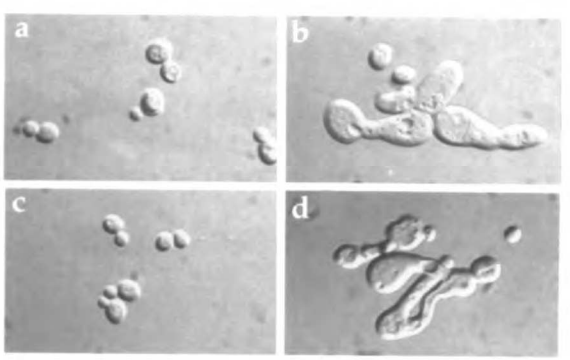
B

WT *kel1Δ* *kel2Δ* *kel1Δ kel2Δ*

C

vector

vector



2 μ *KEL1*

p *GAL KEL2*

Table 3-1

The Effect of Deletion of *KEL1* and *KEL2* On Cell Fusion

genotype	% prezygotes*
WT	2.6 +/- 0.7
kel1Δ	9.4 +/- 1.9
kel1Δ fus1Δ	54.0 +/- 10.6
kel2Δ	3.7 +/- 0.8
kel2Δ fus1Δ	31.9 +/- 3.5
kel1Δ kel2Δ	7.1 +/- 1.4
fus1Δ	22.4 +/- 3.8

* % prezygotes represents the number of prezygotes/(zygotes+prezygotes). Strains were WT (IH3196), *kel1Δ* (JP363), *kel2Δ* (JP371), *fus1Δ* (JP52), *kel1Δ fus1Δ* (JP410), *kel2Δ fus1Δ* (JP412), and *kel1Δ kel2Δ* (JP385) and were mated to a wild-type α strain (IH2350). Values are means of at least four experiments in which a total of at least 900 partnered cells were counted +/- SD.

Table 3-2

The Effect of *PKC1-R398P* on Cell Fusion in *kel1Δ* and *kel2Δ* Mutants

	% prezygotes*	
genotype	vector	*PKC1-R398P*
WT	5.5 +/- 1.0	47.9 +/- 2.6
kel1Δ	30.2 +/- 2.4	79.2 +/- 0.3
kel2Δ	8.7 +/- 2.8	53.9 +/- 4.8
fus1Δ	47.3 +/- 3.7	87.6 +/- 3.6
kel1Δ kel2Δ	19.9 +/- 2.0	69.6 +/- 1.8

* % prezygotes represents the number of prezygotes/(zygotes+prezygotes). Strains were: WT (IH3196), *kel1Δ* (JP363), *kel2Δ* (JP371), *fus1Δ* (JP52), and *kel1Δ kel2Δ* (JP385). Strains were transformed with either YCp50 containing *PKC1-R398P* (pJP67) or YCp50 alone as indicated and were mated to a wild-type α strain (IH2350). More than 200 hundered partnered cells were counted in each experiment to determine % prezygotes. Values are means of three experiments +/- SD.

(see Materials and Methods) in a diploid strain. Haploid segregants were examined for their ability to mate. Unlike *kel1Δ* mutants, *kel2Δ* mutants exhibited normal mating and cell fusion (Tables 3-1 and 3-2). Since there is no detectable requirement for Kel2p in cell fusion in a wild-type strain, the primary function of Kel1p during fusion is not to localize Kel2p. In a *fus1Δ* strain, loss of Kel2p caused a slight but reproducible increase in the number of prezygotes (22.4 +/- 3.8 % for *fus1Δ* mutants versus 31.9 +/- 3.5 % for *fus1Δ kel2Δ* double mutants). We conclude that the role of Kel2p in cell fusion is minor as compared with Kel1p.

Because *kel1Δ* mutants fail to localize Kel2p, one possible cause of the defect in cell fusion in such strains is mislocalization of Kel2p as opposed to the absence of Kel1p or Kel2p at the shmoo tip. If such an explanation were correct, then deletion of *KEL2* in the *kel1Δ* mutant should suppress the defect in cell fusion. When grown in rich medium and mated to a wild-type partner, *kel1Δ* mutants accumulated 9.4 +/-1.9 % prezygotes whereas *kel1Δ kel2Δ* double mutants accumulated 7.1 +/- 1.4 % prezygotes compared with 2.6 +/- 0.7 % prezygotes for wild-type cells (Table 3-1). When cells were grown in minimal medium, *kel1Δ* strains accumulated 30.2 +/- 2.4 % prezygotes whereas *kel1Δ kel2Δ* double mutants accumulated 19.9 +/- 2.0 % prezygotes, compared with 5.5 +/- 1.0 % for wild-type cells (Table 3-2, compare lines 2, 5, and 1, respectively). We conclude that loss of *KEL2* may modestly suppress the fusion defect of *kel1Δ* strains, but that mislocalized Kel2p does not completely account for the fusion defect of *kel1Δ* strains. Consistent with this interpretation, overexpression of *KEL2* did not significantly alter the mating ability of *kel1Δ* mutants (Fig. 3-6A) Taken together, these data indicate that Kel1p has a role in cell fusion which is not solely to localize Kel2p. In contrast, there is little requirement for Kel2p in cell fusion.

kel1Δ Mutants Have a Defect in Cell Morphology

In addition to the defect in cell fusion, *kel1Δ* mutants exhibited a defect in morphology, appearing slightly elongated and heterogeneous in shape compared with wild-type control cells (Fig. 3-6B, panel b). This morphological defect was observed in two different strain backgrounds (data not shown). *kel2Δ* mutants exhibited normal morphology (Fig. 3-6B, panel c). Examination of the *kel1Δ kel2Δ* double mutant revealed the same elongated morphology as seen in the *kel1Δ* single mutant (Fig. 3-6B, panel d), suggesting that, like the fusion defect, the altered morphology of *kel1Δ* mutants is not due to mislocalized Kel2p.

Although loss of Kel2p did not alter morphology, overexpressing Kel2p from the *GAL1,10* promoter caused approximately 5% of cells to display a grossly aberrant morphology (Fig. 3-6C, panel d). When Kel1p was overexpressed from a 2μ plasmid, an equivalent fraction of cells displayed a similar morphology (Fig. 3-6C, panel b). We did not observe this morphology in cells containing vector when grown in galactose (Fig. 3-6C, panel c) or when cells containing p*GAL KEL2* were grown in glucose (data not shown). These data show that although only Kel1p is required for proper morphology, overexpression of either Kel1p or Kel2p disrupts normal cellular morphology.

Because of the potential role of kelch repeats in mediating interaction with cytoskeletal components (see Discussion), we examined actin in *kel1Δ* strains by staining with rhodamine-phalloidin. We did not detect any obvious differences between wild-type and *kel1Δ* mutants. Microtubules were visualized in *kel1Δ*, *kel2Δ*, and *kel1Δ kel2Δ* mutants by indirect immunofluorescence both with and without pheromone treatment. Again, we did not detect any obvious differences (data not shown), but subtle defects

cannot be excluded. Additionally, loss of Kel1p or Kel2p did not affect sensitivity to the microtubule depolymerizing drug benomyl, and *kel1Δ* mutants did not exhibit genetic interactions with *tub1-1* mutants (Carminati, personal communication). Hence, it is currently unclear whether Kel1p interacts with the actin or microtubule cytoskeleton.

Relationship of Kel1p to the Pkc1p Pathway During Mating and Vegetative Growth

KEL1 was identified by its ability to suppress an activated allele of Pkc1p. In principle, Kel1p could be a target of Pkc1p, a negative regulator of Pkc1p, or could act in parallel to promote fusion. To examine the relationship between Kel1p and Pkc1p, we analyzed the phenotype of *kel1Δ* mutants expressing *PKC1-R398P*. If Kel1p were the sole downstream target inhibited by Pkc1p to prevent cell fusion, then we would expect the *PKC1-R398P kel1Δ* double mutant to behave like the *kel1Δ* and *PKC1-R398P* single mutants. Examination of a *kel1Δ PKC1-R398P* double mutant revealed a defect in cell fusion which was worse than that seen in a *kel1Δ* mutant or in a wild-type cell expressing *PKC1-R398P* (Table 3-2). Matings between *kel1Δ* mutants and wild-type partners yielded approximately 30% prezygotes, whereas the *kel1Δ PKC1-R398P* strain exhibited 79% prezygotes, higher than that seen with wild-type strains carrying the *PKC1-R398P* allele (48%). These data suggest that Kel1p is not the sole target of Pkc1p, but may function upstream or parallel to promote cell fusion.

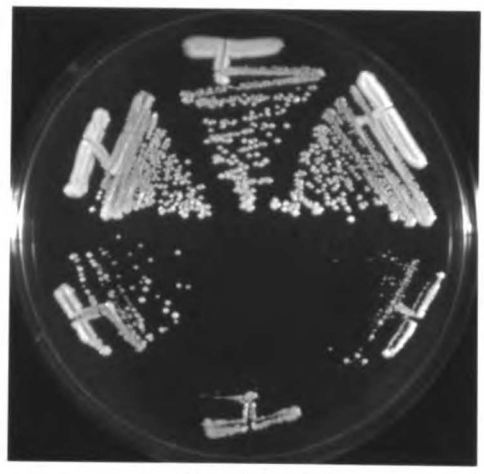
To examine the relationship between Kel1p and Kel2p and the Pkc1p pathway during vegetative growth, we analyzed the phenotype of strains lacking *MPK1* and *KEL1* or *KEL2*. *MPK1* encodes a MAP kinase which appears to function downstream of Pkc1p (Lee and Levin, 1992; Kamada et al., 1995). *mpk1Δ* mutants grow slowly at 34.5°C and fail to grow at 37°C; the

growth defect at high temperature is suppressed by 1 M sorbitol. To determine whether loss of *KEL1* or *KEL2* affects growth of an *mpk1Δ* mutant, *kel1Δ mpk1Δ* and *kel2Δ mpk1Δ* double mutant strains were constructed (see Materials and Methods). All 18 *kel1Δ mpk1Δ* and 13 *kel2Δ mpk1Δ* double mutants grew more poorly than *mpk1Δ* single mutants at 34.5°C (Fig. 3-7A), and the growth defect was suppressed by 1 M sorbitol (Fig. 3-7B). We conclude that loss of *KEL1* or *KEL2* exacerbates the growth defect of *mpk1Δ* mutants.

Fig. 3-7. Deletion of *KEL1* or *KEL2* exacerbates the growth defect of *mpk1Δ* mutants. Wild-type (IH1783), *kel1Δ* (JP445A) , *kel2Δ* (JP446A), *mpk1Δ* (IH3077), *mpk1Δ kel1Δ* (JP490), and *mpk1Δ kel2Δ* (JP491) strains were grown on YEPD (A) or YEPD supplemented with 1 M sorbitol (B) at 34.5°C for 2 days.

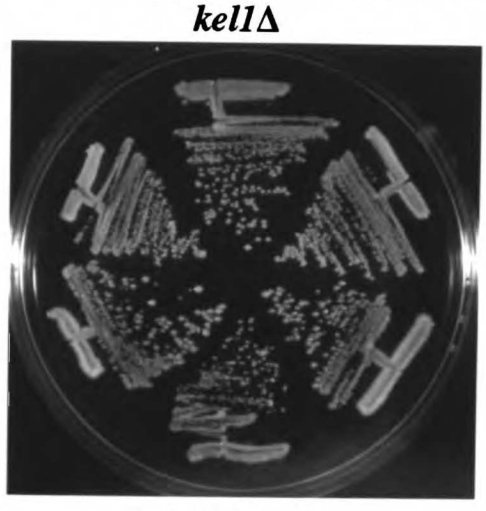
A

YEPD



B

YEPD + 1M Sorbitol



DISCUSSION

Identification and Analysis of Kel1p and Kel2p

We identified a novel gene, *KEL1*, whose overexpression relieved the defect in cell fusion caused by *PKC1-R398P*. Kel1p localizes to the region of the cell where fusion is initiated during mating and to regions of polarized growth in vegetative cells. Mutants lacking Kel1p exhibit defects in cell fusion and also are elongated and heterogeneous in shape, indicating that Kel1p has a role in cell fusion and morphogenesis.

KEL1 and *KEL2* are present in duplicated regions of the genome and encode proteins that are approximately 44% identical. Kel2p is similar to Kel1p in at least three functional respects: overexpression of Kel2p suppresses the mating defect caused by an activated form of Pkc1p; localization of Kel2p is indistinguishable from that of Kel1p; and Kel2p is in a complex with Kel1p. Despite their considerable homology, there are differences between Kel2p and Kel1p. First, Kel2p is unable to localize in the absence of Kel1p, suggesting that Kel1p but not Kel2p can interact with at least one factor at the cell cortex. Second, only *kel1Δ* mutants have detectable phenotypes. In an otherwise wild-type cell, Kel2p is not required for cell fusion or normal cellular morphology. These differences may indicate that Kel1p and Kel2p have diverged such that Kel2p does not play a role in these processes. Another possibility is that Kel1p and Kel2p have a similar function, but the major contributor is Kel1p. Precedent exists for other duplicated genes, such as *GPD1* and *GPD2*, where one gene is responsible for 95% of the detectable activity in the cell (Albertyn et al., 1994). Consistent with such an idea, Kel1p

is more abundant than Kel2p. A minor requirement for Kel2p in cell fusion can be seen in *fus1Δ* mutants.

Even though Kel2p does not normally interact with components involved in cell fusion and morphology, it is capable of interacting with them when overexpressed. Under these conditions, both Kel1p and Kel2p suppressed the mating defect associated with the activated Pkc1p allele. In addition, when Kel1p or Kel2p are overexpressed, a fraction of cells display an aberrant morphology which is strikingly similar in both cases. Overexpression of Kel2p may influence cell fusion and morphology through its interacting with Kel1p. Another possibility is that overproduction of Kel1p and Kel2p influences a common target.

Kel1p and Kel2p Are Members of the Kelch Family of Proteins

Kelch repeats, a motif of approximately fifty amino acids, are found in two to seven copies in proteins from diverse organisms including poxviruses, *Drosophila*, *C. elegans*, and mouse. Based upon sequence similarity to a superfamily of proteins which includes galactose oxidase (Ito et al., 1994) and neuraminidase (Varghese and Colman, 1991), these repeats are thought to form the blades of a β-propeller structure (Bork and Doolittle, 1994). Several kelch-containing proteins are implicated in actin interactions. For example, the *Drosophila* protein, kelch, localizes to ring canals, actin-containing structures which separate nurse cells in the developing egg chamber. In the absence of kelch, actin in the ring canals becomes disorganized (Xue and Cooley, 1993). The *Limulus* protein α scruin bundles actin filaments in the acrosomal process of sperm and is thought to be important at fertilization, when the filaments undergo a conformational change (Sanders et al., 1996). Additional kelch-containing proteins implicated in actin interactions include the actin-fragmin kinase from *Physarum* (Eichinger et al., 1996), spe-26 from

C. elegans (Varkey et al., 1995), and ENC-1 from the mammalian nervous system (Hernandez et al., 1997).

It is not clear whether all kelch domains mediate an interaction with actin. β-scruin from *Limulus* and calicin from bovine sperm are thought to be localized to regions of the cell that lack actin structures (Way et al., 1995; von Bulow et al., 1995). Tea1p, the closest homolog to Kel1p and Kel2p, affects microtubule dynamics rather than actin dynamics and is required in *S. pombe* cells for proper rod-like morphology (Mata and Nurse, 1997). In the absence of Tea1p or when Tea1p is overexpressed, cells exhibit a bent and T-shaped morphology. Kel1p is also required for proper morphology, but there is little evidence in *S. cerevisiae* that microtubules play a role in morphology, whereas actin is thought to be important (for review, see Botstein et al., 1997). Hence, it is currently unclear whether Kel1p interacts with the actin or microtubule cytoskeleton or whether it is involved in some other process.

Kel1p and Kel2p, like Tea1p, have six kelch repeats followed by a coiled-coil domain. Coimmunoprecipitation and two-hybrid analyses demonstrate that Kel1p can associate with itself and with Kel2p (Fig. 3-5). If there are distinct Kel1p-Kel1p and Kel1p-Kel2p complexes, then loss of Kel1p would disrupt both types of complexes. In the absence of Kel2p, Kel1p-Kel1p complexes would remain, perhaps explaining the minor role that Kel2p plays in cell fusion and morphogenesis. The interaction between Kel1p and Kel2p might be mediated by their coiled-coil domains. Some other proteins with kelch repeats appear to dimerize via a BTB/POZ-domain (Robinson and Cooley, 1997), which is absent from Kel1p, Kel2p, and Tea1p.

The Relationship of Kel1p to the Pkc1p Pathway

Hyperactive Pkc1p leads to a block in cell fusion, perhaps because it or a downstream protein kinase phosphorylates and thereby inactivates some

component necessary for cell fusion. If so, we reasoned that overproduction of this component in cells with hyperactive Pkc1p might restore fusion. Is Kel1p such a target? Our results are not conclusive. If Kel1p were the only downstream target of the Pkc1 pathway, we would expect that the fusion defect exhibited by a *PKC1-R398P kel1Δ* double mutant would not be more severe than that exhibited by a strain deleted for *KEL1*. We found, however, that the double mutant had a more severe defect in cell fusion than the single mutants. Thus, if Kel1p is a downstream target of the Pkc1 pathway, it must not be the only target necessary for cell fusion. These observations in no way exclude other possible relationships between Kel1p and the Pkc1 pathway. In particular, Kel1p could function upstream of Pkc1p (where it might affect Rho1p or other potential regulators of Pkc1p; see Kamada et al., 1996; Gray et al., 1997; Zarzov et al., 1996) or in parallel to the Pkc1 pathway. In all of these scenarios, Kel1p appears to function in opposition to Pkc1p to activate cell fusion.

Kel1p also plays a role in vegetative cells that has some functional connection to the Pkc1p pathway. In particular, we observed that loss of either *KEL1* or *KEL2* exacerbates the growth defect of *mpk1Δ* mutants: *kel1Δ mpk1Δ* and *kel2Δ mpk1Δ* mutants grow less well at high temperature than either single mutant. Formally, these results indicate that both Kel1p (or Kel2p) and Mpk1p promote cell integrity. Kel1p could function in parallel to Mpk1p. It is also possible that Kel1p functions upstream to negatively regulate Mpk1p. Loss of Kel1p would lead to increased Mpk1p activity which is necessary to maintain cell integrity in the absence of Kel1p. Although a variety of models are possible, a notable feature of this explanation is that Kel1p acts antagonistically to the Pkc1p pathway, consistent with the

relationship seen during mating. Discriminating among hypotheses will require direct assays of Mpk1p activity in wild-type and *kel1Δ* mutants.

Toda et al. (1993) have made the striking observation that overexpression of *pck2,*$^{+}$ which codes for a Pkc1p-like protein of *S. pombe*, results in production of branched cells similar to those seen in *tea1* mutants (Mata and Nurse, 1997). This relationship is analogous to what we have observed for cell fusion in budding yeast: hyperactivation of protein kinase C leads to a phenotype similar to that due to loss of a kelch protein (Kel1p). Kelch proteins in other organisms may also function in conjunction with protein kinase C pathways. The functional relationship between other kelch proteins and protein kinase C could be explored in organisms without facile genetics using dominant negative forms of the kelch proteins and the dominant, activated forms of protein kinase C.

The Role of Kel1p in Cell Fusion

Kel1p belongs to a class of proteins, including Spa2p, Bni1p, and Fps1p, which is required for cell fusion during mating (Dorer et al., 1997; Philips and Herskowitz, 1997) and which also functions during vegetative growth for morphogenesis and cell integrity (Snyder, 1989; Snyder et al., 1991; Evangelista et al., 1997; Philips and Herskowitz, 1997). One explanation for the defect in cell fusion of mutants lacking Fps1p, Spa2p, or Kel1p is that in their absence the Pkc1p pathway becomes activated, inhibiting cell fusion. *fps1Δ* mutants accumulate high levels of intracellular glycerol which may activate the Pkc1p pathway. Genetic data are consistent with Fps1p functioning upstream to negatively regulate Pkc1p (Philips and Herskowitz, 1997). Several observations link Spa2p and the Pkc1p pathway. Loss of Spa2p results in increased phosphorylation of Swi6p (Sheu et al., submitted), an apparent substrate of Mpk1p (Madden et al., 1997). Spa2p directly interacts with

components of the Pkc1p pathway (Mkk1p/Mkk2p; Sheu et al., submitted). Additionally, loss of Spa2p, Fps1p, or Kel1p exacerbates the growth defect of mutants defective in the Mpk1p MAP kinase pathway (Costigan et al., 1992; Philips and Herskowitz, 1997; Fig. 3-7A). One interpretation of such synthetic lethality is that cells respond to the absence of Fps1p, Kel1p, or Spa2p by activating the Pkc1p pathway. This activation may be essential for cells to remain viable and may also inhibit cell fusion during mating. Thus, the defect in cell fusion exhibited by mutants lacking these proteins could be explained by increased activity of the Pkc1p pathway.

Another explanation for the role of Kel1p, Spa2p, and Bni1p in cell fusion is that they interact with the cytoskeleton to control cell fusion. These proteins localize to regions of polarized growth during mating (Evangelista et al., 1997; Snyder et al., 1991; Fig. 3-3A). *spa2Δ* and *bni1Δ* mutants are defective in shmoo formation (Gehrung and Snyder; 1990; Evangelista et al., 1997), suggesting that Spa2p and Bni1p are involved in organizing the actin cytoskeleton during shmoo formation. Electron microscopic analysis of mating cells shows the presence of clustered vesicles at the zone of cell fusion (Baba et al., 1989; Gammie et al., submitted). Cytoskeletal proteins may be important in directing or maintaining vesicles at this fusion zone that are necessary for cell wall and membrane remodeling. Consistent with the idea that proteins involved in cell fusion might interact with the cytoskeleton to regulate vesicles, Fus1p binds actin by two-hybrid analysis (Amberg et al., 1995), and mutants lacking Fus1p have fewer vesicles at the zone of cell fusion (Gammie et al., submitted). Similarly, in mutants lacking Spa2p, vesicles are more dispersed than in wild-type strains (Gammie et al., submitted). As noted above, some proteins that contain kelch repeats interact with actin. Although *kel1Δ* mutants do not exhibit broadened mating

projections as seen in *spa2Δ* mutants (Fig. 3-4B, panel c), Kel1p might be involved in concentrating vesicles at the shmoo tip. Another possibility is that the actin cytoskeleton plays a role in cell fusion outside of its role in directing secretion. Perhaps in yeast, as in *Limulus* sperm, an actin rearrangement is important in mediating fertilization.

Evidence from *Drosophila* indicates that cytoskeletal components play a role in myoblast fusion. Mutants defective in *myoblast city* (*mbc*) exhibit defects in cytoskeletal organization and myoblast fusion (Rushton et al., 1995; Erickson et al., 1997), failing to localize paired vesicles to the site of cell fusion (Doberstein et al., 1997). Similarly, overexpression of mutant forms of Drac1, a small GTPase known to affect the actin cytoskeleton, disrupts myoblast fusion and morphology (Luo et al., 1994; Doberstein et al., 1997). In vertebrates, cytochalasin B, which disrupts actin filaments, interferes with myoblast fusion (Sanger et al., 1971). It is unclear in myoblast fusion, as in yeast fusion, exactly what role the cytoskeleton plays in the fusion process. As more components become identified, we are in a better position to determine how proteins involved in cell fusion interact with cytoskeletal machinery and the ways in which the protein kinase C pathway governs fusion.

CHAPTER FOUR

CONCLUSION

During mating, haploid yeast cells fuse to form an **a**/α diploid zygote. Cell wall material separating the interacting cells has to be removed to allow the plasma membranes to contact each other. This event is highly regulated, occuring in response to cell-cell contact at the site where cells make contact. The goal of this project was to understand how physical contact between cells elicits the biological response of cell wall degradation. In particular, we would like to know how cell-cell contact generates a signal, and what molecules transduce the signal. Additionally, we want to identify the targets of the signalling pathway, and understand how they are regulated. To begin to address these questions, I took a genetic approach to identify molecules involved in cell fusion, and here I will discuss the progress towards that end. My analysis has provided insight into the signalling pathways that regulate cell fusion, but their relationship to the machinery and the nature of the machinery remains obscure.

Several Signals Control Cell Fusion

To identify mutants defective in cell fusion, we examined mutants which had previously been isolated based upon defective mating to *fus1 fus2* and *far1-c* strains. We predicted that mutants defective in cell fusion would be especially impaired mating to a *fus1 fus2* mutant, itself impaired in cell fusion. The same is true for the *far1-c* mutant which, unbeknownst at the time, is also defective in cell fusion (Philips, unpublished observations). Since we were most interested in mutants which specifically disrupt cell fusion, we examined those mutants that appeared normal for other pheromone responses. Ten of thirteen mutants exhibited substantial defects in cell fusion, indicating that this set of mating-defective mutants was vastly enriched for cell fusion mutants. If we had examined mutants also defective in morphological response to pheromone, *spa2* and *pea2*, we would have

identified two additional cell fusion mutants (Dorer et al., 1997). Thus, mutants defective in mating to *fus1 fus2* and *far1-c* strains provided a large collection of fusion mutants.

Two different classes of mutants were identified - those defective in pheromone production and those exhibiting normal pheromone production. Five of the six mutants defective in pheromone production were specifically defective in **a** cells, and their defect was apparently due to mutations in *AXL1* or *STE6*, genes previously recognized for their role in **a**-factor production (see Chapter 1 and Appendix 1). Work from other labs indicates that high levels of pheromone, probably both **a**- and α-factor, are required for efficient cell fusion (Brizzio et al., 1996). The highest level of pheromone experienced by a cell probably occurs when its mating partner is fully inducing expression of pheromone and secreting pheromone in a polarized manner. It seems likely that when a cell begins to degrade the cell wall, it must have made physical contact with a partner, and the partner must be competent to fuse. The requirement for a high level of pheromone from the partner may be one way to assess that the partner is competent to fuse -- polarized and fully inducing genes required for cell fusion.

A high level of pheromone may be one signal that regulates cell fusion, but it cannot be the only signal. There must be an additional signal which occurs in response to cell-cell contact because cells treated with saturating amounts of exogenous pheromone do not degrade the cell wall, suggesting that pheromone is a necessary, but not sufficient, signal. Consistent with the idea that multiple pathways control cell fusion, no single mutation eliminates cell fusion. Even when screens were done in such a way that both mating partners contained the mutation, no mutants were identified in which fusion was completely blocked (Kurihara et al., 1994). In most cases

examined, more severe defects in cell fusion are observed in strains lacking two genes required for cell fusion compared to the single mutants, further supporting the view that multiple, parallel processes control cell fusion (the one exception being *fus2* and *rvs161*, in which the double mutant behaves as the single mutants; Brizzio et al., submitted).

We found that the glycerol facilitator Fps1p is required for cell fusion. Analysis of *fps1Δ* mutants suggested that intracellular signals can regulate cell fusion. Mutants defective in the *FPS1* gene accumulate intracellular glycerol (Luyten et al., 1995; Philips and Herskowitz, 1997). Their defect in cell fusion can be alleviated if intracellular glycerol concentration is restored to wild-type levels (by deleting *GPD1*) or if 1 M sorbitol is provided extracellularly to osmotically stabilize the cells, suggesting that osmotic imbalance is the cause of their fusion defect. We propose that cells monitor their osmotic state before committing to breaking down their cell wall. Thus, the *fps1Δ* mutant uncovers a checkpoint that inhibits cell fusion if cells are not osmotically balanced. Osmotic imbalance could increase the risk that cells will lyse while trying to fuse. Hence, it makes physiological sense that under such conditions, cells should be exquisitively sensitive to their osmotic state. We conclude that at least three signals appear to regulate cell fusion. Two are extracellular signals -- high pheromone level and cell-cell contact -- and the other is an intracellular signal based on the osmotic state of the cell.

Cell fusion requires a coordinated series of events between the interacting cells. If not properly orchestrated, cells might lyse, so perhaps it is not suprising that overlapping pathways regulate cell fusion and that checkpoints exist to abort the process under adverse circumstances. A similar situation may exist in mammalian fertilization, in which several potential ZP3 receptors have been identified on the sperm, including GalTase, p95, and

sp56, which may indicate multiple levels of control over acrosomal exocytosis. In mammalian fertilization, it is critical that acrosomal exocytosis occurs after ZP binding. If contents of the acrosomal vesicle are released prematurely, then the sperm will not be able to penetrate the ZP to fertilize the egg, and fusogenic molecules will be exposed on the sperm surface prior to interacting with the egg. Additional extracellular signals may also regulate the acrosome reaction. Sperm may be activated sequentially, by progesterone, as sperm transit the reproductive tract, and by ZP3 upon egg-binding (Roldan et al., 1994), suggesting that integration of information from several signalling pathways may occur.

Pkc1p May Mediate an Osmolarity and Morphology Checkpoint

We hypothesized that a checkpoint which inhibits cell fusion in response to osmotic imbalance might be mediated by Pkc1p, previously recognized for its role in responding to conditions that threaten cell integrity, such as hypo-osmotic shock (Davenport et al., 1995; Kamada et al., 1995). Consistent with this possibility, cells expressing an activated allele of Pkc1p exhibit a defect in cell fusion. Pkc1p functions upstream of a MAP kinase module composed of the MEKK, Bck1p, two redundant MEKs, Mkk1p and Mkk2p, and the MAPK, Mpk1p (reviewed by Errede and Levin, 1993). Cells lacking both Fps1p and Mpk1p are inviable, suggesting that the Pkc1p pathway is active in *fps1Δ* mutants since it is required for viability. In addition, genetic data are consistent with Pkc1p acting downstream of Fps1p to negatively regulate cell fusion. Thus, we suggest that Pkc1p inhibits cell fusion under conditions in which cells are not osmotically balanced such as in *fps1Δ* mutants.

The Pkc1p pathway may mediated a cell fusion checkpoint that monitors cell integrity in general, including the status of cellular morphology and not only osmotic state. *spa2* and *kel1* mutants are defective in morphology and

cell fusion, exhibiting similar genetic interactions with the Pkc1p pathway as *fps1Δ* mutants. *kel1Δ* mutants appear elongated and heterogeneous in shape during vegetative growth (Fig. 3-6) and form mating projections that appear narrow compared to those formed by wild-type cells (Philips, unpublished observations). *spa2* mutants exhibit normal morphology during vegetative growth, but form broadened, peanut-shaped projections with pheromone treatment. One possibility is that activation of the Pkc1p pathway is responsible for the defect in cell fusion exhibited by *kel1* and *spa2* mutants. Like *fps1Δ* mutants, loss of Kel1p or Spa2p exacerbates the growth defect of mutants defective in the Pkc1p pathway (Costigan et al., 1992; Fig. 3-7A). One interpretation of such synthetic lethality is that cells respond to the absence of Fps1p, Kel1p, or Spa2p by activating the Pkc1p pathway which is essential for cells to remain viable. In the case of Spa2p, biochemical evidence corroborates this possibility; loss of Spa2p results in increased phosphorylation of Swi6p (Sheu et al., submitted), an apparent substrate of Mpk1p (Madden et al., 1997). Thus, one explanation for the defect in cell fusion of mutants lacking Fps1p, Spa2p, or Kel1p is that in their absence the Pkc1p pathway becomes activated, inhibiting cell fusion.

Perhaps Pkc1p monitors the integrity of the cell, both in terms of morphology and osmolarity, and inhibits cell fusion if defects are detected. If this is the case, then a number of additional mutants, for example *bni1*, *chs5*, *pea2*, *fig1*, and *fig2* mutants, which exhibit defects in morphology in addition to defects in cell fusion, may be explained similiarly to what we propose for *fps1*, *spa2*, and *kel1* mutants. Since the Pkc1p pathway is activated by a variety of environmental stresses and exhibits genetic interaction with numerous genes, it is possible many factors could influence cell fusion indirectly by altering the activity of the Pkc1p pathway. Hence, activation of

this pathway may be one important criterion for distinguishing between those factors which act directly to control cell fusion and those that act indirectly, for example, by alterating cell integrity.

The idea that pathways that regulate cell integrity can also influence cell fusion may have implications for other examples of cell fusion. We presume that maintenance of cell integrity is essential for all cell fusion processes. For example, during myoblast fusion in *Drosophila*, a constitutively activated form of the small GTP-binding protein rac1 ($Drac1^{G12V}$) blocks myoblast fusion (Luo et al., 1994). It has been suggested that in such embryos, membrane ruffling caused by activation of Drac1 interferes with plasma membrane fusion during myotube formation, resulting in lysis of abortively fused cells (Doberstein et al., 1997).

What is the Cell Contact Signal?

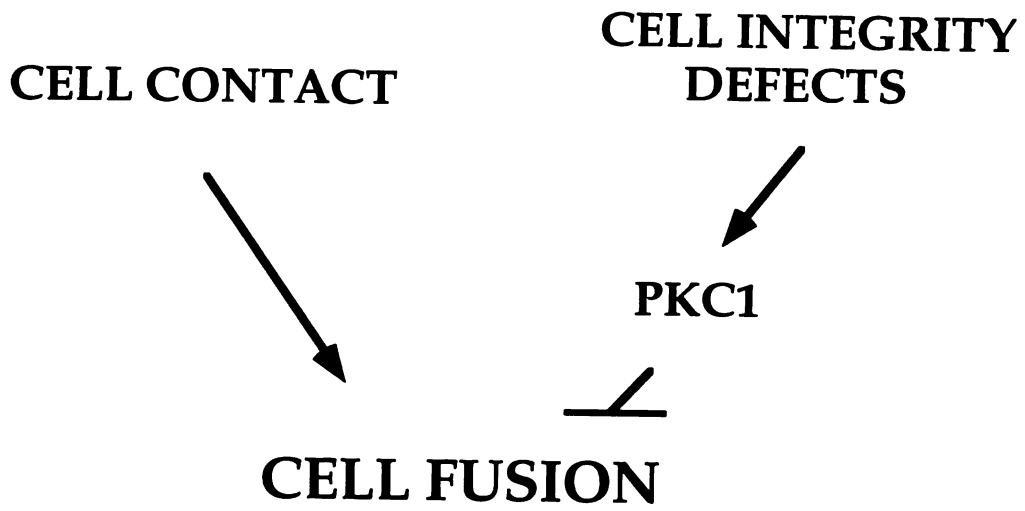
It is still unclear what molecules are involved in sensing cell-cell contact. One possibility is that the Pkc1p pathway regulates cell fusion, perhaps in response to a mechanical signal. Two models could explain the role of Pkc1p in fusion. In the first, Pkc1p negatively regulates cell fusion as part of a checkpoint, in which case Pkc1p is utilized only under certain conditions and plays no role in the cell contact signal (Fig. 4-1A). A second possibility is that Pkc1p is integrally involved in regulating cell fusion and could mediate the cell contact signal (Fig. 4-1B). According to this view, Pkc1p constitutively inhibits cell fusion, so that cell fusion occurs only when Pkc1p is antagonized. Previous work has shown that Mpk1p is activated in response to mating pheromone (Errede et al., 1995; Zarzov et al., 1996; Beuhrer et al., 1997), presumably due to activation of Pkc1p, and is necessary for maintenance of cell integrity under these conditions (Errede et al., 1995). Therefore, one possibility is that Pkc1p is first activated in response to pheromone and

Figure 4-1. Two Schemes for the Regulation of Cell Fusion by Pkc1p

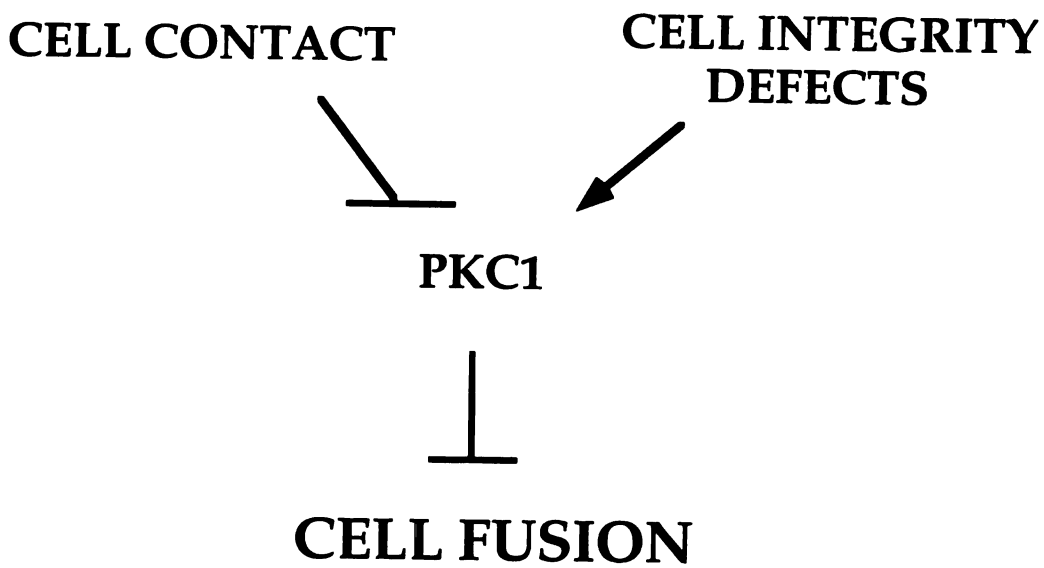
A. Pkc1p is part of a checkpoint that inhibits cell fusion if cells have defects in cell integrity such as defects in osmotic balance or cell morphology.

B. Pkc1p, an integral component in the pathway controlling cell fusion, is a negative regulator of cell fusion that is turned off for cells to fuse.

A


CELL CONTACT
CELL INTEGRITY DEFECTS
PKC1
CELL FUSION

B


CELL CONTACT
CELL INTEGRITY DEFECTS
PKC1
CELL FUSION

thereby inhibits cell wall degradation during initial stages of pheromone response and projection formation. When mating partners come in contact with each other, they generate a mechanical force as the cell walls become irreversibly adhered to each other, which turns off the *PKC1* pathway. Because the Pkc1p pathway regulates transcription of cell wall synthetic enzymes. cell fusion could be promoted by inhibiting production of these enzymes. We suggest that the signal to turn off the *PKC1* pathway could be a mechanical signal because the *PKC1* pathway responds to membrane stretch due to various stimuli, such as low external osmolarity, high temperature, and drug-induced membrane stretch (Kamada et al., 1995). One attractive aspect of a mechanical signal is that it is hard to imagine how receptor-ligand interactions on the outer surface of the cell wall would transduce a signal across the plasma membrane. Receptor-ligand mediated signalling cannot be ruled be out, and there are proteins, such as agglutinins, which are thought to extend from the plasma membrane to the exterior of the cell wall.

To determine whether the activity of Pkc1p regulates cell fusion in a wild-type situation, it would be useful to examine the level of Pkc1p activity during a mating reaction. If the hypothesis is correct, then at the time of cell fusion, Pkc1p activity should decline. If this result is observed, then it would be possible to characterize various mutants based upon Pkc1p activity, thereby distinguishing factors likely to act upstream of Pkc1p, required for the decline in Pkc1p activity, from those that act downstream or parallel to the Pkc1p decline. The technical difficulty in doing this experiment, or any other biochemical studies of cell fusion, is that mating is asynchronous. Thus, only a fraction of cells in a population are fusing at the same time. Presumably one of the main causes of asynchrony is that cells are only competent to mate in the G1 phase of the cell cycle. In an asynchronous population of cells,

various cells will enter G1 at different times. Thus, one way to improve synchrony would be to arrest cells at a particular phase of the cell cycle and then allow them to mate. Even if Pkc1p activity is not a primary determinant of cell fusion, a method to synchronize mating is critical for identifying biochemical markers of fusion which is essential for further dissecting the process.

What other molecules could generate the cell-cell contact signal? Of known molecules, the most attractive possibilities are Fus1p, Fig1p, or Fig2p, the only cell surface proteins implicated in cell fusion. Fus1p has a single transmembrane domain and an intracellular SH3 domain; Fig1p is a member of the four transmembrane (4TM) superfamily of proteins (Erdman et al., 1998); Fig2p contains a GPI-anchor sequence, suggesting that it is extracellular. Fus1p, localized to shmoo tips, is in the right place to mediate a cell contact signal (Erdman et al., 1998). For Fig1p and Fig2p the full-length proteins have not been localized. Transcription of *FUS1*, *FIG1*, and *FIG2* is highly induced by pheromone, suggesting that the gene products function specifically in mating or fusion. Since loss of each of these proteins individually results in only mild defects in cell fusion, none alone are the sensor of cell contact, but they could have overlapping functions. Ste20p, Ste5p, and Fus3p could be involved in transducing a cell contact-induced signal. These molecules are involved in signal transduction in response to pheromone, but evidence suggests they may also play a role in cell fusion. A Fus3p homolog, Kss1p, can substitute for Fus3p for some mating functions, but must not be able to substitute for Fus3p in fusion because *fus3* mutants exhibit defects in cell fusion. There could be a Fus3p-specific target involved in cell fusion, or Fus3p, but not Kss1p, could transduce a cell-contact signal. Overexpression of Ste20p or Ste5p relieves the defect of several mutants defective in cell fusion

(*PKC1-R398P* and *kel1Δ*; Appendix 2), raising the possibility that these molecules play a role in cell fusion in addition to their role in signalling in response to pheromone. Additional evidence that Ste20p may have other functions comes from analysis of Ste20p alleles that do not bind Cdc42p (Peter et al., 1996; Leberer et al., 1997). Strains containing such alleles do not have a defect in signalling in response to pheromone, but they do exhibit a mating defect. They may be defective in adhesion (Leberer et al., 1997), which would be consistent with Ste20p playing a role in a cell contact-mediated event. One possibility is that Ste20p is involved in signalling in response to cell contact to promote adhesion and subsequent fusion.

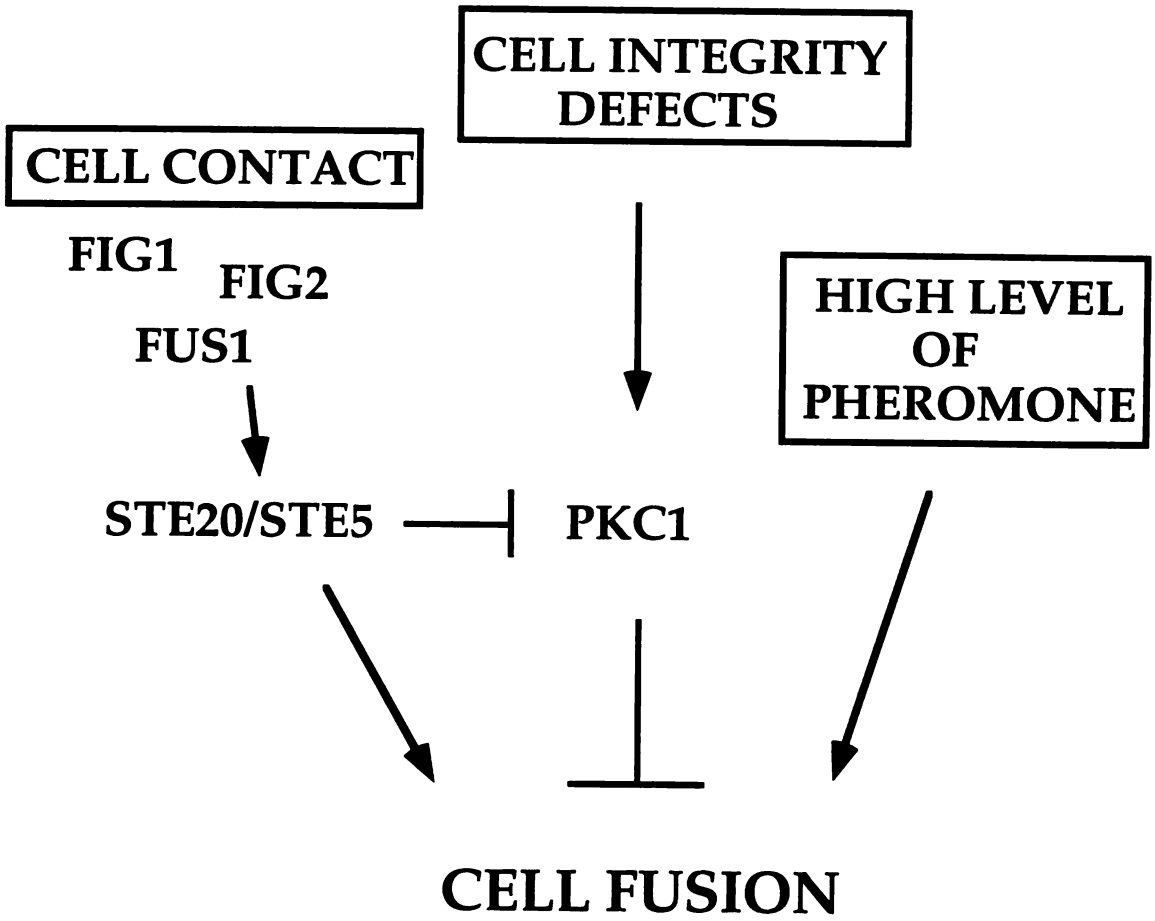
A speculative model for cell fusion is presented in Fig. 4-2. Cell fusion may be subject to multiple controls -- high pheromone insures that the mating partner is also preparing to fuse, the Pkc1p pathway monitors cell integrity to guarantee that cell fusion will not end catastrophically, and a signal induced by cell contact, perhaps generated by Fus1p, Fig1p, or Fig2p and transduced by Ste20p and Ste5p, provides the final trigger. The cell contact signal could activate fusion by inhibiting Pkc1p or acting in parallel to promote cell fusion.

The Role of Kel1p in Fusion

We identified Kel1p, a protein containing kelch repeats, along with Ste20p and Ste5p, as a high copy suppressor of the defect exhibited by *PKC1-R398P* mutants. Genetic data are consistent with Kel1p acting upstream or parallel to Pkc1p to promote cell fusion. There are a number of possible explanations for the role of Kel1p. One possibility raised earlier is that loss of Kel1p causes a morphology defect which activates the Pkc1p pathway, thus inhibiting cell fusion. One caveat is that we do not detect increased phosphorylation of Swi6p, a possible marker for activity of the Pkc1p pathway, in *kel1Δ* mutants.

Figure 4-2. Model of Pathways Controlling Cell Fusion

Cell fusion is subject to multiple controls. Pkc1p monitors cell integrity and inhibits cell fusion if defects are detected. High pheromone levels are required for efficient cell fusion. Cell contact triggers cell fusion, perhaps mediated by the cell surface proteins, Fus1p, Fig1p, or Fig2p, and the signalling molecules Ste20p and Ste5p. The cell contact signal could inhibit Pkc1p or act in parallel to promote cell fusion.


CELL INTEGRITY DEFECTS
CELL CONTACT
FIG1
FIG2
FUS1
HIGH LEVEL OF PHEROMONE
STE20/STE5
PKC1
CELL FUSION

Another possibility is that Kel1p, which localizes to the site of cell fusion, plays a more direct role in cell fusion. Kel1p could function, perhaps along with Ste5p and Ste20p, in cell-contact signalling or could be a component of the cell fusion machinery (see below). Determining what proteins interact with Kel1p and direct measurements of Pkc1p pathway activity in *kel1Δ* mutants should help distinguish among these and other possibilities.

What is the Nature of the Cell Fusion Machinery?

In mammalian fertilization and *Chlamydomonas* mating, a regulated secretory event is important for events which may be analogous to cell wall degradation during yeast fusion. In mammalian fertilization, the acrosome reaction releases enymes to degrade the ZP; in *Chlamydomonas* mating, a serine protease is released which activates a metalloprotease to degrade the cell wall (Snell et al., 1989). In the case of myoblast fusion, it is less clear that a regulated secretory event is involved, but some evidence suggests that secretion plays a role as indicated by the exteriorization of a metalloprotease (Brustis et al., 1994) and the assembly of prefusion complexes, structures composed of paired vesicles (Doberstein et al., 1997)). A regulated secretory event may also be involved in yeast cell fusion. Electron microscopic analysis indicates the presence of clustered vesicles at the zone of cell fusion in mating cells (Gammie et al., submitted); however, since growth and secretion are directed towards the shmoo tip, it is not suprising to see vesicles in this region. Nonetheless, there could be more than one class of vesicles, those undergoing constitutive secretion and others involved in a regulated secretion. This possibility is suggested by several observations. First, a distinct class of vesicles is seen by electron microscopy when cells are treated with pheromone (Baba et al., 1989). Second, fewer vesicles are seen at the fusion junction in mutants lacking Fus1p, suggesting a relationship between

the observed vesicles and cell fusion (Gammie et al., submitted). Additional biochemical and electron microscopic analyses of secretory vesicles in mutants defective in cell fusion may provide a functional correlation between vesicles and fusion. Mutants defective in cell fusion might fail to produce, deliver, or release vesicles in response to cell contact.

The actin cytoskeleton or actin-interacting proteins may be part of the fusion machinery. A number of proteins involved in cell fusion in yeast are also implicated in cytoskeletal interactions: Fus1p binds actin by two-hybrid analysis (Amberg et al., 1995); overexpression of Fus2p partially restores polarized actin distribution to *bem1s* mutants (Leberer et al., 1996); Kel1p is composed of kelch repeats which may be involved in actin interactions. Additionally, *spa2*, *pea2*, and *bni1* mutants are defective in shmoo formation, suggesting that Spa2p, Pea2p, and Bni1p are involved in organizing the actin cytoskeleton during shmoo formation (Gehrung and Snyder, 1990; Valtz and Herskowitz, 1996; Evangelista et al., 1997). Thus, the actin cytoskeleton could play a direct role in fusion or be involved in localizing the machinery. The actin cytoskeleton may be important in localizing the fusion machinery in other examples of fusion as well. For instance in *Drosophila, myoblast city* (*mbc*) mutants exhibit defects in cytoskeletal organization and myoblast fusion (Rushton et al., 1995; Erickson et al., 1997), failing to localize paired vesicles to the site of cell fusion (Doberstein et al., 1997).

APPENDIX 1

IDENTIFICATION OF MUTANTS DEFECTIVE IN CELL FUSION

When this work began, three genes, *FUS1-FUS3*, had been characterized which were required for efficient cell fusion (Trueheart et al., 1987; McCaffrey et al., 1987; Elion et al., 1995; Elion et al., 1990). The identification of *FUS1* and *FUS2* was largely serendipitous. *FUS1* was found in a strain containing a large deletion removing the *FUS1* and *BIK1* genes. Bik1p is required for nuclear fusion (karyogamy) whereas Fus1p is required for efficient cell fusion (Trueheart et al., 1987), and loss of both contributed to a mating defect in this strain (Trueheart et al., 1987). Another group identified *FUS1* because its transcription is higher in α cells than **a**/α cells (McCaffrey et al., 1987). Upon backcrossing the *fus1* mutant, a spontaneous mutation in *FUS2* was identified because it exacerbated the defect of the *fus1Δ* mutant (Trueheart et al., 1987).

We took a systematic approach to identify additional genes required for cell fusion. Mutants were initially identified based upon their defective mating to *fus1 fus2* and *far1-c* mutant strains. *fus1 fus2* double mutants are mildly compromised in mating to a wild-type strain but are severely defective in mating to *fus1* or *fus2* strains (Trueheart et al., 1987; Elion et al., 1995). Hence, mutants defective in mating to a *fus1 fus2* strain should include *fus1Δ*, *fus2Δ*, and perhaps other mutants defective in cell fusion. To determine if any of the mutants were defective in *FUS1* or *FUS2*, plasmids containing these genes were tested for their ability to restore mating to the mutants, which identified one mutant as defective in *FUS2* (Chenevert et al., 1994).

To identify additional mutants defective in cell fusion, we examined the mating-defective mutants microscopically to ascertain whether prezygotes accumulated. We identified mutants of two classes: **a**-cell specific and non-cell type specific. The **a**-cell specific mutants also exhibited defects in **a**-factor production. Plasmids containing either *AXL1/STE22* or *STE6*, genes previously identified for their role in **a**-factor production, restored mating ability to these strains, suggesting that production of wild-type levels of pheromone is required for cell fusion. Two of the non-cell type specific mutants were defective in the *FPS1* gene. A mutation in a third non-cell type specific mutant was mapped, indicating that it identifies a novel gene involved in cell fusion.

METHODS

Yeast Strains, Plasmids, and Media

Yeast strains are described in Appendix 4. Yeast plasmids are described in Appendix 5. Standard yeast growth conditions and genetic manipulations are described in Rose et al. (1990). Cells were grown at 30°C in YEPD medium unless otherwise noted. Yeast transformations were performed by the lithium acetate method (Ito et al., 1983).

Mating Assays

Mating assays scored microscopically were performed by mixing equal numbers of log phase **a** and α cells (6×10^6), collecting cells on 0.45 μm filters (Millipore Corp., Bedford, MA), and incubating on YEPD plates for approximately 4 h at 30°C. Cells were resuspended in 5 ml 70% ethanol by vortexing, washed, and resuspended in 50% glycerol + 1 μg/ml 4,6-diamidino-2-phenylindole (DAPI; Sigma, St. Louis, MO). Samples were sonicated and

viewed with a Zeiss Axioskop microscope at 100X. Prezygotes were scored as structures in which the nuclei of mating partners remained unfused, as evidenced by two distinct DAPI staining structures, and in which a septum was visible between adherent mating partners. Percentage prezygotes was defined as prezygotes/(prezygotes + zygotes). At least 100 partnered cells (zygotes + prezygotes) were counted per sample.

Mating assays scored on plates were performed by spreading a lawn of 9 x 10^6 log phase *fus1 fus2* mating tester cells on a YEPD plate. Patches of cells grown on YEPD (or on minimal medium to select for plasmids) were replica plated to the lawn. After incubation at 30°C for 3.5-4.5 h, plates were replica plated to media selective for growth of diploids.

RESULTS

Identification of Mutants Defective in Cell Fusion

Mutants defective in cell fusion were identified based upon their defect in mating to a *fus1 fus2* mutant or a *far1-c* mutant (Chenevert et al., 1994) and accumulation of prezygotes when mated to a wild-type partner. Chenevert et al. (1994) analyzed the original group of mutants for their ability to respond to pheromone in terms of transcriptional induction, cell cycle arrest, and morphological response. To identify mutants specifically defective in cell fusion, we microscopically examined only those mutants that responded normally to pheromone. The mutants we chose to examine were vastly enriched for cell fusion mutants. The ability of cells to form partners (either zygotes or prezygotes) was also assessed. Mutants defective in partner formation might be defective in events that occur during or prior to cell-cell

contact such as sensitivity to pheromone, polarization or orientation in response to pheromone, or adhesion.

Eight of thirteen mutants exhibited more than a seven-fold increased frequency of prezygotes (Table A1-1; Fig. A1-1A) when mating to a wild-type α strain IH1793. Mutants designated M2, M4, M12, and M13 exhibited an approximately ten-fold increase in prezygotes when mated to a wild-type partner (Table A1-1). Mutants designated M6, M7, M9, and M10 exhibited a similar defect in cell fusion, but they also displayed a substantial defect in partnership, suggesting that they might be defective in finding or adhering to each other. Mutants designated M1 and M5 displayed intermediate defects in cell fusion and ability to form partnered structures.

This initial analysis indicated that mutants M8 and M11 were not defective in cell fusion or partnership. Additional experiments revealed that these strains have a striking defect in cell fusion, but the phenotype depends upon the strain background to which they are mated. M8 and M11 mutants behaved similarly to each other, exhibiting profound defects in cell fusion in matings to IH3186 or IH2350, but little defect in matings to IH1793 (Table A1-2). In contrast, M12 mutants displayed a dramatic defect in matings to IH1793 but appeared essentially wild-type in matings to IH3186, the opposite of that seen for M8 and M11. Therefore, the defect in cell fusion displayed by a given mutant depends upon some uncharacterized attribute of the mating partner. In conclusion, ten of the thirteen mating defective strains exhibited significant defects in cell fusion; the severity of the defect varied depending upon the strain background to which they were mated.

Mutants Defective in Pheromone Production Exhibit Defects in Cell Fusion

Eight of the mutants were backcrossed, revealing they were of two classes: M2, M4, M7, and M10 were **a**-cell specific and M8, M11, M12, and M13 were

Table A1-1

Defect in Cell Fusion of 13 Mating-Defective Mutants

a strain (IH#)	alias	% prezygotes[a]		%partnership[b]	
3160	WT	3	(287)	43	(400)
3172	M1	10	(40)	20	(200)
3173	M2	28	(148)	19	(300)
3174	M3	7	(81)	41	(200)
3175	M4	25	(145)	22	(300)
3176	M5	13	(125)	13	(300)
3177	M6	22	(9)	5	(200)
3178	M7	34	(116)	8	(300)
3179	M8	4	(79)	40	(200)
3180	M9	40	(5)	3	(200)
3181	M10	28	(121)	11	(300)
3182	M11	3	(75)	38	(200)
3183	M12	38	(150)	25	(300)
3184	M13	31	(143)	22	(400)

a strains were mated to α strain IH1793.

[a] % prezygotes represents the number of prezygotes/(zygotes+prezygotes). Numbers in parentheses indicate the total number of partnered cells that were counted.

[b] %partnership represents the number of (zygotes + prezygotes)/total cells. Numbers in parentheses indicate the total number of cells that were counted.

Fig. A1-1. Identification of mutants defective in cell fusion

A. Light Microscopy of Yeast Mating Pairs

(a) Wild-type (IH3160), (b) M2 (IH3173), (c) M10 (IH3181), and (d) M12 (IH3183) strains were mated on filters to wild-type strain IH1793 as described in Materials and Methods.

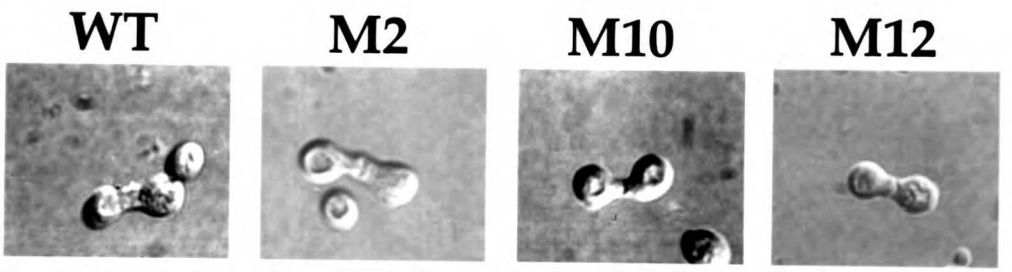
B. p*AXL1* Restores Mating to M2, M4, and M7 Mutants.

A. Wild-type (IH3160), M2 (IH3173), M4 (IH3175), and M7 (IH3178) strains carrying pAXL1 (CY655; *right*) or vector (pRS316; *left*) were mated to a *MATα* *fus1 fus2* strain as described in Materials and Methods.

C. 2μ *STE6* Restores Mating to M9 and M10 Mutants

Wild-type (IH3160), M9 (IH3180), and M10 (IH3181) strains carrying 2μ*STE6* (CY248; *right*) or vector (YEp24; *left*) were mated to a *MATα* *fus1 fus2* strain as described in Materials and Methods.

A



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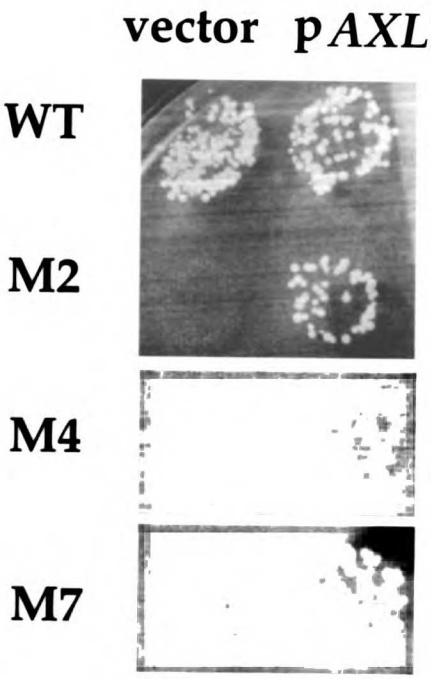


C

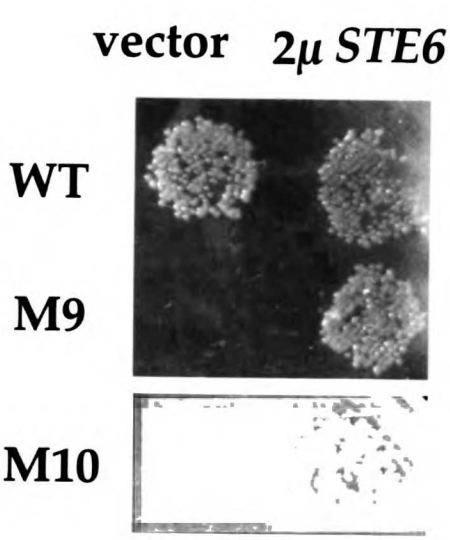


Table A1-2

Defect in Cell Fusion of M8, M11, and M12 Mutants

		% prezygotes in matings to: [a]		
		IH3186	IH2350	IH1793
a strain	alias			
IH3160	WT	1	4	2
IH3179	M8	95	84	4
IH3182	M11	64	68	6
IH3183	M12	2	15	51

a strains were mated to α strains IH3186, IH2350, and IH1793.

[a] % prezygotes represents the number of prezygotes/(zygotes+prezygotes). Data represent the average of at least two experiments in which more than 100 partnered cells were counted.

non-cell-type specific, exhibiting mating defects as both **a** and α cells (Table A1-3). Careful examination of M2, M4, M7, M9, and M10 mutants indicated that they were mildly defective in **a**-factor production (data not shown). It is difficult to detect qualitative differences in pheromone production using a standard halo assay, explaining why this defect was not initially detected. We tested genes involved in **a**-factor production for their ability to complement the mating defect of the **a**-cell specific mutants. The mating defect of M2, M4, and M7 mutants could be restored by providing the *AXL1/STE22* gene, whereas *STE6* restored mating to M9 and M10 mutants (Fig. A1-1). M13 mutants exhibited a pheromone production defect in both **a** and α cells which segregated with the mating defect (Philips, unpublished observations), the genetic basis of which has not been determined. In conclusion, we identified mutants defective in cell fusion which are also compromised in pheromone production, suggesting that pheromone production is important for cell fusion.

Mapping the Mutation in M12 Mutants

The gene responsible for the mating defect of M8 mutants was cloned by complementation, and M11 mutants were found to be defective in the same gene (*FPS1*; see Chapter 2). To determine the feasibility of cloning the gene defective in the M12 mutant by complementation, we assesed whether the mutation was recessive. Because mating is a haploid-specific phenotype, dominance tests were carried out in a *MATα/MATΔ* diploid strain which retains the ability to mate as an α strain. *MATα* strains carrying the M12 mutation were crossed to strains in which the *MAT* locus was deleted (JP9), generating *MATα/MATΔ* M12/+ diploid strains. These diploid strains did not exhibit the mating defect seen in M12 mutants, indicating that the

Table A1-3

Summary of Mutants M1-M13

	Cell Fusion Defect	Partnership Defect	Cell-Type Specificity	Plasmid Complement
Pheromone production defect				
M2	+	+	**a**	*AXL1*
M4	+	-	**a**	*AXL1*
M7	+	+	**a**	*AXL1*
M9	+	+	N.D.	*STE6*
M10	+	+	**a**	*STE6*
M13	+	-	**a** and α	–
No pheromone production defect/Fusion defect				
M8	+	-	**a** and α	*FPS1*
M11	+	-	**a** and α	*FPS1*
M12	+	-	**a** and α	–
No pheromone production defect/Partnership defect				
M1	-	+	N.D.	–
M5	-	+	**a** and α *	–
M6	+	+	N.D.	–
No mating defect				
M3	-	-	N.D.	–

A defect in cell fusion was defined as at least five-fold more prezygotes than seen in wild-type strains and is indicated by +. Strains which accumulated fewer prezygotes are indicated by -.
A defect in partnership was defined as 50% or less partnership than seen in wild-type strains and is indicated by +. Strains which exibited partnership between 50-100% of wild-type are indicated by -.
Cell-type specificity indicates the cell types which exhibit the mating defect.
N.D. indicates that the cell-type specificity was not determined.
Plasmid complement indicates whether plasmids containing *AXL1, FPS1* or *STE6* complemented the mating defect.
* S. O'Rourke and Linda Huang, personal communication

mutation is recessive (data not shown). An attempt to clone the gene defective in the M12 mutant was unsuccessful. The strain was transformed with a pRS306-derived library (C. Boone), and 10,000 transformants were screened for ability to mate with a *fus1 fus2* strain; none exhibited restored mating.

The mutation in the M12 strain was tightly linked to *TRP1*, which is located close to the centromere of chromosome IV. In 58 complete tetrads analyzed, the M12 mutation recombined from *trp1* three times, indicating a genetic distance of approximately 2.4 cM. M12 was further mapped with respect to the temperature-sensitivity conferred by a mutation in *PRP11*. *PRP11* is located approximately 85 kb from *TRP1*, and the genetic distance as determined by Mortimer and Hawthorne (1973) is 17.4 cM. Based upon our segregation analysis, *TRP1* mapped approximately 33 cM from *PRP11* (PD=18; T=20; NPD=1) as did the mutation in M12 (PD=17; T=19; NPD=1). In these crosses, *TRP1* never recombined with the mutation in M12, so we were unable to determine on which side of *TRP1* the M12 mutation is.

Plasmids were obtained that covered approximately 40 kb surrounding *TRP1* (Fig. A1-2). The region between *PTC1* and the *RPL2B* was contained on these plasmids except for the centromere and the *NTH1* gene. However, *nth1Δ* mutants were examined and they did not exhibit a mating defect (data not shown). In addition, *GPD1*, located between *PRP11* and *TRP1;* failed to complement the mating defect of M12 mutants (data not shown). In conclusion, the mutation responsible for the defect in cell fusion in the M12 mutant is on chromosome IV, tightly linked to *TRP1*. It is unlikely that it is located between *PTC1* and *RPL2B*. Since no genes which are known to be required for mating or cell fusion are located near *TRP1*, M12 is a novel cell fusion mutant.

Fig. A1-2 Map of chromosome IV between *PRP11* and *SNQ2*.

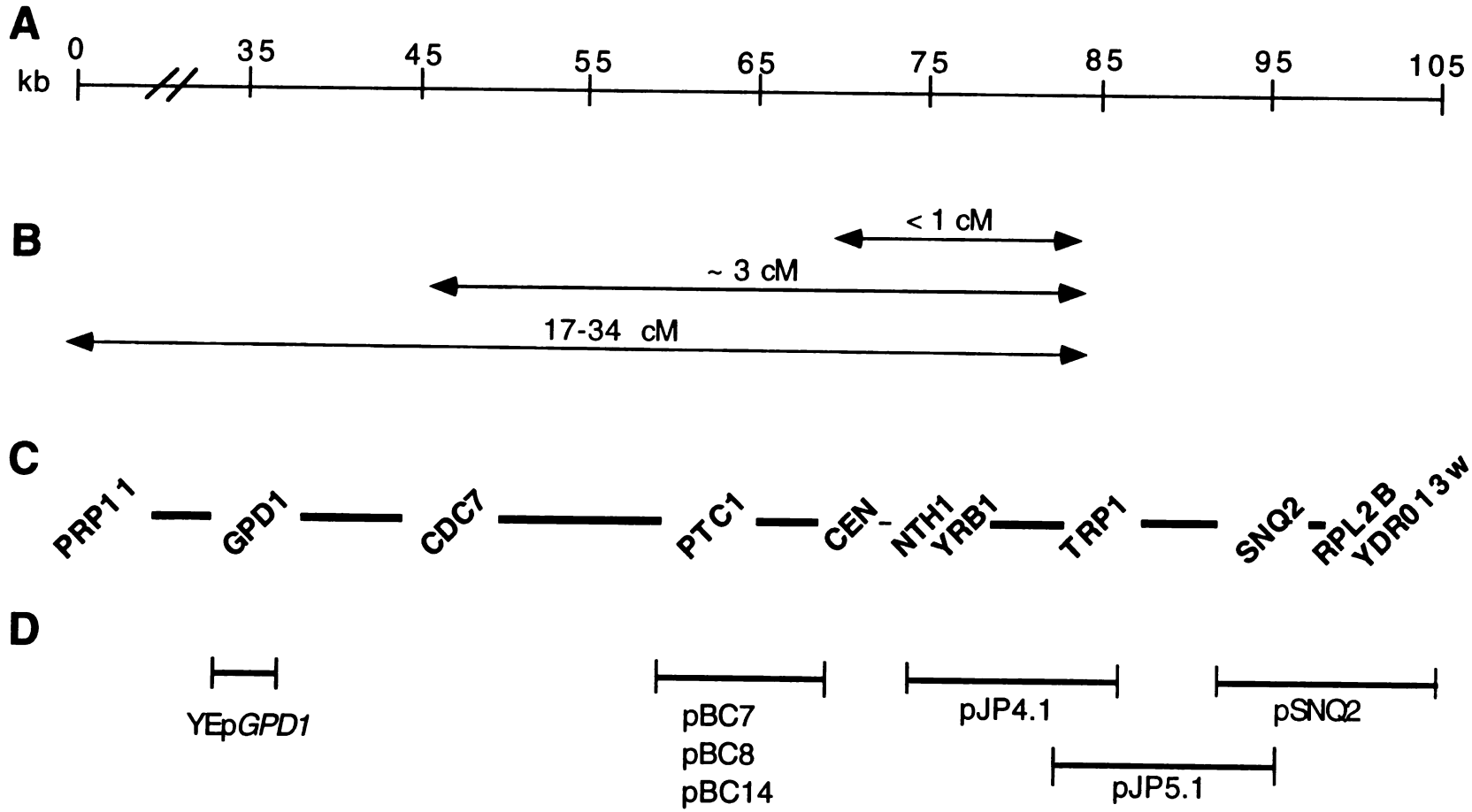
A. Top line indicates physical distance in kb.

B. Lines with arrowheads indicate genetic distance.

C. Map indicates several genes in the 85 kb interval between *PRP11* and *SNQ2*.

D. Plasmids containing DNA in this region are diagrammed with their names indicated underneath. The DNA between *PTC1* and *RBL2B* was contained on these plasmids except for the centromere and the *NTH1* gene. The pBC plasmids contain the *PTC1* gene and extend to the centromere (Bruce Cree, personal communication). pJP4.1, pJP5.1, and pSNQ2 (from Bill Wells) are overlapping plasmids which extend from *YRB1* to within the YDR013w ORF.

YEp*GPD1* contains the *GPD1* gene.


A
kb
0
35
45
55
65
75
85
95
105
B
< 1 cM
~ 3 cM
17-34 cM
C
PRP11
GPD1
CDC7
PTC1
CEN
NTH1
YRB1
TRP1
SNQ2
RPL2B
YDR013w
D
YEpGPD1
pBC7
pBC8
pBC14
pJP4.1
pJP5.1
pSNQ2

DISCUSSION

The Role of Pheromone in Cell Fusion

To identify genes required for cell fusion, we examined mutants which had previously been isolated based upon their defect in mating to a *fus1 fus2* mutant strain (Chenevert et al., 1994). Microscopic examination revealed that ten of thirteen mutants exhibited significant defects in cell fusion. These mutants were of two classes: 5 were **a** cell specific and 5 were non-cell type specific. All of the **a** cell specific and one of the non-cell type specific mutants exhibited defects in pheromone production (M2, M4, M7, M9, M10, and M13). For the **a** cell specific mutants, plasmids containing either Axl1p or Ste6p restored mating. Axl1p and Ste6p are both required for **a**-factor production (Adames et al., 1995; Michaelis and Herskowitz, 1988). Axl1p is required for amino-terminal processing of the **a**-factor precursor (Adames et al., 1995). Unlike Ste6p which is absolutely required for **a**-factor production, *axl1Δ* mutants still produce some **a**-factor because another protease (Ste23p) provides some processing activity (Adames et al., 1995). Axl1p is also required for axial budding in addition to its role in **a**-factor processing (Fujita et al., 1994). Interestingly, the protease activity of Axl1p is required for **a**-factor processing but dispensible for axial budding (Adames et al., 1995). Axl1p governs budding pattern in both **a** and α cells, but is more highly expressed in **a** cells (Fujita et al., 1994), perhaps reflecting its additional function in pheromone processing in **a** cells. The mating defect of M2, M4, and M7 mutants is only seen in **a**-cells, suggesting that the role of Axl1p in cell fusion is due to its function in pheromone processing not bud site selection. Axl1p

was also identified by other labs for its role cell fusion (Brizzio et al., 1996; Dorer et al., 1997).

Ste6p is a member of the ABC-transporter of proteins, which includes the mammalian multidrug resistance (MDR) P-glycoprotein (McGrath et al., 1989). Ste6p is required for export of **a**-factor (Michaelis, 1993; Kuchler et al., 1989); it does not transport α-factor, which is released through the secretory pathway (Julius et al., 1984), explaining why M9 and M10 mutants exhibited defects exclusively in **a** cells. The mutants we identified are able to export some **a**-factor. Thus, they could contain partial loss of function or special alleles of *STE6*. Another possibility is that the mutants are defective in some other gene, and the mating defect is suppressed by overexpression of *STE6*. We think it is likely that they are defective in *STE6* because Elia and Marsh (1996) demonstrated that mutants containing hypomorphic alleles of *STE6* exibit a defect in cell fusion. It is unclear whether the requirement of Ste6p in cell fusion is solely to export **a**-factor or whether it has some additional function, such as exporting some other factor (Elia and Marsh, 1996; Brizzio et al., 1996). Regardless of whether there is another factor, production of high levels of **a**-factor is required for efficient cell fusion. If level the of **a**-factor produced during mating is decreased experimentally, then an otherwise wild-type strain exhibits a defect in cell fusion (Brizzio et al., 1996). Mutants defective in α-factor production also exhibit a defect in cell fusion, suggesting that pheromone, not **a**-factor in particular, plays a role (Brizzio et al., 1996).

There are at least two explanations for the role of pheromone in cell fusion. One possibility is that if a cell fails to produce high levels of pheromone, then the expression of pheromone-induced genes in the mating partner is reduced. Failure to fully induce genes required for cell fusion could account for the defect. A more interesting possibility is that high levels of

pheromones are required for a distinct function during cell fusion outside of their role in gene induction. It has long been noted that different levels of pheromone are required to elicit different biological responses -- for instance, two orders of magnitude more pheromone is required for shmoo formation than for cell cycle arrest or agglutination induction (Moore, 1983; Dorer et al., 1995). Maybe even higher levels of pheromone are required for cell fusion. The requirement for high levels of pheromone could be for localization of fusion components. Localization of Fus1p and Ste2p is seen only when cells are treated with high enough levels of pheromone to induce projection formation. Less pheromone increases expression of these proteins but does not result in localized staining (Jackson et al., 1991).

It is clear that high levels of pheromone are not sufficient to cause cell fusion, because exogenously added pheromone in quantities vastly exceeding the Kd of the pheromone receptor never causes cells to degrade their cell wall. High levels of pheromone may be one signal required for cell fusion, but there must be additional signals generated when cells actually make contact with each other. The existence of several signals would be one way to insure fidelity of the process. When one cell begins to degrade the cell wall, it is critical that there is an adjacent cell preparing to do the same thing. It seems reasonable that a cell will experience the highest level of pheromone when its mating partner secretes pheromone in a polarized manner while fully inducing pheromone-dependent genes. The requirement for a high level of pheromone from the partner might provide a mechanism to assure that the potential partner is competent to fuse -- polarized and fully inducing genes required for cell fusion.

Analysis of M1, M5, M6, M12, and M13 Mutants

The mating defect in M8 and M11 mutants is caused by a mutation in the *FPS1* gene. The role of *FPS1* in cell fusion is described in Chapter 2. The genetic basis of the mating defect in mutants M1, M5, M6, M12, and M13 has not been determined. Analysis of these mutants suggests that M12 and M13 are defective in fusion. The defect in M13 mutants may be a consequence of a pheromone production defect, perhaps caused by a mutation in a gene such as *SPT3*, which is required for production of **a**- and α-factor (Hirschhorn and Winston, 1988). The mutation in M12 mutants maps to a region of chromosome IV to which no known genes involved in mating or cell fusion are located. The mutation is recessive, making it technically feasible to clone the gene by complementation. Our inability to do so may indicate that the gene was not well represented in the library we used, so attempts using different libraries might be fruitful. Alternatively, our mapping data provide a starting point for obtaining cosmids in the region or a candidate gene approach.

It is not clear what is the primary cause of the mating defect in M1, M5, and M6 mutants, which display intermediate defects in cell fusion and partnership. It is possible that these mutants are defective in some other step in mating such as orientation towards a mating partner or in karyogamy. M5, M6, M12 and M13 mutants are mildly compromised in the pheromone confusion assay, which is thought to measure the ability of cells to orient towards their mating partner (Valtz, 1996). The fact that they also display a defect in cell fusion is consistent with a possible role in orientation; *far1-c* mutants, which are known to be defective in orientation, also exhibit defects in cell fusion (Philips, unpublished observations.) Another possibility is that these mutants are defective in genes such as Fig1p, Fig2p, or Rvs161p, which have been identified for their role in mating and cell fusion.

APPENDIX 2

OVEREXPRESSION OF *STE5, STE20,* OR *KEL1* SUPPRESSES *PKC1-R398P*

We showed previously that mutants lacking the glycerol transporter Fps1p exhibit a defect in cell fusion which may be explained by their inability to maintain osmotic balance (Philips and Herskowitz, 1997). *fps1Δ* mutants accumulate intracellular glycerol; if intracellular glycerol is restored to wild-type levels by deleting *GPD1*, which encodes a glycerol-3-phosphate dehydrogenase required for the first step of glycerol biosynthesis, then the cell fusion defect is partially alleviated. Similarly, if 1M sorbitol is provided extracellularly to osmotically support the cell, the defect in cell is relieved. We hypothesized that Pkc1p, which responds to hypo-osmotic shock (Davenport et al., 1995), may mediate the defect in cell fusion in *fps1Δ* mutants. Consistent with such a possibility, strains which contain activated alleles of Pkc1p (Pkc1p-R398P) exhibit a defect in cell fusion. This allele is altered in the pseudosubstrate-binding site of Pkc1p causing a dominant, activated kinase (Nonaka et al., 1995). The defect in cell fusion exhibited by strains containing *PKC1-R398P* is not suppressed by loss of *GPD1*, suggesting that Pkc1p-R398P functions downstream or parallel to glycerol accumulation to inhibit cell fusion (Philips and Herskowtiz, 1997).

We reasoned that if Pkc1p inhibits some target important in cell fusion, then overexpression of this target might relieve the defect in cell fusion imposed by activated Pkc1p. Therefore, we performed a high copy suppressor screen to identify factors that restore mating to cells expressing Pkc1p-R398P

(JP317). In addition to potential targets of Pkc1p, we might identify negative regulators of Pkc1p or factors that act in parallel to Pkc1p to promote cell fusion. Because mutants containing *PKC1-R398P* are not suppressed by loss of *GPD1*, high copy suppressors of JP317 are unlikely to function by altering glycerol balance. We identified four plasmids which suppressed the mating defect of JP317 and characterized those encoding *KEL1*, *STE5*, and *STE20*.

METHODS

Yeast Strains and Plasmids

Yeast strains are described in Appendix 4. Plasmids are described in Appendix 5. DNA manipulations were performed as described in Sambrook et al. (1989).

Microscopic and Plate Mating Assays

Mating assays were performed as described in Philips and Herskowitz (1997).

Quantitation of *FUS1-lacZ* Expression

Strains grown to mid-log phase in SD -Trp Leu were resuspended in YEPD containing 10 μg/ml α-factor or no pheromone. They grew for 1 h before β-galactosidase activities were measured essentially as described in Stern et al. (1984). Data are expressed in Miller units $(OD_{420} \times 1000)/(OD_{600} \times \text{min} \times \text{ml})$.

RESULTS

Identification of High Copy Suppressors of *PKC1-R398P*

Cells expressing an activated allele of Pkc1p (*PKC1-R398P;* JP317) exhibit a mating defect due to impaired cell fusion (Philips and Herskowitz, 1997). The mating defect of JP317 was apparent when cells were mated to a *fus1 fus2* mutant strain, which is also partially defective in cell fusion (see Fig. 3-1A). A high copy suppressor screen was performed to identify genes that improved mating of JP317. JP317 was transformed with a high-copy library, and approximately 4500 transformants were patched to selective media. They were screened by replica plating for mating with a *fus1 fus2* mutant strain. Four plasmids, designated pPHS5/6, pPHS11, pPHS12, and pPHS18, were identified that restored mating when retransformed (Fig. A2-1). Sequence analysis determined that these plasmids contained distinct inserts. The ORF responsible for the suppression of pPHS11 was determined to be *KEL1* and is described in more detail in Chapter 3. Initial attempts at determining which ORF on pPHS18 was responsible for suppression were inconclusive, and this plasmid was not characterized further (see Appendix 5 for a map of pPHS18).

STE5 and *STE20* are likely to account for the suppression of pPHS12 and pPHS5/6, respectively. Plasmids in which *STE5* is the only full length ORF (CY441 and CY442) also suppressed JP317 (data not shown). pPHS5 and pPHS6 were isolated from the same yeast strain and contained *STE20,* but restriction analysis indicated that both plasmids were rearranged compared to the genomic locus. Therefore, it was unclear exactly which ORFs were contained on pPHS5/6. It is likely that *STE20* accounted for the ability of these plasmids to suppress because plasmids containing *STE20* (CY711) under control of the *GAL1,10* promoter suppressed the mating defect caused by *PKC1-R398P.*

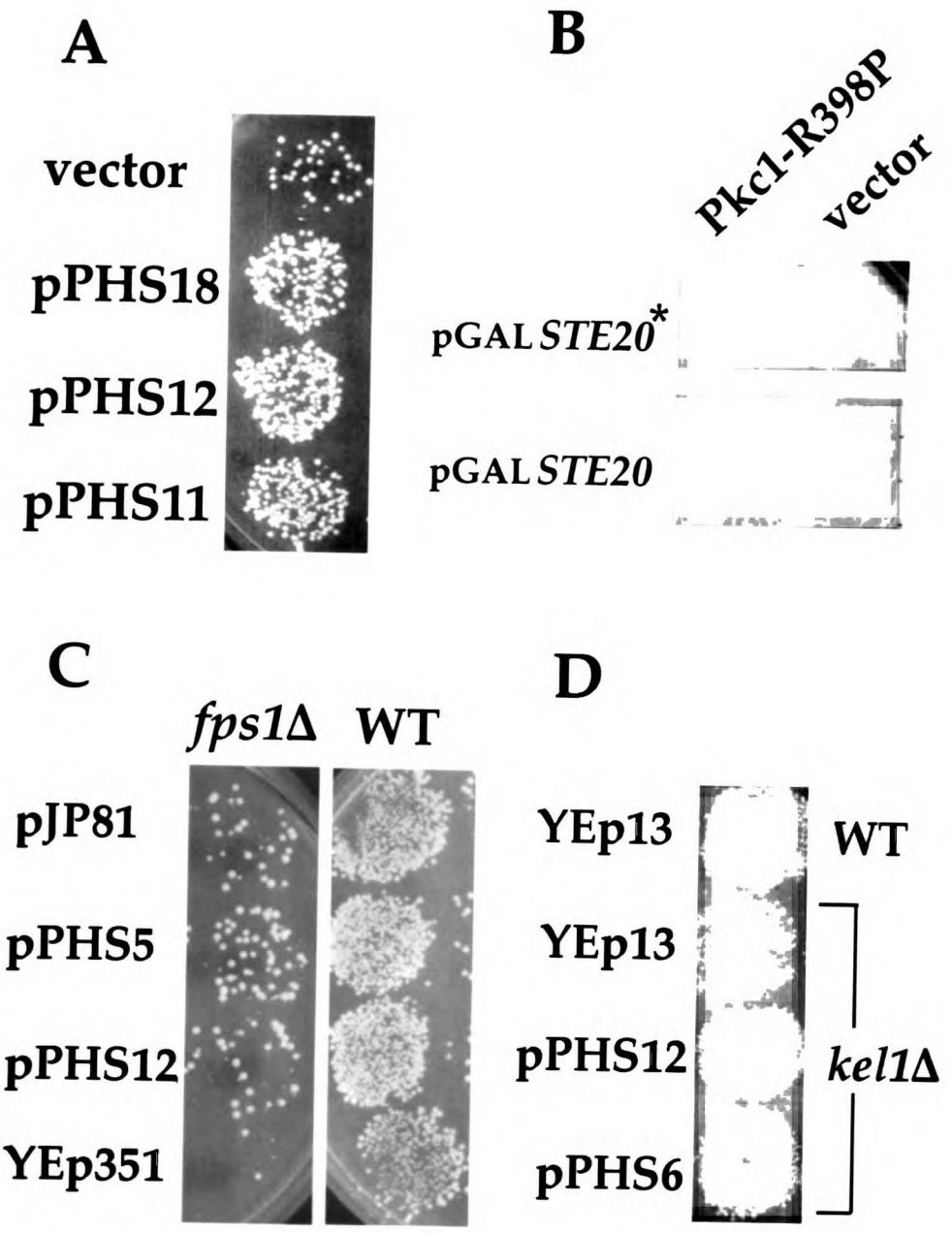
Figure A2-1. Overexpression of *STE5, STE20, KEL1,* or pPHS18 promotes mating in mutants defective in cell fusion

A. pPHS18, *STE5,* and *KEL1* suppress the mating defect caused by *PKC1-R398P.* JP317 (*MAT**a** PKC1-R398P*) carrying vector (YEp13), pPHS18, pPHS12 (*STE5*), or pPHS11 (*KEL1*) was mated to a *MATα fus1 fus2* strain (IH2351) as described in Materials and Methods.

B. p*GAL STE20* suppresses the mating defect caused by *PKC1-R398P.* JP335 carrying pJP106 (*PKC1-R398P*) or vector (YCplac22) was transformed with plasmids containing *STE20* (CY711) or a kinase-inactivated version of *STE20* (CY713) under control of the *GAL1,10* promoter (designated p*GAL STE20* and p*GAL STE20**, respectively). Strains were grown on S raffinose - Trp Ura, and mated to a *MATα fus1 fus2* strain (IH2351) on YEPD.

C. *KEL1, STE20,* and *STE5* suppress the mating defect of *fps1Δ* mutants. JP147 (*fps1Δ::URA3*) containing *KEL1* (pJP81), *STE20* (pPHS5), and pPHS12 (*STE5*) was mated to a *MATα fus1 fus2* strain (IH2351) as described in Materials and Methods.

D. Overexpression of *STE20* or *STE5* suppresses the mating defect of *kel1Δ* mutants. JP432 (*kel1Δ::hisG*) carrying *STE20* (pPHS6), *STE5* (pPHS12), or vector (YEp13) was mated to a *Mat α fus1 fus2* strain (IH2351) as described in Materials and Methods.


A
vector
pPHS18
pPHS12
pPHS11
B
Pkc1-R398P
vector
pGAL STE20*
pGAL STE20
C
fps1Δ
WT
pJP81
pPHS5
pPHS12
YEp351
D
YEp13
WT
YEp13
pPHS12
kel1Δ
pPHS6

Suppression probably requires little overexpression of Ste20p because it was observed when cells were grown on glucose (Fig. A2-1B). Kinase-inactivated forms of Ste20p failed to suppress the mating defect, suggesting that kinase activity is important for suppression (Fig. A2-1B).

KEL1, _STE5_, and _STE20_ Partially Suppress _fps1Δ_ Mutants

We suggested previously that *fps1Δ* mutants exhibit a defect in cell fusion due to elevated Pkc1p activity. If this hypothesis is correct, then plasmids which suppress the mating defect of *PKC1-R398P* strains might also suppress *fps1Δ* mutations. We examined the ability of *STE20* (pPHS5), *STE5* (pPHS12), and *KEL1* (pJP81) to suppress the mating defect of *fps1Δ* mutants and found that they partially restored mating to *fps1Δ* mutants (Fig. A2-1C). *STE5*, *STE20*, and *KEL1* were less effective at suppressing the mating defect of *fps1Δ* mutants compared to the *PKC1-R398P* mutant. They may be less effective because *fps1Δ* mutants exhibit a more severe fusion defect than cells expressing *PKC1-R398P* (see Table 2-5). Perhaps the Pkc1p pathway is more strongly activated in *fps1Δ* mutants than in *PKC1-R398P* mutants, or *fps1Δ* mutants are defective in fusion for reasons in addition to Pkc1p activation.

Does Increased Signalling Through the Pheromone Response Pathway Suppress Pkc1p-R398P?

STE5 and *STE20* are required for signal transduction in response to pheromones. Both Ste5p and Ste20p bind to Gβ and are somehow involved in transducing a signal from Gβ to Ste11p (see Chapter 1). Therefore, we wondered whether Ste5p, Ste20p, or Kel1p suppress the mating defect of JP317 by increasing signalling through the pheromone response pathway. One possibility is that increased transcriptional induction of genes involved in cell fusion is responsible for the suppression. For a variety of reasons, we think this is an unlikely explanation. First, preliminary experiments indicated that

activated alleles of Ste11p (*STE11-1* and *STE11-4*) failed to suppress the mating defect of *PKC1-R398P* mutants, suggesting that pathway activation is not sufficient for suppression. Secondly, strains harboring *PKC1-R398P* on a plasmid do not exhibit altered signalling based upon expression of a pheromone-induced reporter, *FUS1-lacZ*, compared to strains containing vector (Table A2-1). Therefore, we consider it unlikely that *PKC1-R398P* causes a defect in cell fusion due to decreased signalling. Finally, most of the plasmids which suppress JP317 do not affect signalling. Only pPHS12 (*STE5*) significantly alters the basal level of expression of *FUS1-lacZ*. In the presence of pheromone, strains harboring high copy plasmids containing *KEL1*, *KEL2* (which also suppresses the mating defect of JP317; see Chapter 3), *STE5*, or *STE20* do not exhibit altered levels of *FUS1-lacZ*. Hence, we consider it unlikely that *STE5*, *STE20*, or *KEL1* suppress the mating defect of JP317 by increasing signalling of the pheromone response pathway.

STE5 and _STE20_ Suppress _kel1Δ_ Mutants

One possibility is that Ste5p and Ste20p function through Kel1p to suppress the fusion defect of JP317. To examine such a possibility *kel1Δ* mutants were constructed as described in Chapter 3. Consistent with a role for Kel1p in cell fusion, *kel1Δ* mutants are defective in cell fusion. The mating defect of *kel1Δ* mutants could be suppressed by *STE20* (pPHS6) and *STE5* (pPHS12) (Fig. A2-1D), suggesting that *STE20* and *STE5* can promote cell fusion independently of Kel1p.

Table A2-1

Effect of Pkc1p-R398P, Ste5p, Ste20p, Kel1p and Kel2p on *FUS1-lacZ* Expression

	–α factor	+α factor	
	YCplac22	YCplac22	*PKC1-R398P*
1. *KEL1*	0.54	27.7	26.8
2. *KEL2*	0.53	27.5	26.6
3. *STE20*	0.98	28.7	28.4
4. *STE5*	7.8	27.8	31.1
5. *YEp351*	0.62	28.8	27.0

Strain JP334 (*MAT**a** FUS1::lacZ)* was transformed with plasmids containing *KEL1* (pJP81), *KEL2* (pJP92), *STE20* (pPHS6), *STE5* (pPHS12), or vector (YEp351) and either *PKC1-R398P* (pJP106) or vector (YCplac22). β-galactosidase activity was measured as described in Materials and Methods and is expressed in Miller units.

DISCUSSION

Overexpression of *STE5*, *STE20*, and *KEL1* Suppresses *PKC1-R398P*

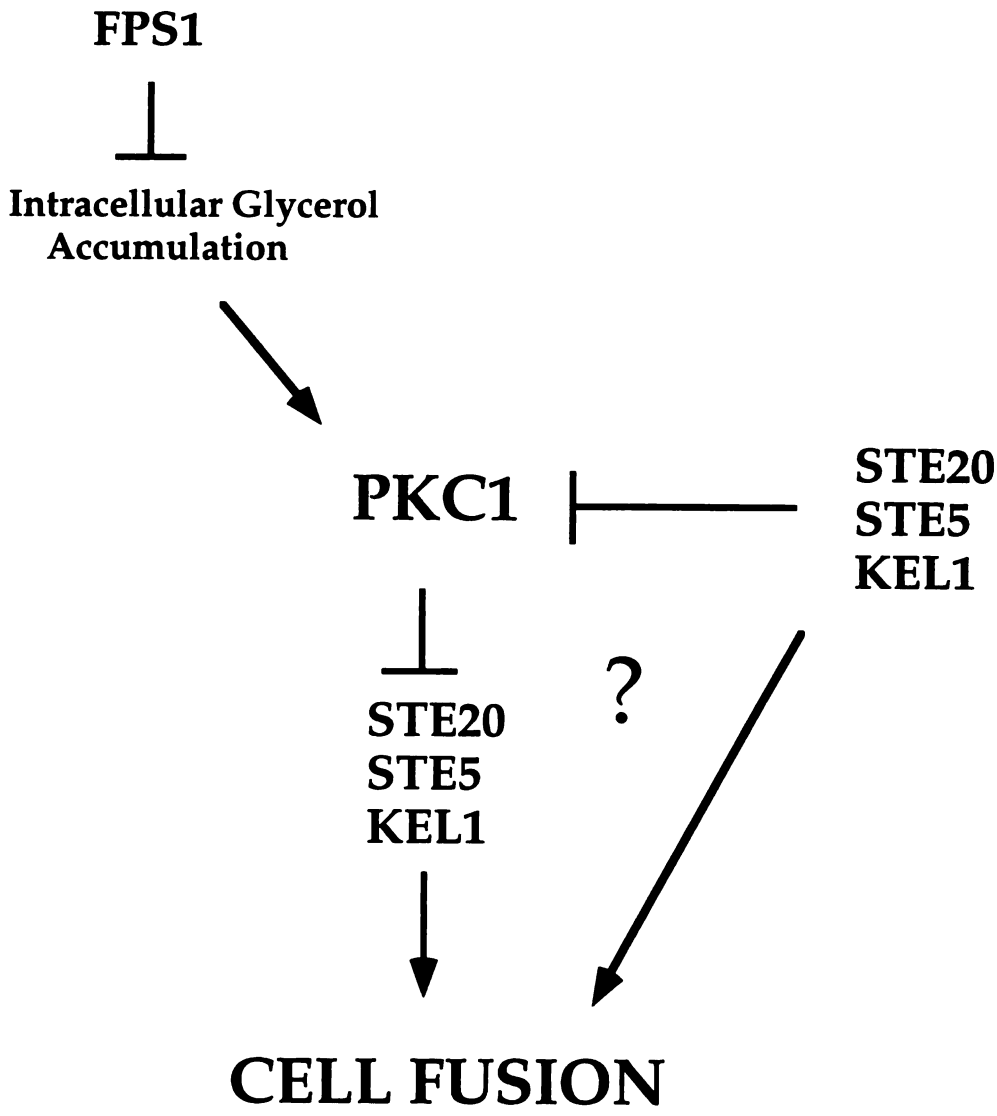
To identify genes involved in cell fusion, we looked for high copy plasmids that restore mating to strains containing an activated allele of Pkc1p. This strategy could identify targets of Pkc1p, negative regulators of Pkc1p, or proteins which act in parallel to promote cell fusion (Fig. A2-2). We identified four plasmids which suppressed the mating defect JP317 and further characterized those encoding *KEL1*, *STE5*, and *STE20*. Consistent with the idea that Pkc1p acts downstream of Fps1, these plasmids also partially suppress the mating defect of *fps1Δ* mutants.

Role of Ste5p and Ste20p In Promoting Cell Fusion

Ste5p and Ste20p are required for signal transduction in response to pheromone, so it is possible that overexpression of Ste5p or Ste20p promotes cell fusion by increasing signalling through the pheromone response pathway. This possibility has not been ruled out. However, preliminary experiments suggest that activation of the pheromone response pathway is not sufficient for suppression. There are several other reasons why we do not favor this explanation. First, activated alleles of Pkc1p do not affect expression of the pheromone-responsive gene *FUS1*, suggesting that gene induction in such a strain is normal. Second, in the presence of pheromone, Ste5p and Ste20p do not alter induction of *FUS1*. An additional argument is that *STE5* and *STE20* suppress the mating defect of *kel1Δ* mutants. Like *PKC1-R398P* mutants, there is no evidence that *kel1Δ* mutants are defective in signalling in response to pheromone, as they induce *FUS1* normally in response to pheromone (Fig. 3-2).

Fig. A2-2. Relationship of Ste5p, Ste20p, and Kel1p to Pkc1p.

Overexpression of Ste5p, Ste20p, or Kel1p suppresses the defect in cell fusion caused by Pkc1p-R398P. Ste5p, Ste20p, and Kel1p could be targets of Pkc1p, negatively regulating Pkc1p, or act in parallel to promote cell fusion.


FPS1
Intracellular Glycerol Accumulation
PKC1
STE20
STE5
KEL1
?
STE20
STE5
KEL1
CELL FUSION

Another possibility is that Ste5p and Ste20p have roles in cell fusion in addition to their function in pheromone signalling. If this is the case, they would have been difficult to identify in the other screens that have been designed to look for loss of function mutants that specifically disrupt fusion. One advantage of the high copy screen was it allowed the identification of genes that are essential for mating or viability. Unless special alleles of Ste5p or Ste20p are identified, it will be difficult to determine if they serve a later role in mating in addition to their early role in pheromone response. Such alleles may already exist for Ste20p, although it is not clear that they result in a defect in cell fusion. Strains containing alleles of Ste20p that do not bind Cdc42p exhibit a mating defect, but are normal for pheromone signalling (Peter et al., 1996; Leberer et al., 1997). They may be defective in adhesion (Leberer et al., 1997), which would be consistent with Ste20p playing a role in a cell-cell contact mediated event. Perhaps Ste20p is involved in signalling in response to cell contact to promote adhesion and subsequent fusion.

The Relationship of Ste5p, Ste20p, and Kel1p

Because Ste20p, Ste5p, and Kel1p behave similarly in suppressing JP317, we wondered what the relationship is among these proteins. It is possible that Kel1p functions by promoting the activity of Ste5p or Ste20p. Since Ste5p and Ste20p are required for mating, it is difficult to determine whether the ability of Kel1p to suppress JP317 depends upon Ste5p or Ste20p. The ability of Ste20p and Ste5p to promote mating cannot depend entirely on Kel1p because Ste20p and Ste5p also suppress the mating defect of *kel1Δ* mutants.
The functional relationships, if they exist, among these proteins remain to be determined.

APPENDIX 3

ANALYSIS OF Kel2p

We identified Kel2p based upon sequence similarity to Kel1p. Kel1p and Kel2p are 44% identical over their entire length. They are composed of two domains: six kelch repeats in the amino-terminal half of the proteins followed by a coiled-coil domain in the carboxy terminus. In addition to sequence similarity, Kel1p and Kel2p are functionally similar. Overexpression of Kel1p or Kel2p alters cellular morphology and suppresses the mating defect caused by an activated allele of Pkc1p (Pkc1p-R398P). Loss of *kel1Δ* or *kel2Δ* exacerbates the growth defect of *mpk1Δ* mutants. Kel1p and Kel2p localize similarly to each other to regions of polarized growth. Two-hybrid and co-immunoprecipitation analyses indicate that Kel1p and Kel2p physically interact, which is significant *in vivo* because Kel2p fails to localize in the absence of Kel1p. To understand better the function of Kel2p, we analyzed the protein by immunoblot and performed a two-hybrid screen to identify interacting proteins.

MATERIALS AND METHODS

Immunoblots Analysis

Two different methods were used to prepare samples for immunoblot analysis. Samples in Fig. A3-1A and 1B were prepared as follows: cells were grown to mid-log phase, microfuged, and resuspended to an OD_{600} of 0.5 in YEPD. Cells were treated with 15 μg/ml α-factor or no pheromone.

Approximately 10 ml of culture was pelleted and resuspended directly in 100 µl 95°C SDS-Laemmli buffer containing protease inhibitors, 80 mM β glycerophosphate, and 1 mM Na_3VO_4. Samples were boiled for five min before resolving by 7.5% SDS-PAGE. For Fig. A3-1C, samples were prepared as follows: after pheromone treatment, 15 ml of culture was pelleted in a microfuge and incubated on ice for 5 min. Pellets were resuspended in 150 µl ice-cold solution 1 (1.85 N NaOH, 7.4% β-mercaptoethanol) and incubated on ice for 10 min. 150 µl ice-cold 50% TCA was added. Cells were incubated on ice for 10 min, followed by centrifugation for 2 min at 4°C. Pellets were washed with 1 ml ice-cold acetone. Samples were centrifuged for 2 min at 4°C. The pellet was resuspended in sample buffer, boiled for 5 min, and resolved by 7.5% SDS-PAGE. Immunoblot analysis was performed with anti-HA (HA11, Babco) or anti-actin antibodies (Boehringer) as described below.

SDS-PAGE was followed by electroblotting onto nitrocellulose filters using the Minigel system (Bio-Rad Labs., Hercules, CA). Blots were incubated in TBST (TBS with 0.1% Triton X-100) with 10% nonfat dry milk for approximately 1 h. Blots were incubated with primary antibody (mAb HA11 diluted 1:10,000 (Babco); mAb anti-actin (Boehringer) diluted 1:2000) in TBST supplemented with 2% nonfat dry milk at 4°C overnight. Blots were washed three times for 5 min in TBST and incubated in secondary antibody (Bio-Rad Labs., Hercules CA) diluted 1:2,000 in TBST with 2% nonfat dry milk for 1 h. Blots were washed twice for 5 min in TBS supplemented with 0.3% Triton X-100 followed by three washes in TBST. Blots were developed using an enhanced chemiluminescence detection kit (Amersham Corp., Arlington Heights, IL).

Two-Hybrid Analysis

Two-hybrid assays were performed as described (Gyuris et al., 1993). Yeast strain EGY48 containing the *LexAop-LacZ* reporter plasmid pSH18-34 was cotransformed with pEG202-based plasmids expressing LexA DNA-binding domain fusions and a library of pJG4-5-based plasmids containing transcriptional activation domain fusions (Gyuris et al., 1993). The two-hybrid screen was performed as described (Golemis et al., 1997).

RESULTS AND DISCUSSION

Immunoblot Analysis of Kel2p

Immunoblot analysis indicated that Kel2p exists in approximately five forms of differing electrophoretic mobility, whereas Kel1p appeared as a single species. We examined the Kel2p isoforms under a variety of situations but obtained no evidence regarding the functional significance of the various isoforms. The relative abundance of the different forms was altered by pheromone treatment with the lower bands becoming less abundant relative to the upper bands. The change was apparent within 30 min to 1 h of pheromone treatment (Fig. A3-1A). The pattern of Kel2p mobility appeared essentially identical in a *kel1Δ* strain, in which Kel2p is not localized, as compared to a wild-type strain (Fig. A3-1B).

The pheromone response pathway and the Pkc1p pathway are activated by pheromone treatment, so we examined the relevance of these pathways to the Kel2p modifications. The pattern of Kel2p mobility was essentially unaltered in *ste20*, *ste11*, *ste7*, *fus3*, and *far1* mutants, indicating that the various isoforms do not depend upon the pheromone response pathway (data not shown). In *ste12* mutants, slightly more of the uppermost form was apparent both in the presence or absence of pheromone (data not shown).

Figure A3-1. Immunoblot Analysis of Kel2p

A. The electophoretic mobility of Kel2p is altered by pheromone treatment. Strain IH3196 harboring plasmids encoding Kel2p-HA (pJP131) or untagged control (pJP123) was treated with α-factor for 0 to 60 min as indicated and analyzed by Western analysis as described in Materials and Methods. + indicates HA-tagged version; - indicates untagged version.

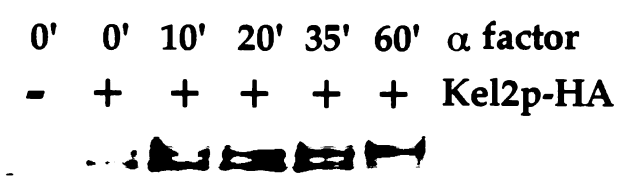
B. Electrophoretic mobility of Kel2p in *kel1Δ* strain.

kel1Δ (JP363) and wild-type (IH3196) strains containing plasmids encoding Kel2p-HA (pJP131) or untagged control (pJP123) were treated with α-factor for 1 hour (indicated +; those not treated with α-factor are indicated by -) and analyzed by Western analysis as described in Materials and Methods. + indicates HA-tagged version of Kel2p; - indicates untagged version.

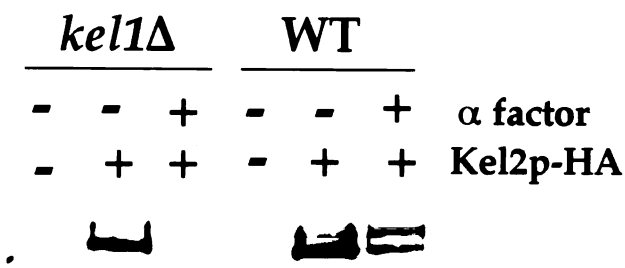
C. Electrophoretic mobility of Kel2p in *PKC1-R398P* strains.

Strains JP317 (PKC1-R398P-pRS306) or JP338 (pRS306) containing JP131 (Kel2p-HA) or JP123 (Kel2p-untagged) incubated in the absence of α-factor (indicated -) or presence of α-factor for 1 h (indicated +) were analyzed by Western analysis as described in Materials and Methods. Kel2p-HA tagged version is indicated by +; - indicates untagged version.

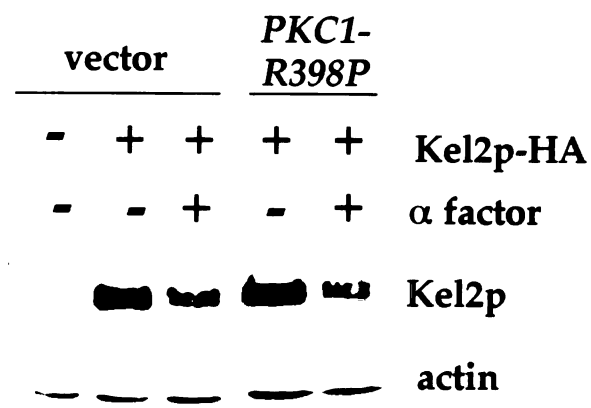
A


0' 0' 10' 20' 35' 60' α factor
- + + + + + Kel2p-HA

B


kel1Δ WT
- - + - - + α factor
- + + - + + Kel2p-HA

C


vector PKC1-R398P
- + + + + Kel2p-HA
- - + - + α factor
Kel2p
actin

When constitutively activated or kinase-inactivated versions of Pkc1p were expressed under control of the *GAL1,10* promoter (pDL242 and pJP163/pDL207, respectively), no detectable differences in Kel2p modifications were observed (data not shown). Similarly, in the presence or absence of pheromone, the electophoretic mobility was unaltered in strains containing *PKC1-R398P* compared to strains containing vector (Fig. A3-1C), suggesting that the Pkc1p pathway does not inhibit cell fusion by affecting Kel2p isoforms. To determine whether the Kel2p isoforms result from phosphorylation state differences, Kel2p was immunoprecipitated and treated with acid phosphatase or alkaline phosphatase. Phosphatase treatment did not alter the electrophoretic pattern of Kel2p. Thus, it is unclear what accounts for the distinct electrophoretic forms of Kel2p and the functional significance of the various forms.

Two-Hybrid Screen to Identify Kel2p-Interacting Proteins

A two-hybrid screen was performed to identify potential Kel2p-interacting proteins (designated Kip). Full-length Kel2p was fused to the lexA DNA-binding domain (Kel2-DBD) to generate pJP158. On its own, this fusion protein did not activate transcription from a reporter construct in which lexA operator sites lie upstream of the *lacZ* gene (pSH18-34; Gyuris et al., 1993). In the strain EGY48, in which lexA operator sites direct expression of the *LEU2* gene, pJP158 on its own did not confer leucine prototrophy. β-galactosidase activity indicated that full-length Kel1p fused to a transcriptional activation domain (Kel1-AD) interacted with Kel2-DBD (Fig. 3-5B), demonstrating that the Kel2-DBD construct was functional. Strain EGY48 harboring Kel2-DBD (pJP158) and the *lacZ* reporter construct (pSH18-34) was transformed with a pJG4-5-based library (S. Elledge) containing transcriptional activation domain fusions expressed under control of the *GAL1,10* promoter. Approximately

225,000-500,000 transformants were screened for interaction with Kel2-DBD as described (Golemis, et al. 1997). Transformants were initially selected for growth on galactose-containing medium lacking leucine and then examined for expression of *lacZ*. Twenty-eight plasmids (predicted to represent 5-10 independent transformants; see Golemis et al., 1997) conferred growth on leucine and *lacZ* expression in a galactose-dependent manner when retransformed. When co-expressed with a Bicoid-lexA DNA-binding domain fusion (pRFHM1; Gyuris et al., 1993), these plasmids did not activate transcription of *lacZ*, indicating that interaction with the Kel2-DBD fusion is specific. Restriction analysis indicated that the plasmids contained at least nine distinct inserts. Thirteen plasmids were sequenced, which identified ten predicted fusion proteins that are listed in Table A3-1. It is not suprising that Kel1p was not identified because expression of Kel1p under control of the *GAL1,10* promoter is toxic.

The relevance of the interactions between Kel2p and the Kip proteins is unclear. It would be useful to distinguish which Kips specifically interact with Kel2p and which also interact with Kel1p. It remains to be determined whether the Kips interact with the kelch or the coiled-coil domain of Kel2p. Of the Kel2p-interacting proteins, the only one with a known role in mating is Ste21p (Akada et al., 1996). Circumstantial evidence raises the possibility that the interaction between Ste21p and Kel2p is meaningful. First, Kel1p and Ste21p were identified in screens which also identified Ste20p and Ste5p, suggesting that there may be a functional relationship among these proteins. *ste21*, *ste20*, and *ste5* mutants were isolated because they exacerbate the mating defect of a *ste4*^{ts}(Gβ) mutant (Akada et al., 1996); overexpression of Kel1p, Kel2p, Ste20p, or Ste5p suppresses the mating defect caused by *PKC1-R398P*

Table A3-1

Kel2p-Interacting Proteins Identified by Two-Hybrid Analysis

Name	Sequence/Gene Name	Comments
Kip6/Kip12	ADH1 (*)	Alcohol dehydrogenase
Kip8	YKL105c	Hypothetical 125 kD protein; similarity to YMR086w
Kip11/Kip13	YNL107w	Similarity to human protein AF-9; similarity to yeast protein Anc1p/TFIIF
Kip15	Pan1p	Required for actin cytoskeletal organization and endocytosis
Kip16	Vma8p	Subunit of the vacuolar H^+-ATPase
Kip23	Ste21p	Mutants are defective in mating; required for *FKS2* expression
Kip29/Kip42	Mds3p	Negative regulator of sporulation
Kip31	Tap42p	Associates with Sit4p and PP2A phosphatase catalytic subunits
Kip33	Rpm2p	Mitochondrial RNaseP
Kip35	Vps30p	Required for sorting and delivery of hydrolases to the vacuole

* of genes in the *S. cerevisiae* database, insert was most homologous to the *ADH1* gene, but there were differences in the sequences.

(see Appendix 2). Ste21p may be important in calcineurin-dependent transcriptional induction of *FKS2* (Alepuz et al., in preparation), suggesting that it functions in the calcineurin pathway. One speculative model is that Kel1p, Kel2p, and Ste21p function in parallel to the Pkc1p pathway, maintaining cell integrity by modulating expression of genes involved in cell wall biosynthesis such as *FKS2*. One caveat to this possibility is that Ste21p is localized to the nucleus (L. Huang, personal communication) whereas Kel1p and Kel2p are localized to the cell periphery (Fig. 3-4). One would have to postulate that a small fraction of Ste21p or Kel1p/Kel2p interact *in vivo* or that their localizations change under certain conditions.

In certain strain backgrounds, loss of Kel2p results in a temperature sensitive growth defect (J. Carminati, personal communication), suggesting that Kel2p has roles outside of mating. One possibility, given the presence of kelch domains in Kel2p, is that Kel2p interacts with cytoskeletal components. Consistent with this idea, overexpression of Kel2p alters cell morphology. Potentially interesting in this regard is the identification of Pan1p as a Kel2p-interacting protein. Pan1p was initially thought to be a polyA-specific ribonuclease, but this turns out not to be the case. Pan1p is essential, required for endocytosis and organization of the actin cytoskeleton, and localizes to cortical actin patches (Tang et al., 1997; Tang and Cai, 1996; Wendland et al., 1996). It is composed of several interesting domains -- potential Ca^{2+} and PIP_2-binding domains, a proline-rich domain, a motif found in Sla1p, and two EH domains, also found in the mammalian Eps15 protein, a component of clathrin coated pits. Consistent with a role in endocytosis, Pan1p physically interacts with End3p (Tang et al., 1997), a protein containing an EH domain, also involved in actin organization and endocytosis (Benedetti, et al., 1994).

One possibility is that Pan1p and Kel2p function in concert to regulate the actin cytoskeleton and/or endocytosis.

The only fusion protein isolated independently on two distinct plasmids is that encoded by YNL107w. The closest yeast homolog to YNL107w is Anc1p, a transcription factor (TFIIF) important in cellular morphogenesis and actin cytoskeletal function (Welch and Drubin, 1994). If the homology between Anc1p and YNL107w is functionally meaningful, then YNL107w might be involved in transcriptional regulation and/or morphogenesis. However, as is the case with Ste21p, if YNL017w is a transcriptional regulator, presumably it would be nuclear, whereas Kel2p is largely cytoplasmic. One fantastical model is that Kel2p is involved in monitoring morphology at the cell periphery and communicating changes to transcriptional machinery such as Ste21p and YNL107w.

APPENDIX 4

YEAST STRAINS

A. The following strains are isogenic to IH3160 whose full genotype is: *HMLa HMRa met1 ade2-101 ura3-52.*

IH#	alias	Genotype/comments
IH3160	W T	*MAT***a** wild-type
IH3172	M1	*MAT***a**
IH3173	M2	*MAT***a**; mating defect complemented by p*AXL1*
IH3174	M3	*MAT***a**
IH3175	M4	*MAT***a**; mating defect complemented by p*AXL1*
IH3176	M5	*MAT***a**
IH3177	M6	*MAT***a**
IH3178	M7	*MAT***a**; mating defect complemented by p*AXL1*
IH3179	M8	*MAT***a** *fps1-1*
IH3180	M9	*MAT***a**; mating defect suppressed by p2µ*STE6*
IH3181	M10	*MAT***a**; mating defect suppressed by p2µ*STE6*
IH3182	M11	*MAT***a** *fps1-2*
IH3183	M12	*MAT***a**
IH3184	M13	*MAT***a**

IH#	Genotype
IH3186	*MAT*α
IH3190	*MAT*α *trp1*Δ*99*
IH3194	*MAT*α *trp1*Δ*99 leu2*Δ*1*

IH3196		*MAT**a** leu2Δ1*
IH3197		*MATα leu2Δ1*
JP#	**IH#**	**Genotype**
JP52	IH3399	*MAT**a** fus1Δ::TRP1 trp1Δ99* (Note: JP52 was published as *leu2Δ1*, but, in fact, it is *LEU2*)
JP54	IH3505	*MAT**a**/MATα*
JP147	IH3400	*MAT**a** fps1Δ::URA3 leu2Δ1*
JP150	IH3401	*MAT**a** FPS1::FPS1-URA3*
JP153	IH3402	*MAT**a** ura3-52::URA3*
JP154	IH3403	*MAT**a** fps1-1::FPS1-URA3*
JP157	IH3404	*MAT**a** fps1-1 ura3-52::URA3*
JP158	IH3405	*MAT**a** fps1-2::FPS1-URA3*
JP161	IH3406	*MAT**a** fps1-2 ura3-52::URA3*
JP163	IH3407	*MAT**a** fps1Δ::LEU2 leu2Δ1*
JP165	IH3408	*MAT**a** fps1Δ::URA3 gpd1Δ::TRP1 leu2Δ1 trp1Δ99*
JP168	IH3409	*MAT**a** gpd1Δ::TRP1 leu2Δ1 trp1Δ99*
JP226	IH3410	*MATα fps1Δ::URA3 trp1Δ99*
JP233	IH3411	*MATα fps1Δ::URA3 gpd1Δ::TRP1 trp1Δ99*
JP236	IH3412	*MATα gpd1Δ::TRP1 trp1Δ99*
JP257	IH3413	*MATα fus2Δ::URA3 trp1Δ99*
JP285	IH3414	*MAT**a** fus1Δ::TRP1 gpd1Δ::TRP1 trp1Δ99*
JP287	IH3415	*MATα fus2Δ::URA3 gpd1Δ::TRP1 trp1Δ99*
JP291	IH3506	*MAT**a** mpk1::TRP1 leu2Δ1 trp1Δ99*
JP317	IH3416	*MAT**a** PKC1::PKC1-R398P-URA3 leu2Δ1*
JP334	IH3507	*MAT**a** FUS1::lacZ-URA3 leu2Δ1 trp1Δ99*
JP335	IH3508	*MAT**a** FUS1::lacZ-LEU2 leu2Δ1 trp1Δ99*
JP338	IH3509	*MAT**a** ura3::URA3 leu2Δ1*

JP358 IH3510 *MATa kel1Δ::hisG-URA3-hisG leu2Δ1*

JP363 IH3511 *MATa kel1Δ::hisG leu2Δ1*

JP370 IH3512 *MATa kel2Δ::LEU2 leu2Δ1*

JP371 IH3513 *MATa kel2Δ::LEU2 leu2Δ1*

JP385 IH3514 *MATa kel1Δ::hisG kel2Δ::LEU2 leu2Δ1*

JP410 IH3515 *MATa kel1Δ::hisG-URA3-hisG fus1Δ::TRP1 trp1Δ99*

JP412 IH3516 *MATa kel2Δ::hisG-URA3-hisG fus1Δ::TRP1 trp1Δ99*

JP423 IH3517 *MATα fus1Δ::TRP1 leu2Δ1 trp1Δ99*

JP424 IH3518 *MATα kel1Δ::hisG-URA3-hisG leu2Δ1 trp1Δ99*

JP432 IH3519 *MATa kel1Δ::hisG ura3::URA3 leu2Δ1*

JP439 IH3520 *MATa kel1Δ::hisG-URA3-hisG leu2Δ1 trp1Δ99*

JP440 IH3521 *MATα kel1Δ::hisG-URA3-hisG fus1Δ::TRP1 leu2Δ1 trp1Δ99*

Strains containing plasmids:

JP199 IH3196 containing YEp*GPD1*

JP200 JP163 containing YEp*GPD1*

JP300 IH3196 containing pJP67

JP301 IH3196 containing YCp50

JP333 IH3194 containing pJW192

JP400 IH3196 containing YCplac195

JP401 JP163 containing YCplac195

B. The following strains are in the EG123 background whose full genotype is: *trp1 ura3 his4 leu2 can1*

IH#	**Genotype**
IH1783	*MAT***a**
IH3077	*MATa mpk1Δ::TRP1*

JP#	IH#	
JP9	IH3522	*matΔ::URA3*
JP10	IH3523	*MAT**a** fus2::URA3*
JP11	IH3524	*MATα fus2::URA3*
JP145	IH3525	*MAT**a** fps1::LEU2*
JP146	IH3526	*MATα fps1::LEU2*
JP325	IH3417	*MAT**a** fps1Δ::LEU2*
JP326	IH3418	*MAT**a** mpk1Δ::TRP1*
JP327	IH3419	*MATα fps1Δ::LEU2 mpk1Δ::TRP1*
JP328	IH3420	*MATα*
JP490	IH3527	*MAT**a** mpk1Δ::TRP1 kel1Δ::hisG-URA3-hisG*
JP491	IH3528	*MATα mpk1Δ::TRP1 kel2Δ::hisG-URA3-hisG*
JP444	IH3529	*MAT**a** kel1Δ::hisG-URA3-hisG*
JP445A	IH3530	*MATα kel1Δ::hisG-URA3-hisG*
JP446A	IH3531	*MATα kel2Δ::hisG-URA3-hisG*

C. The following strains are isogenic:

IH#	Genotype
IH2350	*MATα ura3-52 his4-34 trp1Δ1*
IH2351	*MATα ura3-52 trp1Δ1 fus1Δ1 fus2Δ3*
IH2353	*MAT**a** ura3-52 trp1Δ1 fus1Δ1 fus2Δ3*

APPENDIX 5

PLASMIDS

A. Plasmids generated and used in this study

Additional information regarding the construction of many of these plasmids can be found in the CY collection.

JP#	CY#	Description
pJP2	CY667	*fus1Δ::TRP1*; for description, see Materials and Methods, Chapter 2.
pJP4.1		contains *YRB1*, YDR003w, *RAD57*, *MAF1*, *SOK1*, and *TRP1*
pJP5.1		contains *TRP1*, YDR008c, *GAL3*, YDR010c and part of *SNQ2*
pJP14	CY790	*FPS1*-YEp24; original *FPS1* isolate from Carlson library
pJP17	CY791	*FPS1*-pRS316; SalI-EcoRI fragment containing *FPS1* from pJP14 cloned into SalI-EcoRI sites of pRS316

pJP18-pJP21, pJP23, pJP25, pJP27, and pJP28 are pRS316-derived plasmids containing fragments from pJP17 that were used to determine that *FPS1* complements the mating defect of M8 and M11; see Fig. 2-2 for maps.

JP#	CY#	JP#	CY#
pJP18	CY792	pJP23	CY796
pJP19	CY793	pJP25	CY797
pJP20	CY794	pJP27	CY798
pJP21	CY795	pJP28	CY799

JP#	CY#	Description
pJP30	CY669	*FPS1*-pRS306; BglII-HindIII fragment containing *FPS1* cloned into BamHI-HindIII sites of pRS306
pJP31	CY670	*fps1Δ::LEU2*; for description, see Chapter 2, Materials and Methods and Fig. 2-2.
pJP52	CY671	*fps1Δ::URA3*; for description, see Chapter 2, Materials and Methods and Fig. 2-2.
pJP72	CY677	*PKC1-R398P*-pRS306; SphI fragment from YCpDS1 (pJP67/CY676) blunt cloned into BamHI and SalI sites of pRS306.

The following YEp13-derived plasmids (Nasmyth library) were isolated as high copy suppressors of the mating defect caused by *PKC1-R398P*.

JP#/Designation	CY#	Description
pJP73/pPHS5	CY813	contains *STE20*. pJP73 and pJP74 were isolated from the same yeast colony but are differently rearranged plasmids containing *STE20*.
pJP74/pPHS6	CY814	contains *STE20*.
pJP75/pPHS11	CY815	contains *KEL1*; for a description, see Fig. 3-1.
pJP76/pPHS12	CY816	contains *STE5*.
pJP77/pPHS18	CY817	contains two complete ORFs, YJR124c (homology to metabolite transporters) and *RPS5* (a ribosomal protein), and part of two unknown ORFs, YJR125c and YJR122w.

pJP81- pJP84 are pYEp351-derived plasmids containing fragments from pJP75 that were used to determine that *KEL1* suppresses *PKC1-R398P*; see Chapter 3, Materials and Methods for description; see Fig. 3-1 for maps.

JP#	CY#
pJP81	CY818
pJP82	CY819
pJP83	CY820
pJP84	CY821

JP#	CY#	Description
pJP92	CY823	*KEL2*-YEp351
pJP94	CY825	*kel1Δ::hisG-URA3-hisG*
pJP113	CY832	*kel2Δ::LEU2*
pJP123	CY836	*KEL2*-YEp351
pJP126	CY837	*KEL2-GFP*-YEp351
pJP127	CY838	*KEL1*-YEp351
pJP129	CY840	*KEL1-GFP*-YEp351
pJP131	CY841	*KEL2-HA*-YEp351
pJP138	CY842	*kel2Δ::hisG-URA3-hisG*
pJP139	CY843	*KEL1-GFP*-YIp5
pJP143	CY847	*KEL1*-YIp5
pJP158	CY852	pEG202-*KEL2-DBD*
pJP160	CY853	pRS426-*GAL KEL2*
pJP167	CY857	pJG4-5-*KEL1-AD*
pJP202	CY859	*KEL1-HA*-YEp351
pJP207	CY860	*KEL1*-YCplac111
pJP209	CY861	*KEL1-HA*-YCplac111

The following pJG4-5-derived plasmids (Elledge library) contain sequence encoding a transcriptional activation domain fused to the indicated ORF (see Appendix 3).

Name	CY#	Fusion
pKip6	CY862	*ADH1* ? (of the genes in the *S. cerevesiae* database, *KIP6* was most homologous to *ADH1*, but there were substantial differences)
pKip8	CY863	YKL105c
pKip11	CY864	YNL107w; pKip11 and pKip13 contain the same fusion junction but pKip11 has a larger insert.
pKip13	CY865	YNL107w
pKip15	CY866	*PAN1*
pKip16	CY867	*VMA8*
pKip23	CY868	*STE21*
pKip29	CY869	*MDS3*
pKip31	CY870	*TAP42*
pKip33	CY871	*RPM2*
pKip35	CY872	*VPS30*

B. Plasmids used in this study, generated elsewhere

Name	CY#	Description	Source
pAXL1	CY655	p*AXL1*	IH collection
pBC7		plasmid containing *PTC1*	B. Cree
pBC8		plasmid containing *PTC1*	B. Cree
pBC14		plasmid containing *PTC1*	B. Cree
pDL242	CY675	p*GAL1*[*PKC1-R398A*] (pBM743)	D. Levin

pDL293	CY546	p*GAL1*[*PKC1::HA*] (pBM743)	D. Levin
pDL295	CY550	p*GAL1*[*PKC1-K853R::HA*] (pBM743)	D. Levin
p*GAL STE20*	CY711	p*GAL STE20* (pRD56)	M. Peter
p*GAL STE20-R649*	CY713	p*GAL STE20-R649* (pRD56)	M. Peter
pJP67/YCp50-DS1	CY676	*PKC1-R398P* (YCp50)	Y. Takai
pJW192	CY681	*RAS2-GFP*	J. Whistler
pKOFUS2	CY389	*fus2Δ::URA3*	IH collection
pRFHM1		Bicoid-DBD	Gyuris (1993)
pSH18-34		*LexAop-LacZ*	Gyuris (1993)
pSNQ2		*SNQ2* (YCp50)	B. Wells
2μ STE6	CY248	2μ *STE6* (YEp24)	IH collection
pUC*gpd1Δ::TRP1*	CY678	*gpd1Δ::TRP1*	S. Hohmann
STE5-hyp1	CY441	*STE5-hyp1* (YCp50)	IH collection
STE5-hyp2	CY442	*STE5-hyp 2* (YCp50)	IH collection
YEp*GPD1*	CY679	2μ *GPD1* (YEplac195)	S. Hohmann

YCp50

pRS306

YCplac111

YEp351

YIp5

pJG4-5

pRS426

YCplac195

pRS306, pRS426, YEp351, and YIp5 are described in Guthrie and Fink (1991). pEG202 and pJG4-5 are described in Gyuris et al. (1993). YCplac111 and YCplac195 are described in Gietz and Sugino (1988).

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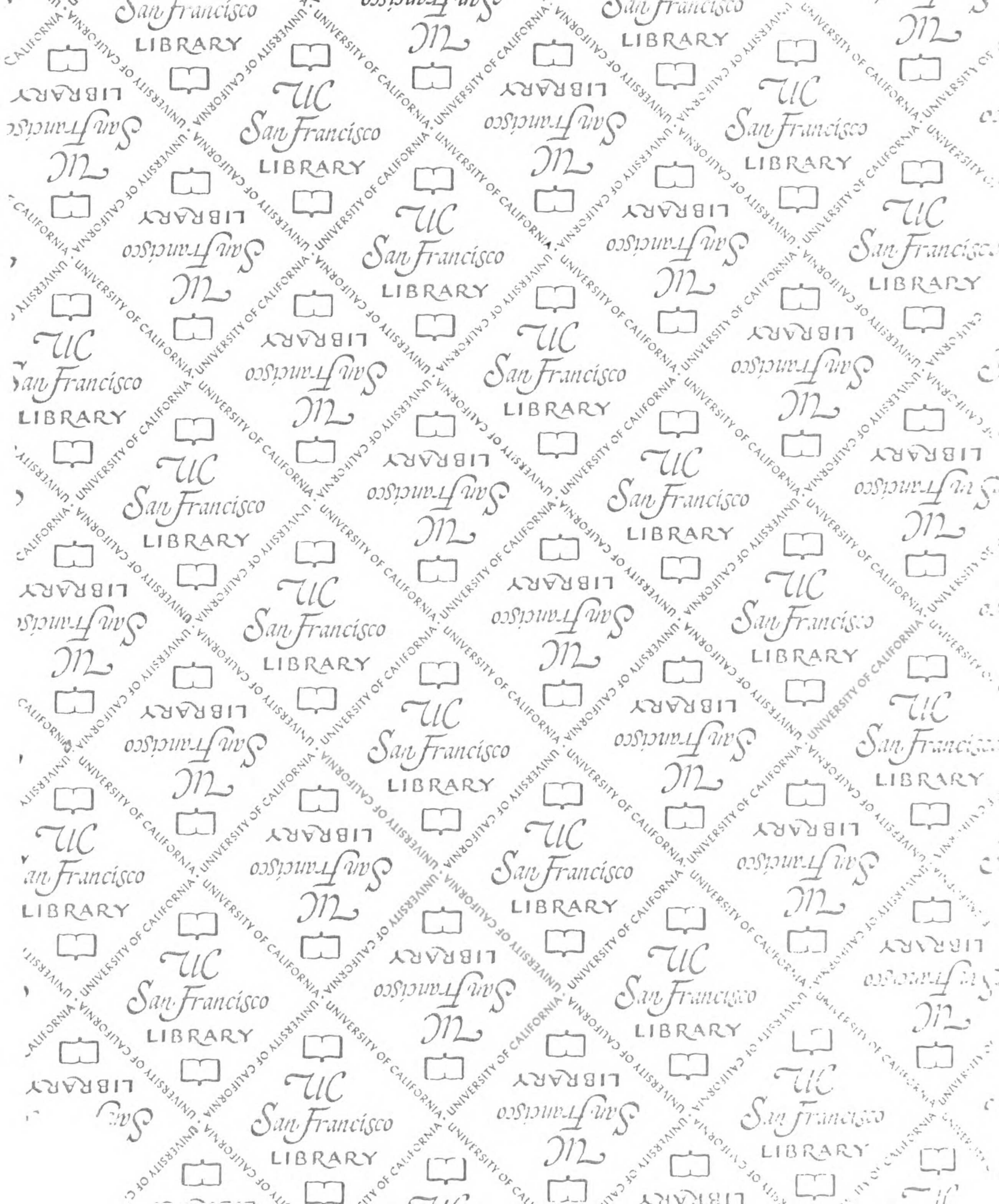
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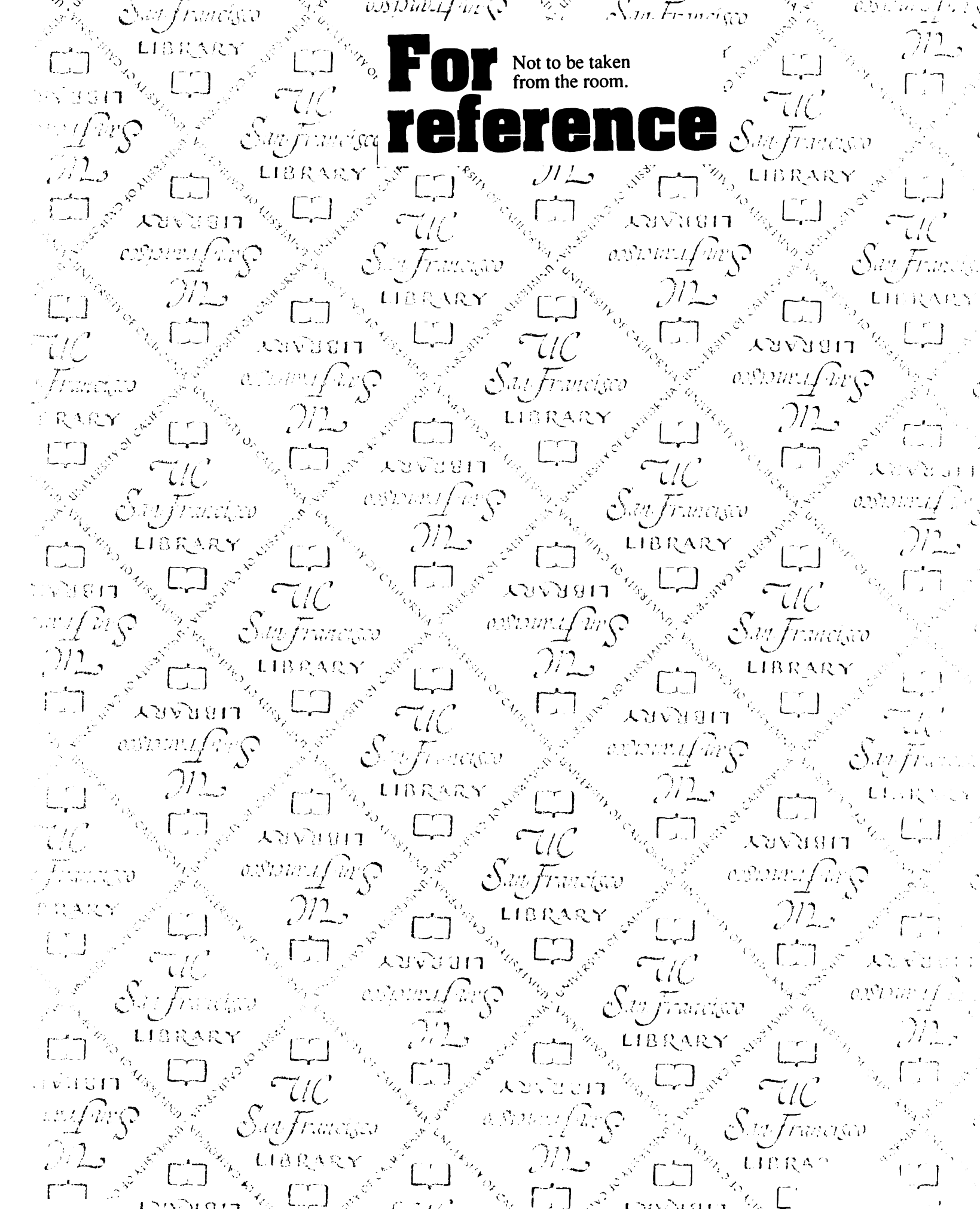
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