ANALYSIS OF NEW GENES INVOLVED IN YEAST MATING AND CELL POLARITY

by

Neil R. Adames

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Name: Neil Robert Adames

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Analysis of New Genes Involved in Yeast Mating and Cell Polarity.

Examining Committee:

Chair: Dr. B. Brandhorst, Professor

Dr. C. Boone, Assistant Professor, Senior Supervisor
Department of Biological Sciences, SFU

Dr. J. Price, Assistant Professor
Department of Biological Sciences, SFU

Dr. R. Cornell, Associate Professor
Department of Chemistry, SFU

Dr. T. Borgford, Associate Professor
Department of Chemistry, SFU

Dr. I. Sadowski, Associate Professor
Department of Biochemistry and Molecular Biology, UBC
Public Examiner

Dr. M. Whiteway, Research Officer
NRC Biotechnology Research Institute, Montreal, Quebec
External Examiner

Date Approved: November 17, 1997

ABSTRACT

Saccharomyces cerevisiae conjugation is a complex process initiated by signaling peptides secreted by haploid cells. These peptide pheromones orchestrate a series of physiological changes that ultimately allow the haploid partners to fuse and form a diploid zygote. A surprisingly large number of the proteins mediating these processes are conserved in higher eukaryotes and have similar functions in other organisms in which they are found. Hence, the study of yeast mating has some relevance to general questions in biology.

My goal was to further characterize the yeast mating reaction at the molecular level by finding new genes involved in this process and assigning functions to the products of these genes. The first step was to mutagenize wild-type yeast cells and to search for mutants that could no longer mate or mated poorly. Interesting mutants were then cloned by complementation of the mating defect with a yeast genomic library. I found one gene, *BNI1*, previously not known to have a role in mating and two new genes, *AXL1* and *SFU1*.

Axl1p is a member of the pitrilysin family of proteases, which include a set of closely related peptidases that cleave oligopeptides such as insulin. We found that Axl1p and another yeast homologue, Ste23p, are required for processing of the peptide pheromone **a**-factor. Furthermore, Axl1p is involved in bud site selection, but its proteolytic activity is not required for this function.

Bni1p has conserved regions characteristic of members of the formin family of proteins. These proteins are implicated in vertebrate limb formation, polarity establishment and cytokinesis. Bni1p is involved in polarizing the actin cytoskeleton during cell growth. It is one of a few proteins shown to be an effector for the highly conserved small GTPases of the rho subfamily which regulate actin structures.

Finally, Sfu1p is a novel protein that appears to be important for signaling, pheromone-induced polarized morphogenesis and cell fusion.

DEDICATION

This tome is dedicated to my family, and especially to my wife Kelly.

I couldn't have done it without you.

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I would like to thank the members of my lab, Pradeep Nair, Steve Ritchie, and particularly Kelly Blundell, for offering help whenever I needed it. Kelly is responsible for cloning *AXL1* and made many of the strains and plasmids used in my research. I also thank the many undergraduates who helped out in the lab over the last four years -- especially Lisa Scouras for her persistent attempts in obtaining an elusive mutant, and Grant Poje for his help with *BNI1* and who cloned *SFU1* and constructed most of the strains and plasmids used to study this gene. Marie Evangelista and Kelly Blundell performed most of the work on *BNI1* that I cite in the Discussion of Chapter 6. I would also like to thank Matt Moreau for his amusement value and Sean (Red Robin) Kelly for his excellent coffee and food while I was writing this thesis. Finally, I thank Charlie Boone for construction of strains and *AXL1* substitution mutant plasmids, the knowledge he has passed on to me and for his guidance. I have learned a great deal of science in his lab..

Most of the work on *AXL1* (Adames et al., 1995) and *BNI1* (Evangelista et al, 1997) has been published.

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ABC-transporter	ATP binding cassette transporter
AIDS	acquired immune deficiency syndrome
ANP	atrial natriuretic peptide
ATP	adenosine triphosphate
ATZ	aminotriazole
CDK	cyclin dependent kinase
CFTR	cystic fibrosis transmembrane conductance regulator
CKI	cyclin dependent kinase inhibitor
CPE	chloroplast processing enzyme
DIC	differential interference contrast
EDTA	ethylenediaminetetraacetic acid
EM	electron microscopy
ER	endoplsamic reticulum
FGF	fibroblast growth factor
FH1 and FH2	formin homology domains 1 and 2
FOA	5-fluoroorotic acid
FTase	farnesyltransferase
G-protein	guanine nucleotide binding protein
GAP	GTPase activating protein
GDI	GDP dissociation inhibitor
GDP	guanosine diphosphate
GEF	guanine nucleotide exchange factor
GGTase	geranylgeranyl transferase
GPI	glycosylphosphatidylinositol
GTP	guanosine triphosphate

HA	hemeagglutinin
hIDE	human insulin-degrading enzyme
HRP	horse radish peroxidase
IDE	insulin degrading enzynme
IL	interleukin
JNK	c-jun N-terminal kinase
MAP kinase	mitogen activated protein kinase
MDR	multidrug resistance protein
MIP	mitochondrial intermediate peptidase
MPP	mitochondrial processing peptidase
N-WASP	neural Wiskott-Aldrich syndrome protein
NPD	nonparental ditype
NRDC	N-arginine dibasic convertase
ORF	open reading frame
PCR	polymerase chain reaction
PAK	p21-activated protein kinase
PD	parental ditype
PEG	polyethyleneglycol
PMSF	phenylmethylsulfonate
PRE	pheromone response element
PSA	pheromone spot assay
ptr	*E. coli* pitrilysin gene
RGS	regulator of G-protein signaling
Rho	ras homologue
SDS	sodium dodecylsulfate
SDS-PAGE	SDS polyacrylamide gel electrophoresis
SPB	spindle pole body

T ..... tetratypes

TAPs ..... transporters involved in antigen processing

TCA ..... trichloroacetic acid

WAS ..... Wiskott-Aldrich syndrome

WASP ..... Wiskott-Aldrich syndrome protein

GLOSSARY OF YEAST GENES

Gene Name(s)	Abbreviation of	Function of Product
ACT1	ACTin	the sole yeast actin gene
ARS	Autonomously Replicating Sequence	yeast origin of replication
AXL1/STE22	AXiaL/STErile	pitrilysin-like protease required for haploid axial budding and for **a**-factor processing
AXL2/BUD10	AXiaL BUD site selection	required for haploid axial budding
BAR1/SST1	BArrier/Super-Sensitive To pheromone	secreted α-factor-degrading peptidase
BEE1/LAS17	Lethal in the Absence of SSD1	component of cortical actin patches
BEM1	Bud EMergence	scaffold protein required for polarized cell growth
BEM3	Bud EMergence	GAP for Cdc42p rho-like GTPase required for polarized growth
BNI1	Bud Neck Interacting	formin homologue required for cytokinesis and cell polarity
BNR1	BNi1 Related	formin involved in cell polarity
BUD1/RSR1	BUD site selection/RaS-Related	Ras-like GTPase required for general bud site selection
BUD2	BUD site selection	GAP for BUD1/RSR1, required for general bud site selection
BUD3	BUD site selection	required for axial bud site selection
BUD4	BUD site selection	required for axial bud site selection
BUD5	BUD site selection	GEF for BUD1/RSR1, required for general bud site selection
BUD6/AIP3	BUD site selection/Actin Interacting Protein	required for bipolar bud site selection
BUD7	BUD site selection	required for bipolar bud site selection
CDC3, 10, 11, 12	Cell Division Cycle	septins, form the bud neck filaments

		required for cytokinesis
CDC24	Cell Division Cycle	GEF for CDC42p, required for polarized growth
CDC42	Cell Division Cycle	rho-like GTPase required for polarized cell growth
CEN	CENtromere	minimal centromeric sequence for chromosome segregation
CHS1	CHitin Synthase	enzyme responsible for chitin synthesis during cell separation
CHS2	CHitin Synthase	enzyme responsible for chitin synthesis in primary septum
CHS3	CHitin Synthase	enzyme responsible for general chitin synthesis and chitin in bud scars
CHS5	CHitin Synthase	protein regulating CHS3 secretion
CTS1	ChiTinaSe	enzyme that degrades chitin
FAR1	Factor ARrest	CKI that inhibits Cln-CDC28 in response to pheromone
FUS1	FUSion	type I transmembrane protein required for cell fusion during mating
FUS2	FUSion	required for cell fusion during mating, shows some homology to FUS1
FUS3	FUSion	MAP kinase required for pheromone signaling, partly redundant with KSS1
GPA1/SCG1	G-Protein Alpha/Suppressor of Constitutive G-protein	G-protein α subunit that interacts with STE4 and STE18
HO	HOmothallism	endonuclease required for mating type switching in homothallic strains
KEX1	Killer toxin EXocytosis	carboxypeptidase involved in killer toxin and α-factor processing
KEX2	Killer toxin EXocytosis	serine protease involved in killer toxin and α-factor processing
KSS1	Kinase Suppressor of SST2	MAP kinase required for pheromone signaling, partly redundant with FUS3
MFA1, 2	Mating Factor **a**	structural genes for **a**-factor pheromones

MFα1, 2	Mating Factor α	structural genes for α-factor pheromones
MYO1	MYOsin	class II myosin, required for secretion and polarized growth
MYO3, 5	MYOsin	partly redundant class I myosins, required for endocytosis and polarized growth
PEA2	PEAnut	required for shmoo growth and bipolar budding, shmoos are peanut-shaped
PFY1	ProFilin Yeast	profilin, binds actin monomers and proline-rich sequences
RAM1/DPR1	RAs Modifier/Defective Pheromone and Ras	FTase β subunit, farnesylates Ras and **a**-factor
RAM2	RAs Modifier	FTase α subunit, farnesylates Ras and **a**-factor
RAX1	Repressor of AXI1 mutant	negative regulator of axial budding
RCE1	Ras Converting Enzyme	CaaX-box protease, modifies Ras and **a**-factor
RGA1	Repressor of G Alpha mutant	GAP for CDC42, has axial budding and pheromone signaling defect
RVS161, 167	Reduced Viability upon Starvation	homologues involved in endocytosis and bipolar budding
SFU1	Shmoo and FUsion defects	required for polarized cell growth and cell fusion during mating
SPA2/PEA1	Spindle Pole Antigen/ PEAnut	required for polarized cell growth, cell fusion and bipolar budding
SST2	Super-Sensitive To mating factor	GAP for Gα subunit, down-regulates pheromone signaling
STE2	STErile	α-factor receptor
STE3	STErile	**a**-factor receptor
STE4	STErile	G-protein β subunit involved in pheromone signaling
STE5	STErile	scaffold protein involved in pheromone signaling
STE6	STErile	ABC-transporter responsible for **a**-factor secretion

STE7	STErile	MAPKK kinase involved in pheromone signaling
STE11	STErile	MAPK kinase involved in pheromone signaling
STE12	STErile	transcription factor involved in pheromone signaling
STE13	STErile	dipeptidyl aminopeptidase involved in killer toxin and α-factor processing
STE14	STErile	carboxylmethyltransferase that modifies **a**-factor
STE18	STErile	G-protein γ subunit involved in pheromone signaling
STE20	STErile	kinase involved in pheromone signaling
STE23	STErile	pitrilysin-like protease involved in **a**-factor processing, Axl1p homologue
STE24	STErile	zinc metalloprotease required for **a**-factor amino-terminal processing
TPM1	TroPoMyosin	actin filament stabilizing protein

INTRODUCTION

Actin' Up During Development

Development and the Cytoskeleton

Defining the process whereby a zygote develops into a multicellular organism containing a multitude of specialized tissues is one of the most compelling problems facing biologists. During development cells are able to change basic properties such as their morphology, physical characteristics and function. How these changes are made and how they are regulated are fundamental questions of biology. Development in metazoans is largely a genetically determined program with rare (but crucial) epigenetic influences. In simple terms, cell differentiation (specialization) is considered to be the result of expressing certain genes needed for a cell's function while preventing the expression of genes that are not required for, or would interfere with, this function. With a few exceptions, differentiation does not entail the selective loss of genes or rearrangement of genes in the differentiating cell. In fact, almost all cells within an adult organism contain all of the genetic information needed for a single cell to develop into a nearly identical adult. This potential for pluripotency has been powerfully demonstrated by the cloning of an adult sheep from a single cell extracted from an adult progenitor (Wilmut et al., 1997).

Although developmental processes are largely regarded as the differential expression of genes leading to specific cell types, there is increasing evidence for the importance of differentially regulating ubiquitous or pre-existing proteins. This is especially true in the case of the cytoskeleton. Many differentiating cells require a specific morphology in order to function. For example, epithelial cells must form tight sheets, often consisting of only one or a few layers that are each one cell thick. These sheets require tight interactions among cells and between cells and the extracellular matrix they secrete. Cytoskeletal components form and regulate these interactions (Sastry and Horwitz, 1993; Mays et al., 1994; Schmidt et al., 1994). Furthermore, the cytoskeleton is involved in cell migration during development - a process that also requires

interactions between cells and the extracellular matrix (Sheetz, 1994; Stossel, 1994). The proper regulation of cytoskeletal proteins is also very important in muscular and neuronal development (Doering, 1993; Ervasti and Campbell, 1993; Trifaro and Vitale, 1993).

Polarity of the cytoskeleton is necessary in many cells that secrete or absorb substances, especially in a regulated manner. In such cells cytoskeletal components are often crucial in the intracellular trafficking of organelles, such as secretory vesicles (Mays et al., 1994; Morris and Frizzell, 1994). Moreover, the cytoskeleton must be polarized to ensure that the materials are secreted or absorbed at the correct site on the surface of the cell (Fath et al., 1993; Mays et al., 1994; Morris and Frizzell, 1994). Cytoskeletal polarity is not only important during morphogenesis and secretion, but also during the earliest stages of development. There are many examples of developmental proteins or their transcripts that are polarized within zygotes or even within the egg prior to fertilization (Bowerman et al., 1993; Klymkowsky and Karnovsky, 1994; Knowles and Cooley, 1994; Erdelyi et al., 1995; Etemad-Moghadam et al., 1995; Guo and Kemphues, 1995; Boyd et al., 1996; Guo and Kemphues, 1996; Watts et al., 1996). Evidence for the role of the cytoskeleton in establishing this polarity is rapidly accumulating (Klymkowsky and Karnovsky, 1994; Knowles and Cooley, 1994; Manseau et al., 1996; Watts et al., 1996). During mitosis developmental determinants are sequestered in one of the daughter cells which then follows a specific developmental pathway, distinct from that followed by the mother cell that received none of the determinant (Way et al., 1994; Guo and Kemphues, 1996; Morgan and Mahowald, 1996).

The developmental programs of metazoan organisms are largely determined through cell-cell or cell-extracellular matrix interactions. Similarly, external conditions are the main influence on developmental programs in unicellular organisms such as the budding yeast *Saccharomyces cerevisiae*. In both systems development is influenced and fine-tuned by factors outside of the cell. The difference lies in the source of the cells' surroundings. While yeast cells are subject to the vagaries of their environment, the environment of most metazoan cells is influenced by

surrounding cells all of which arose from a single egg and, therefore, the environment is genetically determined.

S. cerevisiae Cell-Types

S. cerevisiae exist as two mating types called **a** cells and α cells. The mating type of a strain is determined by a single locus, *MAT*, so that **a** cells are *MAT***a** and α cells are *MAT*α. The *MAT* locus encodes transcriptional regulators that determine the cell type through induction and repression of certain genes (for example, the genes coding for the the mating pheromones **a**-factor and α-factor; Rine et al., 1992). Diploid cells are *MAT***a**/*MAT*α. Coexpression of these loci prevent the expression of haploid-specific genes and the **a**- and α-specific genes, and promotes transcription of genes required in diploids (such as those for sporulation; Rine et al., 1992).

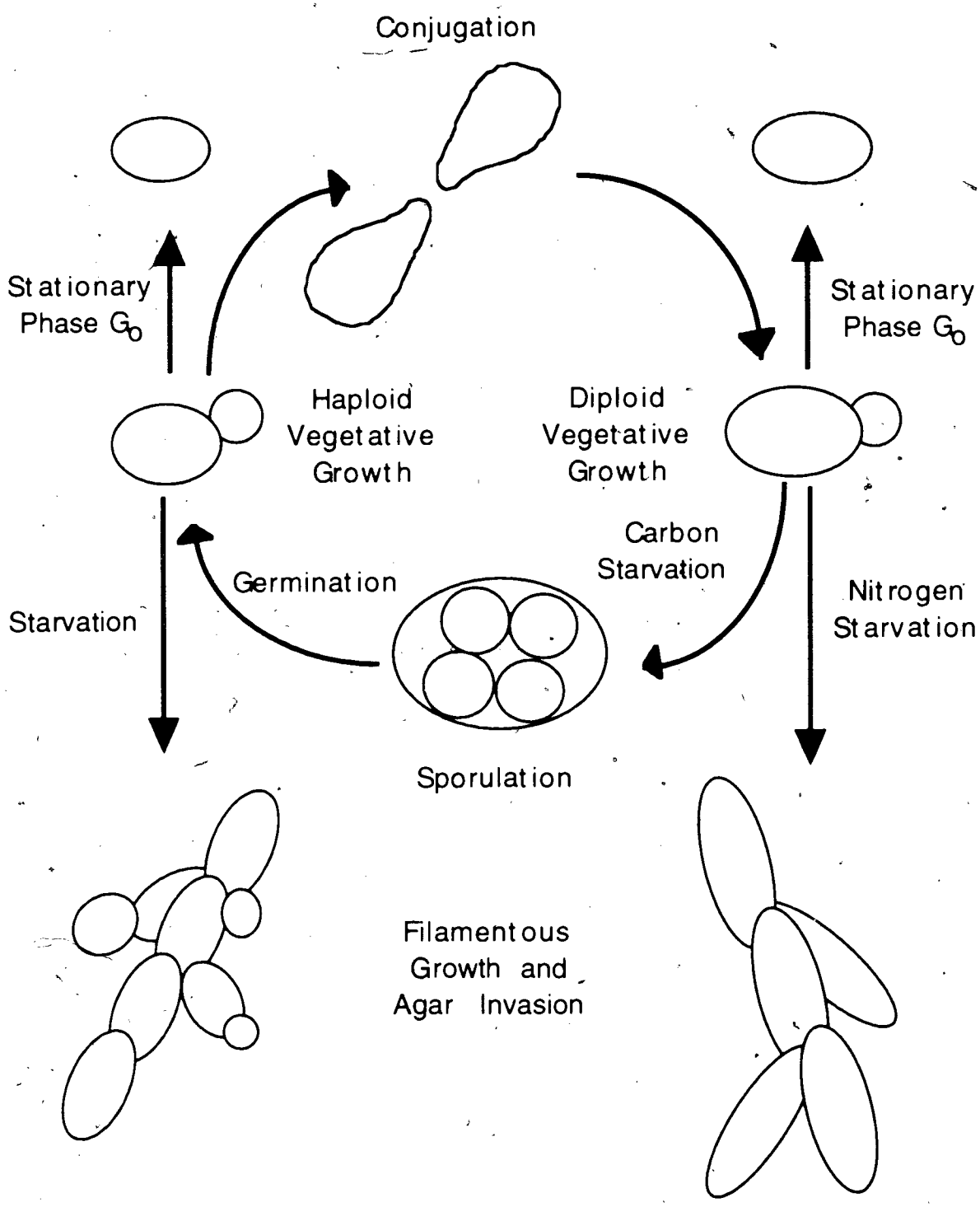
Development in the Budding Yeast

Vegetative Growth

S. cerevisiae is a budding yeast. In order to form a bud the cells must polarize the machinery for plasma membrane and cell wall synthesis so that growth is restricted to the daughter cell bud. This is accomplished by polarizing the actin cytoskeleton. Delivery of the biosynthetic enzymes or products necessary for growth through secretory vesicles is dependent on the actin cytoskeleton (Chant and Pringle, 1991; Drubin, 1991; Bretscher et al., 1994; Mills and Mandel, 1994; Mills et al., 1994; Cid et al., 1995; Govindan and Novick, 1995; Lew and Reed, 1995; Drubin and Nelson, 1996). A great deal of research has focused on how the actin cytoskeleton is regulated and polarized to buds, and how the actin cytoskeleton interacts with secretory mechanisms. Many of the proteins involved in budding have homologues in higher organisms.

Figure 1. Life Cycle of *Saccharomyces cerevisiae*.

Haploid cells divide by budding and can enter a developmental program called haploid invasive growth if starved of nutrients. These cells become slightly elongated and bud only at their ends, in what is called the bipolar budding pattern. In the presence of a cell of the opposite mating type haploid cells grow toward each other, fuse and form a diploid zygote. Diploids also divide by budding and can undergo pseudohyphal growth in the absence of nitrogen and fermentable carbon. These cells bud from only one end (unipolar budding) and become very elongated. In haploid invasive growth and pseudohyphal growth the cells are able to penetrate the agar. These responses to starvation are thought to be nutrient foraging strategies. Diploids sporulate when they are starved for fermentable carbon alone. Sporulation is a specialized developmental pathway in which meiosis is accompanied by packaging of the mitotic products into sac-like asci. Both haploids and diploids are able to enter stationary phase (G_0).


Conjugation
Stationary Phase G_0
Stationary Phase G_0
Haploid Vegetative Growth
Diploid Vegetative Growth
Carbon Starvation
Germination
Starvation
Nitrogen Starvation
Sporulation
Filamentous Growth and Agar Invasion

Mating

Yeast cells can enter several different developmental programs (Fig. 1). One such program is conjugation. Haploid cells of yeast can mate and form diploid cells. Haploids secrete peptide pheromones that cells of the opposite mating type can detect through receptors. *MAT***a** cells secrete **a**-factor and detect α-factor through an α-factor receptor (Sprague and Thorner, 1992). Likewise, *MAT*α cells secrete α-factor and bind **a**-factor via an **a**-factor receptor (Sprague and Thorner, 1992). The genes encoding the pheromones and their receptors are **a** cell- or α cell-specific (Sprague and Thorner, 1992). When cells detect pheromone they arrest in the G_1-phase of the cell cycle before DNA replication commences (Chang and Herskowitz, 1990). This ensures that once mating is accomplished the product has a 2N content of DNA. Cells then polarize growth to the region at which the pheromone concentration is greatest - in the likely direction of the mating partner (Segall, 1993; Dorer et al., 1995; Valtz et al., 1995). This growth results in the formation of a mating projection, otherwise known as a "shmoo" after the Al Capp cartoon figure. These projections enable the mating cells to contact each other if they are not already doing so. Once contact is established, the cell walls and then the cell membranes fuse creating a cytoplasmic bridge between the mating partners (McCaffrey et al., 1987; Trueheart et al., 1987). The nuclei migrate along microtubules into the cytoplasmic bridge, meet and fuse (Kurihara et al., 1994). The dumbell-shaped, fused mating partners become diploid zygotes that bud off diploid cells. The formation of shmoos and cell fusion depend on elements of the actin cytoskeleton, while nuclear migration and fusion depend on microtubule functions.

Filamentous Growth

Haploid yeast cells grow and progress through the cell cycle until they use most of the available nutrients and then form filamentous chains and invade the underlying agar (Roberts and Fink, 1994). This change in growth is termed "haploid invasive growth" and it appears to be a

method of scavenging for more nutrients in the colony's surroundings (Roberts and Fink, 1994). A similar response to nutrient deprivation occurs in diploid colonies (Gimeno et al., 1992; Gimeno and Fink, 1994). In this case, filamentous growth and agar invasion occur in response to nitrogen limitation. This type of growth is called "pseudohyphal growth" to differentiate it from the true hyphal growth found in some other fungi (Gimeno et al., 1992; Gimeno and Fink, 1994). Both forms of filamentous growth make use of similar mechanisms for regulating the actin cytoskeleton and their consequent morphologies.

Sporulation

In the absence of a fermentable carbon source, diploid cells undergo meiosis and sporulation. Each diploid forms four meiotic products encased in a sac-like ascus. The spores within the ascus are usually arranged in a tetrahedral shape and are unordered with respect to the meiotic products (i.e., one cannot infer genetic linkage from the order that the trait is found in spores of the ascus as one can in ordered tetrads such as those of *Sordaria*).

Stationary Phase

Both haploids and diploids will leave the cell cycle at G_1 and enter stationary phase (G_0) if the nutritional conditions will not permit filamentous growth or sporulation. Stationary phase is not simply cell cycle arrest and lack of growth since cells require the expression of specific genes, some of which are unique to stationary phase, to remain viable (as ascertained by return to a nutrient-rich environment; Bauer et al., 1993; Desfarges et al., 1993; Werner-Washburne et al., 1996).

Mating Type Switching

Homothallic strains of *S. cerevisiae* are able to switch mating types (Rine et al., 1992). Switching is controlled by the *HO* gene whose product is an endonuclease that cuts only at the *MAT* locus (Rine et al., 1992). The double-strand break produced by the HO endonuclease is a

signal for DNA repair and conversion of the information at the *MAT* locus to information found at one of the telomeres of the chromosome (Rine et al., 1992). While at the telomeres these genes are kept transcriptionally silent, but once the genes are transposed to the *MAT* locus they are free to be expressed (Rine et al., 1992).

Mating type switching occurs during DNA replication (the S-phase of the cell cycle) and is an asymmetric developmental process. For example, when a *MAT***a** mother cell buds off a daughter, the mother cell will switch to become a *MAT*α cell. The daughter cell remains *MAT***a** until it also buds off a daughter cell (and in turn becomes a mother cell; Bobola et al., 1996; Sil and Herskowitz, 1996). In contrast to the previous developmental programs I mentioned, this asymmetric pattern of mating type switching is genetically controlled and has no epigenetic influence. Daughters are prevented from switching mating types through repression of *HO* transcription by the *ASH1* gene product (Ash1p) (Bobola et al., 1996; Sil and Herskowitz, 1996). Ash1p is localized to daughter cells so that only the mother is able to switch mating types (Bobola et al., 1996; Sil and Herskowitz, 1996). The *ASH1* transcript is localized and translated in daughter cells (Long et al., 1997). Two mutants that prevent both mother and daughter cells from switching were found to be defective in genes encoding cytoskeletal components (Bobola et al., 1996). This indicates that, as in other systems the cytoskeleton underlies an asymmetry or polarity in the cell and, in this case, polarity results in differentiation.

Research Project

The process of conjugation in yeast is a complex response to an extracellular signal, resulting in the activation of an intracellular network of signaling molecules. This process results in arrest of the cell cycle, cellular morphogenesis, cell fusion and nuclear migration and fusion. Many of these events, and the proteins involved, are used for different purposes at other times during the yeast life cycle. Moreover the conservation between organisms displayed by many of the players involved in mating suggests that what is learned about the roles of these proteins in

mating will have applications to their counterparts in higher eukaryotes. This has proven to be the case for many of the signaling and cytoskeletal molecules.

Despite intense scrutiny from researchers, it is obvious that there are large gaps in our understanding of conjugation and the genes involved. My project simply involved a screen for yeast mutants which were unable to mate or mated with low efficiencies. This screen was devised to preclude finding mutations in genes for the signaling molecules involved in detecting pheromone and in pheromone signaling. The reason for this is that this pheromone response pathway has already been intensively studied and is well characterized. Therefore, we hoped to focus the screen on mutants that were defective in components regulated by this signaling pathway. As you will see, I was partly successful in my aim. Three new genes, *AXL1*, *STE23* and *SFU1*, were isolated and characterized and another gene, *BNI1*, was shown to have a function in mating not previously known. Surprisingly, the products of the mutated genes have roles in other processes as well as mating and some have homologues in other organisms. In the following chapters I will describe the search for new genes and the analyses of their products.

CHAPTER 1

Mutant Safari

Introduction

Diploid *Saccharomyces cerevisiae* cells are more resistant to environmental stress and grow more rapidly than haploids. When starved of nitrogen and carbon, diploid cells undergo meiosis and restructure their cell walls, forming haploid spores. Spores are metabolically inactive, have extremely durable cell walls, and are resistant to environmental extremes. Upon return to conditions conducive to vegetative growth, the spores germinate and resume growing as haploids.

To facilitate the production of hardier diploids from haploid cells, haploid yeast are able to mate. This process involves a number of steps initiated by secreted small peptides. Cells of each mating type secrete a specific peptide mating pheromone. *MAT***a** cells secrete **a**-factor, while *MAT*α cells secrete α-factor. These pheromones diffuse into the medium and are detected by cells of the opposite mating type. This detection is accomplished by the α-factor receptor, encoded by *STE2* and expressed only in *MAT***a** cells, and by the **a**-factor receptor, which is encoded by *STE3* and expressed only in *MAT*α cells (Sprague and Thorner, 1992). These two receptors are members of the heterotrimeric guanine nucleotide-binding protein (G-protein) seven transmembrane receptor family to which belong the archetypal β-adrenergic and rhodopsin receptors of mammals (Burkholder and Hartwell, 1985; Hagen et al., 1986; Lefkowitz et al., 1992). Binding of the pheromones to their respective receptors presumably induces a conformational change in the receptor that causes inactive heterotrimeric G-proteins associated with the receptor on the cytoplasmic side of the plasma membrane to exchange bound guanosine diphosphate (GDP) for guanosine triphosphate (GTP) (Konopka et al., 1988; Konopka and Jenness, 1991). This exchange on the Gα subunit causes it to dissociate from the Gβ and Gγ subunits which remain complexed together (Nomoto et al., 1990). In *S. cerevisiae*, the Gα

subunit is encoded by the *GPA1/SCG1* (Dietzel and Kurjan, 1987) gene while *STE4* and *STE18* code for the Gβ and Gγ subunits, respectively (Whiteway et al., 1988).

In *S. cerevisiae*, the Gβγ subunits transduce the pheromone signal to a mitogen activated protein kinase (MAP kinase) cascade. This is shown by the observations that *gpa1* null mutants have a constitutively active kinase cascade, while mutants in either the Gβ or the Gγ subunit genes are unable to activate this cascade (Hartwell, 1980; Miyajima et al., 1989; Cole et al., 1990; Nomoto et al., 1990). These results suggest a model in which the Gα subunit normally prevents activation of the cascade by the Gβγ subunits by remaining complexed with these subunits. In the absence of the Gα subunit, the Gβγ subunits can activate the cascade even without pheromone binding to the receptor . The G-protein encoded by *GPA1*, *STE4* and *STE18*, as well as all of the downstream components of the MAP kinase cascade are common to both *MAT***a** and *MAT*α cells (Sprague and Thorner, 1992). Therefore, the sole cell type-specific determinants of the pheromone response pathway are the pheromone receptors (Bender and Sprague, 1989).

Members of the MAP kinase cascade involved in mating include Fus3p and Kss1p (the MAP kinases, whose functions partially overlap), Ste7p (the MAPK kinase) and Ste11p (the MAPKK kinase) (Sprague and Thorner, 1992; Neiman, 1993). Different MAP kinase cascades also regulate pseudohyphal growth, responses to high extracellular osmolarity, sporulation and are required for the integrity of the cell wall during growth (Levin and Errede, 1995; Schultz et al., 1995; Treisman, 1996). Furthermore, the MAPK cascade involved in the mating response helped to define similar cascades in metazoans. Ste11p activates Ste7p which in turn activates the MAP kinases Fus3p and Kss1p (Neiman and Herskowitz, 1994; Errede et al., 1995; Bardwell et al., 1996). One of the targets of these MAP kinases is the transcription factor Ste12p which is involved in the pheromone-induced transcription of many of the genes required for mating (Elion et al., 1993; Treisman, 1996). Fus3p has been shown to associate with Ste12p and there are residues known to be phosphorylated by Fus3p in response to pheromone (Hung et al., 1997). However, as yet, there are no phosphorylated residues known to regulate the activity of Ste12p. The kinases are tethered together in a complex by Ste5p (Choi et al., 1994; Marcus et al., 1994)

which appears to be a link between the kinases and the G-protein since it physically associates with Ste4p (Whiteway et al., 1995). This scaffolding protein is thought to facilitate the activation of each kinase by keeping it in close association with its activating kinase, and it seems to regulate the specificity of interactions between the kinases and their substrates since deletion of the *STE5* gene results in increased cross-talk between MAP kinase cascades (i.e. an increase in non-specific activation of MAP kinase regulated pathways) (Marcus et al., 1994). Recent work also implicates a motif conserved in Ste7p and in some MAPK kinases from other organisms in determining the specificity of MAPK activation by the kinase's cognate MAPK kinase (Bardwell and Thorner, 1996).

How the Gβγ subunits activate the kinase cascade remains a mystery. The serine/threonine protein kinase encoded by *STE20*, and Ste5p both interact with the β subunit of the G-protein (Whiteway, et al., 1995; Leberer et al., 1997a). Furthermore, Ste20p physically interacts with Ste5p (Leeuw et al., 1995), and Ste20p has been shown to phosphorylate the first kinase in this cascade, Ste11p, although this phosphorylation has yet to be shown to activate Ste11p (Wu et al., 1995). The MAP kinases, Fus3p and Kss1p, are partly redundant for conjugation (Elion et al., 1991). However they do have unique functions. For example, during haploid invasive growth neither protein is required, but the presence of Fus3p alone (in a *kss1* mutant) inhibits agar invasion while the presence of Kss1p alone accentuates this process (Roberts and Fink, 1994). Furthermore, only Fus3p is able to phosphorylate and activate Far1p (Elion et al., 1993; Peter et al., 1993; Tyers and Futcher, 1993). Far1p mediates G_1 arrest of the cell cycle during exposure to pheromone (Chang and Herskowitz, 1990). It does so by binding to and inactivating the complex of G_1 cyclins, Cln1p/Cln2p, with the cyclin dependent kinase (cdk) Cdc28p (Peter et al., 1993; Peter and Herskowitz, 1994). Deletion of *FAR1* prevents G_1 arrest and inhibits mating (Chang and Herskowitz, 1990).

Activation of the pheromone response pathway ultimately results in the transcription of genes required for the mating process. Some of these genes are required early in this process and are expressed in vegetatively growing cells but their expression is increased to facilitate mating.

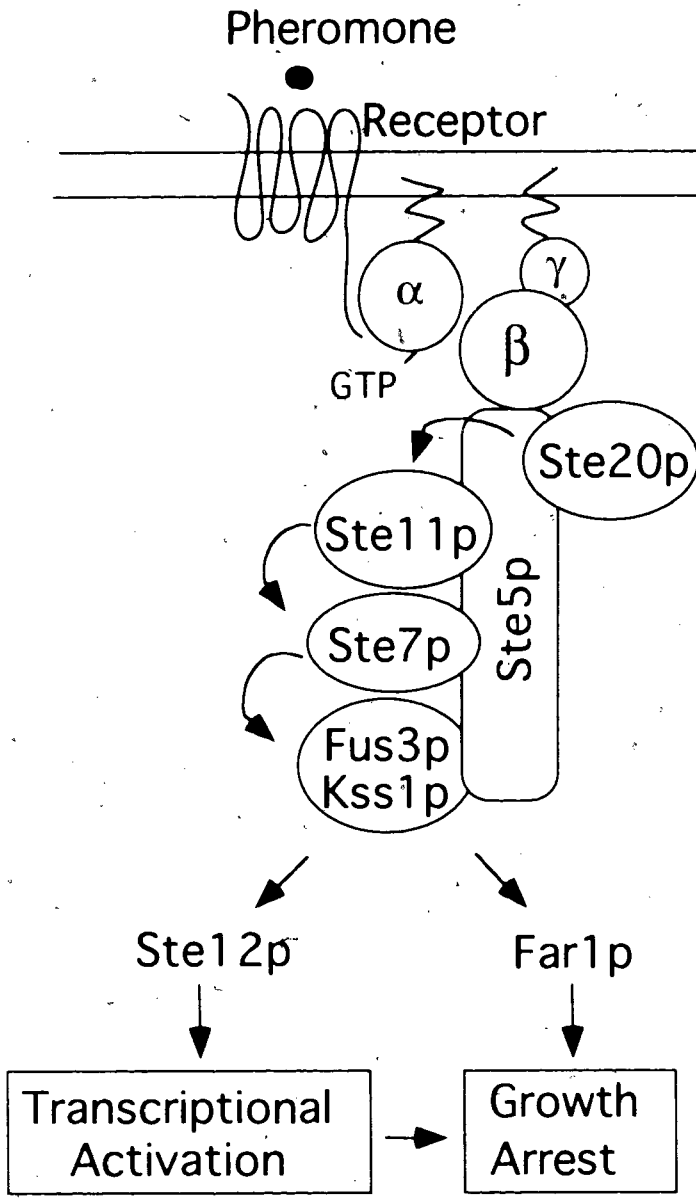
For example, expression of some of the genes involved in pheromone secretion such as the pheromone structural genes *MFa1* and *MFa2* encoding **a**-factor, and *MFα1* and *MFα2* coding for α-factor, as well as the gene for the **a**-factor transporter, *STE6*, are induced several-fold over basal levels (Sprague and Thorner, 1992). Other genes whose expression increase in response to pheromone include those involved in signaling such as *STE2* and *STE3*, *GPA1*, *FUS3*, *STE12*, *BAR1* (encoding a peptidase that degrades extracellular α-factor), and *SST2* (encoding the first member of the RGS (Regulators of G-protein Signaling) protein family which act as GAPs for the Gα subunit of heterotrimeric G-proteins) (Sprague and Thorner, 1992). Finally, many genes required for later events in conjugation, such as *FUS1*, are also induced by pheromone (Sprague and Thorner, 1992). Some of the proteins involved in the pheromone response are shown in Fig. 2.

Detection of pheromone induces cells to polarize towards the source of the pheromone and localize cell growth to this region resulting in the formation of a projection called a shmoo (Lipke et al., 1976). This projection presumably facilitates contact between mating partners - a prerequisite for cell fusion and zygote formation. The growth of this projection requires many of the same proteins involved in polarized cell growth during bud formation (Chenevert et al., 1992; Chenevert, 1994; Chenevert et al., 1994). The main difference between the two processes is that the selection of a bud site is determined by a genetically controlled cellular program, while the choice of location for a mating projection is determined by the direction of the mating partner. The site on the cell surface that is nearest the mating partner, and hence, exposed to the highest concentration of pheromone is chosen for projection formation. This is inferred from experiments in which cells are given a choice between mating with partners that do or do not produce pheromone. In such a situation wild-type cells will mate almost exclusively with the partner that produces pheromone in a process termed mating partner discrimination (Jackson and Hartwell, 1990a; Jackson and Hartwell, 1990b; Jackson et al., 1991). This process requires detection of a gradient of pheromone since addition of exogenous pheromone to pheromoneless cells only poorly restores mating (Jackson and Hartwell, 1990a; Dorer et al., 1995). In fact, exogenous

pheromone reduces the efficiency of mating of wild-type cells to strains that already secrete

Figure 2. Yeast pheromone response pathway.

The basic components of the MAP kinase cascade regulating the response to pheromone are shown. Arrows represent activations (by phosphorylation or otherwise). Binding of pheromone to its receptor causes a change in the receptor's association with a heterotrimeric G-protein. Consequently, the α subunit of the G-protein exchanges GTP for GDP and releases the Gβγ subunits which act together to transduce the signal to intracellular effectors. The β subunit interacts directly with the scaffold protein Ste5p, which, together with the PAK kinase homologue Ste20p, activates the MAP kinase cascade consisting of Ste11p (the MAP kinase kinase kinase), Ste7p (the MAP kinase kinase), and Fus3p and Kss1p (the MAP kinases). The ultimate outcome of activation of this cascade is inhibition of the Cln1,2p-Cdc28p complexes by Far1p and transcriptional activation through the transcription factor Ste12p. Adapted from Leeuw et al. (1995).


Pheromone
Receptor
α
γ
β
GTP
Ste20p
Ste11p
Ste5p
Ste7p
Fus3p
Kss1p
Ste12p
Far1p
Transcriptional Activation
Growth Arrest

pheromone, presumably because the cells are unable to detect a pheromone gradient and, consequently, shmoo in "random" directions (Jackson and Hartwell, 1990a). Moreover, this process of shmooing towards a pheromone source can be directly visualized when a micropipette filled with pheromone is placed in a field of cells (Segall, 1993).

When mating cells first come into contact they stick together through the action of agglutinins (Lipke and Kurjan, 1992). The agglutinins are secreted constituents of the cell wall that are, at least initially, anchored into the external face of the plama membrane through glycosyl-phosphatidylinositol (GPI) modifications (Lipke and Kurjan, 1992). The agglutinin expressed in *MAT***a** cells, encoded by *AGA1* (the core subunit which has its GPI anchor cleaved) and *AGA2* (the plasma membrane anchor for the **a**-agglutinin), recognizes and binds to the α-agglutinin, encoded by *AGα1* (Lipke and Kurjan, 1992).

The mating partners must then break down their cell walls at the point of contact to allow the plama membranes to touch and fuse forming what is called a zygote. The hydrolysis of cell wall material must be tightly regulated, and likely coordinated with wall synthesis. In the absence of such regulation cells would be subject to damage (or lysis in a hypoosmotic environment) if they were to create holes in their walls before making contact with the partner or if the holes were extended beyond the point of contact (Cid et al., 1995). Presumably, some of the enzymes known to be involved in the synthesis and hydrolysis of cell wall materials are regulated both during shmoo formation and during cell fusion. However, little is known about such regulation.

Chitin, which consists of chains of β(1,4)-linked *N*-acetylglucosamine covalently linked to β(1,3)-glucan, is a minor constituent of the cell wall and is most concentrated within bud scars (Cid et al., 1995). There are three genes responsible for chitin synthesis. These are: *CHS1*, which is responsible for repair of the daughter's birth scar during chitin hydrolysis as the daughter bud separates from the mother cell; *CHS2*, which lays down the primary septum between mother and daughter cells during cytokinesis; and *CHS3*, which produces a ring of chitin at the selected bud site before bud emergence and which persists at the mother-bud neck (Cid et al., 1995).

CHS1 has a promoter sequence known as a pheromone response element (PRE) to which activated Ste12p binds in response to pheromone and promotes transcription (Appeltauer and Achstetter, 1989; Hagen et al., 1991). Accordingly, *CHS1* is pheromone-inducible. Although the *CHS3* gene is not transcriptionally regulated by pheromone, the enzyme activity is likely to be regulated by pheromone since Chs3p is responsible for a large increase in the chitin content of mating projections (Cabib et al., 1993; Cid et al., 1995). Despite such regulation, deletions of either *CHS1* or *CHS3* have no measurable effect on mating efficiency (Roncero et al., 1988). This could be explained if the same enzymes from the mating partner were able to complement the activity once the cells have come into contact. However, bilateral matings of *chs3* mutants (both mating partners are *chs3* mutants) also had no defect in mating (Roncero et al., 1988). The absence of an effect on mating by two enzymes that are regulated by pheromone is curious. However, it is possible that Chs1p and Chs3p are functionally redundant during mating. It remains to be seen if *chs1 chs3* double mutants display a unilateral or bilateral mating defect. Moreover, the role in mating and cell fusion of the sole known chitinase, Cts1p, has yet to be studied.

While chitin is a minor constituent in cell walls, the regulation of synthesis and degradation of the major constituents - β(1,3)-glucan, mannoproteins and β(1,6)-glucan - is completely unknown in regards to mating. The functional redundancy of both the biosynthetic and hydrolytic enzymes for these compounds makes it likely that studies using gene deletions will be difficult or inconclusive (Cid et al., 1995). However, the activity of Fks1p, a subunit of the β(1,3)-glucan synthase, has recently been shown to be regulated by Rho1p (Drgonova et al., 1996; Qadota et al., 1996). Rho1p is a small GTPase belonging to the rho subfamily of ras GTPases, and it is necessary for bud growth (Yamochi et al., 1994). The GTP-bound form of Rho1p binds to and activates Fks1p, thus stimulating cell wall synthesis at sites of bud growth, to which both proteins localize (Drgonova et al., 1996; Qadota et al., 1996). The role of Rho1p in mating has not been examined, but it is likely that this GTPase also regulates shmoo growth and, perhaps, cell fusion through Fks1p. The localization of both proteins in cells exposed to

pheromone or in mating mixtures should indicate if such regulation during mating possibly occurs.

Several genes are known to be involved specifically in the cell wall fusion process. Mutations of these genes, *FUS1*, *FUS2* and *FUS3*, result in fusion defects and the accumulation of prezygotes (McCaffrey et al., 1987; Trueheart et al., 1987). When viewed under differential interference contrast (DIC) optics, the mating partners clearly make contact, but a septum between the cells persists (McCaffrey et al., 1987; Trueheart et al., 1987). As shown by electron microscopy, this septum consists of undegraded cell wall material which prevents the cell membranes of the mating partners from contacting (Brizzio et al., 1996; Elia and Marsh, 1996). As mentioned previously, Fus3p is a MAP kinase and is presumably involved in regulating components required for cell fusion. Our lab has recently shown a two-hybrid interaction between Fus3p and Fus1p, thus providing a possible role for the MAP kinase specifically in the fusion process (S. Ritchie and C. Boone, unpublished results). The functions of Fus1p and Fus2p are obscure. The two proteins contain regions of amino acid similarity to each other (Trueheart et al., 1987). Fus1p is predicted to be a membrane protein with a single transmembrane domain, and it contains a src homology 3 (SH3) domain. SH3 domains are protein-protein interaction motifs found in many proteins implicated in regulating the actin cytoskeleton (Carraway and Carraway, 1995). Furthermore, the functions of Fus1p and Fus2p appear to partially overlap since the double mutant has a more severe mating defect than either single mutant and since overexpression of either gene will suppress the fusion defect associated with deletion of the other (Trueheart et al., 1987). There are no mutants known to specifically inhibit plasma membrane fusion once cell wall fusion is successfully completed.

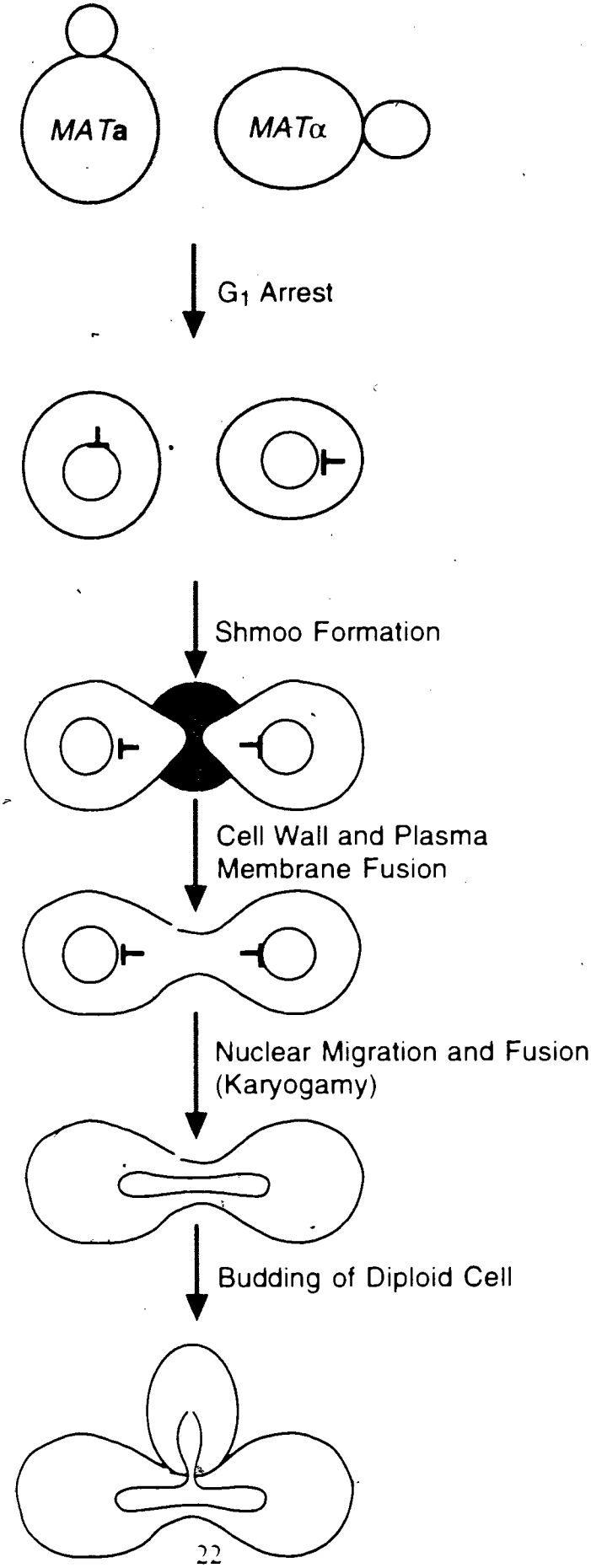
The final step in producing a diploid zygote is nuclear fusion. This process, termed karyogamy, first involves the alignment of the spindle pole body (SPB, the yeast equivalent of the centrosome) to face the direction in which the projection is formed (Berlin et al., 1991; Kurihara et al., 1994). Once cell fusion has occurred, interactions between cytoplasmic microtubules (the equivalent of asters) emanating from each SPB permit migration of the nuclei into the bridge

between the fused cells (Berlin et al., 1991; Kurihara et al., 1994). Lastly, nuclear membrane fusion occurs (Berlin et al., 1991; Kurihara et al., 1994). Screens to identify mutants defective in karyogamy have uncovered a number of genes involved in both karyogamy and cell fusion (Berlin et al., 1991; Kurihara et al., 1994). The karyogamy genes fall into two classes - those involved in SPB or microtubule function, and those involved in nuclear membrane fusion (Kurihara et al., 1994). The nuclei in the first class fail to migrate and remain well separated, while the nuclei of the second class are closely juxtaposed but fail to fuse (Kurihara et al., 1994). Examples of genes involved in nuclear migration are *TUB2*, encoding β-tubulin, *BIK1*, a SPB-associated protein, and *KAR3*, a kinesin-related protein (Kurihara et al., 1994). A gene required for nuclear fusion, *KAR2*, is the yeast BiP/hsp70 homolog and is located in the lumen of the endoplasmic reticulum and nuclear envelope (Kurihara et al., 1994). The precise functions of many of the *KAR* genes are currently unknown.

The process of conjugation in *S. cerevisiae* (summarized in Fig. 3) involves many processes involved in other aspects of its life cycle. For instance, many of the components of the pheromone response pathway are involved in pseudohyphal growth in diploids or in haploid invasive growth (Roberts and Fink, 1994; Mosch and Fink, 1997). Both are responses to nutrient deprivation that optimize the foraging strategy of yeast. Many of the proteins involved in shmoo formation are also necessary for vegetative growth (Chenevert et al., 1994). Furthermore, some of the components of the karyogamy machinery are also involved in vegetative growth during mitosis (Kurihara et al., 1994). In addition, many of the proteins used in these events have homologues that have similar functions in plants and mammals. Therefore, examination of the yeast mating process not only sheds light on the life cycle of this simple eukaryote, but it also illuminates similar processes in higher eukaryotes that are less amenable to genetic manipulation. In this chapter, I describe how I obtained and characterized mutants that were defective for some of the steps involved in mating. Many of these mutants were identified as known genes based on complementation of their mating defects with plamids bearing these genes.

Figure 3. Events in the mating response of yeast.

Detection of pheromone and activation of the pheromone response initiates a series of physiological changes in cells. The cells first G_1 arrest, and then form mating projections towards the source of pheromone. Upon contact, the partners agglutinate, fuse cell walls, and then fuse plasma membranes. At this time the nuclei migrate towards the cytoplasmic bridge and fuse to form a diploid zygote which buds off diploid cells.


MATa
MATα
G1 Arrest
Shmoo Formation
Cell Wall and Plasma
Membrane Fusion
Nuclear Migration and Fusion
(Karyogamy)
Budding of Diploid Cell

Materials and Methods

Strains, Plasmids, Media and Microbiological Techniques

Yeast strains used are listed in Table 1. Plasmids are listed in Table 2. The composition of growth media used are as described (Guthrie and Fink, 1991; Rose et al., 1990). Yeast transformations were by the lithium acetate method (Ito et al., 1983). Standard methods were used to manipulate plasmid DNA (Sambrook et al., 1989).

Crosses and tetrad dissections were performed as described (Guthrie and Fink, 1991). Briefly, *MAT***a** strains were mated to *MAT*α strains overnight on YEPD. Cells were then plated on synthetic media lacking the appropriate amino acids to select for diploids. Diploids were then sporulated, the outer walls of asci were partially digested in β-glucaronidase (Sigma), and asci were dissected onto YEPD using a microdissection arm assembly on a Nikon microscope. Tetrads were grown at 30° C for two days and replica plated or patched onto various media to assay auxotrophy, mating, or other physiological assays.

Strains Y1 and Y5, to which mutants were backcrossed, were constructed by transforming isogenic *BAR1* strains with Hind III-Bam HI-cut plasmid pZV77 (MacKay et al., 1988). This plasmid integrates at *BAR1* and replaces it with a deletion construct containing the *LEU2* gene. Y29 was used in some crosses to test for linkage to *FAR1*. This strain has an integrated plasmid containing the *FAR1* gene and *URA3*, and was constructed by transforming Bam HI-cut pSL2068 into a *BAR1* strain isogenic to SY2625. Y123 was used to test for linkage to *RAM1*. Y141 is Y5 containing plasmid CY919 (vector YCp50 with the *GAL1* promoter driving expression of the *HO* gene).

Table 1. Strain List for Chapter 1.

Strain	Genotype	Source
SY2625	*MAT***a** *bar1Δ his3::FUS1-HIS3 mfa2Δ::FUS1-lacZ ura3-1 leu2-3,-112 trp1-1 ade2-1 can1-100*	C. Boone
SY2014	*MATα ste3Δ306::LEU2 sst2Δ ste2Δ mfa1Δ mfa2Δ::FUS1-lacZ FUS1::HIS ura3-52 leu2-3, 112 ade1*	C. Boone
Y1	*MAT***a** *bar1Δ::LEU2 lys2 his3::FUS1-HIS3 mfa2Δ::FUS1-lacZ ura3-1 leu2-3,-112 trp1-1 ade2-1 can1-100*	N. Adames
Y5	*MATα bar1Δ::LEU2 lys2 his3::FUS1-HIS3 mfa2Δ::FUS1-lacZ ura3-1 leu2-3,-112 trp1-1 ade2-1 can1-100*	N. Adames
Y29	*MAT***a** *FAR1::URA3 his3::FUS1-HIS3 mfa2Δ::FUS1-lacZ ura3-1 leu2-3,-112 trp1-1 ade2-1 can1-100*	N. Adames
Y121	*MAT***a** *RAM1::URA3 bar1Δ::LEU2 leu2-3,-112 his3::FUS1-HIS3 mfa2Δ::FUS1-lacZ trp1-1 ade2-1 can1-100*	K. Blundell
Y62	*MAT***a** *lys1*	I. Herskowitz
Y63	*MATα lys1*	I. Herskowitz
Y66	*MATα far1-c lys1*	I. Herskowitz
70α	*MATα thr3*	G. Sprague
Y141	*MATα* p*GAL1-HO bar1Δ::LEU2 his3::FUS1-HIS3 mfa2Δ::FUS1-lacZ ura3-1 leu2-3,-112 trp1-1 ade2-1 can1-100 lys2*	N. Adames
Y583	*MAT***a** *fus3Δ::URA3 bar1::LEU2t rp1-1 LYS2 his3::FUS1-HIS3 mfa2Δ::FUS1-lacZ ura3-1 leu2-3,-112 ade2-1 can1-100*	K. Blundell

Table 2. Plasmid List for Chapter 1.

Plasmid	Construction	Source
p8	*FAR1* in pRS316 (*CEN ARS URA3*)	G. Sprague
p61	*BEM1* in pRS316 (*CEN ARS URA3*)	J. Chenevert
p62	*PEA2* in pRS316 (*CEN ARS URA3*)	J. Chenevert
p63	*SPA2* in pRS316 (*CEN ARS URA3*)	J. Chenevert
p67	*FUS1* in YCp50 (*CEN ARS URA3*)	N. Valtz
p68	*FUS2* in YCp50 (*CEN ARS URA3*)	N. Valtz
p77	*FUS3* in YCp50 (*CEN ARS URA3*)	C. Boone
p188	*RVS161* in pRS316 (*CEN ARS URA3*)	C. de Hoog
p224	*BNI1* in pRS316 (*CEN ARS URA3*)	N. Adames
p739	*RVS167* in unknown (*CEN ARS URA3*)	B. Andrews
p666	*SFU1* in pRS316 (*CEN ARS URA3*)	G. Poje
p82	*MFA2* in pRS316 (*CEN ARS URA3*)	C. Boone
p3	*STE14* in YEp24 (*2μ URA3*)	G. Sprague
p25	*RAM1* in YCp19 (*CEN ARS URA3*)	F. Tamanoi
p110	*RAM2* in pRS316 (*CEN ARS URA3*)	K. Blundell
p9	*STE6* in YEp352 (*2μ URA3*)	M. Raymond

Mutagenesis

Strain SY2625 was spread onto minimal medium lacking histidine and containing synthetic α-factor (Sigma) at a concentration of 0.05 ng/mL. This amount of α-factor does not cause the cells to G_1 arrest, but does induce *FUS1-HIS3* and permits growth of SY2625 on medium lacking histidine. This regime precluded the isolation of mutations that reduce signaling of the pheromone response pathway, because such mutations will lead to a slower growth rate on medium lacking histidine. Cells were plated at a density of approximately $2x10^3$ cells/plate. Once dry, these plates were mutagenized to 10% survival by exposure to ultraviolet light.

A replica-plating procedure was used to test mutants for their ability to mate (Sprague, 1991). Mutagenized cells were allowed to form colonies and sterile mutants were identified by replica plating to a lawn of the mating tester strain 70α (*MATα thr3*) on minimal medium. Only diploids produced from matings were able to grow on minimal medium. In addition, the *ade2* mutation present in SY2625, causes cells to become red on minimal media as they accumulate an intermediate of adenine biosynthesis that is a substrate for the *ADE2* product. Mating is scored by the appearance of white diploid colonies on a background of dying red cells. Colonies that had an apparent mating defect were tested several times for the ability to mate. Mutants that had a weak mating phenotype were retested using stringent mating conditions. This was accomplished by allowing the mutants to mate for a shorter period before replica plating to minimal media or by mating to the "crippled mater" Y66. Lastly, mutants were tested for threonine auxotrophy since the diploid products of matings between *thr3* mutants and 70α would be unable to grow on minimal medium. *Bona fide* sterile mutants were assayed for several physiological functions involved in mating.

G_1 Arrest Assay

Assays to test for the ability of cells to arrest in response to pheromone were carried out as described (Fink and Styles, 1972). Mutant cells were spread onto rich medium at a density of approximately 100 cells/cm^2. After allowing the plates to dry, various concentrations of α-factor were spotted onto the lawn in a 2 μL volume. The pheromone caused cells to arrest in the G_1 phase of the cell cycle, producing a clear zone of arrested cells surrounded by a lawn of growing cells that were not exposed to high enough concentrations of pheromone to arrest. The size of the clear zone or "halo" can be an indication of how responsive cells are to pheromone.

Pheromone Spot Assay

The lack of G_1 arrest can be due to a dysfunctional pheromone response pathway or due to a specific defect in G_1 arrest (for example, a *far1* mutation). The G_1 arrest assay cannot distinguish between the two possibilities. However, the pheromone spot assay can. This assay employs the same method as the G_1 arrest assay with the distinction that cells are spread onto medium lacking histidine. SY2625 is unable to grow on medium lacking histidine unless there is pheromone present to induce the *FUS1-HIS3* construct. *FUS1* induction occurs at lower concentrations of pheromone than does G_1 arrest. The same concentrations of pheromone used in the G_1 arrest assay are spotted onto the plate. Cells close to the area where pheromone was spotted are able to induce *FUS1-HIS3* but the high concentration of pheromone causes the cells to arrest. Cells farther away experience lower concentrations of α-factor as it diffuses from the source. Eventually cells are able to grow as the levels drop below the threshold required for G1 arrest, but are still high enough to induce *FUS1*. Even further from the source of pheromone the concentration declines to levels that are unable to induce *FUS1-HIS3* and cells cannot grow due to the lack of histidine.

The difference in sensitivity between G_1 arrest and *FUS1* induction to pheromone results in a ring of growing cells surrounding a halo of arrested cells. Again the size of the halo can be an

indication of how well the cells are responding to pheromone. However, the diameter of the ring of growing cells is a better measure of signaling. This growth is a direct consequence of pheromone signaling through activation of the *STE12* transcription factor, whereas G_1 arrest is a pathway that bifurcates from this signaling pathway and is not an absolute requirement for mating.

Because SY2625 cells have a low level of spontaneous (basal) signaling in the pheromone response pathway, I added aminotriazole (ATZ; 1 mM) to the plates used in pheromone spot assays. ATZ is a potent inhibitor of the *HIS3* gene product (Rose et al., 1990), and its addition to plates at low concentrations helps to reduce background growth of SY2625 due to basal signaling.

Pheromone Secretion Assay

I assayed secreted **a**-factor activity by patching *MAT***a** cells onto a lawn of *MAT*α cells, SY2014 (*ste3Δ306 sst2Δ*), which show hypersensitivity to α-factor (Boone et al., 1993). The *ste3Δ306* and *sst2Δ* mutations interfere with negative regulation of pheromone signaling at the level of the α-factor receptor and G protein, respectively, and act synergistically. Secretion of active **a**-factor by the *MAT***a** cells causes the surrounding *MAT*α cells to G_1 arrest, creating a clear halo. The size of the halo corresponds to the amount of secreted **a**-factor.

Mating Projection Assay

When cells detect pheromone they undergo a morphological change, forming a projection, or "shmoo", in an attempt to contact a cell of the opposite mating type. Shmoo formation is a consequence of polarized cell growth, and polarization of the cell towards a mating partner is an important prelude to cell fusion. To assay for the ability to form mating projections, cells were grown overnight in liquid medium. 200 µl from this culture was inoculated into 5 ml of fresh liquid medium and grown to $O.D._{600}$ ~0.5. 500 ng of α-factor was added to 1 ml of this culture which was grown for up to 6h. 250 ml aliquots were removed every hour and cells were fixed in 3.7% formaldehyde, 0.15 M sodium chloride and stored for short periods at 4° C. Fixed cells were observed under DIC optics using an Olympus model microscope.

Backcrosses and Linkage Analysis

Mutants were crossed to Y5 (*MATα lys2 bar1Δ::LEU2*), diploids were sporulated and progeny were examined for 2:2 segregation of the mating defect and pheromone secretion (and any other phenotypes associated with the defect) using the mating tester strains Y62 or Y63, and the pheromone hypersensitive strains, SY2625 or SY2014. The 2:2 ratio indicates that the observed phenotype is due to mutation in a single locus. Several backcrosses to Y1 or Y5 were performed for some mutants to ensure that the mutation resides in a strain as genetically similar to the parent as possible.

Linkage analyses to known genes were performed by crossing the mutant in question to a strain bearing a version of the candidate gene usually disrupted by or closely linked to a nutritional marker. If the mutated gene is an allele of the candidate gene, all of the tetrads will be parental ditypes (i.e., all spore progeny from the cross will carry one or the other of these alleles). Linkage may be determined by testing the progeny for auxotrophies (to follow the marked allele) and mating defects or other phenotypes displayed by the mutant being tested. In general, at least 20 tetrads were analyzed for linkage.

Dominance/Recessiveness Test

*MAT***a**/*MAT***a** or *MATα*/*MATα* diploids for dominance tests were made by crossing mutants to Y141 (*MATα* p*GAL1-HO*). The resulting diploids were grown overnight in liquid medium containing galactose to induce *HO* expression and effect switching of *MAT* loci. Some of these switches result in homozygosis of the *MAT* locus. Diploids from this overnight culture were tested for the ability to mate and produce pheromone (since cell type is determined by the *MAT* locus, *MAT***a**/*MAT***a** cells behave as *MAT***a** cells, and *MATα*/*MATα* cells act like *MATα* cells). Dominant mutations impart to the heterozygous diploid the same phenotype observed in the haploid mutant, but the phenotype of recessive mutations will be masked by the presence of a wild-type allele. All of the mutants that were tested were recessive.

Results

Mutant Yield

Approximately 13,000 colonies were screened for mating defects. From these, I found 33 mutants unable to produce diploid colonies when mated to 70α; 2 of these could not grow on medium lacking threonine and were likely *thr3* mutants. The remaining 31 mutants were assayed for the various mating responses. The 31 mutants were grouped according to their phenotypes (25 are shown in Table 3). Several of the mutants had growth defects in the original isolate that were not linked to the mating defect. For most of these mutants, segregants from backcrosses that have lost these defects were analyzed.

Signaling Mutants

Group I mutants were defective in several, or all assays (one mutant, Y1160, is shown in Fig. 4 and Fig. 5). I obtained 4 such mutants. These mutants are presumably defective in the pheromone response pathway, and were not studied further. Many other mutants also were defective for some of the physiological responses tested. However, the mutants in Group I are particularly likely to have signaling defects since they cannot fully induce *FUS1* (in the pheromone spot assay) or **a**-factor -- two responses that are dependent on Ste12p, a target protein of the Fus3p and Kss1p MAP kinases.

G_1 Arrest Mutants

The mutants in Group II (Y33 and Y935) are unable to G_1 arrest properly (Fig. 4). Y935 had a severe defect in G_1 arrest, and an additional mating projection defect. These defects were linked and segregated as a single mutation. When exposed to pheromone, Y935 cells become slightly larger and more rounded (Fig. 5). Some cells formed small projections but these probably resulted in buds since no shmooing cells were seen after 6 h, but some cells with

Table 3. Mating mutants

Mutant	Group	Phenotype	Identity
Y1160	I	defective in G_1 arrest, shmooing, **a**-factor, and has low pheromone signaling	unknown
Y33	II	defective in G_1 arrest, shmooing	unknown
Y935	II	defective in G_1 arrest, shmooing	*far1*
Y36	III	defective in shmooing, mild arrest defect	*far1*
Y34	III	defective in shmooing, mild arrest defect	unknown
Y1093	III	defective in G_1 arrest, shmooing, cold- and temperature-sensitive growth	*sfu1*
Y37	III	defective in shmooing, mild arrest defect, temperature-sensitive for growth	*bni1-10*
Y932	III	defective in shmooing, mild arrest defect	*fus3*
Y927	III	mild arrest and shmoo defect	unknown
Y1161	III	mild arrest and shmoo defect, cold-sensitive for growth, mating defect partly suppressed by *FAR1*, *BEM1*, and *FUS1* at low copy number	unknown
Y1162	IV	mild shmoo defect, cold-sensitive for growth	unknown
Y32	IV	cold-sensitive for growth, mating defect partly suppressed by *FAR1* and *BEM1* at low copy number	unknown
Y30	IV	temperature-sensitive growth defect	*ram1*
Y31	IV	mating defect only	unknown
Y39	V	defective in **a**-factor secretion	*ram1*
Y40	V	defective in **a**-factor secretion	*ram1*
Y43	V	defective in **a**-factor secretion	*ram1*
Y46	V	defective in **a**-factor secretion	*ram1*
Y38	V	defective in **a**-factor secretion	*ste6*
Y42	V	defective in **a**-factor secretion	*ste6*
Y45	V	defective in **a**-factor secretion	*ste6*
Y47	V	defective in **a**-factor secretion	*ste6*
Y44	V	defective in **a**-factor secretion	*ste14*
Y49	V	defective in **a**-factor secretion	*axl1* (*ste22-1*)
Y939	V	defective in **a**-factor secretion	*axl1* (*ste22-2*)

Figure 4. Mating and arrest defects of mutants in Classes I to IV.

Mutants were tested for several physiological functions required for efficient mating. In the mating assays white diploids produced from the cross between mutant and wild-type cells show against a dark background. Patches of cells were allowed to grow overnight and then replica plated to a lawn of the mating tester strain Y63 (*MATα lys1*). This mixture of cells was mated for 4 h and replica plated onto minimal media to select for growth of diploid colonies. Stringent matings were performed by allowing less time for cells to mate before replica-plating the mating mixtures to minimal media, or by mating to the crippled tester strain Y66 (*MATα far1*-c *lys1*). In the arrest assay, a clear zone or "halo" of G_1-arrested cells forms where pheromone is spotted onto the plate. The size of the halo indicates the extent of arrest. In the pheromone spot assay (PSA) the size of the outer ring indicates how competent cells are for pheromone signaling, while the clear zone within the rings is an indication of how well the mutants arrest in response to pheromone. The wild-type parent strain, SY2625, was used as a control in these assays.

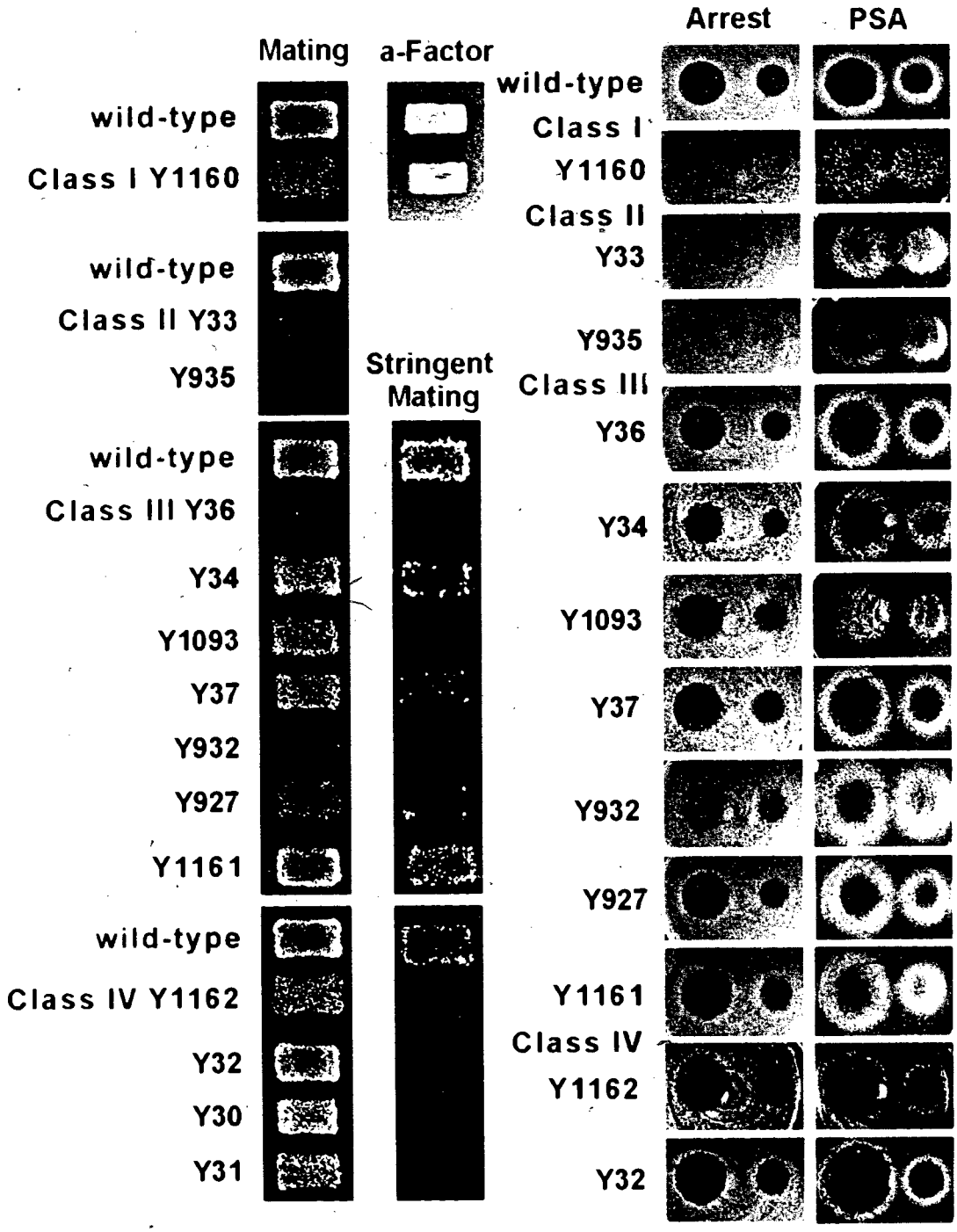
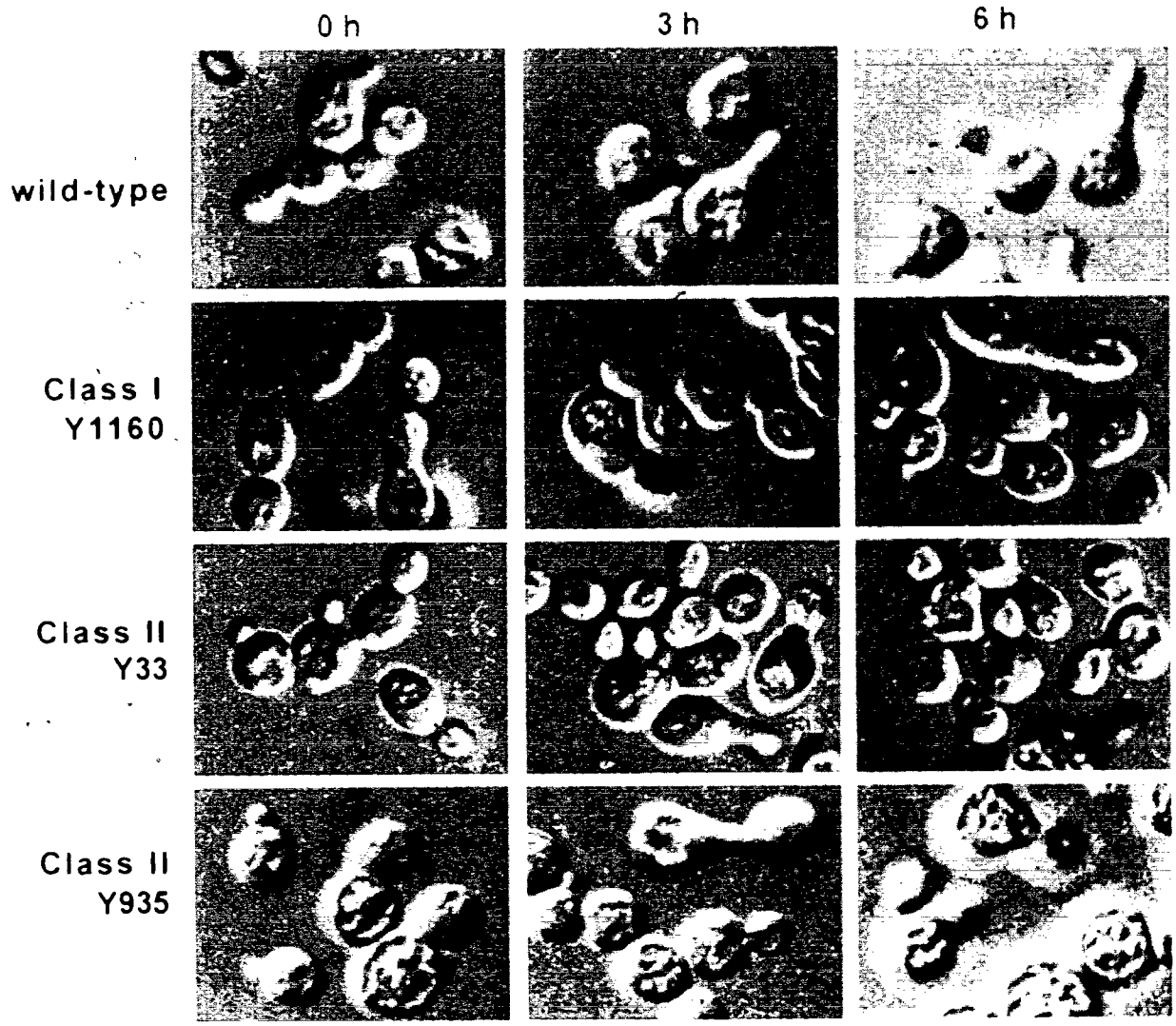

Mating
a-Factor
wild-type
Class I Y1160
wild-type
Class II Y33
Y935
Stringent Mating
wild-type
Class III Y36
Y34
Y1093
Y37
Y932
Y927
Y1161
wild-type
Class IV Y1162
Y32
Y30
Y31
Arrest
PSA
wild-type
Class I
Y1160
Class II
Y33
Y935
Class III
Y36
Y34
Y1093
Y37
Y932
Y927
Y1161
Class IV
Y1162
Y32

Figure 5. Mating projection formation defects of mutants from Classes I and II.

500 ng of α-factor was added to 1 ml of cells grown to mid-log phase. Cells were observed for morphogenesis at 0 h, 3 h and 6 h after the addition of pheromone to the cultures. All assays were done simultaneously in one experiment. Cells were visualized using differential interference contrast (DIC) optics. Assays were performed using wild-type (SY2625) cells and mutants belonging to Class I (Y1160) and Class II (Y33 and Y935). For a summary of the phenotypes of these mutants see Table 3.


0 h
3 h
6 h
wild-type
Class I
Y1160
Class II
Y33
Class II
Y935

shmoo-like projections ending in buds were seen (Fig. 5). A *FAR1* plasmid rescued the mating and arrest defects of this mutant, and allowed the formation of shmoos (Fig. 9A). Linkage analysis with Y29 showed the mutation was linked to *FAR1*.

Projection Formation Mutants

Group III mutants had mild defects in G_1 arrest (Fig. 4), and were either unable to shmoo or formed oddly shaped projections (Fig. 6 and Fig. 7). The mating, arrest and shmoo defects of Y932 were complemented by a plasmid carrying *FUS3* (Fig. 9C). Furthermore, the phenotypes of Y932 closely matched those displayed by a *fus3Δ::URA3* mutant, Y583 (Fig. 9C). The mating defect of this mutant appears to be linked to *fus3*.

Mutants Y36, Y34, Y1093, and Y37 similarly had strong defects in projection formation (Figs. 4 and 6). The mating defects all segregated as single loci. In addition, Y34 cells clumped together indicating that they had a cytokinesis or cell separation defect (Fig. 6) that segregated with the mating defect. Y37 showed a mild cell separation defect (Fig. 6, evident in the 0 h photograph). Y1161 had a weak defect in projection formation. This mutant formed shmoos, but appeared to be slower in doing so than wild-type cells and no cells with multiple projections were seen at 6 h (Fig. 7), whereas wild-type cells often had more than one shmoo by this time. Moreover, Y1093, Y37, and Y1161 all had growth defects (Fig. 8). Y1093 was temperature- and cold-sensitive (Fig. 8). Y37 showed a temperature-sensitive growth defect, while Y1161 was cold-sensitive (Fig. 8). Y927 had a weak G_1 arrest defect and mild shmoo defect similar to Y37 and Y1093. These last two mutants, Y37 and Y1093, are discussed at length in Chapter 6.

Miscellaneous Mutants

The only observable phenotypes in the Group IV mutant, Y32, were its mild mating (Fig. 4) and cold-sensitive growth defects (Fig. 8). The mating defect in this mutant segregated 2:2. Y1162 also arrested normally (Fig. 4) but few cells were able to form projections and these tended to be rather broad at early hours of exposure (Fig. 7). This mutant also had a strong separation

Figure 6. Mating projection formation defects of Class III mutants.

Mating projection assays were performed with Class III mutants (Y36, Y34, Y1093, Y37 and Y932) as described in Materials and Methods and in Fig. 5. Assays were performed simultaneously with those shown in Fig. 5.

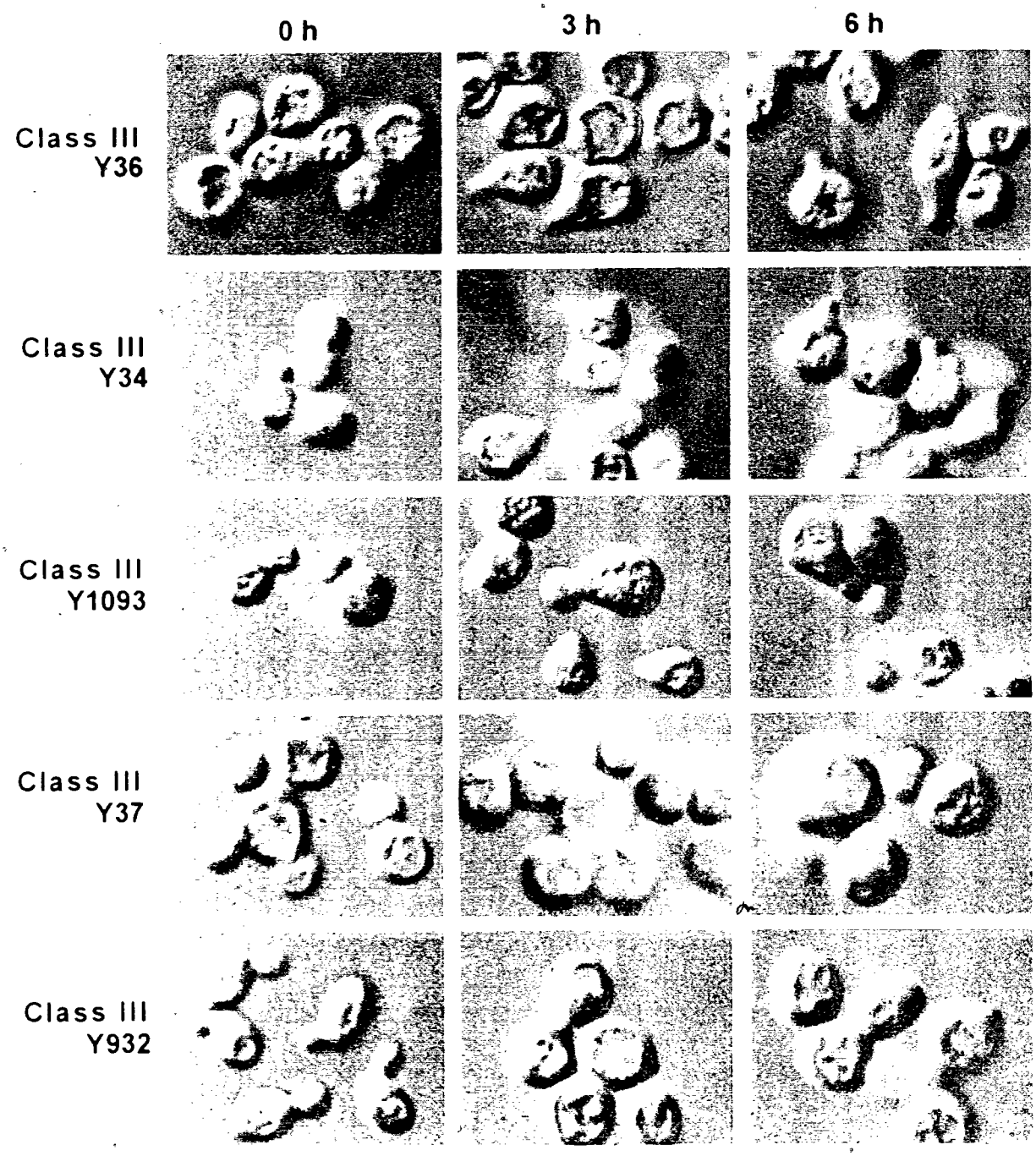

0 h
3 h
6 h
Class III
Y36
Class III
Y34
Class III
Y1093
Class III
Y37
Class III
Y932

Figure 7. Mating projection formation in Class III and IV mutants.

Mating projection assays were performed with Class III mutants (Y927 and Y1161) and with the Class IV mutants (Y1162 and Y32) as described in Materials and Methods and in Fig. 5. Assays were performed simultaneously with those shown in Fig. 5 and Fig. 6.

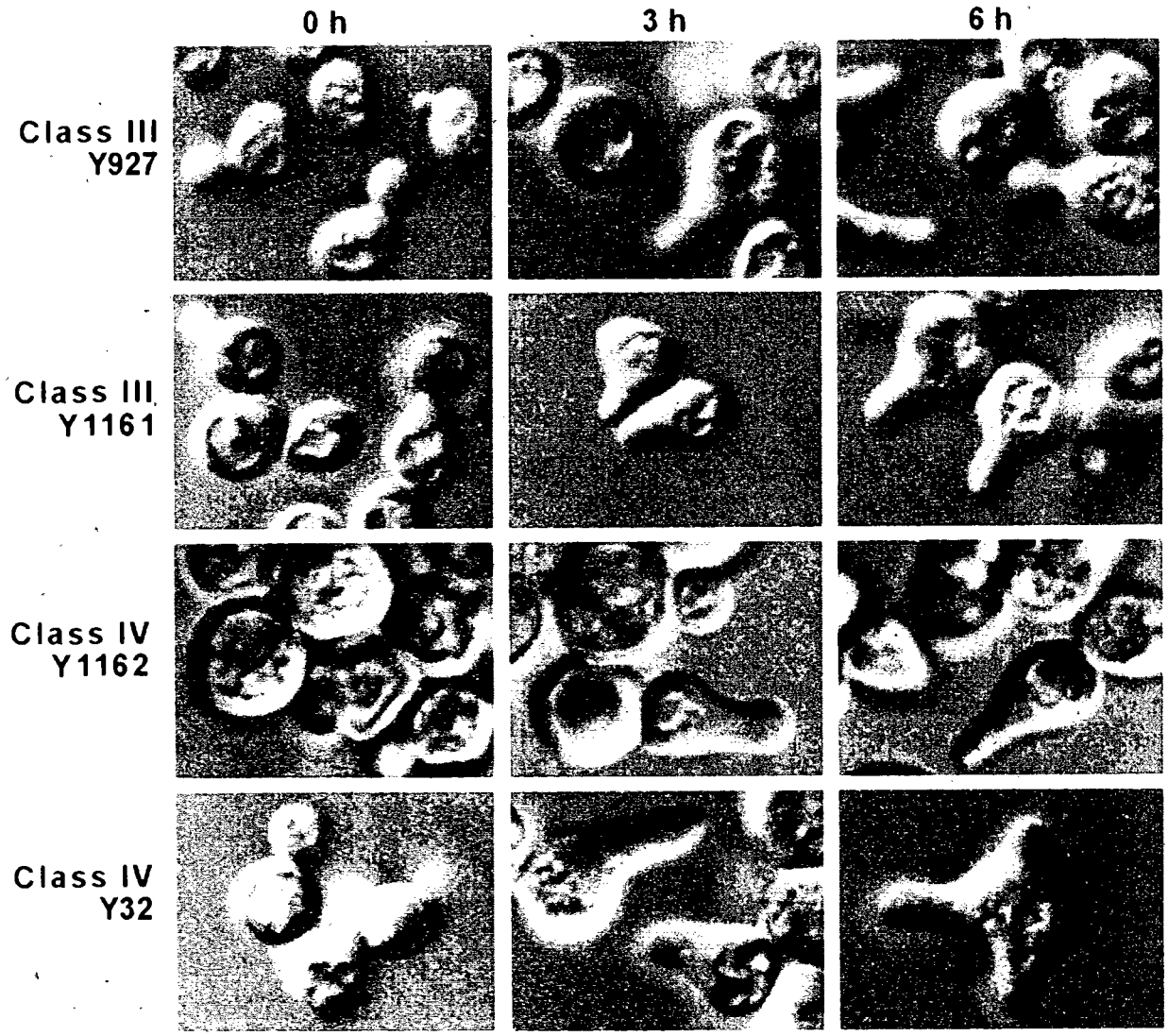

0 h
3 h
6 h
Class III
Y927
Class III
Y1161
Class IV
Y1162
Class IV
Y32

Figure 8. Temperature-sensitive or cold-sensitive growth of mutants.

Cells were grown on rich medium at the indicated temperatures until the wild-type cells (SY2625) had formed colonies 2-3 mm in diameter. The Class III mutants, Y1093, Y37, Y932, Y927, and Y1161, and the Class IV mutants, Y1162 and Y32, were tested for growth defects at various temperatures. Y927 serves as a control for these mutants and its lack of growth defects indicates that defects in shmooing or in G_1 arrest do not necessarily lead to defects in vegetative growth.

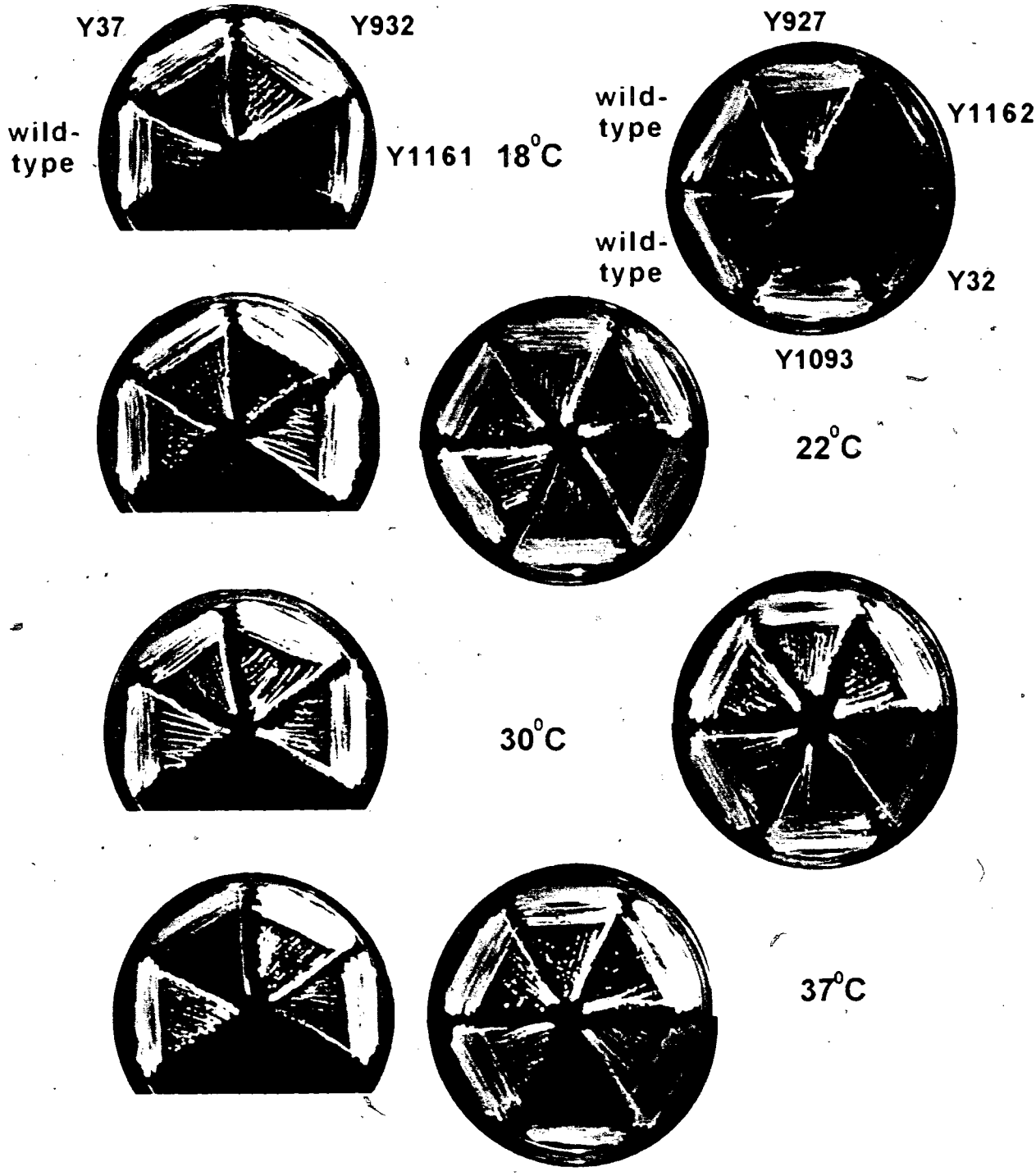

Y37
Y932
wild-
type
Y1161
18°C
Y927
wild-
type
Y1162
wild-
type
Y32
Y1093
22°C
30°C
37°C

Figure 9. Y935 contains a mutant allele of *FAR1* and Y932 is mutated in *FUS3*.

(*A*) Mating assays, pheromone spot assays (PSA), and mating projection (shmoo) assays were performed, as described in Materials and Methods, on Y935 transformed with empty vector (top panels) or transformed with p*FAR1* (p8; bottom panels). (*B*) These assays were also done with strain Y932 transformed with vector (top panels), with p*FUS3* (p68; middle panels), or with the wild-type strain, SY2625 (mating only), with Y932 (not transformed; mating only) and with the *fus3Δ::URA3* mutant, Y583 (bottom panels).

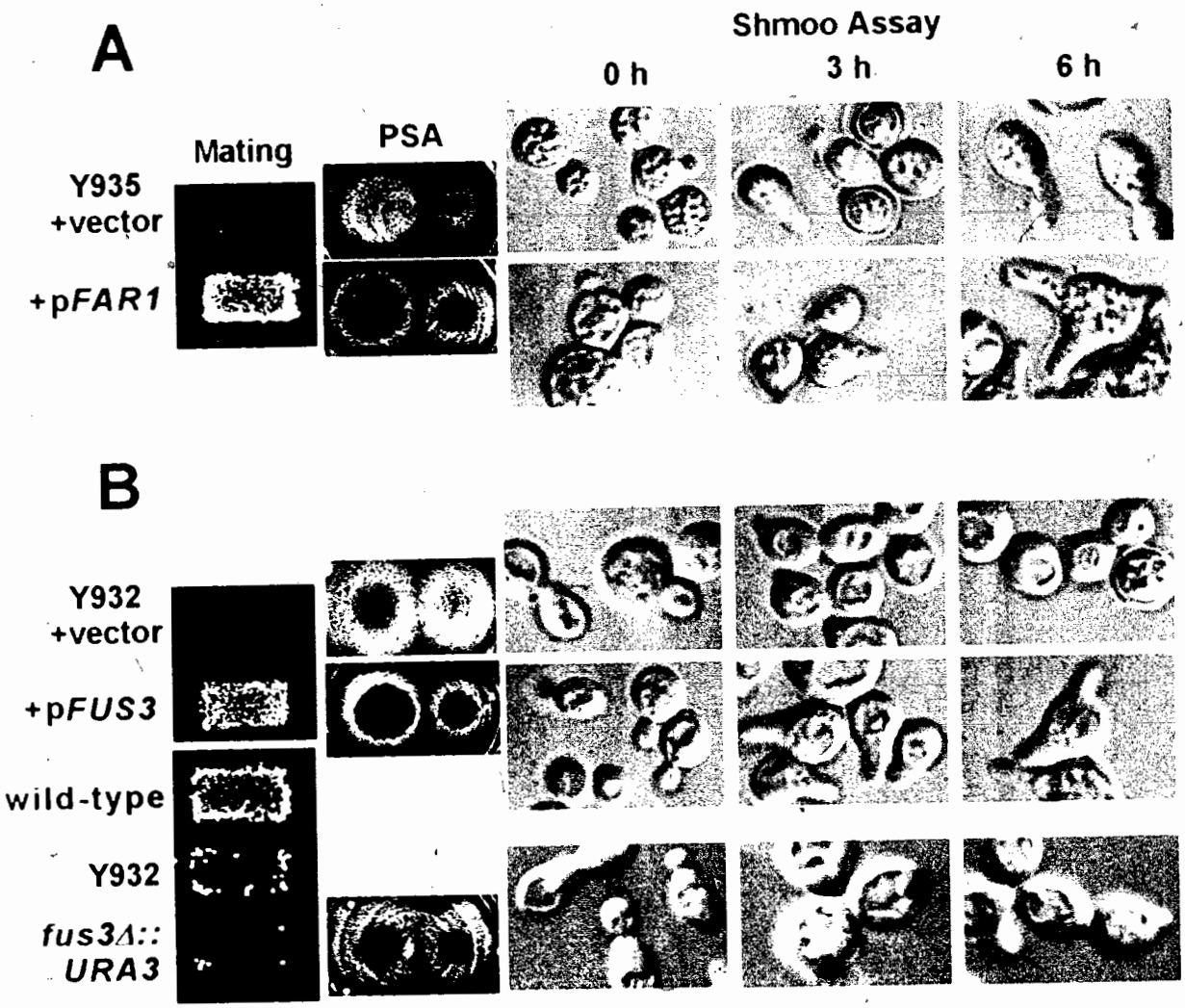
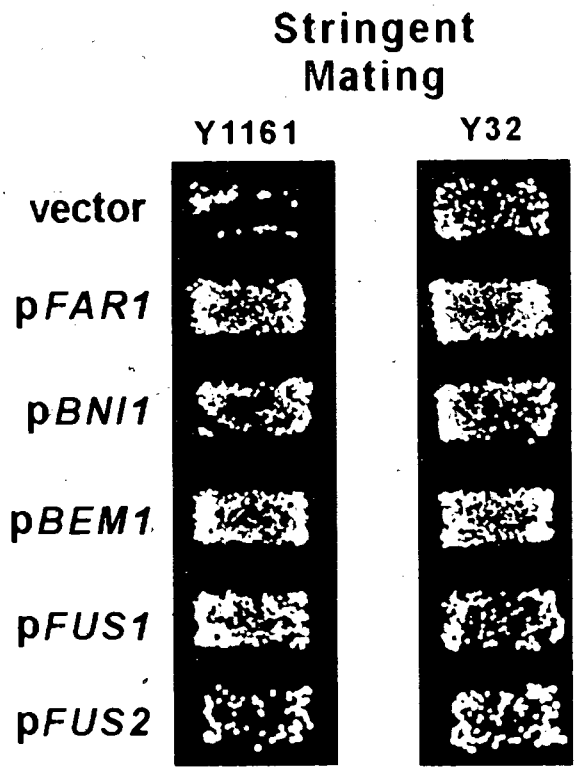

A
Shmoo Assay
0 h
3 h
6 h
Mating
PSA
Y935
+vector
+pFAR1
B
Y932
+vector
+pFUS3
wild-type
Y932
fus3Δ::
URA3

Figure 10. Partial rescue of the mating defects of mutant strains Y1161 and Y32.

Strains Y1161 and Y32 were transformed with plasmids p*FAR1* (p8), p*BNI1* (p224), p*BEM1* (p61), p*FUS1* (p67) and p*FUS2* (p77). Mating assays were performed, as described in the Materials and Methods, on the transformants to test for complementation of the mating defects of Y1161 and Y32 with these genes.


Stringent
Mating
Y1161
Y32
vector
pFAR1
pBNI1
pBEM1
pFUS1
pFUS2

defect and clumpy appearance (Fig. 7) that was tightly linked to its mating defect. Y1162 also had a cold-sensitive growth defect (Fig. 8). Y30 had a weak mating defect (Fig. 4) and a temperature sensitive growth defect associated with the mating defect (data not shown). Y31 showed only a mating defect (Fig. 4).

The mutants in Groups III and IV were transformed with plasmids containing genes known to affect G1 arrest, morphogenesis and cell fusion (*FAR1*, *BEM1*, *PEA2*, *SPA2*, *FUS1*, *FUS2*, *FUS3*, *RVS161*, *BNI1*, *RVS167*, *SFU1*, *MFA2*, *STE14*, *RAM1*, *RAM2*, *STE6*). The mating defect of Y1161 appeared to be partly suppressed by *FAR1*, *BEM1* and *FUS1* (Fig. 10). Similarly, mating in Y32 also seemed to improve with an extra copy of *FAR1* or *BEM1* (Fig. 10).

a-Factor Mutants

Group V strains secreted low levels of bioactive **a**-factor (Fig. 11). One mutant, 10-2-39-1, had such a severe mating defect that I was not able to backcross it. This mutant also has a severe growth defect, so the reduced secretion of **a**-factor may be due to the slow growth. The defect in another mutant, Y48, did not segregate as a single mutation. Y48 was not examined further. The mating defects in this group of strains were evident only in *MAT***a** cells, and α-factor secretion was unaffected (for example, see Chapter 4, Fig. 18). The mutations segregated as single loci. This analysis was complicated by the **a**-specific nature of these defects, but half of the *MAT***a** spores from each cross had mating defects. All of the strains tested had recessive mutations. Therefore, we transformed into these strains plasmids carrying all of the genes known to be involved in processing and secreting **a**-factor.

The defects in four mutants were complemented by *RAM1* (Fig. 12A), four were complemented by *STE6* (Fig. 12B), and one was complemented by *STE14* (Fig. 12C). *RAM1* on a plasmid (Fig. 12A) or integrated into the genome (Fig. 12D) also boosted mating and **a**-factor secretion in wild-type cells, but none of the other genes did so. This observation raised the possibility that *RAM1* complementation could occur in non-*ram1* mutants. However, linkage analysis revealed that Y39, Y40, Y43 and Y46 were all *ram1* mutants. Two remaining mutants,

Figure H. Mating and **a**-factor defects of Class V mutants.

Mating and **a**-factor assays were performed using Class V mutants. Assays were done as described in the Materials and Methods. For the **a**-factor assays, cells were patched onto a lawn of *MATα* cells (SY2014) that are hypersensitive to **a**-factor. Cells of the lawn surrounding the patch arrest and fail to grow, leaving a clear zone. The size of this clear zone is an indication of the quantity of pheromone exported by cells in the patch.

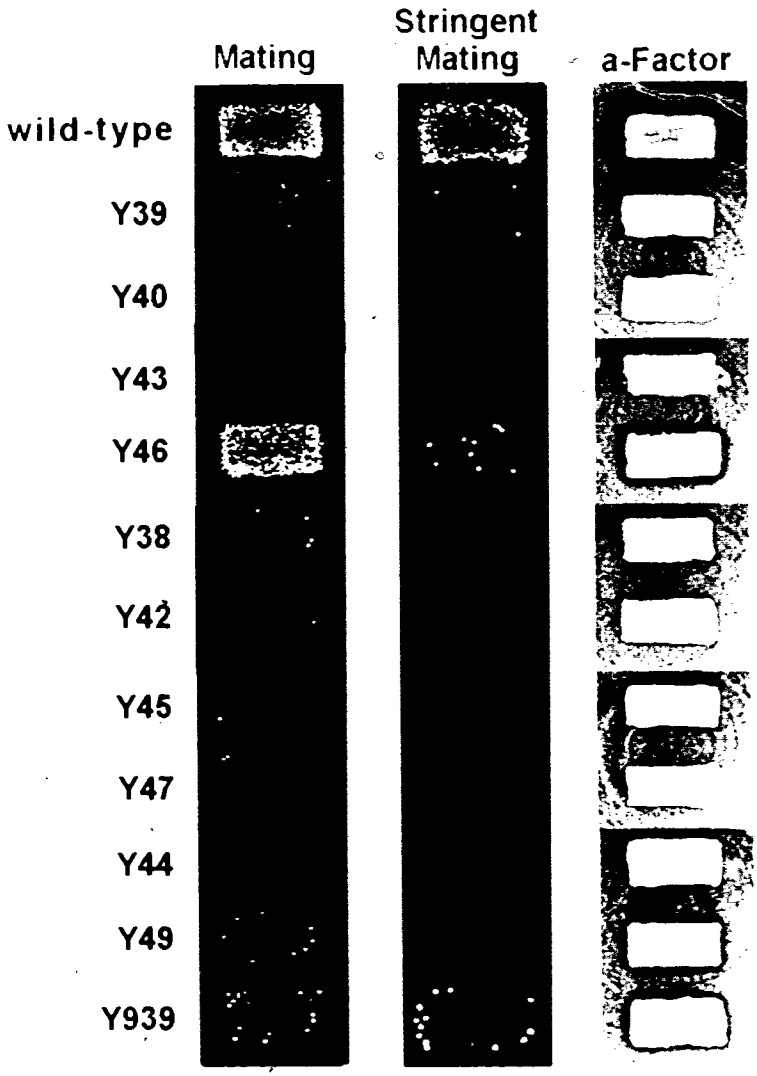
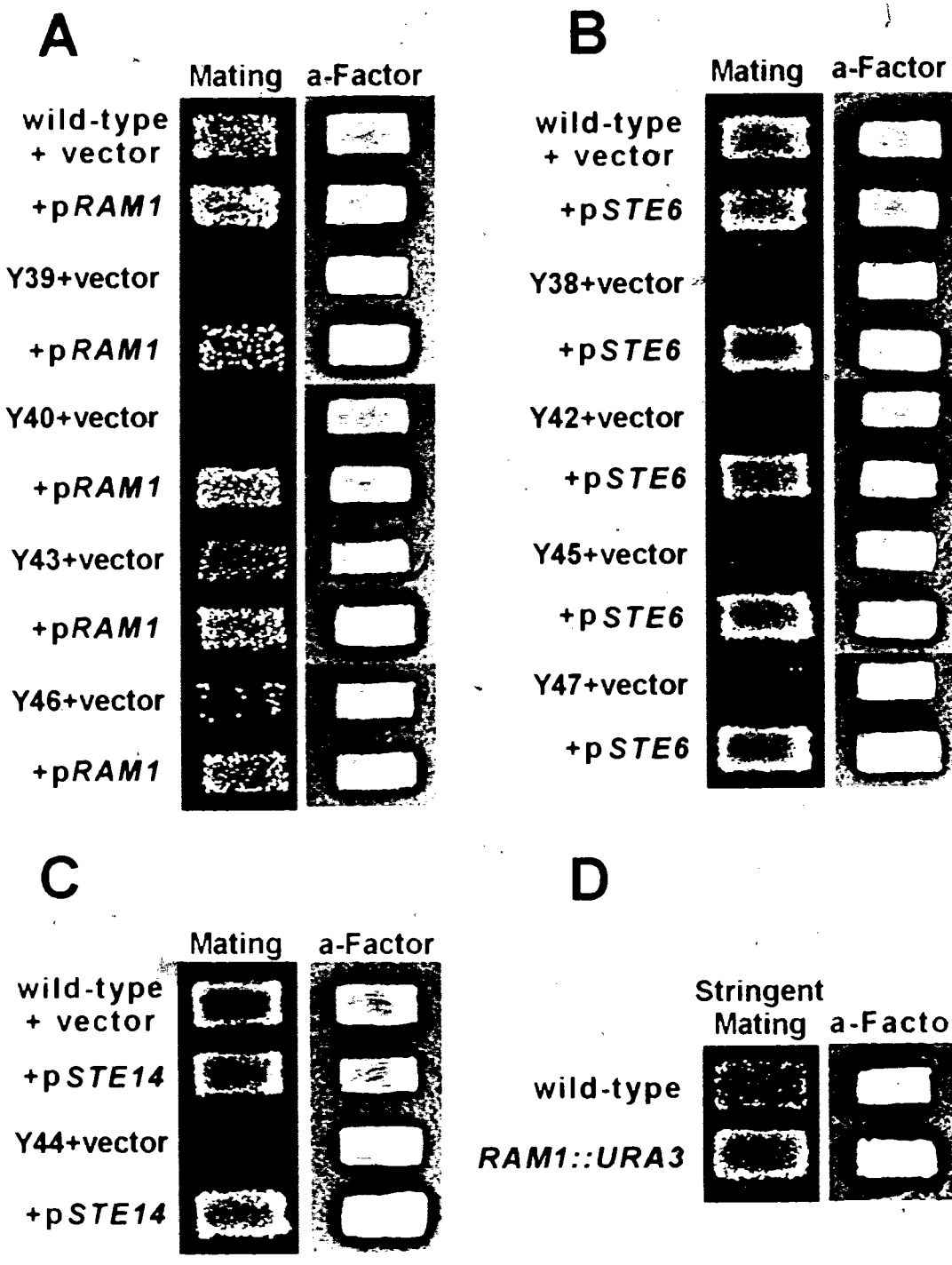

Mating
Stringent Mating
a-Factor
wild-type
Y39
Y40
Y43
Y46
Y38
Y42
Y45
Y47
Y44
Y49
Y939

Figure 12. Complementation of Class V mutants with genes required for **a**-factor secretion. Mutants were transformed with plasmids (*A*) p*RAM1* (p25), (*B*) p*STE6* (p9), or (*C*) p*STE14* (p3). Mating and pheromone secretion in these tranformants were compared to mutants transformed with empty vector. The wild-type strain, SY2625, was also transformed with these plasmids and empty vector and assayed for mating and **a**-factor production. (*D*) *RAM1* was integrated into the parental strain SY2625. The resulting strain, Y121 (*MAT***a** *RAM1::URA3*), was tested for its ability to mate and secrete **a**-factor and compared to the wild-type strain, SY2625.


A
Mating
a-Factor
wild-type + vector
+pRAM1
Y39+vector
+pRAM1
Y40+vector
+pRAM1
Y43+vector
+pRAM1
Y46+vector
+pRAM1
B
Mating
a-Factor
wild-type + vector
+pSTE6
Y38+vector
+pSTE6
Y42+vector
+pSTE6
Y45+vector
+pSTE6
Y47+vector
+pSTE6
C
Mating
a-Factor
wild-type + vector
+pSTE14
Y44+vector
+pSTE14
D
Stringent
Mating
a-Factor
wild-type
RAM1::URA3

Y49 and Y939, are both defective in the *STE22/AXL1* gene. Y49 was cloned as described in Chapter 3, and the Y939 mating and pheromone defects were complemented by a plasmid bearing *STE22/AXL1*.

Discussion

Many other mutant screens performed in other laboratories had narrow parameters and isolated mutants affecting specific aspects of mating. For example, some screens concentrated on mating projection morphogenesis (Chenevert et al., 1994). Several have screened specifically for mutants defective in cell fusion and karyogamy (Kurihara et al., 1994; Polaina and Conde, 1982). Another was designed to isolate mutants defective in **a**-factor secretion (Fujimura-Kamada et al., 1997). Despite the small size of my mutant screen, the simplicity of it allowed me to uncover a wide variety of mutations affecting many diverse aspects of yeast conjugation. Mutants were found that affected **a**-factor secretion, pheromone signaling, G_1 arrest, cellular morphogenesis, and other mating processes. Unlike other screens, I did not attempt to isolate specific classes of mutants. Furthermore, I included mutants that had very weak, but reproducible mating defects.

This screen was similar in breadth to that first conducted in Léland Hartwell's laboratory, in which many of the *ste* (sterile) mutants were isolated (Hartwell, 1980). The major distinction here is that my screen theoretically precluded isolation of many of the *ste* mutations which affect pheromone signaling. I required that the cells be competent for signaling in order to grow. Therefore, while I did not restrict the screen to a certain class of mutants, I did attempt to exclude mutants that adversely affect the pheromone response pathway. We designed our screen this way because the components of this signaling pathway have been intensively studied, and there was little likelihood of contributing more to this field.

My efforts to exclude signaling mutants were somewhat successful, since only four mutants were recovered that had severe signaling defects in our assays. Two of these mutants grew on minimal medium lacking histidine even in the absence of pheromone. One of these two grew just as well as it did on rich medium and was likely either a *MATα* contaminant of a *HIS3*

strain, a bypass mutant of the *his3* mutation, or a revertant. It is unlikely that the strain could have been a revertant in the normal sense since the *FUS1-HIS3* construct is integrated at the *his3* mutant locus of SY2625. However, it is possible that a mutation in the promoter of this construct could cause constitutive expression of the wild-type *HIS3* gene. The second of these two mutants displayed the odd phenotype of being able to grow very slowly on minimal medium lacking histidine, but was unable to grow at all on rich medium. It is possible that this mutant prefers a low pH, since synthetic media is acidic and rich medium has neutral pH. The other two mutants were probably true signaling mutants with leaky alleles since they were able to respond to pheromone, but not very well. The leakiness of the phenotypes possibly allowed these mutants to grow slowly on the mutagenized plates.

Far1p is a Fus3p activated inhibitor of Cln-Cdc28p complexes and mediates G_1 arrest in response to pheromone (Chang and Herskowitz, 1990; Peter et al., 1993; Peter and Herskowitz, 1994). The defects for G_1 arrest in the mutants of Group II are consistent with mutations in *FAR1*. *far1* mutants also have defects in projection formation, presumably due to the inability to arrest (Chang and Herskowitz, 1990). Y935 is likely a *far1* mutant which displays all of the characteristics of a *far1* null allele.

The arrest, mating and shmooing phenotypes of Y932 are all consistent with a *fus3* mutation. Fus3p phosphorylates and activates Far1p as well as mediating the transcriptional regulation of genes required for mating (Chang and Herskowitz, 1992; Elion et al., 1993; Tyers and Futcher, 1993). Therefore, they are expected to have defects in all aspects of mating, as does Y932. A *fus3Δ::URA3* mutant has all of the same phenotypes as Y932 and *FUS3* rescues these defects in this mutant. Moreover, the mating defect in this mutant is closely linked to *fus3*.

Mutants that have only shmoo defects, like those in Group III, are expected to affect the actin cytoskeleton or secretion. The actin cytoskeleton is polarized at sites of cell growth, and directs secretion of membrane and cell wall components to this region (Cid et al., 1995; Govindan and Novick, 1995). The finding that 5 of the 9 mutants in groups III and IV have associated growth defects is consistent with defects in the cytoskeleton or vesicular transport. Such proteins

are expected to be used for polarized growth during projection formation as well as vegetative growth. In fact, this screen should specifically affect proteins that are more important for projection formation or produce alleles of these proteins that have specific defects in the mating pathway. Possible examples of potential targets are proteins that link activated pheromone receptors to the cytoskeleton or proteins that are redundant during vegetative growth but not during shmoo growth.

The Group IV mutants, did not have any serious morphological defects but could affect processes not assayed in the screen, such as cell fusion and karyogamy. This group is likely to represent a variety of mutations. The mutations in two mutants, Y34 and Y1162 may be related to chitin synthase function. These mutants display cell separation defects similar to that found in mutations of chitin synthase 2 (Chs2p) or its regulators (Sburlati and Cabib, 1986; Shaw et al., 1991; Cabib et al., 1993; Cid et al., 1995). This enzyme produces the chitin layer of the primary septum between mothers and buds. A chitinase, encoded by *CTS1*, digests the primary septum during cell separation (Cid et al., 1995). Absence of this layer of chitin prevents cell separation. As you might expect, *cts1* mutants have a similar cell separation defect (Cid et al., 1995). The mutants were also unable to shmoo effectively, a process in which chitin incorporation into the cell wall is increased. Chs3p is responsible for the high chitin content in walls of mating projections and *CHS2* is pheromone-inducible, but neither *chs3* nor *chs2* mutants have any apparent mating defects (Cid et al., 1995). The mating defects in Y1162 and Y34 cells may simply be due to topological constraints on the cells because only cells on the outside of clumps can come into contact with cells of the opposite mating type.

I transformed into the class II and IV mutants various plasmids containing genes known to be involved in shmoo formation and cell fusion. Polarized growth during mating requires proteins, such as Spa2p and Pea2p since mutations in these genes lead to defects in projection formation (Snyder, 1989; Gehrung and Snyder, 1990; Chenevert et al., 1994; Valtz and Herskowitz, 1996). Curiously, these proteins are also necessary for the diploid budding pattern but not for axial budding (Valtz and Herskowitz, 1996; Zahner et al., 1996). *RVS161* and

RVS167 were found to be synthetically sterile with *sst2* (T. Favero and C. Boone, unpublished) and, oddly enough, also confer random budding on diploids homozygous for mutations in these genes (Sivadon et al., 1995; Zahner et al., 1996). *BEM1* was first isolated as a gene required for bud emergence in all cells, but some alleles are specifically defective in shmooing (Chenevert et al., 1994). Similarly, some alleles of *CDC24*, also required for bud emergence, affect only mating projection formation (Chenevert et al., 1994). Far1p also has a role in mating, separable from its arrest function, which I will discuss further in Chapter 2. *FUS1*, *FUS2* and *FUS3* have unspecified roles in cell fusion. *BNI1* and *SFU1* are also needed for morphogenesis during mating and will be discussed in Chapter 6. The apparent partial suppression of both Y1161 and Y32 by *BEM1* and *FAR1* is intriguing because these proteins interact with each other (Lyons et al., 1996). Perhaps the normal products of the genes that are mutated in Y1161 and Y32 also interact with these proteins. Surprisingly, Y1161 can undergo haploid invasive growth, whereas the wild-type parent, SY2625, cannot (data not shown). The invasive growth of Y1161 is not, however, accompanied by filamentous growth. Wild-type cells can be induced to invade agar by exposing them to pheromone, implying that Y1161 is somehow constitutively signaling to components that regulate agar invasion (and perhaps is a downstream component of nutrient sensing molecules). The nature of the mating defect in the class IV mutants is puzzling since they have no obvious shmoo deficiency. Perhaps they are defective for fusion or for proper orientation of the shmoos in gradients (chemotropism).

I obtained mutants in many, but not all of the genes involved in **a**-factor biogenesis. of the 14 mutants in Group V, 4 were complemented by plasmids bearing *STE6*. *ste6* mutants seemed to represent a high proportion of the mutants, probably because its open reading frame (ORF) is large (3.8 kb). On the other hand, no *MFA1* mutants were obtained (*MFA2* is disrupted in SY2625), probably due to the very small size of this ORF (108 bp). The most numerous mutants were those complemented by *RAM1* (5 mutants). This ORF is not particularly large (2.0 kb), which begs the question of why it is so over-represented in my screen. The answer probably lies in the observation that SY2625 appears to have a mild *ram1* mutation. In comparison to strains

with other genetic backgrounds, SY2625 secretes low amounts of bioactive **a**-factor (data not shown). Moreover, transformation of *RAM1* plasmids into SY2625 boosts **a**-factor secretion (Fig. 12A). Integrating *RAM1* so that it is in single copy also enhances mating and **a**-factor secretion in the wild-type strain (Fig. 12D). Finally, in searches for high dosage suppressors of SY2625-derived mating mutants, members of our lab often find that *RAM1* can boost mating in many mutants, but not to wild-type levels. The possibility that SY2625 is already compromised for Ram1p function implies that mutations in *RAM1* that would otherwise have little effect on the function of this enzyme are able to severely impair its activity in SY2625; hence, *ram1* mutants will be more common.

Some of the mutants uncovered in this screen are in new genes, or in genes that were not known to have a role in mating. These mutants are discussed further in the following chapters. Even some of the mutants in known genes may prove useful in extending knowledge of their functions. Recovering the mutant alleles for some of these may prove useful. Alternatively, some of these mutants could be used in further mutant screens, or in library screens to find genetically interacting proteins. In addition, the wide array of mutations and the conspicuous absence of some well known proteins involved in mating indicates that the mutagenesis was far from being saturated. There are probably many more genes involved in yeast conjugation waiting to be uncovered.

CHAPTER 2
Red Herrings

Introduction

The Far1p cyclin-dependent kinase inhibitor (cki) mediates G_1 arrest during mating (Chang and Herskowitz, 1990). This protein is phosphorylated and activated by the MAP kinase, Fus3p, in response to pheromone (Chang and Herskowitz, 1992; Elion et al., 1993). Active Far1p binds to, and inhibits the activity of the cyclin-dependent kinase (cdk) Cdc28p complexed with the G_1 cyclins Cln1p or Cln2p (Peter et al., 1993; Peter and Herskowitz, 1994). Cells carrying *far1* null alleles are unable to arrest in response to pheromone and continue dividing their nuclei (Chang and Herskowitz, 1990). These cells also have abnormal mating projections, likely because the cells continue to attempt to bud while shmooing (Chang and Herskowitz, 1990). These null mutants have severe mating defects (Chang and Herskowitz, 1990).

The role in mating of the farnesyltransferase β-subunit, Ram1p, is in the maturation of the *MAT***a** mating pheromone, **a**-factor. The first step in pro-**a**-factor maturation is its farnesylation and subsequent targeting to membranes (Goodman et al., 1990; Schafer et al., 1990; He et al., 1991; Chen et al., 1997). The subsequent events in processing and secretion all occur on membranes and are dependent on farnesylation of pro-**a**-factor (Chen et al., 1997). Pro-**a**-factor farnesylation is directed by a CaaX box motif at the carboxy-terminus of the peptide (Moores et al., 1991). In addition to **a**-factor, CaaX boxes are found in many of the small GTPases that regulate nuclear import, vesicle trafficking, and growth in yeast and higher organisms (Schafer et al., 1990; Finegold *et al.*, 1991; Rossi *et al.*, 1991). Moreover, the γ subunits of heterotrimeric G proteins are prenylated (Whiteway and Thomas, 1994). In yeast the Gγ subunit of the G protein associated with the pheromone receptors is farnesylated by Ram1p/Ram2p (Whiteway and Thomas, 1994). In light of the large number of proteins that are farnesylated in yeast, some of

which are crucial to cellular processes, it is surprising that the only severe effect associated with *ram1* mutants seems to be in **a**-factor secretion.

In this study, I have cloned two mutants from my mutagenic screen that were defective in the *FAR1* and *RAM1* genes, but did not display the phenotypes expected for such mutations.

Materials and Methods

Strains, Plasmids, Media and Microbiological Techniques

The strains used in this study are shown in Table 4. Plasmids used are described in Table 5. Media and genetic methods that were used are described (Guthrie & Fink, 1991; Rose et al., 1990) and in Chapter 1. Mating and other assays are also described in Chapter 1. Methods used to manipulate DNA are as outlined (Sambrook et al., 1989). Transformation of yeast cells was by the lithium acetate method (Ito et al., 1983). Mating and **a**-factor production assays were performed as described in Chapter 1.

Strain Y29 (*MATα FAR1::URA3*) was used for linkage analysis with Y36, and strain Y123 (*MATα RAM1::URA3*) was used in linkage analysis with Y30. Strains Y62, Y63 or Y66 were used in mating assays. SY2014 was used as the pheromone hypersensitive strain for **a**-factor assays.

Cloning Y30 and Y36

The mutant strains were transformed with a yeast genomic library (Boone et al., 1993) in the plasmid pRS316, which is a yeast shuttle vector having the *URA3* gene for selection in yeast and the ampicillin resistance gene for selection in *Escherichia coli* (Sikorski and Hieter, 1989). The cells were spread onto SD plates lacking uracil to give a density of approximately 200 colonies/plate. Once the colonies reached a sufficient size, they were scored for complementation of the mating defect by mating to Y63. The mating defect of Y30 was so weak that a weakly mating *MATα* tester strain, Y66 (*far1-c*), was used to clone this mutant (Chenevert et al., 1994).

Table 4. Strain List for Chapter 2.

Strain	Genotype	Source
SY2014	*MATα ste3Δ306::LEU2 sst2Δ ste2Δ mfa1Δ mfa2Δ::FUS1-lacZ FUS1::HIS ura3-52 leu2-3, 112 ade1*	C. Boone
Y62	*MAT***a** *lys1*	I. Herskowitz
Y63	*MATα lys1*	I. Herskowitz
Y66	*MATα far1-c lys1*	I. Herskowitz
Y29	*MAT***a** *FAR1::URA3 his3::FUS1-HIS3 mfa2Δ::FUS1-lacZ ura3-1 leu2-3,-112 trp1-1 ade2-1 can1-100*	N. Adames
Y123	*MATα RAM1::URA3 bar1Δ::LEU2 lys2 his3::FUS1-HIS3 mfa2Δ:FUS1-lacZ trp1-1 ade2-1 can1-100*	N. Adames
Y36	*MAT***a** *far1 his3::FUS1-HIS3 mfa2Δ::FUS1-lacZ ura3-1 leu2-3,-112 trp1-1 ade2-1 can1-100 bar1Δ*	N. Adames
Y30	*MAT***a** *ram1 his3::FUS1-HIS3 mfa2Δ::FUS1-lacZ ura3-1 leu2-3,-112 trp1-1 ade2-1 can1-100 bar1Δ*	N. Adames
Y1163	*MATα ram1 his3::FUS1-HIS3 mfa2Δ::FUS1-lacZ ura3-1 leu2-3,-112 trp1-1 ade2-1 can1-100 bar1Δ*	N. Adames

Table 5. Plasmid List for Chapter 2.

Plasmid	Construction	Source
p8	*FAR1* in pRS316 (*CEN ARS URA3*)	G. Sprague
p49	cloned genomic fragment containing *FAR1* in pRS316 (*CEN ARS URA3*)	N. Adames
p50	cloned genomic fragment containing *FAR1* in pRS316 (*CEN ARS URA3*)	N. Adames
p25	*RAM1* in YCp19 (*CEN ARS URA3*)	F. Tamanoi
p59	cloned genomic fragment containing *RAM1* in pRS316 (*CEN ARS URA3*)	N. Adames
p60	cloned genomic fragment containing *RAM1* in pRS316 (*CEN ARS URA3*)	N. Adames

Colonies that mated were tested to determine if the mating increase was plasmid-dependent and not due to reversion of the mutations. These transformants were plated onto 5-fluoro-orotic acid (FOA) to cure the cells of plasmids. FOA is lethal to cells expressing the *URA3* product due to its conversion by this enzyme to a toxic compound. Transformants that lost the ability to mate after plating on FOA (because they had lost the complementing plasmid) were then examined further.

Plasmids were isolated from transformants by extracting total DNA and transforming it into *E. coli* to amplify plasmid DNA (Hoffman and Winston, 1987). Minipreps and test restriction digests were performed on 6-12 transformants. Plasmids containing an insert were transformed again into the mutant strain to test for complementation of the mating defect.

Physical Mapping of Clones

Fragments from the cloned complementing plasmids were isolated, Genecleaned (BIO101) and labeled with horse radish peroxidase (HRP) using a chemiluminescent nucleic acid labeling kit (Amersham) for use as probes.

These probes were hybridized to a blot containing ordered λ phage clones covering most of the yeast genome (constructed by M. Olson and available through ATCC) using standard methods for hybridization and washing (Sambrook et al., 1989). A control probe included with the genomic blot was labeled the same way and included in the hybridization. Probes were visualized as instructed in the chemiluminescent kit.

Sequencing

The ends of the insert in the smallest complementing subclone from Y30 were sequenced. This plasmid was prepared for sequencing from *E. coli* strain DH5α using a modified version of the alkaline-lysis miniprep method (Sambrook et al., 1989). Before precipitation of the DNA the samples were treated with 50 μg of RNAse A for 1h, and protein was phenol-chloroform extracted. After ethanol precipitation the DNA was further purified by polyethylene glycol (PEG)

precipitation. Sequencing was performed by the University of Calgary Core DNA Services. The ends of subclones were sequenced using primers complementary to flanking regions in the vector.

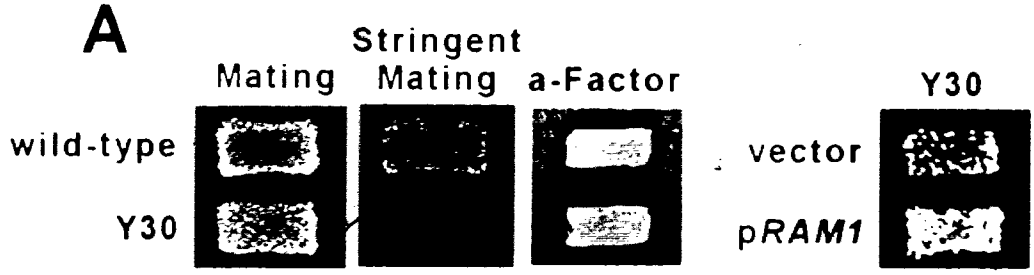
Results

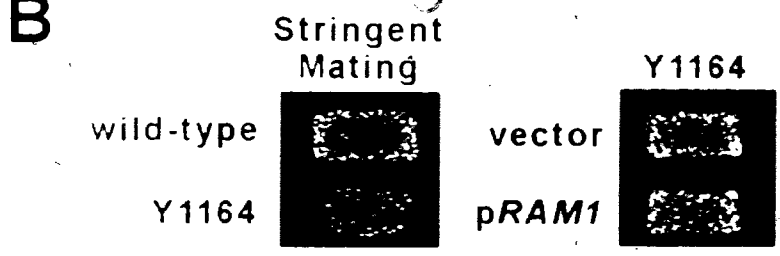
Y30 is a ram1 Mutant

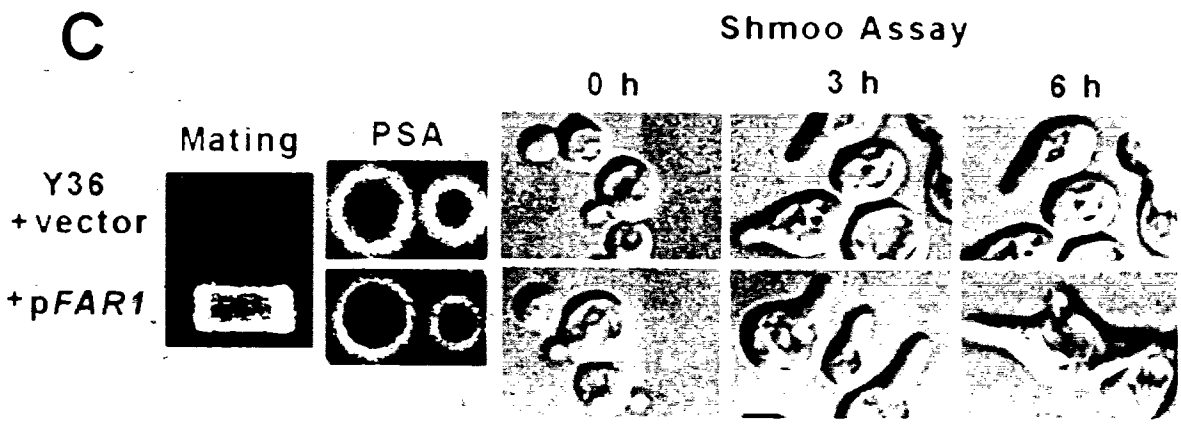
The mutant Y30 had a weak mating defect that was the result of a single recessive mutation. Two clones, p59 and p60, were found to complement the mating defect of this strain. These clones were 7.8 kb and 9.8 kb in size, respectively, and according to their restriction maps shared a common region of 6.0 kb. A fragment of the insert was used to probe a yeast genomic blot to physically map this region. This fragment hybridized to three overlapping λ clones, from strongest to weakest hybridization, λPM4114 (ATCC 70256), λPM6605 (ATCC 70751), and λPM6235 (ATCC 70700). These clones are located on the left arm of chromosome IV close to the centromere.

I made subclones and deletions of p59 and tested these for complementation of the mating defect of Y30. The restriction map of the smallest complementing clone was compared to maps of the known genes in this region, deduced from the sequences of these genes. The restriction map appeared to correspond to that for *RAM1*, but there were a few discrepancies in the positions of sites. Sequencing of the ends of this clone put to rest any doubts, because the sequences were identical to those flanking *RAM1* on either side. Plasmids used to test the **a**-factor mutants in Chapter 1 were transformed into this strain to test for complementation of the mating defect. Only those containing *RAM1* alleviated the defect (Fig. 13A). Upon closer inspection of Y30, there was a very subtle defect in **a**-factor secretion that was rescued by *RAM1* (Fig. 13A). This was unexpected because this mutant did not have an **a**-specific mating defect. *MAT*α segregants from the backcross that gave Y30 showed weak, but reproducible mating defects that were alleviated by *RAM1* (Fig. 13B). Finally, linkage analysis confirmed that the mutation in Y30 was in *RAM1*.

Figure 13. Rescue of Y30 with a plasmid carrying *RAM1*, and of Y36 with *FAR1*. (*A*) Mating and pheromone assays were performed on Y30 with empty vector or with p*RAM1* (p25). (*B*) A *MAT*α segregant from Y30 (Y1163) also shows a mating defect. (*C*) The mating, G_1 arrest and shmoo defects of Y36 were complemented by *FAR1*. Assays using Y36 transformed with vector (top panels) are compared to those using Y36 transformed with p*FAR1* (p8; bottom panels).


A
Mating
Stringent Mating
a-Factor
wild-type
Y30
Y30
vector
pRAM1


B
Stringent Mating
Y1164
wild-type
Y1164
vector
pRAM1


C
Shmoo Assay
0 h
3 h
6 h
Mating
PSA
Y36
+vector
+pFAR1

Y36 is a far1 Mutant

Mutant Y36 had a mild "halo fill-in" phenotype in which a halo of the proper dimensions was visible but cells were growing slowly within the perimeter of the halo, making the normally clear zone of arrest look turbid. Backcrossing the original mutant indicated that the mutant phenotype is due to a single recessive mutation. I recovered from the genomic library two plasmids of 8.0 kb (p49) and 7.5 kb (p50) that rescued the mating defect of this mutant. According to their restriction maps, the inserts in these plasmids were nearly identical. These restriction maps bore a remarkable resemblance to the restriction map of *FAR1* and *FAR1* did, indeed, complement all of the defects of Y36 (Fig. 13C). Linkage analysis with Y29 confirmed that Y36 is an allele of *FAR1* and is not simply suppressed by an extra copy of this gene.

Discussion

At the time that I cloned *FAR1*, the existence of a *far1* allele that did not produce a defect in G_1 arrest but did result in a mating defect was surprising. However, since this time these types of alleles have been well characterized (Chenevert et al., 1994; Valtz et al., 1995). Alleles of *far1* that were first isolated, such as those discussed in Chapter 1, had both mating and arrest phenotypes. The defect in mating of *far1* mutants is not a result of the failure to G_1 arrest because there are *far1* mutants which retain the ability to mate but are unable to arrest (Peter et al., 1993). T[redacted]utants have only a mild mating defect, probably due to the defect in projection formation (see below). The mutations in these alleles reside in the amino-terminus of the protein (Peter et al., 1993; Valtz et al., 1995). The amino-terminal portion of Far1p is necessary and sufficient for G_1 arrest (Peter et al., 1993; Valtz et al., 1995).

There also exist *far1* alleles, like Y36, that are defective in mating but normal (or nearly so in Y36) for arrest and shmoo formation (Valtz et al., 1995). These Arrest+ Mating- alleles carry mutations in the carboxy terminus and within an amino-terminal LIM domain (a zinc-binding

motif involved in protein-protein interactions; Valtz et al., 1995) Interestingly, alleles of each type are able to complement each other to rescue both mating and G_1 arrest (Valtz et al., 1995).

These fascinating alleles of *FAR1* demonstrate that G_1 arrest, and efficient projection formation are not absolute requirements for mating although they do affect the process. It is not clear how the failure to arrest affects the formation of shmoos; however, *far1* mutants do eventually form shmoos with unusual morphologies due to continued budding of the cells (see Fig. 5; Chang and Herskowitz, 1990). Perhaps the defect in projection formation seen in *far1* mutants is due to competition between the machinery for shmoo formation and for bud formation, which use many of the same proteins (Chenevert, 1994). Consistent with this interpretation is the observation that the severity of shmoo defects in my *far1* mutants corresponded to the severity of the arrest defects. Y935 had severe shmoo and arrest defects (Chapter 1, Fig. 5) but Y36 had mild defects in both assays (Chapter 1, Fig. 6).

The mechanism of pheromone-induced G_1 arrest is well understood. However, the role of Far1p in mating is not. It is known that functional Far1p is required for orienting mating projections towards a source of pheromone (chemotropism), which is presumably important for efficient mating (Dorer et al., 1995; Valtz et al., 1995). This function in chemotropism is independent of Far1p's role in arrest (Dorer et al., 1995; Valtz et al., 1995). Arrest+Mating- *far1* alleles are unable to orient projections towards a needle filled with α-factor and instead shmoo in random directions (Valtz et al., 1995). These cells shmoo at their presumptive bud sites as shown by the observation that zygotes of these mutants almost always fuse next to the previous bud site (at the axial position) (Dorer et al., 1995; Valtz et al., 1995). Furthermore, while these mutants have low mating efficiencies, addition of high levels of exogenous pheromone to these cells does not further inhibit mating as it does in wild-type cells (Dorer et al., 1995). This lack of mating inhibition by exogenous pheromone is similar to that seen in strains hypersensitive to pheromone and is an indication that the mutant strains are already defective for chemotropic orientation of projections and are using the incipient bud sites in a default mating pathway (Dorer et al., 1995).

How Far1p is involved in reorienting the polarity establishment proteins from axial bud sites to sites experiencing higher pheromone concentrations is a mystery. It is possible that Far1p is involved in erasing the axial bud sites as sites for cell growth. Consistent with this possibility is the observation that *far1* cells exposed to low levels of pheromone continue to bud axially, but wild-type cells switch to a bipolar budding pattern (Dorer et al., 1995). Alternatively, Far1p could promote chemotropic growth and over-ride the bud site selection machinery. The molecular mechanism for the role of Far1p in promoting chemotropic growth may involve the bud emergence protein, Bem1p, which is required for polarized growth. These two proteins physically interact (Lyons et al., 1996). There is an intriguing stretch of homology between Far1p and Ste5p (Leberer et al., 1993), and since both proteins interact with Bem1p this domain may be involved in binding Bem1p (Leeuw et al., 1995). Far1p could possibly determine which proteins associate with Bem1p and specifically prevent interaction with the bud site selection proteins or promote association with Ste5p and other proteins of the pheromone response pathway.

The finding that Y30 was a *ram1* mutant was surprising since it had not been classified as an **a**-factor mutant. Upon reinspection, Y30 did have a very mild defect in bioactive pheromone production. Since farnesylation is a prerequisite for secretion of **a**-factor and for the bioactivity of this pheromone, the pheromone levels seen in the assays represent normal mature pheromone (Chen et al., 1997). It is likely that the assay we employed was not sensitive enough to accurately reflect absolute levels of pheromone. Even a modest reduction in **a**-factor could possibly lead to a mating defect, since very high levels of pheromone are necessary to induce cell fusion during conjugation (Brizzio et al., 1996). A reduction in **a**-factor, however, does not explain why *MATα* versions of Y30 (such as Y1163) also display reduced mating. The modest mating defects in these mutants could represent the small effects of *ram1* mutations on pheromone signaling through inactivation of Gγ. The reason *ram1* null mutants do not have a more severe effect on signaling is apparently due to crossprenylation of Gγ by geranylgeranyl transferase (GGTase; Whiteway and Thomas, 1994). However, this crossprenylation cannot fully complement the

ram1 mutation, since geranylgeranylated Gγ suppresses the constitutive signaling caused by deletions of the Gα gene, *GPA1* (Whiteway and Thomas, 1994).

In addition to the mating defect, Y30 and Y1163 had growth defects. To the best of my knowledge, *ram1* mutants have never been reported to inhibit growth. Some of the GTPase proteins that regulate growth, such as Ras2p, and budding, Rho3p and Rho4p, are farnesylated. Since Ram1p determines the specificity of substrate recognition, the Y30 mutant could specifically inhibit isoprenylation of such proteins and retard their functions in cell growth, while having little effect on its recognition of pro-**a**-factor. Alternatively, the mutation may alter the type of modification and permit the Ram1p/Ram2p farnesyltransferase (FTase) to act as a GGTase. Specific mutations of Ram1p have been shown to permit this protein to act both as a FTase and as a GGTase (Mtsuzawa et al., 1995; Del Villar et al., 1997). In this respect it is interesting to note that alteration of the CaaX box of human Ras from a farnesylation target to a geranylgeranylation target results in a growth defect (Cox et al., 1994). Perhaps geranylgeranylated Ras1p and Ras2p have a similar inhibitory effect on cell growth. Since geranylated a-factor is still secreted and active, I would not expect such a mutation to affect a-factor production (Caldwell et al., 1994). In addition to Y30, two of the *ram1* mutants characterized in chapter 1 also had growth defects (data not shown), although whether these were linked to the mating defect was not determined. In short, the Y30 *ram1* mutation appears to be unique in its effects on mating in both haploid cell types. If it does affect the farnesylation of only certain substrates such as Ras or Rho, this mutant could be useful in designing farnesyltransferase inhibitors that specifically prevent Ras or Rho modifications and would be useful in chemotherapeutic treatment of some cancers.

CHAPTER 3

The Axl is Broken and Next to the Kar

Introduction

Haploid yeast are able to conjugate to form diploids. Cells secrete peptide mating pheromones to attract partners of the opposite mating type. These peptides bind to their cognate receptors and activate a MAP kinase cascade that causes cell cycle arrest and also leads to the transcription of many of the genes required for mating (Sprague and Thorner, 1992). The **a**-factor pheromone is first synthesized as a pro-**a**-factor oligopeptide with an amino-terminal extension and a CaaX-box motif (where C is cysteine, a is an aliphatic amino acid and X is any of several possible residues) (Caldwell et al., 1995; Chen et al., 1997). This motif directs farnesylation of the sulfhydryl group of the cysteine which targets the peptide to the cytosolic surface of a membrane and permits the proteolytic removal of the three C-terminal residues (Schafer et al., 1990; Caldwell et al., 1995; Chen et al., 1997). After cleavage of these amino acids, the carboxy-terminal S-farnesyl-cysteine is methylated (Volker et al., 1991; Hrycyna and Clarke, 1992; Hrycyna et al., 1994; Chen et al., 1997). Finally, the amino-terminal extension of pro-**a**-factor is proteolytically cleaved in two steps to yield mature **a**-factor which is then secreted (Chen et al., 1997).

I isolated a mutant, called *ste22-1* (Y49) that is defective in mating due to a defect in **a**-factor secretion (see Chapters 1 and 4). We cloned the *STE22* gene, sequenced the open reading frame, and mapped the gene to the right arm of chromosome XVI. During the course of our work, another group published a paper in which they isolated *STE22* as a gene involved in axial bud site selection and called the gene *AXL1* (Fujita et al., 1994). Therefore, *STE22* will hereafter be referred to as *AXL1*.

Materials and Methods

Strains, Media and Microbiological Techniques

The strains used in cloning and mapping *AXL1* are listed in Table 6. Plasmids are listed in Table 7. Media and techniques used are described (Rose et al., 1990; Guthrie and Fink, 1991). Standard methods were used for manipulating DNA (Sambrook et al., 1989). Transformations of yeast were by the lithium acetate method (Ito et al., 1983).

A plasmid for disrupting the putative *AXL1* gene with a nutritional marker, p98 (Fig. 14A), was constructed by inserting a Bgl II fragment of *URA3* into the Sst I site of p79 into which we had introduced a Bgl II linker. This insertion interrupts the *AXL1* ORF at codon 206 within the conserved active site of Axl1p. Disruption of the cloned gene in Y142 was accomplished by transforming p98 cut with Bam HI-Eco RI into the parental strain, SY2625, and selecting for growth on media lacking uracil. To confirm the presence of integrated *URA3* in the genome, Southern analysis was performed on one colony which exhibited the same phenotypes as the Y49 strain.

A strain containing a complete deletion of *AXL1* marked with *LEU2* was also constructed (Fig. 14B). A 5.0-kb Sal I fragment from p79 was cloned into pUC19, and an internal 4.0-kb Hpa I-Xho I fragment was replaced with a *LEU2* fragment. The *axl1Δ::LEU2* plasmid, p114, was cut with Sal I and tranformed into SY2625 to make the strain, Y173. Replacement of the AXL1 ORF with LEU2 was confirmed by PCR analysis. A *sge1::URA3* strain was made for meiotic mapping to *AXL1* by transforming SY2625 with Hind III-cut p95.

Testing Linkage of Cloned Gene to the ste22-1 Mutation

The *axl1::URA3* strain, Y142, was crossed to a *MATα axl1* strain, Y278 (obtained from backcrossing Y49), and tetrad analysis was performed on the sporulated diploid to test for linkage as described in Chapter 1.

Table 6. Strain List for Chapter 3.

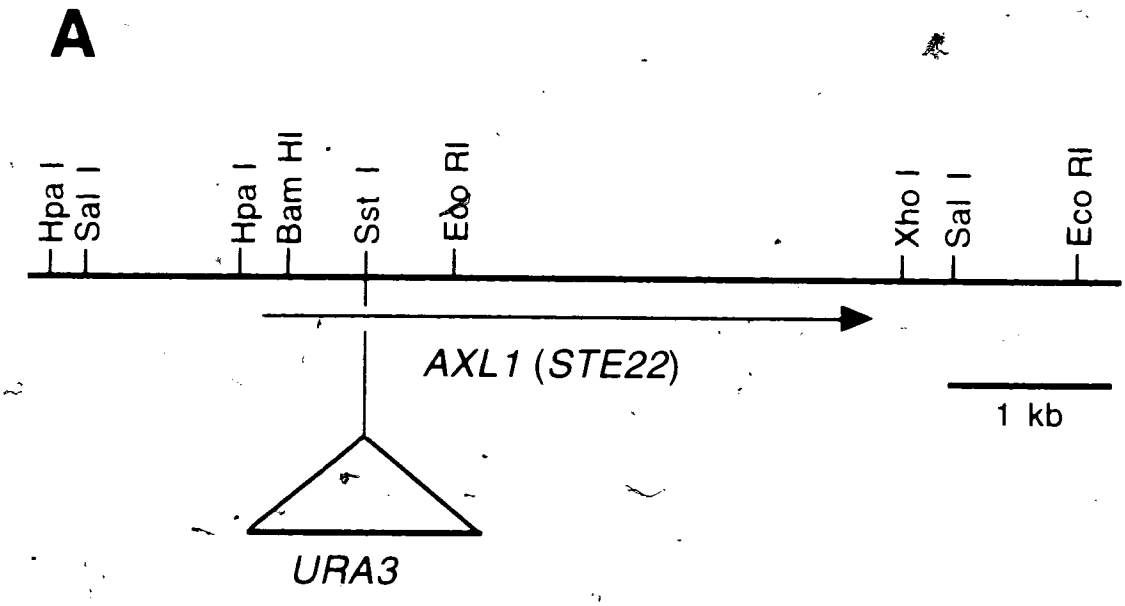
Strain	Genotype	Source
SY2625	*MAT***a** *bar1Δ his3::FUS1-HIS3 mfa2Δ::FUS1-lacZ ura3-1 leu2-3,-112 trp1-1 ade2-1 can1-100*	C. Boone
Y49	*MAT***a** *ste22-1 allele 10-2-46-1 his3::FUS1-HIS3 mfa2Δ::FUS1-lacZ ura3-1 leu2-3,-112 trp1-1 ade2-1 can1-100 bar1Δ*	N. Adames
Y278	*MATα ste22-1 allele 10-2-46-1 his3::FUS1-HIS3 mfa2Δ::FUS1-lacZ ura3-1 leu2-3,-112 trp1-1 ade2-1. can1-100 bar1Δ*	N. Adames
Y142	*MAT***a** *axl1::URA3 his3::FUS1-HIS3 mfa2Δ::FUS1-lacZ ura3-1 leu2-3,-112 trp1-1 ade2-1 can1-100 LYS2 bar1Δ*	K. Blundell
Y173	*MAT***a** *axl1Δ::LEU2 bar1Δ his3::FUS1-HIS3 mfa2D::FUS1-lacZ ura3-1 leu2-3,-112 trp1-1 ade2-1 can1-100*	K. Blundell
Y196	*MATα axl1Δ::LEU2 bar1Δ his3::FUS1-HIS3 mfa2Δ::FUS1-lacZ ura3-1 leu2-3,-112 trp1-1 ade2-1 can1-100*	N. Adames
Y133	*MATα ura3-52 leu2-3,112 Ty1-48::LEU2*	M. Rose
Y134	*MATα ura3-52 leu2-3,112 trp1-Δ1 tef1-∇1324::URA3*	M. Rose
Y136	*MAT***a** *ura3 leu2-3,112 ade2-101 ade6 trp1-Δ1 lys2 aro7 kar3-1::pM806 (URA3)*	M. Rose

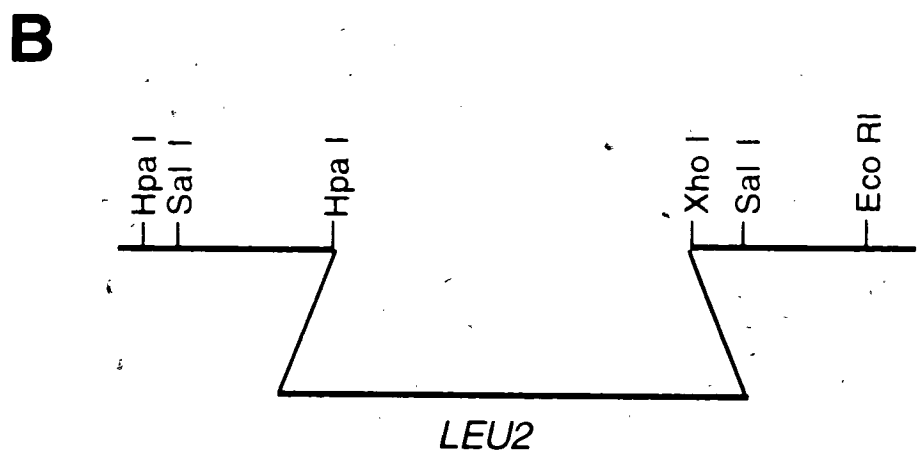
Table 7. Plasmid List for Chapter 3.

Plasmid	Construction	Source
p79	cloned genomic fragment containing *AXL1* in pRS316 (*CEN ARS URA3*)	K. Blundell
p80	cloned genomic fragment containing *AXL1* in pRS316 (*CEN ARS URA3*)	K. Blundell
p98	*axl1::URA3* in pUC19	K. Blundell
p114	*axl1Δ::LEU2* in pUC19	K. Blundell
p95	*sge1::URA3* in unknown vector	H. Amakasu

Figure 14. Gene disruption plasmids for *AXL1*.

Plasmids were designed and targeted as described in the Materials and Methods. (*A*) The *axl1::URA3* construct (p98) inserts the *URA3* gene at codon 206 of *AXL1* which occurs within the conserved region containing the putative active site. (*B*) The *axl1::LEU2* construct (p114) replaces the entire *AXL1* ORF with the *LEU2* gene.


A
Hpa I
Sal I
Hpa I
Bam HI
Sst I
Eco RI
Xho I
Sal I
Eco RI
AXL1 (STE22)
1 kb
URA3


B
Hpa I
Sal I
Hpa I
Xho I
Sal I
Eco RI
LEU2

Cloning AXL1

The *axl1* mutant, Y49, was transformed with a genomic library as described in Chapter 2. Transformants displaying increased mating compared to Y49 transformed with empty vector were tested for plasmid-dependence. Transformants that lost the ability to mate after plating on FOA were then examined further. Plasmids were isolated and transformed again into Y49 to test for complementation of the mating defect.

Sequencing

Restriction maps of the genomic insert in two complementing plasmids (p79 and p80) were independently made. These were compared and proved to be identical for a common 6.5-kb region. The restriction map for one clone guided the construction of subclones made to determine the region in which the complementing open reading frame (ORF) lay.

Once I determined the approximate position of the ORF, I subcloned fragments of this region into vectors and made a series of deletions using most of the restriction sites available in the region of the ORF. Sequencing was performed as described in Chapter 2. There were sufficient restriction sites present in the genomic DNA to permit sequencing in both strands of most of the ORF. Where gaps in the sequence occurred, I designed sequencing primers flanking the gaps based on sequence I had previously obtained.

Physical Mapping

A probe of *AXL1* was made by digesting a pRS316 plasmid (Sikorski and Hieter, 1989) containing an 8.5 kb *AXL1* genomic fragment with Hpa I and Xho I and subjecting the digest to electrophoresis in a 1% agarose gel. A 4.0-kb insert fragment, corresponding to the *AXL1* ORF, was used as a probe against the yeast genomic blot described in Chapter 2. A clone to which the

AXL1 probe hybridized was identified, and its chromosomal position was determined using the software provided with the genomic blot.

Crosses and Tetrad Dissection

Crosses and tetrad dissection were performed as described in Guthrie and Fink (1991) and as described in Chapter 2. Approximately 80 tetrads from each cross were analyzed for linkage. Y142 was used in crosses to Y133 (*Ty1-48::LEU2*) and Y173 was used in crosses to Y134 (*tef1-Δ1324::URA3*). The *MAT***a** version of Y173, Y196 (obtained by backcrossing Y173), was crossed to the *sge1::URA3* mutant and to the *kar3::URA3* mutant, Y136.

Calculation of Genetic Distance Between Genes

Genetic distance between markers was calculated according to Guthrie and Fink (1991). Tetrads were scored as being Parental Ditype (PD), Non-Parental Ditype (NPD) or Tetratype (T). PD tetrads occur when no crossovers or an even number of crossovers occur between the same two chromatids between the genes of interest. NPD tetrads are the result of a single crossover event between two pairs of chromatids (i.e., a 4-strand double crossover), while T tetrads are a product of one or an odd number of crossover events within a single pair of chromatids. The expected ratio of PD:NPD:T for unlinked genes is 1:1:4. The ratio for linked genes is >1:<1:<4 and approaches 1:0:0 for closely linked genes (i.e., crossovers between the genes are rare). For linked genes, the calculation for genetic distance, given in centiMorgans (cM), is as follows:

$$cM = \frac{100}{2} \times \frac{T+6NPD}{T+NPD+PD}$$

Results

The Cloned Gene is Allelic to AXL1 and Encodes a Protease Homologue

The *AXL1* gene was cloned by complementation of the *axl1* mating defect with a yeast genomic library. Five different plasmids complementing the mating defect of the *axl1* strain, Y49, were recovered. As determined by restriction digests, these plasmids all overlapped in a region of approximately 5.5 kb. Subclones were made to determine in what region the complementing ORF resided. According to these results, the ORF appeared to cover most of the 5.5-kb overlap amongst the genomic library clones.

Analysis of tetrads from the cross between Y142 and Y278 revealed that all were parental ditypes. This analysis was complicated by the fact that the axl1 mating defect was specific to *MAT***a** cells, however we recovered no *MAT***a** cells that were able to mate. In crosses between Y49 and Y5 (a *MAT*α wild-type strain), mating competent *MAT***a** spores were recovered, showing that the *axl1* mutation is not closely linked to the *MAT***a** locus. Therefore, all spores from Y142 crossed to Y278 carried either the *axl1* or the *axl1::URA3* allele, and so the cloned gene is closely linked and probably identical to the gene mutated in axl1 cells.

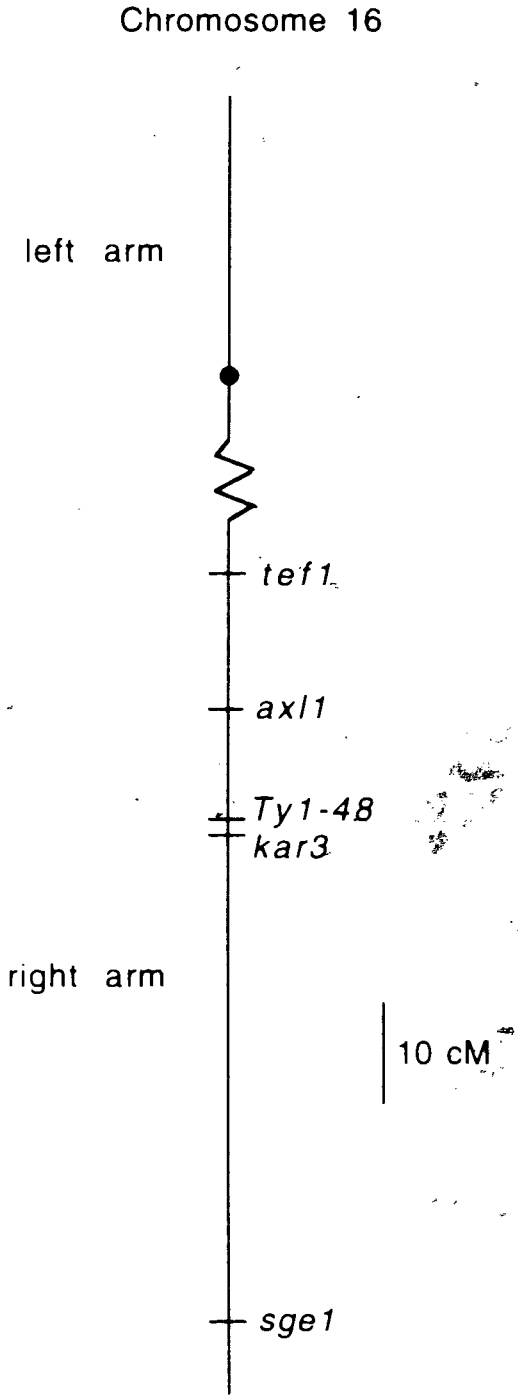
Sequencing of *AXL1* revealed a large open reading frame, YPR122w, 3,624 bp in length, encoding a protein of 1208 amino acids. This protein shows homology to members of the pitrilysin family of endoproteases (analysis of this sequence is described in Chapter 4). Sequencing also revealed an ORF corresponding to the sequence for the *CTR1* gene encoded on the same strand and 3' to *AXL1*. This gene was isolated due to its involvement in the regulation of copper and iron uptake by yeast (Dancis et al., 1994).

Table 8. Mapping data for markers linked to *AXL1*.

	--------Ascus type---------			
Markers	PD	NPD	T	Map distance (cM)
axl1::LEU2--sgel::URA3	18	14	48	82.5
axl1::LEU2--kar3::URA3	68	1	26	16.8
axl1::URA3--Ty1-48::LEU2	61	24	0	14.1
axl1::LEU2--tef1-∇1324::URA3	55	0	32	18.4
tef1-∇1324::URA3--Ty1-48::LEU2	42	0	37	23.4

Abbreviations: PD, parental ditype; NPD, non-parental ditype; T, tetratype

Figure 15. Genetic map showing position of *axl1* relative to *tef1*, *Ty1-48*, *kar3* and *sge1*. The map was constructed from the data in Table 8 and from edition 13 of the *S. cerevisiae* genetic map. The map is drawn to scale using the map distances from Table 8. The relative positions of *sge1* and *kar3* differ from those of the genetic map, but agree with our data and with the physical map of these genes.


Chromosome 16
left arm
tef1
axl1
Ty1-48
kar3
right arm
10 cM
sge1

AXL1 Maps to the Right Arm of Chromosome XVI

The *AXL1* fragment hybridized to clone λPM5832 (ATCC 70613) which is part of the right arm of chromosome XVI and maps close to *kar3* between it and *aro7* . We used this information to select genes on this chromosome which we could use to genetically map *AXL1*.

A three point cross between *axl1*, *tef1* and *Ty1-48* was performed. In addition, we did two point crosses between *axl1* and *kar3*, and between *axl1* and *sge1*. The results of the crosses between the marked *axl1* strains and other genes previously mapped to the right arm of chromosome XVI are shown in Table 8. Based on these results and map positions reported for these genes, we have mapped *axl1* to a position between *tef1* and *Ty1-48* (Fig. 15).

Discussion

The *axl1* mutant was isolated because of its mating defect. This defect was found only in *MAT***a** *axl1-1* cells. The low mating efficiency of this mutant is probably due to a defect in **a**-factor secretion since mutants produce smaller halos in **a**-factor secretion assays.

AXL1 was cloned for its ability to completely rescue the mating defect of *axl1* cells and it also completely complements the pheromone secretion defect of this strain (Chapter 4). The homology of Axl1p to endoproteases suggests that this protein is involved in proteolytic processing of pro-**a**-factor.

AXL1 maps to the right arm of chromosome XVI both physically and genetically. Linkage analysis has allowed us to establish a gene order, starting closest to the centromere, of *tef1-axl1-Ty1-48-kar3-sge1*. This order contrasts with that shown in the yeast genetic map edition 12, in which *sge1* is shown closer to the centromere than *kar3* (Cherry et al., 1997). However, the physical map of these genes agrees with our data, suggesting that an error was made in mapping the relative positions of *kar3* and *sge1* (Cherry et al., 1997). Completion of the *S. cerevisiae* genome sequencing project has confirmed the accuracy of the physical map and our mapping data (Cherry et al., 1997).

CHAPTER 4

How Do You Make a Pheromone?

Introduction

Many organisms secrete peptide hormones that regulate diverse physiological processes. Most, if not all of these hormones are translated as larger forms of modified peptides that are eventually released from cells (Fuller et al., 1989; Bresnahan et al., 1990; Seidah et al., 1991; Seidah et al., 1993; Lledo, 1997). The mature hormones are produced by the action of proteases which cleave at specific residues of their substrates (Fuller et al., 1989; Bresnahan et al., 1990; Seidah et al., 1991; Seidah et al., 1993; Lledo, 1997). Similarly, yeast cells also secrete peptide "hormones" called mating pheromones. *MAT***a** cells secrete **a**-factor and *MAT*α cells secrete α-factor (Sprague and Thorner, 1992). The study of how these pheromones are processed and secreted has illuminated hormone secretion in higher organisms.

The 13-residue α-factor is encoded by two genes, *MFα1* and *MFα2*, which differ in some respects (Kurjan and Herskowitz, 1982; Brake et al., 1983; Emter et al., 1983; Julius et al., 1983; Singh et al., 1983). The primary translation products of both genes are precursors (prepro-α-factor) that have a hydrophobic signal or leader sequence (pre-region), a hydrophilic pro-region containing three consensus sequences for N-linked glycosylation, and a carboxy-terminal region containing multiple copies of the mature α-factor sequence separated from each other and the prepro-region by "spacer" sequences (Kurjan and Herskowitz, 1982; Brake et al., 1983; Emter et al., 1983; Julius et al., 1983; Singh et al., 1983). The spacers begin with a pair of basic residues (KR) followed by two or three dipeptides consisting of -x-A- (where x is any amino acid residue). *MFα1* contains four copies of α-factor while *MFα2* has two (Kurjan and Herskowitz, 1982; Brake et al., 1983). However, deletion of either gene alone has no effect on mating efficiency, indicating that the genes are functionally redundant (Sprague and Thorner, 1992). This functional redundancy may indicate that the ability of yeast to mate is crucial for their survival.

The signal sequence of prepro-α-factor targets the protein for transport into the endoplasmic reticulcum where the leader is proteolytically removed and mannose-rich core oligosaccharides are added onto the pro-domain (Sprague and Thorner, 1992). The protein undergoes additional glycosylation of these cores in the Golgi stacks (Sprague and Thorner, 1992). Mature α-factor is produced in the late Golgi through the action of a series of proteases. The *KEX2* gene product, Kex2p, is an endoprotease that cleaves after the KR residues in the spacer regions, thus liberating the pheromone repeats (Leibowitz and Wickner, 1976; Mizuno et al., 1988; Cunningham and Wickner, 1989; Mizuno et al., 1989; Redding et al., 1991; Zhu et al., 1992). Kex2p is a member of the subtilisin family of proteases with specificity for doublets of basic residues (Mizuno et al., 1988; Mizuno et al., 1989). The Kex2p endoprotease is the founding member of a class of prohormone convertases involved in processing hormones and other precursor proteins in organisms such as *Caenorhabditis elegans* and humans (Bresnahan et al., 1990; Hatsuzawa et al., 1990; Misumi et al., 1990; Seidah et al., 1990; Bennett et al., 1992; Hatsuzawa et al., 1992; Nagle et al., 1993; Rangaraju and Harris, 1993; Seidah et al., 1994).

The KR residues remaining at the carboxy-termini of the pheromone precursors are cleaved by the Kex1p KR-carboxypeptidase (Leibowitz and Wickner, 1976; Dmochowska et al., 1987). The final step in α-factor maturation is removal of the -x-A- residues at the amino-termini by dipeptidyl aminopeptidase A, encoded by *STE13* (Anna-Arriola and Herskowitz, 1994). All of these proteases are integral membrane proteins having their active sites within the lumen of the Golgi compartment. Mature α-factor is transported outside the cell via secretory vesicles.

In contrast to secretion of α-factor, **a**-factor does not enter the classical secretory pathway (ER to Golgi to secretory vesicles) (Kuchler and Thorner, 1992). This was first suggested by structural analysis of the precursor and mature forms of this pheromone. The **a**-factor precursors are very short, do not have a hydrophobic signal sequence, and lack sites for N-glycosylation (Chen et al., 1997). Furthermore, the mature form is isoprenylated and methylated (Caldwell et al., 1995; Chen et al., 1997). None of these features are typical of proteins that traverse the classical secretory pathway. Evidence supporting this conclusion includes the observation that

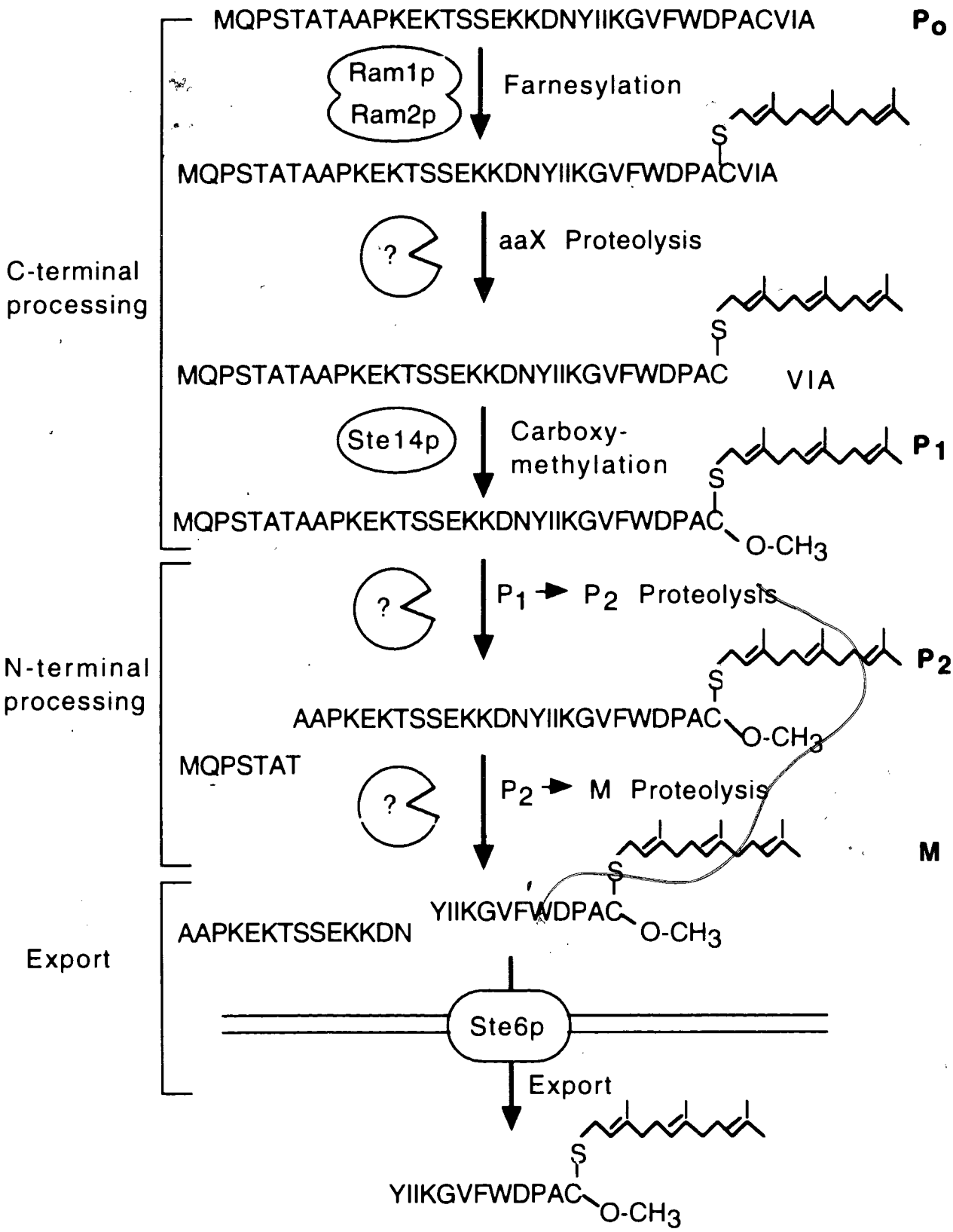
kex2 and *ste13* mutations have α cell-specific mating defects and affect α-factor secretion but not **a**-factor secretion, indicating that **a**-factor is not processed in the same way as is α-factor (Leibowitz and Wickner, 1976; Anna-Arriola and Herskowitz, 1994). In addition, subcellular fractionation and protease accessibility studies indicate that pro-**a**-factor does not enter intracellular membrane-bound compartments (Sprague and Thorner, 1992). More convincing, however, is the finding that temperature-sensitive secretion (*sec*) mutants prevent exocytosis of α-factor at the restrictive temperature but have no effect on **a**-factor secretion (McGrath and Varshavsky, 1989).

There are two **a**-factor precursors that differ slightly in amino acid sequence and are encoded by two genes. *MFA1* produces a 36-residue pro-**a**-factor precursor (MQPSTATA APKEKTSSEKKDN**YIIKGVFWDPAC**VIA) which contains only one copy of the mature **a**-factor sequence (shown in bold lettering) (Brake et al., 1985; Chen et al., 1997). The precursor encoded by *MFA2* (MQPITTASTQATQKDKSSEKKDN**YIIKGLFWDPAC**VIA) is 38 residues long and differs from *MFA1* in the mature **a**-factor sequence by one residue (L29 instead of V27, shown underlined) (Brake et al., 1985; Chen et al., 1997). The products from both genes contribute almost equally to **a**-factor secretion and are functionally redundant (Chen et al., 1997).

The proteins involved in processing and secreting **a**-factor are shown in Fig. 16. The first modification of pro-**a**-factor is farnesylation of a motif called a CaaX box (where C is cysteine, a is an aliphatic residue, and X can be a variety of amino acids) at the carboxy-terminus (Moores et al., 1991). This motif directs prenylation of small GTPases such as Ras, as well as the Gγ subunit of heterotrimeric G proteins (Finegold et al., 1990; Schafer et al., 1990; Moores et al., 1991; Caldwell et al., 1995). Two types of isoprenoids are known to modify proteins -- farnesyl groups (C_{15} isoprenoids) and geranylgeranyl groups (C_{20} isoprenoids; Moores et al, 1991; Caldwell et al., 1995). The last amino acid of the protein (the X of the CaaX box) determines which lipid is attached to the protein. Farnesyl transferase (FTase) preferentially adds a farnesyl group to the protein if this residue is methionine, serine, alanine, cysteine, or glutamine (Moores et al, 1991; Caldwell et al., 1995). Geranylgeranyl transferase I (GGTase) prefers the last residue to be leucine or phenylalanine (Moores et al, 1991; Caldwell et al., 1995). These

Figure 16. Biogenesis of **a**-factor.

The *MAT***a** mating pheromone, **a**-factor, is a dodecapeptide first synthesized as a propeptide. The pro-**a**-factor protein undergoes a number of post-translational modifications and is secreted via a non-classical secretory pathway. Ram1p and Ram2p are cytosolic proteins and are subunits of a farnesyltransferase that adds a C15 hydrocarbon farnesyl group to the last cysteine of pro-**a**-factor. After farnesylation, all of the processing events take place at cellular membranes. Ste14p methylates the carboxyl group of the *S*-farnesyl-cysteine after the last three amino acids following the cysteine are removed. Modification of the amino terminus of pro-**a**-factor involves two sequential proteolytic clips that remove the pro-domain amino-terminal extension to yield mature pheromone. **a**-factor is then pumped out of the cell by Ste6p, an ABC transporter.


MQPSTATAAPKEKTSSEKKDNYIIKGVFWDPACVIA
Po
Ram1p
Ram2p
Farnesylation
S
MQPSTATAAPKEKTSSEKKDNYIIKGVFWDPACVIA
?
aaX Proteolysis
C-terminal processing
S
MQPSTATAAPKEKTSSEKKDNYIIKGVFWDPAC
VIA
Ste14p
Carboxy-methylation
P1
S
MQPSTATAAPKEKTSSEKKDNYIIKGVFWDPAC
O-CH3
?
P1 → P2 Proteolysis
N-terminal processing
P2
S
AAPKEKTSSEKKDNYIIKGVFWDPAC
O-CH3
MQPSTAT
?
P2 → M Proteolysis
M
S
YIIKGVFWDPAC
O-CH3
AAPKEKTSSEKKDN
Export
Ste6p
Export
S
YIIKGVFWDPAC
O-CH3

isoprenoids are linked to the protein via a thioether bond with the cysteine in the CaaX box (Schafer et al., 1990).

Prenylation of proteins occurs in the cytosol and helps target them to membranes, presumably by insertion of the lipid portion of the protein into the membrane bilayer or through interaction with a specific isoprenoid receptor. Such modifications may also aid in protein-protein interactions at membranes (Omer and Gibbs, 1994; Caldwell et al., 1995). Many other lipid modifications similarly target proteins to membranes (Casey, 1995). Why there is such a multiplicity of mechanisms for protein lipidation is unknown. In fact some proteins function normally when the type of lipid modification they receive is altered. For example, the two yeast Ras homologues are farnesylated and are required for growth, but cells are still viable in the absence of the yeast FTase (Trueblood et al., 1993). In the absence of FTase, yeast Ras proteins can still be modified by GGTase and still function for growth (Trueblood et al., 1993). Moreover, **a**-factor can be geranylgeranylated without affecting its export or activity (Caldwell et al., 1994). Crossprenylation also occurs in the other direction since a GGTase mutant strain farnesylates Rho1p and Cdc42p (Ohya et al., 1993). Evidence indicates that cross-prenylation of targets by prenyltransferases may be common (Caldwell et al., 1995).

The yeast FTase is encoded by *RAM1* (coding for the β subunit which confers substrate specificity on the enzyme) and *RAM2* (coding for the catalytic α subunit) which has mammalian counterparts (Goodman et al., 1990; He et al., 1991; Kohl et al., 1991). Ram2p is also a subunit of the GGTase I along with the Cdc43p β subunit (Mayer et al., 1992). Mutations in either *RAM1* or *RAM2* prevent Ras modification and result in **a** cell-specific sterility due to the lack of **a**-factor secretion (He et al., 1991). Farnesylation is a prerequisite for pro-**a**-factor maturation since *ram1* mutants, and wild-type cells depleted of mevalonate (the isoprenoid precursor) accumulate unprocessed pro-**a**-factor within the cell (Sprague and Thorner, 1992). Prenylation also targets pro-**a**-factor to membranes within the cell and the pheromone remains associated with membranes until it is secreted (Chen et al., 1997; Sapperstein et al., 1994).

The next step in carboxy-terminal processing is cleavage of the three amino acids following the farnesylated cysteine (the aaX). The enzyme activity responsible for this proteolytic step resides on membranes, as one might expect since the **a**-factor precursor is in membranes (Boyartchuk et al., 1997). After removal of the aaX residues, the exposed carboxyl group of the *S*-farnesyl-cysteine is methylated by the Ste14p carboxyl methyltransferase using *S*-adenosyl-methionine as the methyl donor (Hrycyna and Clarke, 1990; Volker et al., 1991; Hrycyna and Clarke, 1992; Hrycyna et al., 1994). The predicted amino acid sequence of Ste14p has five or six potential transmembrane domains and the enzyme activity is also associated with membrane fractions (Hrycyna et al., 1991; Ashby et al., 1993; Sapperstein et al., 1994). Methylation is a requirement for efficient transport of mature **a**-factor out of the cell, but unlike farnesylation, is not a prerequisite for the remaining maturation steps (Sapperstein et al., 1994; Chen et al., 1997).

Upon completion of the three carboxy modifications, the amino-terminal extension of pro-**a**-factor is removed. Unexpectedly, the removal of the amino-terminus is accomplished in two sequential proteolytic steps rather than in a single cleavage (Chen et al., 1997). The first event removes the first seven amino acids (MQPSTAT) from the precursor encoded by *MFA1* (Chen et al., 1997). The next protease cleaves between N21 and Y22 (numbering corresponds to full length pro-**a**-factor) of the precursor to yield fully mature **a**-factor (Chen et al., 1997). The genes coding for these proteases and for the CaaX box protease were unknown when I started this project.

Mature **a**-factor is finally secreted by the adenosine triphosphate (ATP)-binding cassette (ABC) transporter Ste6p (Kuchler et al., 1989; McGrath and Varshavsky, 1989). These transporters are integral membrane proteins having twelve transmembrane domains and two ATP-binding motifs (Kuchler et al., 1989; McGrath and Varshavsky, 1989; Michaelis, 1993). The transporter can be a single polypeptide with all these features, such as Ste6p, or it can consist of two polypeptides, each consisting of six membrane domains and one ATP-binding motif (Michaelis, 1993). Ste6p is highly selective and is able to transport only fully modified **a**-factor (Michaelis, 1993). How this transporter interacts with **a**-factor and discriminates against the various precursor forms is unknown. Ste6p is a homologue of the mammalian multiple drug

resistance (MDR) P-glycoprotein implicated in resistance of cancer cells to a variety of hydrophobic chemotherapeutic agents (Kane et al., 1990; Cornwell, 1991; Becker et al., 1992; Biedler, 1992; Michaelis, 1993). The cystic fibrosis transmembrane conductance regulatory (CFTR) protein and transport proteins associated with antigen processing (TAPs) in antigen presenting cells are also members of the ABC transporter superfamily (Higgins et al., 1990; Harris, 1992; Hughes, 1994; Hill and Ploegh, 1995). Therefore, Ste6p has homology to other proteins involved in transport of hydrophobic compounds and peptides. Although MDR1 seems to be fairly promiscuous in its ability to transport hydrophobic compounds, the mouse *mdr2* gene product is a "flippase" that specifically transports phosphatidylcholine from the cytosolic side of membranes, where it is synthesized, to the opposite side (Smit et al., 1993). The specificity of *mdr2* for phosphatidylcholine has been demonstrated by expression in yeast cells (Ruetz and Gros, 1994). There is growing evidence that other MDR proteins may have similar flippase activity for lipids, lipid-modified proteins, and drugs and may be more selective than MDR1 (Caldwell et al., 1995). It is possible that Ste6p uses a similar flipping mechanism to transport **a**-factor. This possibility is borne out by the observation that mouse *mdr3* can functionally complement **a**-factor transport in cells deleted for the *STE6* gene (Raymond et al., 1992; Caldwell et al., 1995). Studies on the mechanism of pheromone transport by Ste6p has implications for a number of important transporters.

In the same vein, the carboxy-terminal processing of **a**-factor is identical to modifications in such medically relevant proteins as the proto-oncogene Ras, mutations in which are implicated in 90% of pancreatic cancers and 50% of colon cancers (Barbacid, 1987). In fact, the transforming ability of oncogenic forms of Ras is dependent on farnesylation (Schafer, 1990; Gibbs et al., 1994). Much attention has been given to the design of drugs that inhibit Ras by blocking its farnesylation, and the yeast system has been used to search for such potential chemotherapeutic compounds (Gibbs et al., 1994). Clearly, studies on **a**-factor secretion have been of great importance in describing some crucial cellular processes. In this chapter I describe the assignment of a function for the *AXL1* gene product in proteolytic processing of **a**-factor.

Strains, Media and Microbiological Techniques

Yeast strains used in this study are listed in Table 9. The genetic techniques and media used are as described in Rose et al. (1990) and Guthrie and Fink (1991). The lithium acetate method was used to transform yeast strains (Ito et al., 1983). All strains were generated by means of standard genetic or molecular methods involving the appropriate constructs described below. In particular, the *axl1 ste23* double mutants (Y220 and Y231) were created by crossing the appropriate *MAT***a** *ste23* (Y221 and Y233) and *MAT*α *axl1* (Y196 and Y197) mutants, followed by sporulation of the resultant diploid and isolation of the double mutant from non-parental ditype tetrads. Gene disruptions were confirmed by polymerase chain reaction (PCR) or Southern analysis. Mating and **a**-factor assays were performed as described (see Chapter 1; Sprague, 1991). The construction of Y142 and Y173 was described in Chapter 3.

Plasmid Constructions

Plasmids are listed in Table 10. To delete the entire *STE23* ORF and create the *ste23*Δ::*URA3* mutation, PCR primers (5'-TCGGAAGACCTCATTCTTGCTCATTTTGATATT-GCTCTGTAGATTGTACTGAGAGTGCAC-3'; and 5'-GCTACAAACAGCGTCGACTTGAA-TGCCCCGACATCTTCGACTGTGCGGTATTTCACACCG-3') were used to amplify the *URA3* sequence of pRS316, and the reaction product was transformed into yeast for one-step gene replacement (Guthrie and Fink, 1991). To create the *ste23*Δ::*LEU2* allele (a deletion corresponding to 931 amino acids) carried on p153 (Fig. 17), a *LEU2* fragment was used to replace the 2.8-kb Pml I-Ecl136 II fragment of *STE23*, which occurs within a 6.2-kb Hind III-Bgl II genomic fragment carried on pSP72 (Promega). To create YEp*MFA1* (p128), a 1.6-kb Bam HI fragment containing *MFA1*, from pKK16 (Kuchler et al., 1993), was ligated into the Bam HI sit of YEp351 (Hill et al., 1986). p129 is a YEp352 plasmid (Hill et al., 1986)

Table 9. Strain List for Chapter 4.

Strain	Genotype	Source
SY2625	*MAT***a** *bar1Δ his3::FUS1-HIS3 mfa2Δ::FUS1-lacZ ura3-1 leu2-3,-112 trp1-1 ade2-1 can1-100*	C. Boone
Y83	*MATα bar1Δ his3::FUS1-HIS3 mfa2Δ::FUS1-lacZ ura3-1 leu2-3,-112 trp1-1 ade2-1 can1-100*	N. Adames
SY2014	*MATα ste3Δ306::LEU2 sst2Δ ste2Δ mfa1Δ mfa2Δ::FUS1-lacZ FUS1::HIS ura3-52 leu2-3, 112 ade1*	C. Boone
Y62	*MAT***a** *lys1*	I.Herskowitz
Y63	*MATα lys1*	I.Herskowitz
Y49	*MAT***a** *ste22-1 his3::FUS1-HIS3 mfa2Δ::FUS1-lacZ ura3-1 leu2-3,-112 trp1-1 ade2-1 can1-100 bar1Δ*	N. Adames
Y278	*MATα ste22-1 his3::FUS1-HIS3 mfa2Δ::FUS1-lacZ ura3-1 leu2-3,-112 trp1-1 ade2-1 can1-100 bar1Δ*	N. Adames
Y142	*MAT***a** *axl1::URA3 his3::FUS1-HIS3 mfa2Δ::FUS1-lacZ ura3-1 leu2-3,-112 trp1-1 ade2-1 can1-100 LYS2 bar1Δ*	K. Blundell
Y197	*MATα axl1::URA3 his3::FUS1-HIS3 mfa2Δ::FUS1-lacZ ura3-1 leu2-3,-112 trp1-1 ade2-1 can1-100 LYS2 bar1Δ*	N. Adames
Y115	*MAT***a** *bar1Δ his3::FUS1-HIS3 mfa1Δ::LEU2 mfa2Δ::FUS1-lacZ ura3-1 leu2-3,-112 trp1-1 ade2-1 can1-100*	K. Blundell
Y195	*MATα bar1Δ his3::FUS1-HIS3 mfa1Δ::LEU2 mfa2Δ::FUS1-lacZ ura3-1 leu2-3,-112 trp1-1 ade2-1 can1-100*	N. Adames
Y173	*MAT***a** *axl1Δ::LEU2 his3::FUS1-HIS3 mfa2Δ::FUS1-lacZ ura3-1 leu2-3,-112 trp1-1 ade2-1 can1-100 LYS2 bar1Δ*	K. Blundell
Y196	*MATα axl1Δ::LEU2 his3::FUS1-HIS3 mfa2Δ::FUS1-lacZ ura3-1 leu2-3,-112 trp1-1 ade2-1 can1-100 LYS2 bar1Δ*	N. Adames
Y220	*MAT***a** *axl1::URA3 ste23Δ:URA3 his3::FUS1-HIS3 mfa2Δ::FUS1-lacZ ura3-1 leu2-3,-112 trp1-1 ade2-1 can1-100 LYS2 bar1Δ*	K. Blundell

Table 9 (cont'd). Strain List for Chapter 4.

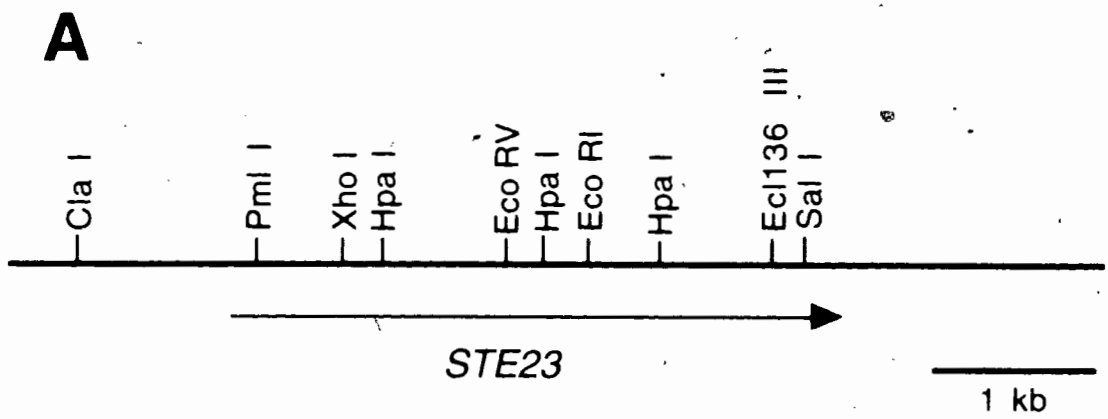
Strain	Genotype	Source
Y221	*MATa ste23Δ::URA3 his3::FUS1-HIS3 mfa2Δ::FUS1-lacZ ura3-1 leu2-3,-112 trp1-1 ade2-1 can1-100 LYS2 bar1Δ*	K. Blundell
Y231	*MATa axl1Δ::LEU2 ste23Δ:LEU2 his3::FUS1-HIS3 mfa2Δ::FUS1-lacZ ura3-1 leu2-3,-112 trp1-1 ade2-1 can1-100 LYS2 bar1Δ*	C. Boone
Y233	*MATa ste23Δ::LEU2 his3::FUS1-HIS3 mfa2Δ::FUS1-lacZ ura3-1 leu2-3,-112 trp1-1 ade2-1 can1-100 LYS2 bar1Δ*	C. Boone

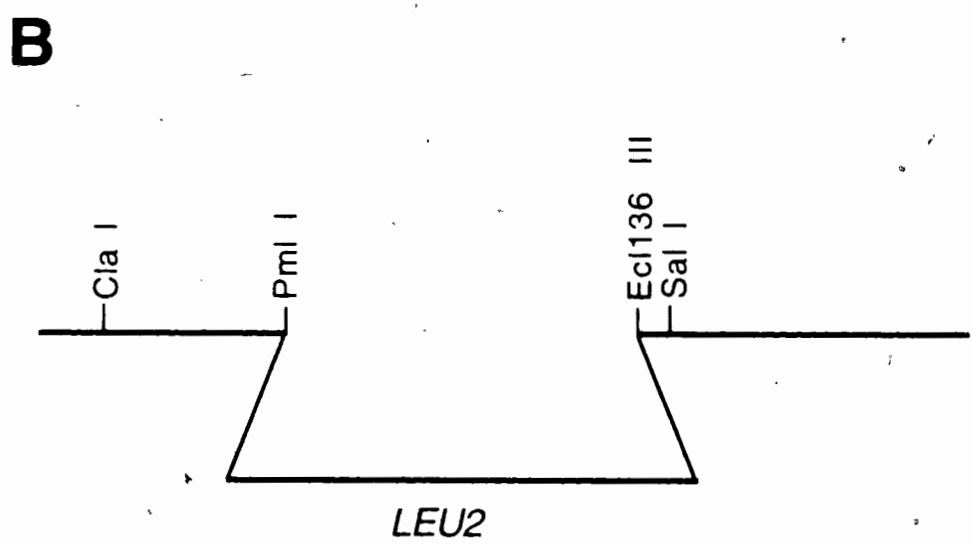
Table 10. Plasmid List for Chapter 4.

Plasmid	Construction	Source
p79	cloned genomic fragment containing *AXL1* in pRS316 (*CEN ARS URA3*)	K. Blundell
p128	*MFA1* in YEp351 (2μ *LEU2*)	C. Boone
p25	*RAM1* in YCp19 (*CEN ARS URA3*)	F. Tamanoi
p82	*MFA2* in pRS316 (*CEN ARS URA3*)	C. Boone
p129	*AXL1* in YEp352 (2μ *URA3*)	C. Boone
p126	*axl1(H68A)* in pRS316 (*CEN ARS URA3*)	C. Boone
p138	*axl1(E71A)* in pRS316 (*CEN ARS URA3*)	C. Boone
p139	*axl1(E71D)* in pRS316 (*CEN ARS URA3*)	C. Boone
p151	*AXL1-HA* in YEp352 (2μ *URA3*)	C. Boone
p162	*axl1(H68A)-HA* in YEp352 (2μ *URA3*)	C. Boone
p161	*axl1(E71A)-HA* in YEp352 (2μ *URA3*)	C. Boone
p163	*axl1(E71D)-HA* in YEp352 (2μ *URA3*)	C. Boone
p153	*ste23Δ::LEU2* in pUC19	C. Boone

Figure 17. A gene disruption plasmid for *STE23*.

(*A*) A restriction enzyme map of the *STE23* ORF and flanking regions. (*B*) Construction of a *ste23* deletion plasmid and strains is described in the Materials and Methods. The *ste23Δ::LEU2* plasmid replaces 931 amino acids of *STE23* with the *LEU2* gene.


A
Cla I
Pml I
Xho I
Hpa I
Eco RV
Hpa I
Eco RI
Hpa I
Ecl136 III
Sal I
STE23
1 kb


B
Cla I
Pml I
Ecl136 III
Sal I
LEU2

containing a 5.5-kb Sal I fragment of p*AXL1* (p79). p151 was derived from p129 by insertion of a linker at the Bgl II site within *AXL1*, which led to an in-frame insertion of the hemagglutinin (HA) epitope (DQYPYDVPDYA) (Kolodziej and Young, 1991) between amino acids 854 and 855 of Axl1p. pC225 is a KS+ (Stratagene) plasmid containing a 0.5-kb Bam HI-Sst I fragment from p*AXL1* (p79).

Site-Directed Mutagenesis of AXL1

Substitution mutations of the proposed active site of Axl1p were created with the use of pC225 and site-specific mutagenesis involving appropriate synthetic oligonucleotides (*axl1-H68A*, 5'-GTGCTCACAAAGCGCTGCCAAACCGGC-3'; *axl1-E71A*, 5'-AAGAATCATGTG-CGCACAAAGGTGCGC-3'; and *axl1-E71D*, 5'-AAGAATCATGTGATCACAAAGGTGCGC-3'). The mutations were confirmed by sequence analysis. After mutagenesis, the 0.4-kb Bam HI-Msc I fragment from the mutagenized pC225 plasmids was transferred into p*AXL1* to create a set of pRS316 plasmids carrying different *AXL1* alleles, p126 (*axl1-H68A*), p138 (*axl1-E71A*), and p139 (*axl1-E71D*). Similarly, a set of HA-tagged alleles carried on YEp352 were created after replacement of the p151 Bam HI-Msc I fragment, to generate p162 (*axl1-H68A*), p161 (*axl1-E71A*), and p163 (*axl1-E71D*).

*Pulse-Chase Labelling and Preparation of Intracellular and Extracellular **a**-Factor*

Metabolic labelling of **a**-factor, immunoprecipitation, and SDS-polyacrylamide gel electrophoresis (SDS-PAGE) were performed as described (Chen et al., 1997). Yeast cells containing *MFA1* on a high copy plasmid, p128, were grown in synthetic media to $O.D._{600}$ 0.2-0.5 and 10 $O.D._{600}$ U of cells were used for each sample. Cells in synthetic media lacking cysteine and methionine were incubated with 150 µCi [^{35}S]cysteine for each time point. The chase was initiated by addition of 10 µl of chase mix (1 M cysteine, 1 M methionine) for each time point. Immediately after labeling the cells were split into polypropylene tubes representing each time point. This was necessary to ensure efficient recovery of **a**-factor, because the pheromone

sticks to these tubes. Consequently, separate tubes must be used for each of the time points. The chase is terminated by adding ice-cold sodium azide and samples were kept on ice until processed.

Intracellular samples were prepared from cell pellets while extracellular **a**-factor was prepared from the supernatant and the tubes in which the chase was performed. Cells were lysed with ice-cold NaOH and β-mercaptoethanol and proteins were trichloroacetic acid (TCA) precipitated. The protein pellet was resuspended in 2X Laemmli sample buffer (20% glycerol, 10% β-mercaptoethanol, 4.3% SDS, 0.125 M Tris-HCl, pH 6.8, and 0.2% bromophenol blue), neutralized with 2 M Tris base when necessary, and boiled for 5 min. This protein made up the intracellular fraction.

Proteins of the extracellular fraction from the supernatant were TCA precipitated and resuspended in 2X Laemmli sample buffer. Proteins from the tubes were extracted with isopropanol and dried in a speed vacuum with heat. This protein was suspended in 1X Laemmli sample buffer, combined with the proteins from the supernatant, and boiled for 5 min.

*Immunoprecipitation of **a**-Factor*

For immunoprecipitation, the intracellular and extracellular samples were diluted in immunoprecipitation buffer (50mM Tris-HCl, pH 7.5, 1.0% Triton X-100, 150 mM NaCl, 1 mM PMSF, 0.5% aprotinin) cleared of insoluble material by centrifugation, and supernatant was transferred to a new tube. To this was added 10 µl of **a**-factor rabbit antiserum (gifts from S. Michaelis and J. Becker), and the samples were incubated at 4° C overnight. Immunoprecipitates were collected with protein A-sepharose CL-4B beads (Sigma). Beads were collected and washed four times in wash buffer A (50mM Tris-HCl, pH 7.5, 0.1% Triton X-100, 0.02% SDS, 150 mM NaCl, 5 mM EDTA, 1 mM PMSF, 0.5% aprotinin) and once in wash buffer B (50mM Tris-HCl, pH 7.5, 0.1% Triton X-100, 150 mM NaCl, 5 mM EDTA, 1 mM PMSF, 0.5% aprotinin). After the final wash, bound immune complexes were released from the beads by the addition of 30 µl 2X Laemmli sample buffer. Samples were boiled for 5 min before SDS-PAGE.

*SDS-PAGE Analysis of **a**-Factor*

A modification of the SDS-PAGE method (Laemmli, 1970) was used to resolve the very small **a**-factor species, which range in molecular mass from ~1.6 to 4.5 kD. Moreover, the farnesyl group confers unusual migration properties on these peptides. Therefore, some of the conditions were optimized for the best separation of the various **a**-factor intermediates as described (Chen et al., 1997). An acrylamide percentage of 16% at a acrylamide/BIS ratio of 30:0.8 was used. A pH of 8.8 for the 4X Laemmli lower buffer was also found to work best. A third parameter was that gels had to be run at a relatively high current of 50-65 mA to achieve good separation. Finally, the amount of protein added was crucial -- amounts of protein derived from >1 $O.D._{600}$ U of cells produced fuzzy bands. Electrophoresis was carried out using the above modifications, using prestained low molecular weight markers (Gibco BRL).

After electrophoresis, gels were fixed in 10% acetic acid, thoroughly rinsed in distilled water, and soaked in 0.7 M sodium salicylate, pH 7.0. Gels were then dried and exposed to x-ray film.

Western Blots

Proteins expressed in yeast were extracted as described (Peter et al., 1996). Briefly, Y231 cells containing p129, p151, p161, p162 or p163 were grown to $O.D._{600}$ ~0.5 and beaten with glass beads in the presence of protease inhibitors (0.2 mM PMSF, 2 mM leupeptin, 2 mM benzamide and 2% aprotinin) and 1:1 mixture of 20% TCA/TCA buffer (20 mM Tris, pH8.0, 50 mM ammonium acetate and 0.5 mM EDTA). The liquid portion from this tube was centrifuged and the protein pellet was resuspended in TCA-Laemmli buffer (2% SDS, 10% glycerol, 0.1 M Tris base, 10 mM EDTA). The proteins were boiled then centrifuged and the soluble portion was retained for SDS-PAGE analysis (Laemmli, 1970). Equal amounts of protein from each sample were electrophoresed on an 8% polyacrylamide gel and tranferred to nitrocellulose membranes (Schleicher & Schuell, Inc.) by electroblotting using the BioRad minigel system. Membranes

were incubated with a mouse anti-HA antibody (Berkeley Antibody Company) and probed with HRP-conjugated anti-mouse IgG (Boehringer Mannheim). Proteins were visualized using a chemiluminescent detection system (Amersham) and membranes were exposed to X-ray film.

Results

axl1 Mutants Affect **a***-Factor Secretion*

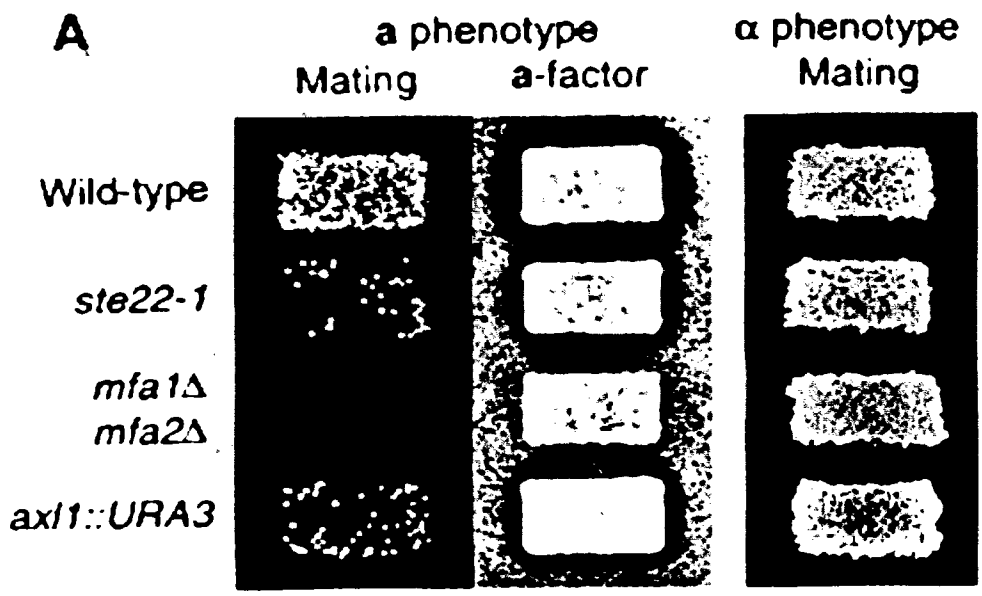
The original *ste22-1* mutant displayed a defect in **a**-factor secretion and an **a**-specific mating defect (Fig. 18A). Both defects were remedied by introduction of the cloned plasmid containing *AXL1* (Fig. 18B). As discussed in Chapter 3, *AXL1* is identical to *STE22*. The disruption allele, *axl1::URA3*, had phenotypes indistinguishable from those of the original mutant, indicating that the *ste22-1* mutation is a null allele (Fig. 18A). These mutants produced residual amounts of **a**-factor, as can be seen in comparison to a strain deleted for both **a**-factor structural genes. The *mfa1Δ mfa2Δ* strain produced no **a**-factor and is completely sterile (Fig. 18A).

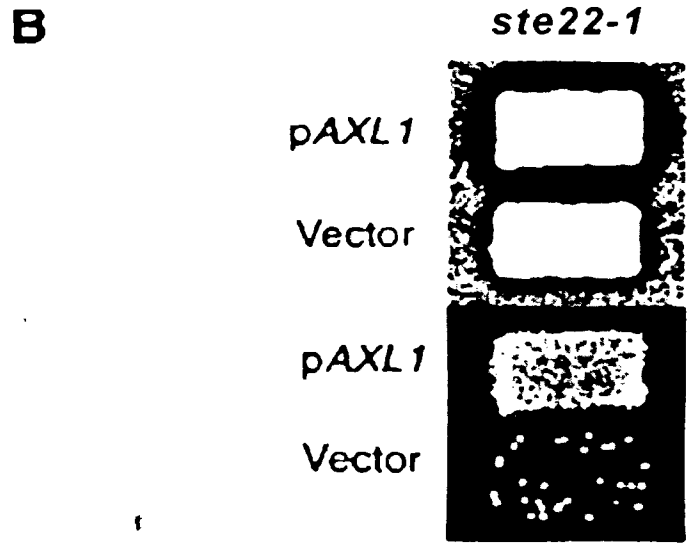
AXL1 Encodes a Pitrilysin Homologue

Axl1p shares sequence similarity to members of the pitrilysin family of endoproteases whose archetypes are human insulin-degrading enzyme (hIDE), or insulysin (EC 3.4.24.56), and *E. coli* protease III, or pitrilysin (EC 3.4.24.55) (Affholter et al., 1988; Rawlings and Barrett, 1991) - metalloproteases with a preference for small peptide substrates (Rawlings and Barrett, 1991). Another member of the hIDE family, rat *N*-arginine dibasic convertase (NRDC), is proposed to function as a prohormone processing enzyme (Pierotti et al., 1994). A highly conserved domain is present in hIDE-like sequences that is likely to be important both for proteolysis and for metal binding (Fig. 19).

Figure 18. Mating and **a**-factor defects of *ste22-1* (*axl1*) cells.

(*A*) Mating and **a**-factor assays were performed as described in Chapter 1, Materials and Methods. In mating assays, white diploid colonies produced by successful matings appear against a dark background. *MAT***a** cells were mated to the tester strain Y63 (*MAT*α *lys1*), while *MAT*α cells were mated to Y62 (*MAT***a** *lys1*). In pheromone assays *MAT***a** cells were patched onto a lawn of *MAT*α cells consisting of a strain (SY2014) that is hypersensitive to **a**-factor. Cells of the lawn surrounding the patch arrest and fail to grow, leaving a clear zone. The size of the zone is indicative of the amount of pheromone produced by cells within the patch. Assays were performed using wild-type (SY2625), *ste22-1* (Y49), *mfa1Δmfa2Δ* (Y115), and *axl1::URA3* (Y142) strains. The *MAT*α equivalents to these strains were Y83, Y278, Y195, and Y197, respectively. (*B*) Mating and pheromone secretion in *ste22-1* cells (Y49) transformed with empty vector or with p*AXL1* (p79).


A
a phenotype
Mating
a-factor
α phenotype
Mating
Wild-type
ste22-1
mfa1Δ
mfa2Δ
axl1::URA3


B
ste22-1
pAXL1
Vector
pAXL1
Vector

STE23 Codes for a Structural and Functional Homologue of Axl1p

axl1 null mutants were able to secrete residual levels of bioactive **a**-factor (Fig. 18A), and extra copies of *MFA2* and *RAM1* were able to partially rescue the mating and pheromone defects in these mutants (Fig. 20A). These observations implied that *S. cerevisiae* contains a functional homologue of Axl1p. The chromosome XII sequencing project has identified an ORF, YLR389C, which we call *STE23*, encoding a new member of the pitrilysin family (Fig. 19). To assess the function of *STE23*, we created a *ste23Δ::URA3* mutation. When introduced into a wild-type *AXL1* strain, the *ste23Δ::URA3* mutation failed to perturb mating efficiency or secreted **a**-factor activity (Fig. 20B). In contrast, experiments with the *axl1::URA3 ste23Δ::URA3* double mutant clearly showed that *ste23Δ::URA3* accentuated both the mating and pheromone secretion defects of *axl1::URA3* cells (Fig. 20B). Moreover, extra copies of *MFA2* and *RAM1* were unable to alleviate the **a**-factor defect of the *axl1Δ::LEU2 ste23Δ::LEU2* double mutant, indicating a complete block in the biosynthesis of this peptide pheromone (Fig. 20A).

*Axl1p and Ste23p are Required for the Final Event in Pro-**a**-Factor Maturation*

Because insulysin homologues can function as specific endoproteases, Axl1p and Ste23p may act as propheromone processing enzymes. An *axl1* mutant was tested for defects in carboxy-terminal CaaX-proteolysis and was found to be unaffected (M. Ashby, personal communication). To address the possibility that Axl1p is involved in amino-terminal processing, **a**-factor peptides were labeled with [^{35}S]cysteine in a pulse-chase protocol, then immunoprecipitated from both intracellular and extracellular fractions and subjected to SDS-PAGE. Three different intracellular **a**-factor peptides were observed in cells containing a functional *AXL1* gene (Fig. 21A). The largest form, designated P1 , has been assigned to a propheromone molecule with a completely modified carboxy-terminus, yet retains an unprocessed amino-terminal extension (Fig. 16 and Fig. 21D) (Chen et al., 1997). The P2 propheromone is derived from P1 through proteolytic

Figure 19. Alignment of Axl1p and Ste23p with members of the pitrilysin family of endopeptidases.

Some representative proteins belonging to the pitrilysin subfamily of metallopeptidases (M16 family) were manually aligned using the MACDNASIS Pro v3.0 program. Yeast proteins are denoted by Sc, *Caenorhabditis elegans* proteins by Ce, human by Hs, rat by Rn, and *E. coli* by Ec. The aligned sequences are Axl1p (Genbank accession no. D17787), Ste23p (Genbank accession no. U19729), insulin-degrading enzyme (IDE; Genbank accession no. M21188), *C. elegans* ORF C02G6.1 (Genbank accession no. U55372), *C. elegans* ORF C28F5.4 (Genbank accession no. U23180), *N*-arginine dibasic convertase (NRDC; Swiss-Prot accession no. P47245), protease III (ptr; Genbank accession no. X06227), *C. elegans* ORF C05D11.1 (Genbank accession no. AF01642), yeast ORF YOL098c (EMBL accession no. Z74840), and yeast ORF YDR430c (SGD ID no. S0002839). Numbers to the left and right of the alignments show the positions from the start codon of the first and last residue in the indicated line of sequence. Residues that are identical in at least five of the ten proteins have dots over them. Residues that are conserved (where I=L=M=V, H=K=R, D=E, S=T, and F=Y) in at least five proteins are indicated with commas over them. Residues that are identical in all ten of the proteins are shown in bold print. The conserved putative active site residues are shown by asterisks. Large gaps in the sequence of a protein is indicated by *(X)n*, where n is the number of residues omitted from the alignment. The 90 amino acid gap in NRDC consists of an extremely acidic domain inserted in the conserved region. The function of this domain is not known. C05D11.1, YOL098c and YDR430c show greatest similarity to each other and little similarity, outside of the core active site residues, to the other pitrilysin-like proteins. These three proteins possibly constitute a distinct subgroup of the pitrilysin subfamily.

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                    .;.  . .   .. ; . ..;   ; ..;...        ..  ..    . .. ; ..
Sc Axl1          14 YIPLSYSNRT HKVCKLPNGI LALIISDPTD ----SSSCSL TVCTGSHNDP  60
Sc Ste23         64 FLKPDLDERS YRFIELPNKL KALLIQDPKA ----KAAASL DVNIGAFEDP 109
Hs IDE           54 ITKSPEDKRE YRGLELANGI KVLLMSDPTT ----KSSAAL DVHIGSLSDP  99
Ce C02G6.1       16 IVKGAQDDRE YRGLELTNGI RVLLVSDPTT ----KSAAAL DVKVGHLMDP  61
Ce C28F5.4       17 IVKGSQDTKK YRGLELTNGL RVLLVSDSKT ----VSAVAL DVKVGHLMDP  63
Rn NRDC         100 IIKSPSDPKQ YRYIKLQNGL QALLISDLSN (x)91 QSAAAL CVGVGSFADP 236
Ec ptr           34 IRKSDKDNRQ YQAIRLDNGM VVLLVSDPQA ----KSLSAL VVPVGSLEDP  79
Ce C05D11.1       6 LWSCTETVLN GGIKLFLYSS KNTKLRVAIG ----VPGPMV HGAVSFVTEA  51
Sc YOL098c        6 LVSFQPDYVP QYHITKYISE RTKLQLVHIN ----KTSPLV HGYFAVPTEC  51
Sc YDR430c       30 EVRRILPVPE LRLTAVDLVH SQTGAEHLHI ----RDDKNN VFSIAFKTNP  75

                      *    **
                    ;; ...:;. :..... ... ... .  ,    ;.   ..    .. . ;. .
Sc Axl1          61 KDIAGLAHLC EHMILSAGSK KYPDPGLFHT LIAKNNGSQ- NAFTTGEQTT 109
Sc Ste23        109 KNLPGLAHFC EHLLFM-GSE KFPDENEYSS YLSKHGGSS- NAYTASQNTN 157
Hs IDE           99 PNIAGLSHFC EHMLFL-GTK KYPKENEYSQ FLSEHAGSS- NAFTSGEHTN 147
Ce C02G6.1       61 WELPGLAHFC EHMLFL-GTA KYPTENEYSK FLTDNAGHR- NAVTASDHTN 109
Ce C28F5.4       63 WELPGLAHFC EHMLFL-GTA KYPSEREYFK YLAANNGDS- NAYTDTDHTN 111
Rn NRDC         236 DDLPGLAHFL EHMVFM-GSL KYPDENGFDA FLKKHGGSD- NASTDCERTV 284
Ec ptr           79 EAYQGLAHYL EHMSLM-GSK KYPQADSLAE YLKMHGGSH- NASTAPYRTA 127
Ce C05D11.1      51 DSDDGLPHTL EHLVFM-GSK KYPFKGVLDV IANRCLADGT NAWTDTDHTA 100
Sc YOL098c       51 LNDSGAPHTL EHLIFM-GSK SYPYKGLLDT AGNLSLSNT- NAWTDTDQTV 99
Sc YDR430c       75 PDSTGVPHIL EHTTLC-GSV KYPVRDPFFK MLNKSLANFM NAMTGPDYTF 124

                    . .;.  .  .   ..  .... ... .  ..    ;..  .    ..
Sc Axl1         110 FYFELPNTQN NGEFTFESIL DVFASFFKEP LFNPLLISKE IY------AI 153
Sc Ste23        158 YFFEVNHQHL FG------AL DRFSGFFSCP LFNKDSTDKE IN------AV 195
Hs IDE          148 YYFDVSHEHL EG------AL DRFAQFFLCP LFDESCKDRE VN------AV 185
Ce C02G6.1      110 YHFDVKPDQL RG------AL DRFVQFFLSP QFTESATERE VC------AV 147
Ce C28F5.4      112 YSFEVRSEKL YG------AL DRFAQFFLDP QFTESATERE VC------AV 149
Rn NRDC         285 FQFDVQRKYF KE------AL DRWAQFFIHP LMIRDAIDRE VE------AV 322
Ec ptr          128 FYLEVENDAL PG------AV DRLADAIAEP LLDKKYAERE RN------AV 165
Ce C05D11.1     101 YTLSTVGSDG FL-----KVL PVYINHLLTP MLTASQFATE VH------HI 139
Sc YOL098c      100 YTLSSAGWKG FS-----KLL PAYLDHILHP TLTDEACLTE VY------HI 138
Sc YDR430c      125 FPFSTTNPQD FA-----NLR GVYLDSTLNP LLKQEDFDQE GWRLEHKNIT 169

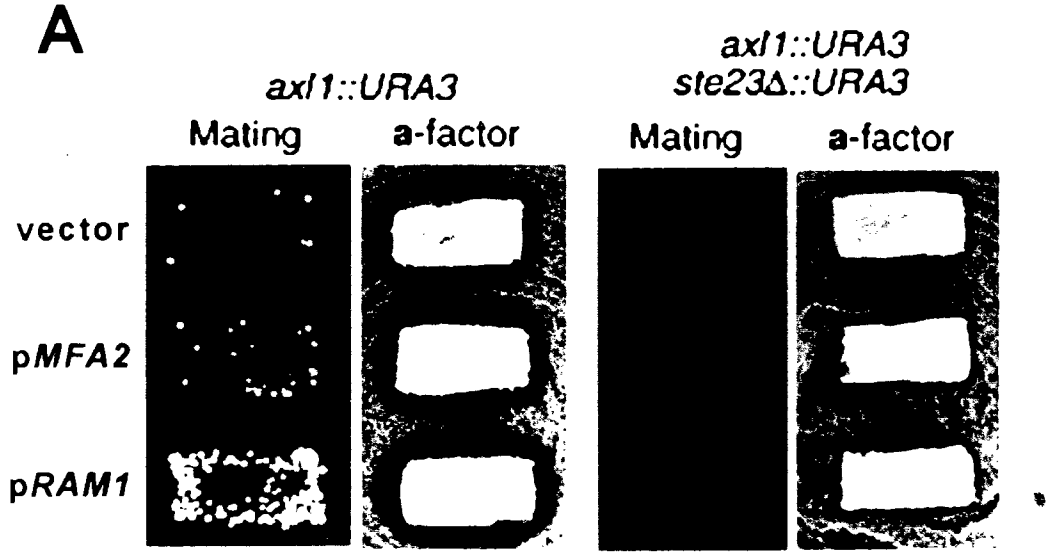
                     *
                    ...  .;   .; . .; ;  .. .. ..;   ..  ..  ..    .  .;
Sc Axl1         154 QSEHEGNISS TTKIFYHAAR ILANPDHPFS RFSTGNIHSL SSIPQLKKIK 203
Sc Ste23        196 NSENKKNLQN DIWRIYQLDK SLTNTKHPYH KFSTGNIETL GTLPKENGLN 245
Hs IDE          186 DSEHEKNVMN DAWRLFQLEK ATGNPKHPFS KFGTGNKYTL ETRPNQEGID 235
Ce C02G6.1      148 DSEHSNNLNN DLWRLSQVDR SLSKPGHDYA KFGTGNKKTL LEEARKKGVE 297
Ce C28F5.4      150 NCEYLDKVNE DFWRCLQVER SLSKPGHDYS KFAIGNKKTL LEDPRTKGIE 199
Rn NRDC         323 DSEYQLARPS DANRKEMLFG SLARPGHPMG KFFWGNAETL KHEPKKNNID 372
Ec ptr          166 NAELTMARTR DGMRMAQVSA ETINPAHPGS KFSGGNLETL SDKP---GNP 212
Ce C05D11.1     140 TGEG--NDAG VVYSEMQDHE SEMESIMDR- KTKEVIYPPF NPYAVDTGGR 186
Sc YOL098c      139 DPEN-LGDKG VVFSEMEAIE TQGWYISGLE KQ-RLMFPEG SGYRSETGGL 186
Sc YDR430c      169 DPESNIVFKG VVYNEMKGQI SNANYYFWS- KFQQSIYPSL -NNSGGDPMK 217

                    ,  .  .    ;.. .;.  ..;.;.;..      . ;  ..  . . ... .
Sc Axl1         204 LKSSLNTYFE NNFFGENITL CIRGPQSVN(x)673 LTLVPDLR NKKQIGYIVL 924
Sc Ste23        246 VRDELLKFHK NFYSANLMKL CILGREDLD(x)546 EPCFDTLR TKEQLGYVVF 838
Hs IDE          236 VRQELLKFHS AYYSSNLMAV CVLGRESLD(x)547 EPCFNTLR TKEQLGYIVF 829
Ce C02G6.1      198 PRDALLQFYK KWYSSNIMTC CIIGKESLD(x)541 EPVFDTLR TNEALGYIVW 785
Ce C28F5.4      200 PRDALLDFYK NWYSSDIMTC CIVGKESLD(x)373 NPAYTILR TNEALGYNVS 619
Rn NRDC         373 THARLREFWM RYYSAHYMTL VVQSKETLD(x)551 EPCFDFLR TKQTLGYHVY 970
Ec ptr          213 VQQALKDFHE KYYSANLMKA VIYSNKPLP(x)538 PWFYNQLR TEEQLGYAVF 797
Ce C05D11.1     187 LKNLRESCTL EKVRDYHKKF YHLSNMVVT(x)560 GPLWRAIR -GDGLAYGAN 792
Sc YOL098c      187 TKNLRTLTND EIRQFHKSLY SSDNLCVIV(x)637 GPFWKGIR -GAGLAYGAS 869
Sc YDR430c      218 ITDLRYGDLL DFHHKNYHPS NAKTFTYGN(x)594 KHLHREVR -EKGGAYGGG 857

                    .  .    ; ; ;.    . ..   ..  ..
Sc Axl1         925 GGLRVLTDT- -VGIHITVMS GSSGHN-LET RINEYL  957
Sc Ste23        839 SSSLNNHGT- -ANIRILIQS EHTTPY-LEW RINNFY  871
Hs IDE          830 SGPRRANGI- -QSLRFIIQS EKPPHY-LES RVEAFL  863
Ce C02G6.1      786 TGCRFNCGA- -VALNIFVQG PKSVDYVLE- RIEVFL  819
Ce C28F5.4      620 TESRLNDGN- -VYLHVIVQG PESADHVLE- RIEVFL  653
Rn NRDC         971 PTCRNTSGIL GFSVTVGTQA TKYNSETVDK KIEEFL 1004
Ec ptr          798 AFPMSV-GR- QWGMGFLLQS NDKQPSFLWE RYKAEF  831
Ce C05D11.1     793 VFVKPDRKQI TLSLYRCAQP AVAYERTRDI IRKIVE  826
Sc YOL098c      870 MLKLCEINSW GFNIYRGADI IKCYEVGKQI VQDYAS  903
Sc YDR430c      858 ASYSALAGIF SFYSYRDPQP LKSLETFKNS GRYILN  891
```

Figure 20. Ste23p is a functional as well as structural homologue of Axl1p.

(*A*) Mating and **a**-factor assays of mutant *axl1Δ::LEU2* (Y173) transformed with empty vector, p*MFA2* (p82), or with p*RAM1* (p25). The *axl1Δ::LEU2 ste23Δ:::LEU2* double mutant (Y231) was also transformed with these plasmids and tested for mating and pheromone secretion. (*B*) Mating and pheromone assays were done with the following strains: wild-type (SY2625), *axl1::URA3* (Y142), *ste23Δ::URA3* (Y221), and *axl1::URA3 ste23Δ::URA3* (Y220).


A
axl1::URA3
Mating
a-factor
axl1::URA3
ste23Δ::URA3
Mating
a-factor
vector
pMFA2
pRAM1

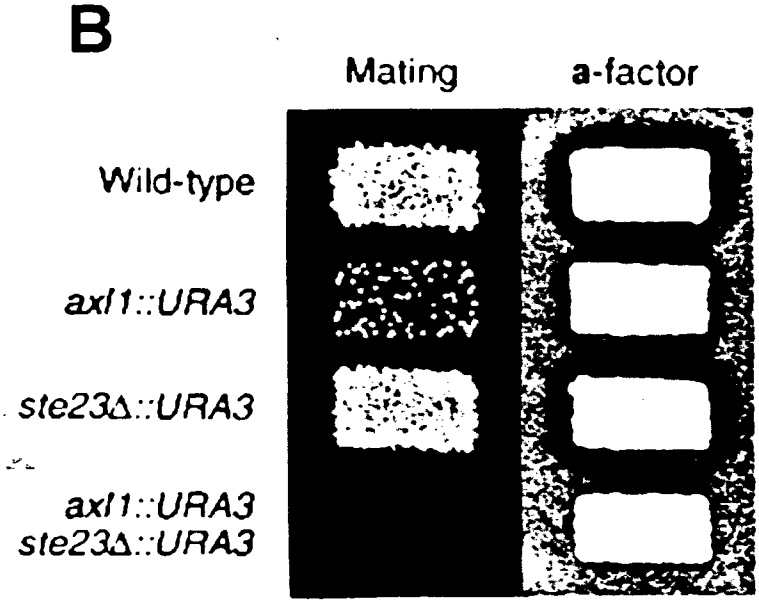
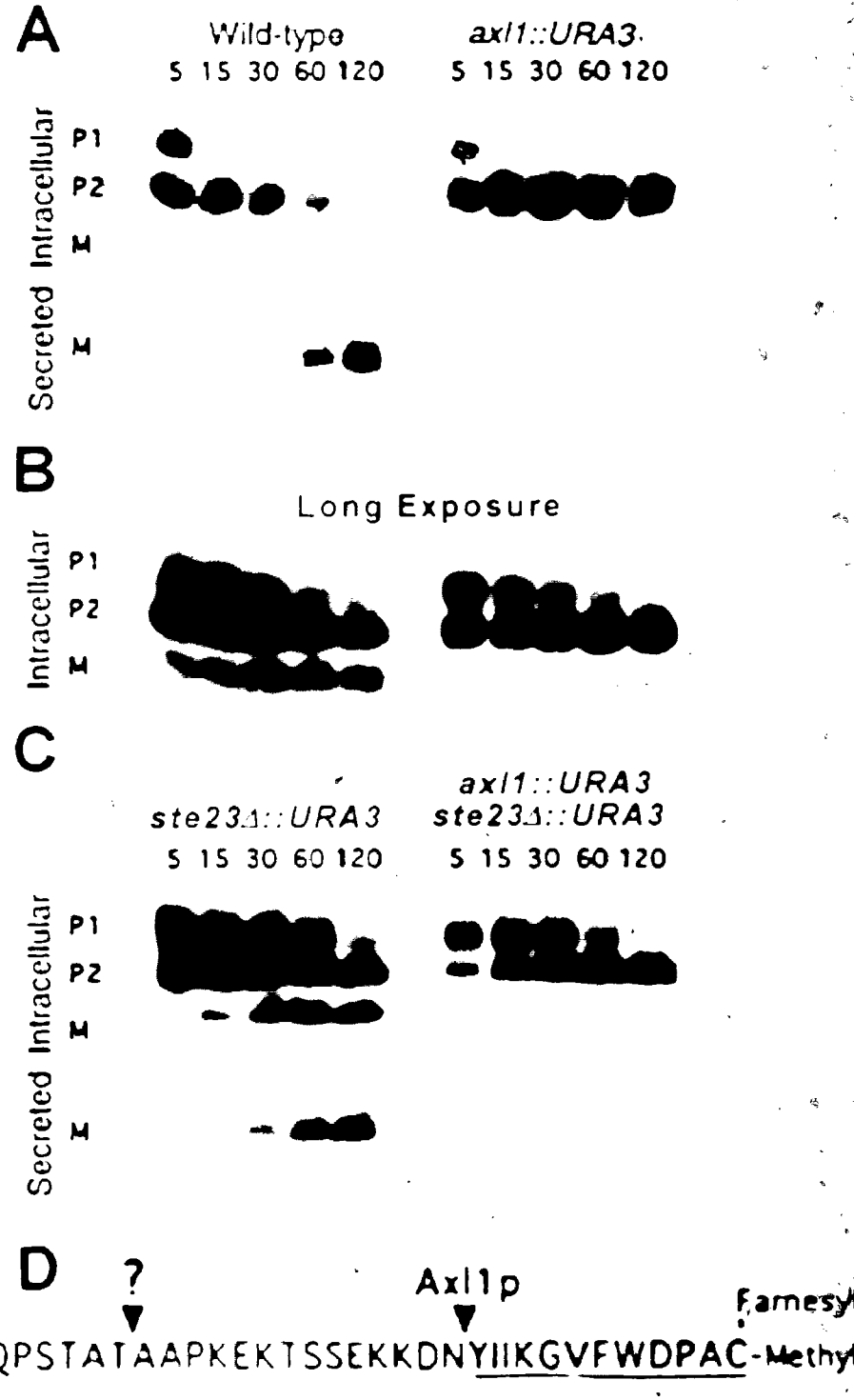

B
Mating
a-factor
Wild-type
axl1::URA3
ste23Δ::URA3
axl1::URA3
ste23Δ::URA3

Figure 21. *axl1* mutants accumulate an **a**-factor precursor that retains the amino-terminal extension.

Cells were pulse-labelled with [^{35}S]cysteine and label was chased with unlabelled methionine and cysteine. At the times indicated (min.), cells were pelleted and extracellular **a**-factor from the supernatant was immunoprecipitated with polyclonal antibodies against mature **a**-factor. Cells were broken open and intracellular **a**-factor was similarly immunoprecipitated. **a**-factor-antibody complexes were collected with protein A-sepharose beads, washed, and prepared for SDS-PAGE. Equal amounts of protein from each sample were loaded onto SDS-polyacrylamide gels and electrophoresed along with prestained marker protein used to follow the progress of the samples. Gels were treated with sodium salicylate, dried and exposed to x-ray film. (*A*) Three forms of **a**-factor are seen in the intracellular fractions of wild-type (SY2625) cells; P1, corresponding to pro-**a**-factor that is fully modified at the carboxy terminus but retains an intact amino-terminal extension, P2, in which the first seven amino acids have been removed, and the fully modified mature (M) **a**-factor. Only mature pheromone is efficiently exported into the media and seen in the secreted fractions. The *axl1::URA3* mutant (Y142) was simultaneously treated along with the wild-type cells. (*B*) A longer exposure of the gel shown in (*A*). (*C*) Pulse-chase experiments were performed simultaneously on *ste23Δ::URA3* (Y221) and *axl1::URA3 ste23Δ::URA3* (Y220) double mutant cells.


A
Wild-type
axl1::URA3
5 15 30 60 120
5 15 30 60 120
Secreted
Intracellular
P1
P2
M
M
B
Long Exposure
Intracellular
P1
P2
M
C
ste23Δ::URA3
axl1::URA3
ste23Δ::URA3
5 15 30 60 120
5 15 30 60 120
Secreted
Intracellular
P1
P2
M
M
D
?
Axl1p
Farnesyl
MQPSTATAAPKEKTSSEKKDNYIIKGVFWDPAC-Methyl

cleavage within the amino-terminal domain of the precursor (Fig. 16 and Fig. 21D; see also Chen et al., 1997). The species designated M corresponds to mature **a**-factor, which results from a second cleavage event within the amino-terminal region of P2 (Fig. 16 and Fig. 21D; Chen et al., 1997). Only the mature pheromone is efficiently exported across the plasma membrane and recovered from the extracellular fraction (Chen et al., 1997).

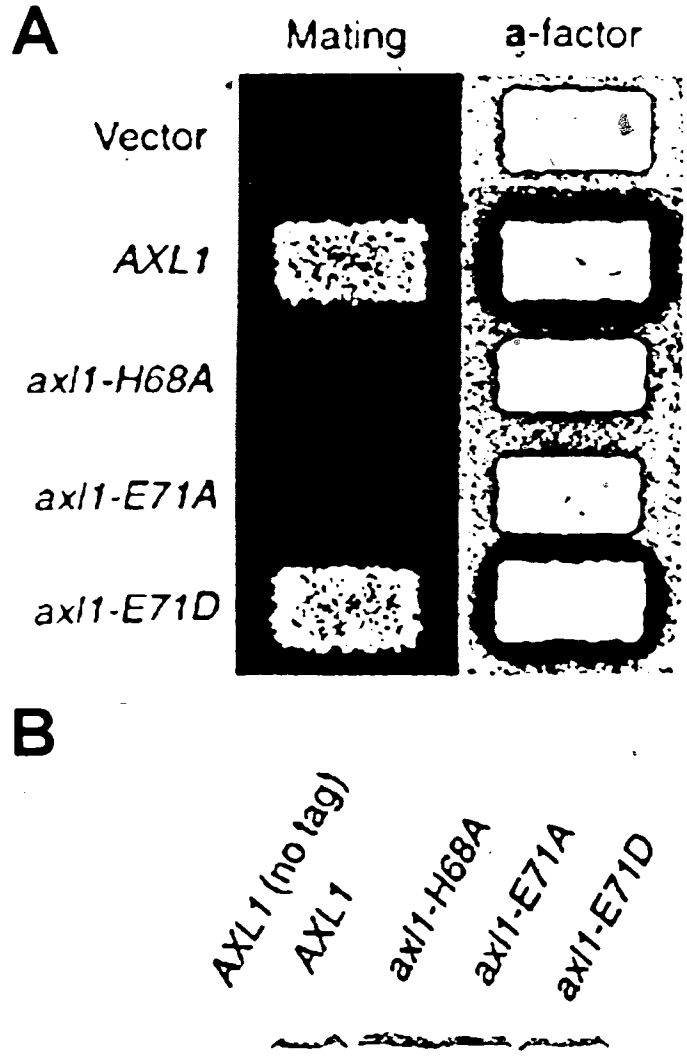
In *axl1::URA3* cells, the P1 propheromone was processed to the P2 form at a normal rate, but the P2 intermediate was processed inefficiently and remained relatively stable for the duration of the chase period (Fig. 21A). Longer exposure of the gels revealed small amounts of mature **a**-factor much lower than levels seen in *AXL1* cells (Fig. 21B). Consequently, *axl1::URA3* mutants produced very little **a**-factor for export (Fig. 21A and B).

Pulse-chase studies with *ste23Δ::URA3* cells indicated that the **a**-factor precursor was processed normally (Fig. 21C); however, the *axl1::URA3 ste23Δ::URA3* double mutant accumulated the P2 propheromone intracellularly (Fig. 21C), and possibly processed the P2 form less efficiently than *axl1::URA3* cells since no mature species was evident even after long exposures to film.

*The Putative Active Site of Axl1p is Required for **a**-Factor Processing*

Proteins of the pitrilysin family contain a sequence motif, $HXXEH(X)_nE$ with two invariant histidine residues and a glutamate, and a downstream glutamate (Becker and Roth, 1992; Hooper, 1994). These histidine residues of hIDE and protease III, have been implicated in metal binding and proteolytic activity (Becker and Roth, 1993; Gehm et al., 1993; Perlman et al., 1993; Perlman and Rosner, 1994). The first glutamate residue is also important for proteolysis (Perlman et al., 1993). By site-specific mutagenesis, we generated three variants of the HXXEH sequence found within Axl1p that correspond to substitutions of the first histidine (*axl1-H68A*), and the glutamate residue (*axl1-E71A* and *axl1-E71D*). Both the *axl1-H68A* and *axl1-E71A* alleles failed to restore pheromone production and mating to an *axl1 ste23* double mutant, whereas the more conservative substitution associated with *axl1-E71D* complemented these mutant

Figure 22. The putative active site of Axl1p is required for its proteolytic activity. *AXL1* constructs were made in which the first histidine of the putative active site was replaced with an alanine (*axl1-H68A*; p162), and in which the first glutamate was replaced with an alanine (*axl1-E71A*; p161) or with an aspartate (*axl1-E71D*; p163). An in-frame hemagglutinin (HA) epitope was also inserted between amino acids 854 and 855 of *AXL1* in these constructs, and also in p151, containing wild-type *AXL1*. (*A*) Mating and **a**-factor assays were performed on an *axl1Δ::LEU2 ste23Δ::LEU2* strain (Y231) transformed with empty vector, *AXL1* (p151), *axl1-H68A* (p162), *axl1-E71A* (p161), and *axl1-E71D* (p163). (*B*) Expression of the mutant proteins was analyzed by probing Western blots of protein extracted from the same transformants, with a monoclonal antibody against the HA epitope. Protein from Y231 cells transformed with a plasmid carrying an untagged *AXL1* (p129), indicated as *AXL1* (no tag), was also analyzed as a negative control.


A
Mating
a-factor
Vector
AXL1
axl1-H68A
axl1-E71A
axl1-E71D
B
AXL1 (no tag)
AXL1
axl1-H68A
axl1-E71A
axl1-E71D

phenotypes (Fig. 22A). The loss of function associated with *axl1-H68A* and *axl1-E71A* was not due to reduced product expression or stability, because epitope-tagged versions of the altered proteins were detected at steady-state levels that were identical to those of the wild-type protein (Fig. 22B).

Discussion

Mutants in *AXL1* displayed **a**-specific mating defects and reduced **a**-factor secretion. Genes that were known to be involved in pro-**a**-factor processing or secretion failed to rescue the phenotypes associated with the original *ste22-1* isolate, suggesting that the defective gene was unknown. The mutated gene, *AXL1*, encodes a homologue of the pitrilysin family of endopeptidases.

The pitrilysin family consists of two groups of proteins, the pitrilysin subfamily, of which Axl1p is a member, and the mitochondrial processing peptidase (MPP) subfamily. Members of the MPP subfamily include α- and β-MPP subunits from several organisms (Braun and Schmitz, 1995). These enzymes reside on the matrix side of the inner mitochondrial membrane and cleave the amino-terminal mitochondrial targeting sequence from imported proteins (Braun and Schmitz, 1995). Only the β-MPP subunits retain the conserved $HXXEH(X)_nE$ motif and are presumed to be the catalytic subunit, while the α-subunit has lost one or more of the zinc-binding residues and is proposed to have an essential function in presenting signal peptides to the β subunit (Braun and Schmitz, 1995). The MPP subfamily also includes the core proteins of cytochrome *c* reductase which have no proteolytic function as subunits of this enzyme but are necessary for its function (Braun and Schmitz, 1995). Interestingly, some of these proteins have dual roles as core proteins and as MPP proteins (Braun and Schmitz, 1995).

Why are the core proteins of the respiratory chain related to proteases? It is possible that the mitochondrial proteins in the pitrilysin family evolved from endosymbionts which contained pitrilysin-like endopeptidases. These endosymbionts were derived from non-sulphur purple bacteria, in the case of mitochondria. Bacteria have no core protein subunits in cytochrome *c*

reductase (Braun and Schmitz, 1995). It has been proposed by Braun and Schmitz (1995) that a pitrilysin-like protease evolved into an ancestral MPP by association with the cytochrome *c* reductase complex in the mitochondrial progenitors. This new function for the protease evolved as many of the mitochondrial genes moved to the nucleus and, therefore, required transport of their products back into the organelle.

Subsequently, a gene duplication of the protease resulted in an α-MPP and β-MPP protease heterodimer that was specific for imported proteins. Eventually, one of these proteases, the α-MPP subunit, lost one or more of the zinc ligands of the $HXXEH(X)_nE$ motif, and the proteases became indispensible for cytochrome *c* reductase activity. The β-MPP/core I, α-MPP/core II combination corresponds to the situation found in the mitochondria of higher plants (Braun and Schmitz, 1995). Further gene duplications gave rise first to separate core II and α-MPP genes, the arrangement found in *Neurospora*, and then separate β-MPP and core I genes, resulting in a core I/core II complex and a separate β-MPP/α-MPP heterodimer in the matrix (Braun and Schmitz, 1995). The mitochondria of *S. cerevisiae* and mammals have this last arrangement of proteins (Braun and Schmitz, 1995). In support of this theory, the pitrilysin and MPP subfamilies were calculated to have diverged approximately 3500 million years ago, at about the time of the eukaryote/eubacterium divergence (Rawlings and Barrett, 1995). In contrast, the insulysin and pitrilysin proteins are estimated to have diverged only 1400 million years ago, suggesting that the eukaryotic members of this subfamily are derived from endosymbionts rather than bacteria (Rawlings and Barrett, 1995).

Another member of the pitrilysin family is the chloroplast processing enzyme (CPE) from peas (Vander Vere et al., 1995). CPE cleaves the amino-terminal signal peptides of chloroplast proteins as they enter the stroma (Vander Vere et al., 1995). This enzyme doesn't appear to be a member of either the pitrilysin or MPP subfamilies. It is most related in sequence to bacterial enzymes and seems to show an equal degree of relatedness to the two subfamilies (Vander Vere et al., 1995). Moreover, its localization in chloroplasts is distinct from the other pitrilysin-like proteases. This protease serves a role in chloroplast function analogous to that of the MPP

proteins in mitochondrial function. Presumably, this enzyme evolved from a pitrilysin-like protease that was present in the cyanobacterial precursor to chloroplasts. CPE may be the first example of a family of chloroplast proteases.

Members of the pitrilysin subfamily include the aforementioned proteases and some others, including three yeast homologues in addition to Axl1p (Fig. 19). Of these yeast proteins, the product of the gene we have named *STE23* shows the most homology to Axl1p (31% identity over 293 amino acids in the amino-terminus). The similarity in sequence between all of the pitrilysin-related proteins resides in the amino-terminal part of the protein with an additional small region of homology at the carboxy terminus (Fig. 19). Cells deleted for *STE23* had no **a**-factor or mating defects but the *axl1 ste23* double mutants were completely sterile and produced less pheromone than the *axl1* mutants. Thus, our findings are consistent with the possibility that Ste23p is responsible for the residual propheromone processing displayed by *axl1* mutants.

The similarity of Axl1p and Ste23p to endopeptidases suggested that they might be required for proteolytic processing of **a**-factor. We found that *axl1* mutants were defective for the final proteolytic step in pro-**a**-factor maturation. This step involves the removal of the amino-terminal extension remaining after the first seven residues are cleaved (Fig. 16 and Fig. 21D). Only the mature form of **a**-factor is efficiently secreted by Ste6p; therefore, the failure to remove the amino-terminal extension results in the accumulation of the P2 form of pro-**a**-factor within the cell. Hence, the sterility of *axl1* cells largely resulted from a defect in P2 propheromone processing and reduced secretion of mature **a**-factor. The *axl1 ste23* double mutants also accumulated the P2 precursor, indicating that the two enzymes functionally overlap, executing the same step in pro-**a**-factor maturation. Ste23p is likely to have a lower affinity for pro-**a**-factor than Axl1p since increasing the levels of pro-**a**-factor with extra copies of *MFA1* or *RAM1* (which is likely mutated and catalyzes a rate-limiting step in SY2625) resulted in increased **a**-factor secretion in *axl1* null mutants.

The pitrilysin proteases have a conserved motif, HXXEH, that is essential for proteolytic activity (Fig. 19). This motif is an inversion of the HEXXH motif found in the bacterial

endopeptidase thermolysin and, like the thermolysin motif, is presumed to constitute the active site of the pitrilysins. In thermolysin, the two histidines and a downstream glutamate coordinate a zinc ion in the active site, while the glutamate of the motif polarizes a water molecule with the aid of the zinc ion (Roques, 1993).

Each molecule of pitrilysin or hIDE binds one molecule of zinc, but when any of the conserved histidines or the downstream glutamate are replaced with other residues the protein can no longer bind zinc and loses its proteolytic activity (Becker and Roth, 1993; Gehm et al., 1993; Perlman et al., 1993; Perlman and Rosner, 1994). Changing the glutamate of the HXXEH motif also prevents proteolysis, but has no effect on zinc-binding (Gehm et al., 1993; Perlman et al., 1993; Perlman and Rosner, 1994). In addition, it was shown that the mutant hIDE proteins were still able to bind their substrate, insulin, indicating that these conserved residues are not involved in substrate recognition (Perlman et al., 1993).

Mutants of Axl1p in which the conserved residues, histidine-68 or glutamate-71, were replaced with alanine were unable to complement the mating and pheromone defects of *axl1 ste23* cells; however, the mutant Axl1p containing the conservative change of aspartate in place of glutatmate-71 was able to function in pro-**a**-factor processing. These results show that the conserved HXXEH motif of Axl1p is important for proteolysis and is consistent with the proposal that this motif forms part of the protease active site.

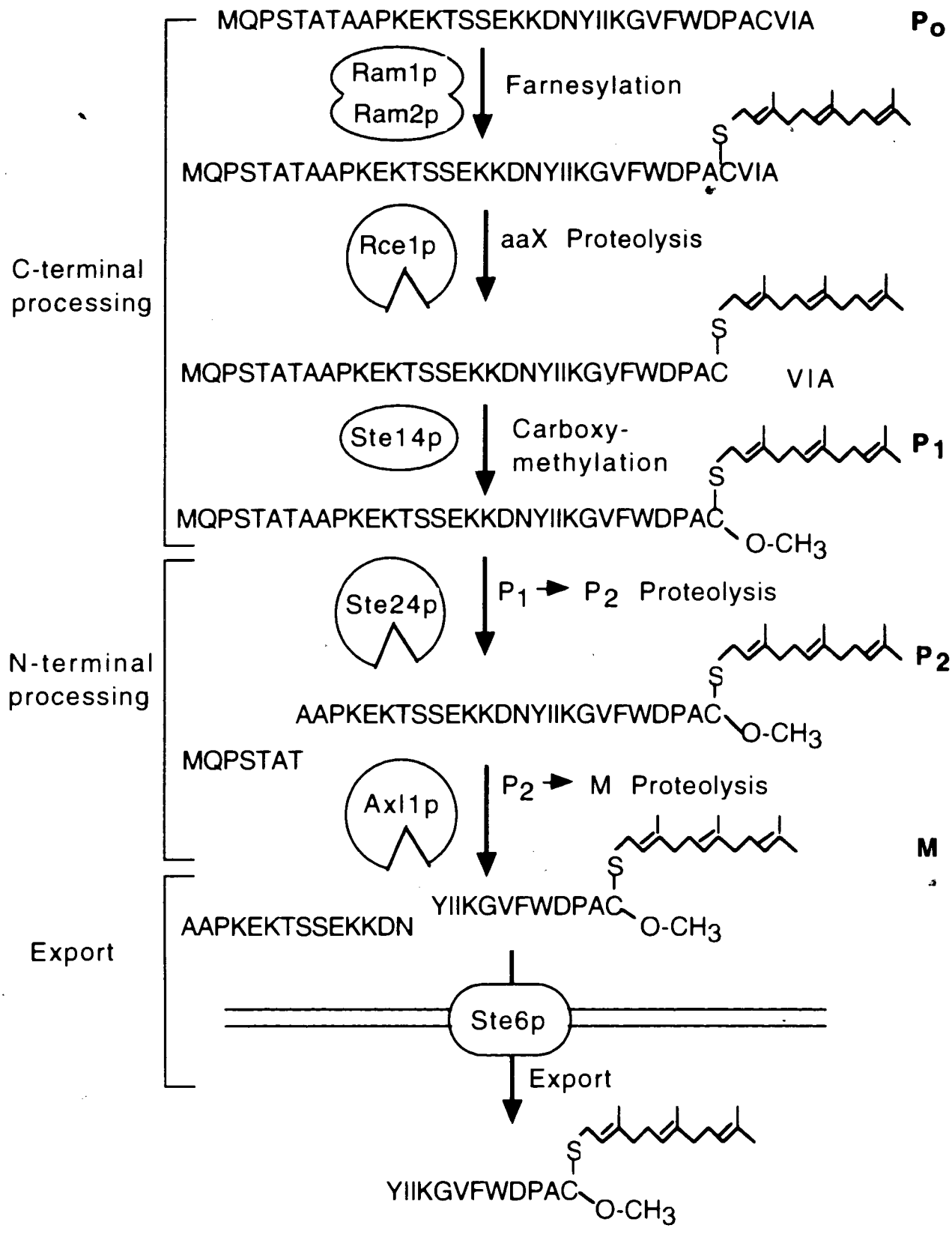
The physiological substrates of the pitrilysin-like proteins are unknown. *In vivo*, hIDE is implicated in the degradation of intracellular insulin; however, the broad range of tissues in which hIDE is present and substrates which can be cleaved by hIDE suggests that this protease has other functions (Akiyama et al., 1988; Rosner, 1990; Fagan and Waxman, 1991; Muller et al., 1991). For example, hIDE has a high affinity for and degrades atrial natriuretic peptide (ANP), a hormone implicated in nitric oxide regulation of vasodilation (Muller et al., 1991). There is evidence that hIDE might also degrade the signal sequence of peroxisomal proteins (Authier et al., 1995). This latter function is consistent with localization of hIDE within peroxisomes (Authier et al., 1994; Kuo et al., 1994). The human, rat and *Drosophila* IDEs all contain a carboxy-terminal

consensus sequence for peroxisomal targeting, A/S-K-L, suggesting that they may all have similar functions in the cell. However, insulin does not enter peroxisomes and most of the cellular hIDE is found in the cytoplasm (Akiyama et al., 1988; Kuo et al., 1994). It has been proposed that the insulin-degrading activity of hIDE requires only the cytoplasmic form and that the location of the protease is regulated (Kuo et al., 1994). Accordingly, mutation of the peroxisomal targeting sequence of hIDE prevents peroxisomal localization but has no effect on insulin degradation (V. Chesneau, R. Perlman, W. Li, G.-A. Keller and M. Rosner, personal communication).

Another endopeptidase in the pitrilysin subfamily is rat *N*-arginine dibasic convertase (NRDC). This protein is found in high abundance in the testes, and has been shown to cleave several hormone precursors (particularly somatostatin) at physiologically relevant sites - on the amino-terminal side of an arginine that is part of a doublet of basic residues (Pierotti et al., 1994). In addition, NRDC has a signal sequence for transport into the ER and, presumably, is a lumenal protein since it has no transmembrane domain (Pierotti et al., 1994). These observations suggest that NRDC is a prohormone convertase in the secretory pathway.

Our findings imply that the proteolytic activity of Axl1p is required for the final step in **a**-factor maturation. The genes encoding the CaaX-box protease, Rce1p, and the amino-terminal protease that removes the first seven residues, Ste24p, have both recently been isolated (Boyartchuk et al., 1997; Fujimura-Kamada et al., 1997). Molecular identities have now been assigned to all of the enzymatic activities known to be involved in pro-**a**-factor processing (Fig. 23). Ste24p is a metalloprotease containing a thermolysin-like $HEXXH(X)_nE$ active site motif (Fujimura-Kamada et al., 1997). Interestingly, *STE24* mutants accumulate the P1 form of pro-**a**-factor, indicating that Axl1p is unable to cleave pro-**a**-factor with a complete amino-terminal extension (Fujimura-Kamada et al., 1997). It is puzzling why yeast evolved the necessity for two sequential clips of the pro-**a**-factor amino-terminal extension. Ste24p is present in all cell types (Fujimura-Kamada et al., 1997) and Axl1p is expressed in *MAT***a** and *MAT*α cells (Fujita et al., 1994; see Chapter 5) suggesting that these proteases are involved in other processes as well as **a**-factor processing. Perhaps these proteins act together in the proteolysis of other substrates, and

Figure 23. All of the proteins necessary for **a**-factor maturation and secretion are now known. Until recently, none of the proteases involved in processing of the **a**-factor precursor had been assigned to genes. However, it is now known that the CaaX-box protease is encoded by *RCE1*, and amino-terminal processing is accomplished, sequentially, by the products of the *STE24* and *AXL1* genes. Each step of **a**-factor maturation requires completion of the previous step, with the exception that Ste24p and Axl1p can cleave unmethylated propheromone (although Ste6p is unable to transport this form of **a**-factor).


MQPSTATAAPKEKTSSEKKDNYIIKGVFWDPACVIA
Po
Ram1p
Ram2p
Farnesylation
MQPSTATAAPKEKTSSEKKDNYIIKGVFWDPACVIA
Rce1p
aaX Proteolysis
C-terminal processing
MQPSTATAAPKEKTSSEKKDNYIIKGVFWDPAC
VIA
Ste14p
Carboxy-methylation
P1
MQPSTATAAPKEKTSSEKKDNYIIKGVFWDPAC
O-CH3
Ste24p
P1 → P2 Proteolysis
N-terminal processing
P2
AAPKEKTSSEKKDNYIIKGVFWDPAC
O-CH3
MQPSTAT
Axl1p
P2 → M Proteolysis
M
AAPKEKTSSEKKDN
YIIKGVFWDPAC
O-CH3
Export
Ste6p
Export
YIIKGVFWDPAC
O-CH3

this sequential processing permits tighter regulation of these events. It is interesting that proteolytic processing of mitochondrial targeting signals also occurs in two sequential steps, catalyzed by MPP and mitochondrial intermediate peptidase (MIP) (Rawlings and Barrett, 1995).

Rce1p and Ste24p, as well as the carboxymethyltransferase, Ste14p, are predicted to be membrane proteins and these proteins all process a farnesylated and membrane-bound form of pro-**a**-factor to generate mature pheromone for secretion; therefore, we suggest that Axl1p may also localize to the cytoplasmic surface of membranes. To do so, Axl1p would likely need to associate with a membrane protein since it has no apparent transmembrane or membrane targeting domains. It is possible that Axl1p associates with the **a**-factor transporter Ste6p. Complexes of proteases and transporters for their substrates or products would permit direct exchange of the molecule and would increase the efficiency of transport. An extreme example of association between a protease and transporter is found in a *Dictyostelium* ABC transporter, tagB/tagC, which contains serine protease domains (Shaulsky et al., 1995). This direct coupling of proteolysis and transport probably confers a high degree of specificity on the protease and transporter so that both domains recognize only proteins on which the other domain can act.

The processing and secretion of **a**-factor involves many highly conserved proteins that are of biological significance. CaaX-box processing is of great interest to researchers studying signal transduction and cancer. Farnesyltransferase inhibitors have been studied as potential chemotherapeutic agents (Gibbs et al., 1994), and perhaps inhibitors of CaaX-box proteases will follow suit. Moreover, secretion of **a**-factor may utilize mechanisms similar to other proteins, such as interleukin-1α (IL-1α), IL-1β, fibroblast growth factor-1 (FGF-1), and FGF-2, that are secreted through a non-classical pathway (Howard et al., 1991; Kuchler and Thorner, 1992; Rubartelli et al., 1992; Forough et al., 1993; Rubartelli et al., 1993; Higgins et al., 1994). Finally, the role of Axl1p in pro-**a**-factor maturation supports the notion that some insulinase homologues, such as NRDC, function as eukaryotic propeptide convertases.

CHAPTER 5

You Separate Him From His Mummy and Make Him Bud Off

Introduction

Budding in yeast requires highly polarized cell surface growth directed to the bud. Growth is mediated by the actin cytoskeleton, which serves as tracks for vesicular and organellar movement (Mills et al., 1994; Welch et al., 1994; Govindan and Novick, 1995). In particular, actin directs the movement of secretory vesicles carrying cargoes of plasma membrane and cell wall consituents (Mills et al., 1994; Welch et al., 1994; Govindan and Novick, 1995). Mutations in *ACT1* (encoding the only actin in yeast) and many genes whose products regulate or form actin structures often result in mislocalized actin structures and growth defects (Adams et al., 1989; Amatruda et al., 1992; Liu and Bretscher, 1992; Adams et al., 1993; Amberg et al., 1995; Drees et al., 1995; Govindan et al., 1995; Munn et al., 1995). Actin structures in yeast consist of long bundles of filaments called "cables" and short filaments of actin clustered in cortical patches (Mulholland et al., 1994; Welch et al., 1994). Both of these structures are necessary for efficient secretion and endocytosis (Liu and Bretscher, 1992; Cid et al., 1995). Actin structures are by no means static and cortical patches are especially dynamic and display high rates of movement (Doyle and Botstein, 1996; Waddle et al., 1996). This movement is thought to evenly distribute the incorporation of membrane and wall components (Doyle and Botstein, 1996; Waddle et al., 1996). Despite the dynamic nature of patches, they and actin cables are constrained in their localization to regions of cell growth in a cell-cycle regulated manner (Doyle and Botstein, 1996; Waddle et al., 1996).

During the G_1-S transition, actin patches and growth are restricted to a small area on the surface of the mother cell at the presumptive bud site and continue to be polarized to the apex of the growing bud throughout S phase (Adams and Pringle, 1984; Kilmartin and Adams, 1984;

Ford and Pringle, 1991). Apical growth is promoted by the G_1 cyclins Cln1p and Cln2p (Adams and Pringle, 1984; Kilmartin and Adams, 1984; Ford and Pringle, 1991).

At M phase, actin structures are depolarized and growth becomes uniform in both cells in what is termed "isotropic growth" (Adams and Pringle, 1984; Kilmartin and Adams, 1984; Ford and Pringle, 1991; Lew and Reed, 1995). The apical-isotropic switch is mediated by the Clb1p and Clb2p cyclins (Adams and Pringle, 1984; Kilmartin and Adams, 1984; Ford and Pringle, 1991; Lew and Reed, 1995). This switch is delayed during starvation and pseudohyphal growth, resulting in prolonged apical growth and elongated cells (Lew and Reed, 1995). Overexpression of the Cln1p or Cln2p cyclins similarly leads to hyperpolarized cell growth. Accordingly, *grr1* mutants, which accumulate Cln2p, are constitutively pseudohyphal (Barral et al., 1995). Pseudohyphal cells have a longer G_2 phase, permitting growth of the bud until it reaches the same size as the mother (Kron et al., 1994). In this manner pseudohyphal cells undergo synchronous budding cycles since both daughter and mother cells can enter S phase immediately upon cytokinesis (Kron et al., 1994).

During cytokinesis, growth is redirected to the mother-bud neck from both cells (Adams and Pringle, 1984; Ford and Pringle, 1991; Kilmartin and Adams, 1984; Lew and Reed, 1995). This change is brought about through the destruction of Clb1p and Clb2p resulting in inactivation of Cdc28p (Lew and Reed, 1995). At this time, chitin is layed down in a ring around the mother-bud neck on the mother cell and a layer is deposited in the primary septum separating the two cells (Cid et al., 1995). Cell wall material is then layed down by the mother and daughter on both sides of the primary septum and separation of the cells occurs by degradation of the chitin in the primary septum (Cid et al., 1995). The mother bud retains a bud scar with the chitin ring, while the daughter bud has a birth scar devoid of chitin (Cid et al., 1995). Upon separation, the mother cell is able to almost immediately commence another cycle of budding, but the smaller daughter cell must first undergo further isotropic growth in G_1 before becoming large enough to enter S phase (Kron et al., 1994).

Yeast cells do not bud in random locations on the cell surface. Instead they have distinct patterns of bud site selection that are determined by the cell type and nutritional state of the cells. Haploid cells bud in an axial pattern, in which buds form at a site immediately adjacent to the site of the last bud (Chant, 1996; Chant and Pringle, 1991). Diploid cells bud at one or the other pole of the long axis in a bipolar pattern (Chant and Pringle, 1991; Chant, 1996; Zahner et al., 1996). These patterns are not regulated by ploidy but are genetically determined (Chant and Pringle, 1991; Chant, 1996).

Much progress has been made in recent years on determining how budding and bud site selection are regulated. Many genes have been uncovered in screens for mutants that bud in abnormal patterns. Mutations in several genes lead to random bud site selection both in diploids and in haploids, indicating that the two patterns utilize some common components. These genes, referred to as general bud site selection genes, are *RSR1* (also known as *BUD1*), *BUD2*, and *BUD5* (Table 11; Chant et al., 1991; Chant and Herskowitz, 1991).

It turns out that these genes encode proteins of a small GTPase cycle. Rsr1p is a small GTPase related to Ras (Ruggieri et al., 1992). Small GTPases act as molecular switches, and are active or "on" when in the GTP-bound state and are "off" when the GTP is hydrolyzed to GDP (Hall, 1992). Small GTPases have a low intrinsic GTPase activity and require cofactors called GTPase activating proteins (GAPs) to efficiently stimulate hydrolysis of GTP and effectively inactivate the GTPase (Hall, 1993). Proteins called guanine nucleotide exchange factors (GEFs) help exchange GDP bound to the GTPase for GTP, thus stimulating the GTPase . A third class of proteins, guanine nucleotide exchange inhibitors (GDIs), bind to the GDP-bound GTPase and prevents its activation and possibly also inhibits membrane localization (Hall, 1993; Ridley, 1995). Bud2p is a GAP while Bud5p is a GEF for Rsr1p (Chant et al., 1991; Chant and Herskowitz, 1991; Chant and Stowers, 1995).

The function of Rsr1p in bud site selection requires cycling between its active GTP-bound state and its inactive GDP-bound form. Mutants of either *BUD2* or *BUD5* have the same random-budding phenotype as *rsr1* mutants (Chant and Herskowitz, 1991). Furthermore, mutant

versions of Rsr1p that are unable to hydrolyze GTP (and are therefore constitutively active) or are unable to exchange bound GDP for GTP (and remain inactive) also lead to random bud site selection (Chant and Pringle, 1991; Chant and Stowers, 1995).

Another set of genes is responsible for polarity establishment in yeast (Table 11). Mutations in *CDC24*, *CDC42*, *BEM1* and *BEM3* prevent budding and cause misdirected growth throughout the cell, resulting in large round cells (Adams et al., 1990; Chenevert et al., 1992; Chenevert et al., 1994; Peterson et al., 1994; Zheng et al., 1994). Despite the presence of a cell cycle checkpoint that delays mitosis in the absence of bud formation, these mutants are unable to build a bud so that most cells in a population are multinucleate (Lew and Reed, 1995). The products of *CDC24*, *CDC42*, *BEM1* and *BEM3* are required to polarize the actin cytoskeleton and, thus, restrict growth to defined regions of the cell surface. Cdc24p, Cdc42p and Bem3p are consituents of another small GTPase cycle. Cdc42p is a member of the Rho/Rac subfamily of GTPases implicated in regulation of the actin cytoskeleton in all organisms in which they have been studied (Hall, 1993). This protein is necessary for actin polymerization in permeabilized yeast cells (Li et al., 1995). Cdc24p is the GEF and Bem3p is a GAP for Cdc42p (Chant and Stowers, 1995). Again, cycling of the GTPase between the GTP-bound and GDP-bound states is important for GTPase function, since null mutations of *CDC24* or *BEM3* and activated mutants of *CDC42* all produce the same lethal polarity defect (Chant and Stowers, 1995).

Components of the general bud site selection GTPase cycle interact with the polarity establishment proteins. *RSR1* was originally isolated as a multicopy suppressor of a temperature-sensitive *cdc24* mutation (Bender and Pringle, 1989). Moreover, the GTP-bound form of Rsr1p physically interacts with Cdc24p *in vivo* and *in vitro* (Zheng et al., 1994; Zheng et al., 1995); however, Rsr1p-GTP does not stimulate the *in vitro* GEF activity of Cdc24p for Cdc42p (Zheng et al., 1994; Zheng et al., 1995). Both Rsr1p and Cdc24p localize throughout the surface of cells, while Cdc42p localizes to sites of polarized growth (the incipient bud, the bud tip, and the bud neck at cytokinesis) (Chant and Stowers, 1995). Bud2p apparently localizes to bud sites (cited in Park et al., 1997), while the cellular location of Bud5p is not known at present. These data

Table 11. Genes involved in budding.

Genes	Gene Product	Mutant Phenotypes
Septins		
CDC3,CDC10,	10 nm filament ring proteins	Bipolar budding in haploids
CDC11,CDC12	GTP-binding domain	Cytokinesis defects
Axial bud-site selection genes		
BUD3	unknown	Bipolar budding in haploids
BUD4	GTP-binding domain	"
AXL1	**a**-factor protease	"
AXL2	Type I membrane glycoprotein	"
Genes affecting axial budding		
MYO1	Class II myosin	Bipolar budding in haploids
HKR1	Type I membrane glycoprotein	"
RGA1	GAP for Cdc42p	"
TPM2	Tropomyosin	Overexpression causes bipolar budding in haploids
General bud-site selection genes		
RSR1(BUD1)	Ras-related protein	Random budding in all cell types
BUD2	GAP for Rsr1p	Random budding in all cell types, *bud2-21* allele randomizes budding in diploids only
BUD5	GEF for Rsr1p	Random budding in all cell types, *Bud5-4* allele randomizes budding in diploids only
Polarity establishment genes		
CDC42	Rho/Rac GTPase	Round, multinucleate cells that are unable to bud. Overexpression randomizes bud site selection
CDC24	GEF for Cdc42p	Round, multinucleate cells that are unable to bud, *cdc24-3* and *cdc-24-4* alleles bud randomly at permissive temperature
BEM1	Two SH3 domains	Round, multinucleate cells that are unable to bud
BEM2	GAP for Rho1p	At restrictive temperature round, multinucleate cells that are unable to bud Random budding at permissive temperature
MYO2	Class V myosin	Round, multinucleate cells that are unable to bud.

continued on next page

Table 11 (cont'd). Proteins involved in budding.

Genes	Gene Product	Mutant Phenotypes
Bipolar bud-site selection genes		
ACT1	Actin	Random budding in diploids
SPA2	Coiled-coil domain	"
RVS161,RVS167	Homologues, SH3 domain in *RVS161*	"
FEN1, SUR1	Homologues involved in ergosterol biosynthesis, mutations in either can suppress *RVS161* and *RVS167* mutants	"
SEC3	Required for secretion, exocyst complex component	"
SEC4	Required for secretion, Rab GTPase	"
SEC9	Required for secretion, SNAP protein	"
BNI1	Formin homologue	"
PEA2, BUD6, BUD7	Unknown	"
BUD8	Unknown	Proximal budding
BUD9	Unknown	Distal budding

Adapted from Roemer et al. (1996)

suggest a model for selection of bud sites in which the bud site selection machinery interacts with cellular cues to localize and possibly activate Cdc42p at the incipient bud site, resulting in polarization of the actin cytoskeleton and cell growth. Since Rsr1p is present throughout cells it is likely activated at the bud site, possibly through localization and/or activation of Bud2p and Bud5p. Rsr1p-GTP could then recruit Cdc24p which, in turn, could recruit or activate Cdc42p at the bud site. Overexpression of either *CDC42* or *CDC24* leads to random bud site selection in all cell types, as do null mutations in the general site selection genes (Bender and Pringle, 1989). Thus, random bud site selection could be a result of delocalized Cdc42p, a consequence of both situations. What cue(s) Cdc42p then uses to polarize growth is unknown. Mutations in *BUD2* have been demonstrated to be synthetically lethal with *cln1 cln2* double mutations (Chant, 1996). This lethality may reflect a role for G_1 cyclins in activation of Cdc42p at random sites during random budding. Presumably, random bud site selection induced by overproduction of Cdc24p or Cdc42p in *cln1 cln2* mutants would also be lethal. The Rho proteins (Rho1p, Rho2p, Rho3p and Rho4p) are required for maintenance of bud growth and are possibly downstream effectors for Cdc42p (Cid et al., 1995). Rho1p in particular has been shown to directly regulate both Pkc1p, a protein kinase required for bud growth and maintenance, and Fks1p, the catalytic subunit of β(1,6)-glucan synthase (Nonaka et al., 1995; Drgonova et al., 1996; Qadota et al., 1996).

The cortical cues that direct bud site selection are currently the subject of much interest. The axial budding pattern suggests that the previous bud site is a template for building a new bud. A group of proteins called septins, and associated proteins are components of this cytokinesis tag (Chant, 1996; Longtine et al., 1996). The septins, Cdc3p, Cdc10p, Cdc11p and Cdc12p, are the initial axial cue and are assembled into a ring on the cell surface at the incipient bud site before bud emergence (Chant, 1996; Longtine et al., 1996). Bud growth is confined within the septin ring so that this ring eventually circles the neck between mother and bud (Chant, 1996; Longtine et al., 1996). Electron microscopy (EM) of sections through the bud neck reveals multiple rings composed of 10 nm filaments that encircle the neck just below the plasma membrane (Longtine et

al., 1996). Immunofluorescent, immunoEM, and studies with septin mutants indicate that these filaments are likely composed of the septins (Longtine et al., 1996). Moreover, *Drosophila* homologues of the yeast septins are able to form filaments *in vitro* (Field et al., 1996). During mitosis, the septin ring splits in two and is divided into the mother and daughter cells at cytokinesis (Chant, 1996). These rings persist for most of G_1 and then disappear at late G_1 about the time that a new ring assembles immediately adjacent to the old ring at the next bud site (Chant, 1996).

G_1 cyclins play an early role in establishing bud sites through the septins. *cdc12* mutations, like *bud2* mutations, are lethal in *cln1 cln2* mutants. Septin mutants also exhibit synthetic lethality amongst themselves, indicating that they play a crucial role in budding (Longtine et al., 1996). Temperature-sensitive mutations in septin genes result in loss of all four septins from the bud neck and prevent cytokinesis leading to cell death at the restrictive temperatures (Chant, 1996; Longtine et al., 1996). These defects implicate septins in cytokinesis, and septins from other organisms seem to play similar roles during cytokinesis. The *Drosophila* homologue Pnut is required for cytokinesis and it and another homologue, Sep1, both localize to cleavage furrows during normal cytokinesis and during cellularization of the syncitial blastoderm (Neufeld and Rubin, 1994; Fares et al., 1995). Despite their filamentous structure, the yeast septins are not part of a contractile apparatus since the rings do not decrease in size at any time (Longtine et al., 1996).

The neck filaments could conceivably act as a framework for the cytokinesis apparatus. They may do so by directing actin to the bud neck, since the repolarization of actin cable and patches towards the neck at cytokinesis is perturbed in septin mutants (Longtine et al., 1996). Unfortunately, it is not known if any of this actin serves a contractile function analogous to cytokinesis in animal cells. Mutants in *MYO1* (encoding the only conventional class II myosin in yeast) have defects in cytokinesis, but this phenotype can be attributed to defects in cell separation since these mutants fail to deposit chitin in the primary septum in a manner similar to *chs2* (chitin synthase II) mutants (Longtine et al., 1996). Therefore, Myo1p may be involved in delivery of

secretory vesicles containing Chs2p to the bud neck. Alternatively, actin and Myo1p may, indeed, form a contractile apparatus and the cell division that does occur along with aberrant septum structure may result from the accumulation of cell wall material directed to the mother bud neck and consequent constriction of the neck (Longtine et al., 1996).

Since yeast (unlike animal cells) have a cell wall, it is possible that there is no need for contractile actin in the bud neck and that cytokinesis is normally accomplished by directing cell wall and plasma membrane deposition to the neck region as I just described. Therefore, the role of the septins would be to serve as an initial target for this polarized secretion. Consistent with this interpretation, septin mutants have elongated buds due to hyperpolarization of actin to the bud tip (Longtine et al., 1996). Moreover, buds normally have very little chitin in their walls until cytokinesis occurs, but septin mutants mislocalize chitin diffusely throughout the lateral wall of the bud (Longtine et al., 1996). These observations indicate that the septin mutants do not properly undergo the apical/isotropic switch in growth or repolarize the actin cytoskeleton to the bud neck.

In addition to cytokinesis defects, septin mutants have altered budding patterns. Haploid mutants bud in the bipolar pattern instead of the normal axial pattern, but the budding pattern in diploid cells is unaffected (Longtine et al., 1996). Along with the persistence of neck filament localization until just before the formation of a new septin ring and bud site right next to the old site, this observation suggests that the neck filaments act as a template for the construction of a new bud (Chant, 1996; Longtine et al., 1996). Further support for this "cytokinesis tag" model for axial budding comes from work on two more bud mutants, *bud3* and *bud4*. Both of these mutants are specifically defective for axial bud site selection and haploids bud in the bipolar pattern (Chant et al., 1995; Sanders and Herskowitz, 1996). Both proteins also localize as single rings to the mother-bud neck during mitosis, split into two rings during cytokinesis, and finally disappear at the same time as do the neck filaments (Chant et al., 1995; Sanders and Herskowitz, 1996). Moreover, localization of Bud3p and Bud4p is dependent on the septins (Chant et al., 1995; Sanders and Herskowitz, 1996).

The septins, Bud3p and Bud4p act together as a cortical cue for the general bud site complex. This cue is short lived, as indicated by several experimental observations. Treatment of cells with low levels of pheromone results in the selection of bipolar bud sites (Chant et al., 1995; Sanders and Herskowitz, 1996; Dorer et al., 1997). Similarly, starving haploid cells choose bipolar positions for budding and resume axial budding at one of the poles once nutrients are reintroduced into the cultures (Cid et al., 1995). Both of these treatments lead to a delay in the G_1-S transition of the cell cycle and the loss of Bud3p (and possibly also Bud4p) (Chant et al., 1995; Sanders and Herskowitz, 1996). The existence of mutants that specifically affect axial budding indicates that axial site selection employs a different set of proteins than does bipolar site selection. Furthermore, the bipolar cortical cues are present even in haploid cells but the axial cues normally override these more persistent markers.

It seems that the bud neck filaments have a conserved role in cytokinesis and budding yeast have evolved a mechanism for bud site selection based on this role. How this cytokinesis tag interacts with the general bud site selection and polarity establishment proteins is still not understood and remains the focus of much research. Also, all of the proteins I have discussed are expressed in diploid as well as haploid cells (Chant et al., 1995; Longtine et al., 1996; Sanders and Herskowitz, 1996). The question remains of how haploids choose to use the axial bud site components while diploid cells do not. Here I discuss the role of Axl1p in determining the haploid pattern of bud site selection.

Material and Methods

Strains, Media and Microbiological Techniques

Yeast strains used are listed in Table 12. The genetic techniques and media used are as described in Rose et al. (1990) and Guthrie and Fink (1991). All strains were generated as outlined in Chapter 4 using strains Y212 (*MAT***a**) and Y214 (*MAT*α). Y212 was tranformed with Msc I-cut p153 to create Y234 (*MAT***a** *ste23Δ::LEU2*). Y293 (*MAT*α *axl1Δ::LEU2*) was made by

Table 12. Strain List for Chapter 5.

Strain	Genotype	Source
SY2625	*MATa bar1Δ his3::FUS1-HIS3 mfa2Δ::FUS1-lacZ ura3-1 leu2-3,-112 trp1-1 ade2-1 can1-100*	C. Boone
W3031A	*MATa ura3-1 leu2-3,-112 his3-11,-15 trp1-1 ade2-1 can1-100*	J. Hersch
W3031B	*MATα ura3-1 leu2-3,-112 his3-11,-15 trp1-1 ade2-1 can1-100*	J. Hersch
Y179	*MATa/MATα ura3-1/ura3-1 leu2-3,-112/leu2-3,-112 his3-11,-15/his3-11,-15 trp1-1/trp1-1 ade2-1/ade2-1 can1-100/can1-100*	K. Blundell
Y212	*MATa leu2 ura3 trp1 can1 his4-519*	G. Sprague
Y214	*MATα leu2 ura3 trp1 can1 his4-519*	N. Adames
Y175	*MATa axl1Δ::LEU2 leu2 ura3 trp1 can1 his4-519*	K. Blundell
Y293	*MATα axl1Δ::LEU2 leu2 ura3 trp1 can1 his4-519*	N. Adames
Y234	*MATa ste23Δ::LEU2 leu2 ura3 trp1 can1 his4-519*	C. Boone
Y272	*MATa axl1Δ::LEU2 ste23Δ::LEU2 leu2 ura3 trp1 can1 his4-519*	C. Boone
Y274	*MATa LYS2::lexAop-HIS3 URA3::lexAop-lacZ ade2 trp1 leu2 GAL4 gal80*	A. Bender
Y704	*MATa LexA-LEU2 ura3::URA3-lexAop-LacZ barΔ1 his3 trp1 ura3-52 leu2 GAL+*	K. Blundell

Table 13. Plasmid List for Chapter 5.

Plasmid	Construction	Source
p120	*AXL1-lacZ* in pVT105U (2μ *URA3*)	K. Blundell
p151	*AXL1-HA* in YEp352 (2μ *URA3*)	C. Boone
p162	*axl1(H68A)-HA* in YEp352 (2μ *URA3*)	C. Boone
p161	*axl1(E71A)-HA* in YEp352 (2μ *URA3*)	C. Boone
p163	*axl1(E71D)-HA* in YEp352 (2μ *URA3*)	C. Boone
p153	*ste23Δ::LEU2* in pUC19	C. Boone
p114	*axl1Δ::LEU2* in pUC19	K. Blundell
p479	*BUD5* in pBTM116NLS	J. Brown
p1129	*BNR1* (297-789) in pJG4-5	S. Ritchie
p1063	*AXL1* in pGAD424	N. Adames
p681	*AXL1*(codons 1-416) in pGAD424	N. Adames
p422	*AXL1*(codons 378-1208) in pGAD424	N. Adames
p1064	*AXL1* in pEG202	N. Adames
p864	*AXL1-HA* in pEG202	N. Adames
p611	*AXL1*(codons 1-206) in pEG202	N. Adames
p719	*AXL1*(codons 1-416) in pEG202	N. Adames
p898	*AXL1*(codons 378-1208) in pEG202	N. Adames

transforming Y214 with Sal I-cut p114. Bud scars were stained for observation as described (Pringle, 1991). Briefly, cells were grown overnight at 30° C so that the density reached an $O.D._{600}$ ~1.0 to ensure that most cells had budded multiple times. Cells were washed once in water and resuspended in an equal volume of water. 1 ml was removed and pelleted, resuspended in 100 µl of calcofluor (1 mg/ml) and allowed to sit for 5 min. The cell suspension was washed in water three times and allowed to sit for 30 min. The cells were washed once more, vortexed, and observed for fluorescence using an Olympus model 2H microscope with an ultraviolet filter set.

The microcolony assay for bud site selection was used as described (Chant and Herskowitz, 1991). In this assay, single cells are allowed to grow on solid media for approximately two generations. At this time, microscopic analysis of microcolonies containing four cells should reveal several different arrangements of cells. Cells which are using the bipolar pattern of budding will form either a line of four cells or a line of three cells with a bud branching off of the mother cell at one end. Cells which are using axial sites will produce clusters of four cells. The parent strain used in the mutant search, SY2625, buds randomly. Therefore, to examine bud site selection we constructed strains derived from strain EG123. These derivatives were constructed using standard genetic or molecular methods. Gene deletions were confirmed by PCR analysis.

For two-hybrid experiments (Phizicky and Fields, 1995) DNA-binding domain fusion constructs were cotransformed with activation domain fusion plasmids into either Y704 for pEG202 and pJG4-5 constructs, or into Y274 for pBTM116- and pGAD424-derived plasmids.

Plasmids

Plasmids used in this chapter are listed in Table 13. The *lacZ* reporter construct, p120, was constructed by inserting a 3.0 kb *lacZ* fragment at the Bam HI site of the genomic fragment of *AXL1* in p79. This construct was then inserted into pVT105 digested with Hind III-Xho I. To construct two-hybrid plasmids containing *AXL1* fused with either the *GAL4* transcriptional activation domain or fused to the *lexA* DNA binding domain, a Bgl II site was introduced into the

promoter just before the start codon by PCR of nucleotides 1-2713 using the primers 5'-GTGGCGGGGCAGATCTTAGTTAAATGTC-CTTGAGAGAAGTA-3' and 5'CACCAATAGCGAATTCTGAG-3'. The product was ligated into the Bgl II-EcoR I site of pSP72 (Promega) containing the EcoR I-XhoI carboxy-terminal fragment of *AXL1* either from p79 (no HA tag) or from p151 (HA-tagged).

The Bgl II-XhoI fragment encoding the entire *AXL1* ORF was then cloned into the BamH I-XhoI site of pGAD424 (Chien et al., 1991) in which the Pst I site was replaced with Xho I to create p1063 (no tag), or cloned into the BamH I-Xho I site of pEG202 (Phizicky and Fields, 1995) to create p1064 (no tag) or p864 (tagged). Two fragments encoding amino-terminal portions of *AXL1* were also introduced into these vectors. A 1.2-kb Bgl II-SnaB I fragment encoding amino acids 1-416 was inserted in the BamH I-Pst I sites of pGAD424 and pEG202 to create p681 and p719, respectively. A 0.6-kb Bgl II-Not I fragment (the Not I replaced the Sst I site) encoding residues 1-206 was inserted into the BamH I-Not I sites of pEG202 to make p611. The carboxy-terminal half of *AXL1* was cloned into the EcoR I-Xho I site of pGAD424 (p422) and pEG202 (p898) as a 2.8-kb EcoR I-Xho I fragment. Constructs containing full length Axl1p were tested for their ability to complement the **a**-factor defect of *axl1* mutants. The other constructs were sequenced to ensure that the fusion proteins were in-frame. pBTM116NLS (Chien et al., 1991) containing the DNA-binding domain fusion with Bud5p (p479) was a gift from J. Brown. The *BNR1* fusion with the *GAL4* activation domain (p1129) was obtained by S. Ritchie from a two hybrid library in pJG4-5 (Gyuris et al., 1993).

β-Galactosidase Assays

To assay induction of *lacZ* from the *AXL1* promoter, cells carrying p120 were grown to O.D.$_{600}$ ~0.5 in synthetic medium lacking uracil and harvested for β-galactosidase assays as described previously (Hagen et al., 1991). Assays were also performed to measure transcriptional induction in two hybrid experiments (Phizicky and Fields, 1995). These were done in triplicate and the data shown are the averages of three independent experiments.

Results

AXL1 Expression is Haploid-specific

Haploid *MAT***a** and *MAT*α cells, as well as diploid cells containing p120 were examined for expression of *lacZ* from the *AXL1* promoter. Haploid cells were also exposed to pheromone to test for pheromone-inducibility of *AXL1*. The results are shown in Table 14. *AXL1* promoter activity was restricted to haploid cells and was ~2-fold inducible by pheromone in both haploid cell types.

The Proteolytic Active Site is Not Required for Axial Budding

Fujita et al. (1994) previously reported the isolation of *AXL1* as a gene involved in axial budding. We deleted *AXL1* in the EG123 background and confirmed the defect in axial budding (Fig. 24A). The *AXL1* active site mutants were tested for their ability to rescue the axial budding defect of *axl1* mutants. Surprisingly, all of the active site mutants (including those on a *CEN* plasmid; data not shown) were competent for axial bud-site selection despite the finding that the *axl1(H68A)* and *axl1(E71A)* mutants were defective for proteolysis of **a**-factor (Fig. 24B; and Chapter 4).

Axl1p Interacts with Bud5p and Bnr1p in Two Hybrid Assays

To determine what proteins Axl1p interacts with to effect axial budding, I tested a series of two hybrid fusion proteins against fusions of Axl1p to either the activation domain of Gal4p or the DNA-binding domain of *lex*A. Since Axl1p is involved in bud site selection, I tested for interactions with proteins known to affect budding. I found that Axl1p interacted with full length Bud5p, the GEF for Rsr1p (Table 15). This interaction was strongest with full length Axl1p but both the carboxy- and amino-terminal halves weakly interacted.

Table 14. Cell type and pheromone-inducible expression of *AXL1*.

Strain[a]	Pheromone[b]	*lacZ* expression[c]
*MAT***a**	-	15.8 ± 0.8
*MAT*α	-	10.4 ± 0.5
*MAT***a**/α	-	0.6 ± 0.4
*MAT***a**	+	40.5 ± 1.3
*MAT*α	+	20.1 ± 0.8

a Co-isogenic strains W3031A (*MAT***a**), W3031B (*MAT*α), and Y179 (*MAT***a**/*MAT*α) carrying p120 (*AXL1-lacZ*)

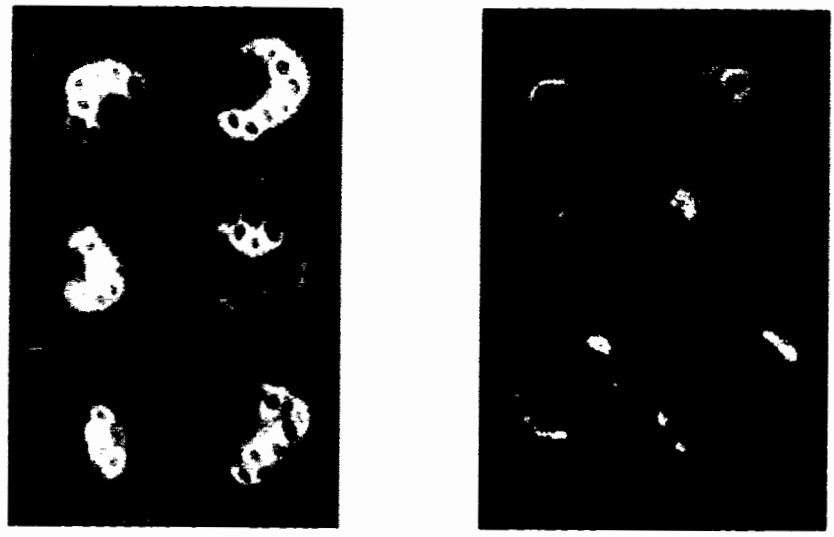
b Cells were grown to $O.D._{600}$ ~0.5 and then exposed to pheromone or an equal volume of liquid medium. Pheromone induction was achieved by the addition of **a**-factor (100 ng/ml final volume) to *MAT*α cells or α-factor (1μg/ml final volume) to *MAT***a** cells for one hour.

c β-galactosidase activity was determined as described previously (Hagen et al., 1991). Values shown are in Miller units with standard deviations. The results shown are the averages of three independent experiments performed in triplicate.

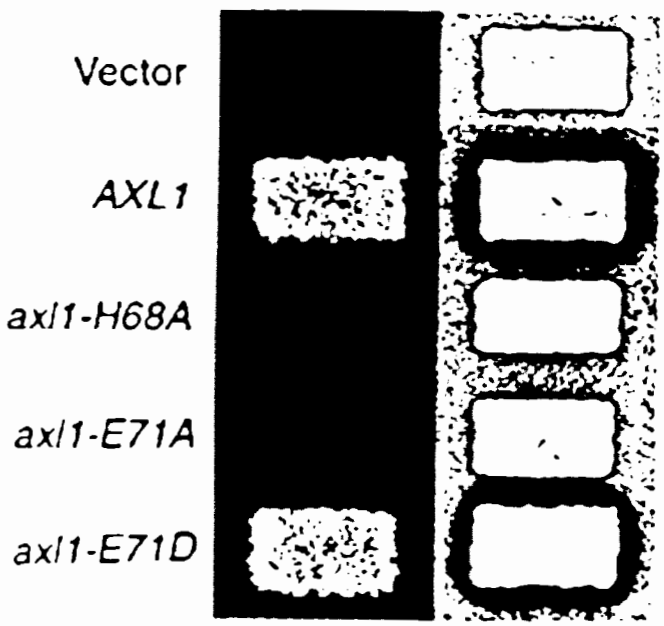
Figure 24. Proteolytic activity of Axl1p is not required for axial bud site selection.

(*A*) Bud scar staining patterns of haploid wild-type (Y212) and *axl1Δ::LEU2* (Y175) cells. Cells were stained with calcofluor white and observed under uv light to visualize bud scars which are preferentially stained due to their high chitin content. (*B*) Mating and microcolony budding assays were performed on *axl1Δ::LEU2 ste23Δ::LEU2* (Y272) cells transformed with vector, *AXL1* (p151), *axl1-H68A* (p162), *axl1-E71A* (p161), *axl1-E71D* (p163). Microcolonies containing four cells were scored as axial, bipolar or random according to the pattern of cells in the colony. Axial budding gives rise to four cells clustered together so that they are all in contact with each other. Bipolar budding produces four cells arranged linearly or three cells in a row with one cell branched out. Randomly budding cells produces none of these arrangements. 600 colonies were scored in three independent trials and the results were expressed as percentages of the total number of scored colonies.

A



B



	Bud site selection (%)		
	Axial	Bipolar	Random
Vector	35	62	3
AXL1	91	8	1
axl1-H68A	90	9	1
axl1-E71A	92	7	1
axl1-E71D	92	7	1

Table 15. Bud5p and Bnr1p interact with Axl1p.

DNA-binding domain fusion[a]	Activation-domain fusion[b]	*lacZ* expression[c] (Miller units)
Bud5p	vector	0.3 ± 0.0
Bud5p	Axl1p	12.0 ± 0.9
Bud5p	Axl1p(1-416)	1.2 ± 0.2
Bud5p	Axl1p(378-1208)	2.0 ± 0.1
Axl1p	vector	0.1 ± 0.0
Axl1p	Bnr1p(297-789)	0.1 ± 0.1
Axl1p-HA	vector	0.1 ± 0.0
Axl1p-HA	Bnr1p(297-789)	20.9 ± 1.4
Axl1p(1-206)	vector	0.1 ± 0.0
Axl1p(1-206)	Bnr1p(297-789)	48.1 ± 2.1
Axl1p(1-416)	vector	0.1 ± 0.0
Axl1p(1-416)	Bnr1p(297-789)	0.1 ± 0.0
Axl1p(378-1208)	vector	0.1 ± 0.1
Axl1p(378-1208)	Bnr1p(297-789)	0.1 ± 0.0

a The vector carrying *BUD5* was pBTM116 NLS. *AXL1* was carried by pEG202. The plasmids used for DNA-binding domain fusions were p479 (Bud5p), p1064 (Axl1p), p864 (Axl1p-HA), p611 [Axl1p(1-206)], p719 [Axl1p(1-416)], and p898 [Axl1p(378-1208)].

b For Bud5p, the vector control was pGAD424. The control for Axl1p was pJG4-5. The plasmids used for activation-domain fusions were p1063 (Axl1p), p681 [Axl1p(1-416)], p422 [Axl1p(378-1208)], and p1129 [Bnr1p(297-789)].

c Assays were done as described in Materials and Methods using Y274 for the DNA-binding domain-Bud5p fusions and Y704 for DNA-binding domain-Axl1p fusions. Values shown are with standard deviations. The results shown are the averages of two independent experiments performed in triplicate.

A protein that causes an axial budding defect when depleted from cells, Bnr1p, also interacts with Axl1p in the two-hybrid system (Table 15). Bnr1p is a member of the formin family of proteins implicated in cytokinesis, cell polarity and limb formation and will be discussed further in the next chapter (Imamura et al., 1997). The construct I used contains a fragment of *BNR1* that encodes most of the amino terminus up to and including part of a conserved sequence called the formin homology domain 1 (FH1). In this case, Bnr1p interacted strongly with the first 206 amino acids of Axl1p (Table 15). Surprisingly, Bnr1p also interacted strongly with the HA-tagged version of Axl1p but not with untagged Axl1p. No interactions were seen with any other fragments of Axl1p.

Discussion

We had cloned *AXL1* as a pro-**a**-factor protease involved in processing this pheromone precursor, but Fujita et al. (1994) had cloned this gene by virtue of its role in axial bud site selection. Like *bud3* and *bud4* cells, *axl1* mutants specifically disrupt the axial budding pattern but have no effect on bipolar budding (Fujita et al., 1994). Ectopic expression of *AXL1* in diploid cells causes the majority of these cells to bud axially (Fujita et al., 1994). Furthermore, *AXL1* is expressed only in haploid cells (Fujita et al., 1994). Hence, Axl1p appears to be the major determinant for axial budding since it is the only protein known to affect axial budding that is haploid-specific.

I similarly found that *AXL1* was expressed only in haploid cells and was slightly more abundant in *MAT***a** cells. Moreover, *AXL1* is pheromone-inducible and has two potential upstream regulatory sequences known as pheromone response elements (PREs). Thus, expression of Axl1p correlates well with a function in haploid bud site selection and an additional role in **a**-factor maturation. Whether *AXL1* is the sole haploid-specific determinant for axial budding is debatable since expression in diploids was unable to completely convert bipolar budding to the axial pattern (Fujita et al., 1994). I have similarly been unable to convert diploids

to a completely axial pattern using a number of constructs in which *AXL1* expression is driven by several different promoters of different strengths (data not shown). Perhaps Axl1p is not the sole determinant for axial budding and ectopic expression in diploids of another haploid-specific gene is required. Alternatively, the level of expression of this protein may be crucial for proper function. This seems unlikely since overexpression in *axl1* haploids still rescues the bud site defect of these cells (Fig. 24B and data not shown). Another possibility is that a diploid-specific protein strengthens the bias for polar budding or weakens the axial sites. This protein would be a bipolar equivalent of Axl1p. Therefore, to completely convert the bipolar pattern to the axial pattern one might have to do so in a mutant background.

Fujita et al. (1994) suggest that, by token of its homology to proteases, Axl1p may process and activate a protein required for axial budding, or may inactivate a repressor of axial budding. Consistent with the latter possibility, they have cloned a gene, *RAX1*, that when mutated suppresses the *axl1* budding defect (cited in Fujita et al., 1994). They suggest that this gene is a substrate for Axl1p (they claim it shows some homology to insulin) and is a repressor of axial budding (Fujita et al., 1994). However, we have shown that the proteolytic active site of Axl1p is dipensible for this protein's function in axial bud site selection. When one of the zinc binding residues of Axl1p or the conserved glutamate in the presumed active site are mutated, the protein is unable to process **a**-factor, but these same proteins rescued the budding defect of *axl1* mutants to wild-type levels. The *rax1* mutation may have a non-specific effect on diploid budding and could weaken the bipolar signals to the point where the dominant signal for bud site selection remains the axial sites even in *axl1* mutants. For example, one mutant, *bud7*, disrupts bipolar budding and many of these diploids have long chains of bud scars reminiscent of axial budding (Zahner et al., 1996). Intriguingly, *bud3 bud7* haploid double mutants bud in a pattern very much like axial budding, forming chains or clusters of bud scars (Zahner et al., 1996). It is possible that *axl1 bud7* cells would behave similarly, opening the possibility that *RAX1* functions in a similar manner to *BUD7* and may, in fact be identical to *BUD7*.

The observation that Axl1p interacts with Bud5p in two hybrid experiments suggests that Axl1p may function in bud site selection by localizing or activating Bud5p at axial sites. This would, in turn, locally activate the general bud site selection GTPase, Rsr1p, which would subsequently activate the polarity establishment proteins to promote polarized growth to this region on the cell's surface. Axl1p is unlikely to proteolytically cleave Bud5p since no cases have ever been reported of zymogen forms of GEFs or GAPs. Moreover, Bud5p does not appear to be proteolytically processed (J. Brown, personal communication).

Some mutant alleles of *BUD5* and *BUD2* have been found to deter bipolar budding, but have no effect on axial budding (Zahner et al., 1996). These mutations presumably affect the interaction of Bud5p with components specific to bipolar budding. It is likely that mutations of these genes will be found that specifically affect axial budding. Such a set of mutants will be useful for determining how proteins interact with Bud5p and whether the interactions are specific for a budding pattern. Presumably, Axl1p would only interact with the mutants that affect bipolar budding.

Axl1p was also found to interact with the formin, Bnr1p, which has some role in axial site selection. This interaction was mediated through the amino-terminus of Axl1p which included all of the zinc ligands of the active site. Bnr1p is a large protein (Mr ~157 kD) and is unlikely to serve as a substrate for a protease that specifically cleaves a peptide 26 residues long and is a member of a family of endopeptidases that are specific for short peptides. The observation that HA-tagged full length Axl1p interacted with Bnr1p, whereas the untagged version did not, is difficult to explain. I have observed that the tagged protein does not rescue the **a**-factor and budding defects of *axl1* mutants as well as the untagged protein (data not shown). Possibly, the tagged protein has a slightly different conformation from wild-type. The tag is inserted just before a conserved region at the carboxy-terminus. This conserved domain contains several hydrophobic residues including a tyrosine (see Fig. 19). Several pitrilysin homologues have a preference for cleaving at tyrosine residues that are followed by hydrophobic amino acids (including Axl1p and Ste23p). This region could act as a pseudosubstrate for the active site and thereby regulate the

enzyme's activity in a manner akin to the regulatory domains of many kinases. Insertion of an HA tag before this region could conceivably perturb its interaction with the active site and unmask the amino-terminal region of the protein, thus permitting interaction with Bnr1p when only the isolated amino-terminus would otherwise interact.

Apparently, Axl1p also interacts with Axl2p (Bud10p; J. Chant, personal communication), although I was unable to detect an interaction with full length Axl2p fused to the *GAL4* activation domain (a gift from J. Brown). Bnr1p was also found to interact with Fus1p and with Bud2p (S. Ritchie, personal communication) and may act as a scaffolding protein bringing together Axl1p, Fus1p, Bud5p and Bud2p to regulate Rsr1p and polarize growth to the vicinity of this complex. In this context, it is of note that *bnr1* null haploid cells are not only defective in axial site selection, but are also unable to choose bipolar sites which are normally the default pathway (Imamura et al., 1997). As a result, the haploids bud randomly; however, diploid cells are unaffected. This differentiates *BNR1* from the general bud-site selection genes which randomize budding in all cell types, and from axial bud-site selection genes, which use default bipolar sites. Therefore, *bnr1* mutants must somehow prevent Rsr1p from functioning, but in a haploid-specific manner.

I should point out that protein-protein interactions seen in two-hybrid assays can be artificial in nature since the proteins are not in their native environments. For example, fusing proteins can alter their native conformations. Also, the proteins are overproduced and targeted to the nucleus where they often would not normally be found. These manipulations could lead to spurious interactions between proteins that would not normally even be present in the same region of the cell, or could artificially heighten interactions that would normally be too weak to be of physiological significance. This said, two-hybrid assays have the advantage over *in vitro* binding assays of presumably occuring under more physiological conditions. However, protein-protein interactions are not generally accepted as having been rigorously tested unless both *in vivo* and *in vitro* assays are performed. Therefore, it is imperative to show that purified Axl1p and Bud5p or

Bnr1p bind in a tube. The genetic data linking these proteins together in the axial budding pathway clearly suggests that these proteins do, in fact, interact in a meaningful way.

AXL2 has recently been cloned as a dosage suppressor (i.e., it can suppress the defect when it is highly expressed) of synthetic lethality between *spa2* and *cdc10* (the septin), and independently as a protein that is required for axial but not bipolar budding (Halme et al., 1996; Roemer et al., 1996). Axl2p is a type I transmembrane protein that first appears at the presumptive bud site as a patch and mostly remains at the neck as the bud grows out (Halme et al., 1996; Roemer et al., 1996). Its localization does not depend to a great extent on Bud3p, nor does Bud3p's localization depend on Axl2p (Halme et al., 1996; Roemer et al., 1996). Because of its topology and role in polarized growth, this protein has been proposed to function analogously to integrins, the extracellular matrix receptors of animals. Interestingly, mutation of *HKR1*, a type I membrane protein of unknown function, results in reduced β(1,3)-glucan content in the cell wall and a defect in axial budding. Similarly, *myo1* mutants, which are defective in delivering chitin to the bud neck, also have an axial budding defect and haploids bud at bipolar sites (Cid et al., 1995).

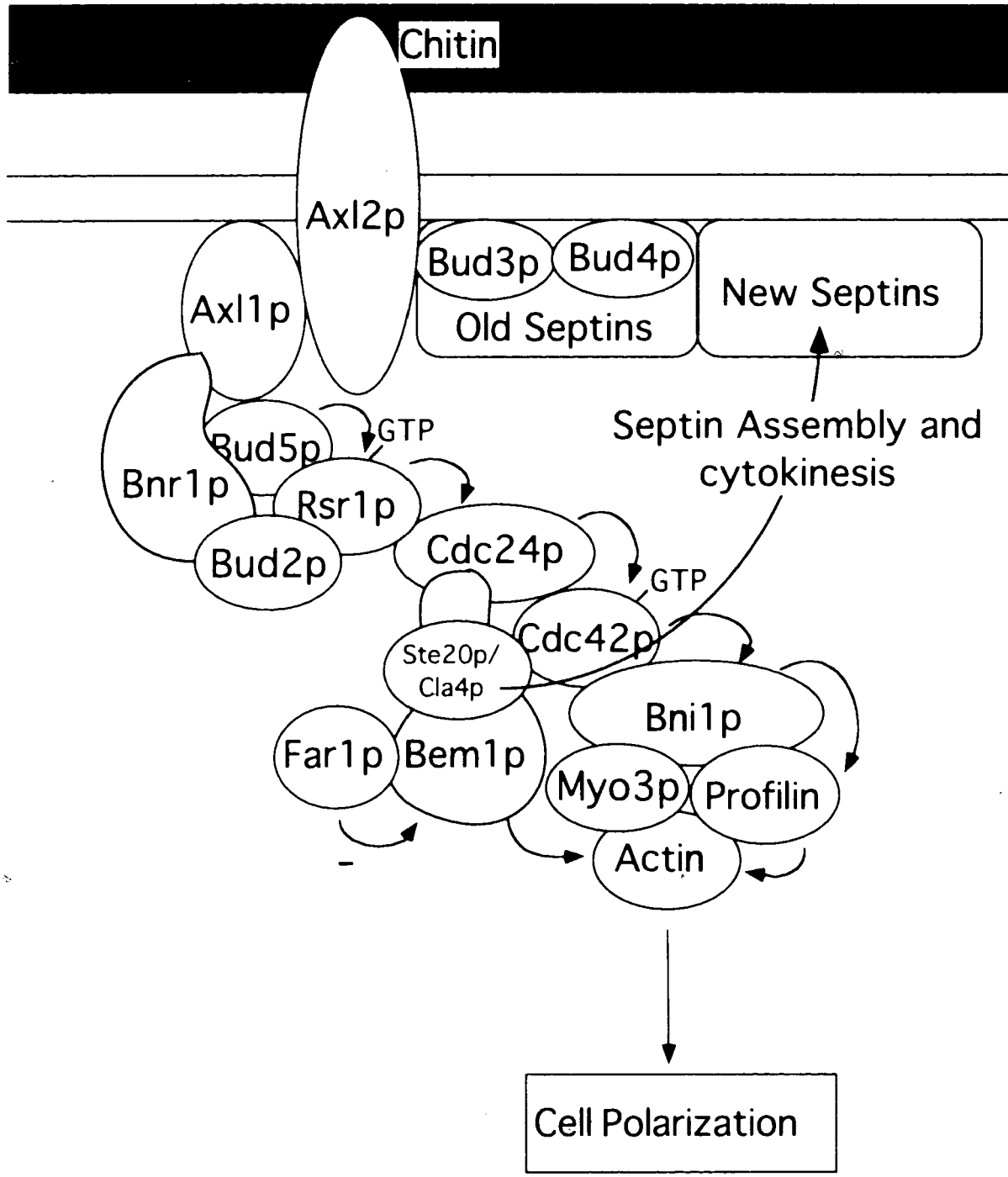
It seems that axial budding is a cyclical process involving the assembly of septins and associated bud-site selection proteins which direct the axial bud-site selection proteins, the polarity establishment proteins, and finally the actin cytoskeleton and polarized growth to the new bud site (Fig. 25). Proper assembly of the neck filaments depends on the Cdc42p-regulated kinases Ste20p and Cla4p in what appears to be a positive reinforcement loop (Cvrckova et al., 1995). Surprisingly, certain *rga1* mutants have an axial budding defect (Stevenson et al., 1995), which suggests that proper regulation of Cdc42p through GTP binding and hydrolysis is important for this process. Repolarization of the actin cytoskeleton and secretion to the bud neck region during mitosis and cytokinesis is also dependent on the septins. This permits insertion of new membrane between mother and daughter, and secretion of cell wall constituents and enzymes required for cell separation to the septum. Thus the septins are responsible for the first and last steps in bud formation.

Secretion at the septum appears to be essential for axial budding. Perhaps a cell wall constituent acts as an extracellular marker for the next round of budding. This constituent may be β(1,3)-glucan. Another likely candidate is chitin since it is localized at high concentrations at the bud scar, is covalently linked to the cell wall through β(1,3)-glucan, and is delocalized in *myo1* mutants and absent from the primary septum (Cid et al., 1995). I would predict that if chitin is such an extracellular marker, *chs3* and possibly *chs5* mutants which are responsible for the bulk of chitin in cells, including the bud scar ring, should also be defective for axial bud site selection. A potential receptor for the cell wall marker is Axl2p; however, no interaction between Axl2p and chitin has been revealed as yet (J. Chant, personal communication).

The previous observations along with the two-hybrid interactions I have observed, suggest the following model for axial bud site selection (Fig. 25). Following cytokinesis, a complex of the neck filaments with Bud3p, Bud4p, and Axl2p direct Axl1p and Bnr1p to a region adjacent to the most recent bud site. Components of the cell wall, such as chitin, which are enriched at the bud scar may aid in targeting Axl2p to this region. Axl1p and Bnr1p (which associates with Fus1p and Bud2p in two hybrid assays; S. Ritchie, unpublished observations) act together to recruit Bud5p and Bud2p to this region and locally activate Rsr1p. The Rsr1p GTPase then interacts with the polarity extablishment proteins Cdc24p and Cdc42p (and possibly also Rga1p) to localize the GTPase activity of Cdc42p. Cdc42p could then activate Ste20p and Cla4p possibly resulting in the polymerization of septins and the stabilization of a new ring of neck filaments at a site adjacent to the previous ring. The complex of Ste20p/Cla4p with Bem1p could also promote actin polymerization since Bem1p interacts with actin in whole cell extracts (Leeuw et al., 1995) and p21-activated kinase (PAK) proteins (including Ste20p and Cla4p) are able to phophorylate myosin I heavy chain and presumably facilitate the formation of filamentous actin structures (Wu et al., 1996). Moreover, Cdc42p could recruit and/or activate proteins, such as Bni1p, which are involved in polarizing the actin cytoskeleton. In the case of Bni1p, this is probably accomplished through its interaction with profilin and Myo3p which could help polymerize actin and move along actin filaments, respectively (see Chapter 6).

Figure 25. A model for axial bud site selection.

The prevalent theory for axial bud site selection proposes a "cytokinesis tag" in which remnants of the previous bud site direct cell growth to an adjacent region of the cell surface. The remnants are thought to consist of the septin ring which is required for cytokinesis. Several proteins, Bud3p, Bud4p and Axl2p, associate with the neck filaments prior to cell separation and act as cortical cues or tags directing the general bud site selection proteins, Bud2p, Bud5p and Rsr1p, to this region. I propose that Axl1p and Bnr1p mediate the interaction between the cortical cue proteins and the general bud site selection complex. The general bud site selection proteins, in turn, direct the activity of the polarity establishment proteins, Cdc24p and Cdc42p, to this site. Consequently, the Cdc42p GTPase activates or localizes proteins, such as Ste20p and Cla4p, involved in the assembly or maintenance of a new septin ring adjacent to the old one. Cdc42p also polarizes the actin cytoskeleton and secretory vesicles for cell surface growth to the new bud site. It does this through proteins such as Bem1p and Bni1p which interact with and organize actin filaments. Far1p might inhibit the association of Bem1p with the bud site selection complex.


Chitin
Axl2p
Bud3p
Bud4p
Old Septins
New Septins
Axl1p
Septin Assembly and
cytokinesis
Bud5p
GTP
Bnr1p
Rsr1p
Bud2p
Cdc24p
GTP
Cdc42p
Ste20p/
Cla4p
Bni1p
Far1p
Bem1p
Myo3p
Profilin
Actin
Cell Polarization

Clearly, uncovering the signals responsible for activating the Rsr1p, Cdc42p, and the Rho proteins have implications for other GTPase signaling cascades. For example, Rho/Rac GTPases in mammalian cells are regulated by and, in turn, regulate integrins (Hildebrand et al., 1996). These GTPases also regulate transcription and the cell cycle through Cdc42p and the Ste20p homologue, PAK (Bagrodia et al., 1995; Coso et al., 1995; Hill et al., 1995; Minden et al., 1995; Polverino et al., 1995; Zhang et al., 1995; Brown et al., 1996; Crespo et al., 1996; Teramoto et al., 1996; Atfi et al., 1997; Clark et al., 1997). Perhaps, yeast proteins will help to reveal other functions for GTPases of the Rho family and new proteins with which they interact.

CHAPTER 6
Formin' a Connection

Introduction

Rho-like GTPases interact with and organize the actin cytoskeleton in all cells in which they have been studied (Hall, 1994; Ridley, 1995). The type of actin structure produced depends on the extracellular signal and the GTPases activated (Hall, 1994; Chant and Stowers, 1995; Ridley, 1995). Moreover, GTPases are known to activate MAP kinase cascades, leading to transcriptional regulation of specific genes (Coso et al., 1995; Davis, 1995; Hill et al., 1995; Minden et al., 1995; Frost et al., 1996; Lim et al., 1996). How these GTPases are regulated and how they regulate actin and transcription are important questions with wide-ranging implications.

In yeast, Ste20p is the first characterized member of the p21 activated kinase (PAK) family of protein kinases. PAKs all contain a conserved domain involved in binding to and regulation by the Rho-related proteins Cdc42 and Rac (Burbelo et al., 1995). Ste20p is required for activation of the MAP kinase cascade of the pheromone response pathway (Leberer et al., 1992; Wu et al., 1995). Interestingly, temperature sensitive mutants of the yeast Cdc42p and Cdc24p lead to a pheromone signaling defect (Simon et al., 1995; Zhao et al., 1995). Mutations in the gene *RGA1* encoding a GAP for Cdc42p cause constitutive signaling (Stevenson et al., 1995). In addition, Cdc24p physically associates with the Gβ subunit (Zhao et al., 1995). These observations suggest a model in which Gβγ associates with Cdc24p and locally activates Cdc42p which in turn activates Ste20p and the MAPK cascade. Similarly, in mammalian cells Rho proteins promote transcription through activation of MAP kinase pathways. For example, Rac and Cdc42Hs activate c-jun through a kinase cascade terminating with the c-jun N-terminal kinase (JNK) MAP kinase (Bagrodia et al., 1995; Zhang et al., 1995; Brown et al., 1996; Teramoto et al., 1996; Atfi et al., 1997). This activation is mediated through a Ste20p homologue hPAK1 (Brown et al., 1996).

These observations indicate that Cdc42p is involved in Ste20p activation of the pheromone response pathway. However, a Ste20p mutant in which the conserved Cdc42p-binding motif has been removed functions normally *in vitro* and in cells, suggesting that Cdc42p regulation of Ste20p is not necessary for pheromone signaling (Peter et al., 1996; Leberer et al., 1997a; Leberer et al., 1997b). Therefore, it appears that Ste20p activation of the MAPK pathway is independent of Cdc42p, and this GTPase has some indirect effect on signaling. Moreover, overexpression of Cdc42p can boost mating in cells deleted for *STE20* (Leberer et al., 1997b). This effect may be mediated by one of the other Ste20p homologs, Cla4p or Skm1p. In fact, a mutant version of Skm1p, in which the regulatory amino-terminus is deleted, is able to suppress the signaling defect of *ste20* cells (Martin et al., 1997). On the other hand, the interaction between Cdc42p and Ste20p is paramount for the processes of cytokinesis and filamentous growth (Mosch et al., 1996), of which the latter utilizes some of the components of the pheromone response pathway (Liu et al., 1993; Roberts and Fink, 1994).

In mammalian cells, Rho proteins promote the formation of several actin-based cellular structures and behaviours. Cdc42Hs induces finger-like extensions of the plasma membrane, called filopodia, that are filled with bundles of long actin filaments (Nobes and Hall, 1995). Rac activation results in the formation and extension of thin sheets of plasma membrane, known as lamellipodia, which are dragged back to the center of the cell over the top surface in a process called ruffling (Nobes and Hall, 1995; Westwick et al., 1997). Rho controls the formation of stress fibers -- long actin fibers anchoring cells to their substrate via integrins (Nobes and Hall, 1995). Yeast cells have a set of five Rho-related GTPases that organize the actin cytoskeleton and coordinate cell surface growth. Cdc42p localizes to growth sites (Ziman et al., 1993) and is required for polarized morphogenesis during both budding and mating (Chant, 1996). Yeast Cdc42p, like its mammalian counterpart, regulates actin assembly (Li et al., 1995), but little is known about its targets in this regulation. In this chapter I examine the roles of two genes in polarized growth. One of these genes, *BNI1* was previously characterized based on synthetic

lethality of a *bni1* mutant with a temperature sensitive *cdc12* mutant (H. Fares and J.R. Pringle, unpublished data). The other gene, *SFU1*, encodes a novel protein of unknown function.

Materials and Methods

Strains, Media and Microbiological Techniques

Strains used are listed in Table 16. Media and genetic techniques were performed as described in Rose et al. (1990) and Guthrie and Fink (1991). Strains were constructed using standard molecular and genetic techniques. Y285 is a spore progeny of a *MATα* segregant of Y37 crossed to SY2585. Y587 was made by tranformation into SY2625 of p321 cut with Hind III and Xho I. Y483 was similarly made using SY2585. Y425 was made by transforming Not I-digested p1036 (*sst2Δ::LEU2*) into Y483. Strain Y822 was produced by transforming into Y483 Sma I-cut p317 to switch the marker from *URA3* to *LEU2*. The homozygous *bni1::URA3/bni1::URA3* mutant, Y1164, was produced by crossing Y859 and Y858. Y837 was made by transforming into SY2625 p1055 cut with Hind III-Sst I to make this *bud6::URA3* strain. Y845 was similarly made using Y83.

Y881 is a spore progeny from a cross between SY2585 and a *MATα* segregant of Y1093. Bam HI-Sal I-cut p793 was transformed into Y1 to create the *sfu1::URA3* strain Y892. Y961 was similarly made using strain SY2585. Y961 was transformed with Not I-cut p1036 to make Y1010.

Plasmids

Plasmids used in this chapter are listed in Table 17. The *bni1::URA3* construct, p321, was made by inserting the *URA3* gene into an 8.5-kb Bam HI-Not I fragment of *BNI1* carried on pBKS+. This truncates the gene before the region coding for the FH1 domain at amino acid 1229. A Bam HI site was introduced into *BNI1* in-frame and just before the start codon by PCR amplification of *BNI1* with primers (5'-TGGATCCGCGAAATGTTGAAGAATCTAGGCTCC-

Table 16. Strain List for Chapter 6.

Strain	Genotype	Source
SY2625	*MATa bar1Δ his3::FUS1-HIS3 mfa2Δ::FUS1-lacZ ura3-1 leu2-3,-112 trp1-1 ade2-1 can1-100*	C. Boone
SY2585	*MATα BAR1 his3::FUS1-HIS3 mfa2Δ::FUS1-lacZ ura3-1 leu2-3,-112 trp1-1 ade2-1 can1-100*	C. Boone
Y1	*MATα bar1Δ::LEU2 his3::FUS1-HIS3 mfa2Δ::FUS1-lacZ ura3-1 leu2-3,-112 trp1-1 ade2-1 can1-100*	N. Adames
Y83	*MATα bar1Δ his3::FUS1-HIS3 mfa2Δ::FUS1-lacZ ura3-1 leu2-3,-112 trp1-1 ade2-1 can1-100*	N. Adames
Y213	*MATa/MATα leu2 /leu2 ura3/ura3 trp1/trp1 can1/can1 his4-519/his4-519*	N. Adames
Y62	*MATa lys1*	I. Herskowitz
Y63	*MATα lys1*	I. Herskowitz
Y37	*MATa bni1-10 bar1Δ his3::FUS1-HIS3 mfa2Δ::FUS1-lacZ ura3-1 leu2-3,-112 trp1-1 ade2-1 can1-100*	N. Adames
Y285	*MATa bni1-10 BAR1 his3::FUS1-HIS3 mfa2Δ::FUS1-lacZ ura3-1 leu2-3,-112 trp1-1 ade2-1 can1-100*	N. Adames
Y587	*MATa bni1::URA3 bar1Δ::LEU2 his3::FUS1-HIS3 mfa2Δ::FUS1-lacZ ura3-1 leu2-3,-112 trp1-1 ade2-1 can1-100*	K. Blundell
Y483	*MATa bni1::URA3 BAR1 his3::FUS1-HIS3 mfa2Δ::FUS1-lacZ ura3-1 leu2-3,-112 trp1-1 ade2-1 can1-100*	K. Blundell
Y425	*MATa bni1::URA3 sst2Δ::LEU2 his3::FUS1-HIS3 mfa2Δ::FUS1-lacZ ura3-1 leu2-3,-112 trp1-1 ade2-1 can1-100*	C. Boone
Y859	*MATa bni1::URA3 trp1-Δ99 leu2-Δ1bar1-1 met1-1 ade2-101 ura3-52*	N. Valtz
Y858	*MATα bni1::URA3 trp1-Δ99 leu2-Δ1bar1-1 met1-1 ade2-101 ura3-52*	N. Valtz
Y1164	*MATa/MATα bni1::URA3/bni1::URA3 trp1-Δ99/trp1-Δ99 leu2-Δ1/leu2-Δ1bar1-1/bar1-1 met1-1/met1-1 ade2-101/ade2-101/ura3-52*	N. Adames

Table 16 (cont'd). Strain List for Chapter 6.

Strain	Genotype	Source
Y822	*MAT***a** *BAR1 bni1::LEU2 his3::FUS1-HIS3 mfa2Δ::FUS1-lacZ ura3-1 leu2-3,-112 trp1-1 ade2-1 can1-100*	K. Blundell
Y837	*MAT***a** *bud6::LEU2 bar1Δ his3::FUS1-HIS3 mfa2Δ::FUS1-lacZ ura3-1 leu2-3,-112 trp1-1 ade2-1 can1-100*	K. Blundell
Y845	*MATα bud6::LEU2 bar1Δ his3::FUS1-HIS3 mfa2Δ::FUS1-lacZ ura3-1 leu2-3,-112 trp1-1 ade2-1 can1-100*	K. Blundell
Y1093	*MAT***a** *sfu1-1bar1Δ his3::FUS1-HIS3 mfa2Δ::FUS1-lacZ ura3-1 leu2-3,-112 trp1-1 ade2-1 can1-100*	N. Adames
Y881	*MAT***a** *sfu1-1 BAR1 his3::FUS1-HIS3 mfa2Δ::FUS1-lacZ ura3-1 leu2-3,-112 trp1-1 ade2-1 can1-100*	N. Adames
Y892	*MAT***a** *sfu1::URA3 bar1Δ::LEU2 his3::FUS1-HIS3 mfa2Δ::FUS1-lacZ ura3-1 leu2-3,-112 trp1-1 ade2-1 can1-100*	G. Poje
Y961	*MAT***a** *sfu1::URA3 BAR1 his3::FUS1-HIS3 mfa2Δ::FUS1-lacZ ura3-1 leu2-3,-112 trp1-1 ade2-1 can1-100*	G. Poje
Y1010	*MAT***a** *sfu1::URA3 sst2Δ::LEU2 his3::FUS1-HIS3 mfa2Δ::FUS1-lacZ ura3-1 leu2-3,-112 trp1-1 ade2-1 can1-100*	G. Poje

Table 17. Plasmid List for Chapter 6.

Plasmid	Construction	Source
p224	cloned genomic fragment containing *BNI1* in pRS316 (*CEN ARS URA3*)	N. Adames
p321	*BNI1::URA3* in pBKS+	S. Kim
p317	*URA3* to *LEU2* switcher plasmid	F. Cross
p527	*GAL1*-3XHA (*CEN ARS URA3*)	C. Boone
p915	*GAL1*-3XHA-*BNI1* (*CEN ARS URA3*)	K. Blundell
p1020	*GAL1*-3XHA-*BNI1ΔFH1* (*CEN ARS URA3*)	K. Blundell
p827	*GAL1*-3XHA-*BNI1ΔC*(codons 1-1214) (*CEN ARS URA3*)	C. Boone
p1025	*GAL1*-3XHA-*BNI1ΔN*(codons 1215-1953) (*CEN ARS URA3*)	K. Blundell
p1037	*GAL1*-3XHA-*BNI1ΔNΔFH1*(codons 1215-1953) (*CEN ARS URA3*)	K. Blundell
p666	cloned genomic fragment containing *SFU1* in pRS316 (*CEN ARS URA3*)	G. Poje
p793	*sfu1::URA3* in pBKS+	G. Poje
p1306	*sst2Δ::LEU2* in unknown vector	S. Givan
p1055	*bud6::LEU2* in pBKS+	K. Blundell

3' and 5'-AGCGGCCGCTTAAGTGGCCATTTTCCTTGTAGCCAGTTTCGTAGAAAGTAA-ACC-3') incorporating a Bam HI site and an MscI site, respectively. The product was cloned into p224 cut with Bam HI and Msc I to make p182. p915 was constructed by inserting a Bam HI-Not I fragment containing the entire *BNI1* ORF into p527. p1020 was made by deleting a 110 bp Bgl II fragment from p915. p827 was made by cloning a Bam HI-Eco47 III fragment of *BNI1* into p527. To create p1025, a Bam HI linker was introduced into p182 at the Eco47 III site and a Bam HI-Not I fragment from this plasmid was inserted into p527. p1037 was made by deleting the FH1-encoding Bgl II fragment of p1025. p793 was made by inserting *URA3* at the Bgl II site within *SFU1* (p666). This truncates the protein at amino acid 63. p1055 was constructed by inserting a Hind III-Sst I fragment of *BUD6* into pBKS+ and inserting *URA3* into the Sna BI site, truncating the product at its ninth amino acid.

Cloning and Physical Mapping

Y37 and Y1093 were cloned as described for other strains in Chapter 2. One complementing clone was obtained for each strain. Physical mapping of *BNI1* was performed as described for *AXL1* in Chapter 3.

Results

sjo1 mutants are Defective in Apical Growth

We called the mutant gene in Y37 *SJO1* (Shmooless Joe) due to its marked shmoo defect (Fig. 26A). Few cells were able to shmoo and those that did formed very broad and short shmoos. Many of the cells exposed to pheromone were enlarged and round. These observations imply that *SJO1* is required for normal apical growth for morphogenesis during mating. The mutant also had a mild separation or cytokinesis defect as evidenced by multiply budded cells (see Chapter 1, Fig. 6). Vegetative cells also appeared to be more rounded than usual and the bud scars were quite heterogeneous in size with many of the scars being much larger than normal (Fig.

28A and B). Apparently, *SJO1* is also required for normal morphogenesis during bud growth and for cytokinesis.

SJO1 Maps to Chromosome XIV L and is Identical to BNI1

The plamid complementing Y37 was restriction mapped and found to have an insert of ~8 kb. No subclones were obtained which were able to rescue the mating defect of Y37. A Hind III fragment from this clone mapped to the overlapping clones λPM5770 (ATCC 70609) and λPM6425 (ATCC 70712). These clones are located on the left arm of chromosome XIV between *met2* and *rad50*. Sequencing of the ends of the clone revealed that the clone contained a single open reading frame encoding *BNI1*, a gene that was previously isolated based on synthetic lethality between *cdc12* and *bni1* mutations (H. Fares and J.R. Pringle, unpublished observations). This gene is 5862 bp in length and the product is 1953 amino acids long. The open reading frame is YNL271c (PIR accession number S48523).

A strain carrying a disruption of the *BNI1* gene displays all of the phenotypes that are associated with *sjo* (Fig. 26A and Fig. 28A). Furthermore, *sjo* was closely linked to *bni1::URA3*. *BNI1* did not, however, rescue the ts growth defect in Y37, and the *bni1::URA3* strain did not display temperature-sensitive growth inhibition (Fig. 26C) suggesting that this growth defect was due to a second mutation in Y37 that was not lost during backcrosses.

BNI1 Encodes a Formin Homologue and Has Another Homologue in Yeast

Bni1p shows similarity to a family of proteins called the formins, named after the founding member, mouse *limb deformity* locus (Zeller et al., 1989; Vogt et al., 1993). The formins include the *Drosophila* genes *diaphanous* (Castrillon and Wasserman, 1994) and *cappuccino* (Emmons et al., 1995), the *Aspergillus nidulans* gene *figA* (*sepA*; Marhoul and Adams, 1995), the *Schizosaccharomyces pombe* genes *fus1* (Petersen et al., 1995) and *cdc12* (Chang et al., 1997), and mammalian p140mDia (Watanabe et al., 1997). These proteins have roles in cytokinesis, the establishment of cell polarity, and vertebrate limb formation. Formins

share two regions of homology, designated FH1 and FH2. The FH1 domain (amino acids 1230 to 1330 in Bni1p) consists of proline-rich sequences and the FH2 domain (amino acids 1516-1616 in Bni1p) is characterized by a consensus sequence of ~100 amino acids.

bni1 is Synthetically Sterile with bar1 and sst2

A *BNI1* mutant was also isolated in another mutant screen performed in our laboratory (T. Favero, unpublished results). The basis of this screen was to find mutations that showed synthetic sterility with *sst2*. The mating defect of *bni1* mutants is exacerbated by mutations in either *bar1* or *sst2* (Fig. 26B). *BAR1 SST2* strains barely had any mating defect. The severity of the mating defect corresponded to the degree of sensitivity of cells to pheromone since *sst2* mutants are the most sensitive to pheromone and show the strongest mating defect (Fig. 26B). We also obtained *BAR1* as a multicopy suppressor of *bni1-10* (data not shown).

Overexpression of BNI1ΔN is Toxic to Cells

Expression of Bni1p lacking its amino terminus, Bni1p(1215-1953), leads to cell death (Evangelista et al., 1997). Cells expressing this protein are large, round and multinucleate (Evangelista et al., 1997). Overexpression of wild-type protein has no discernible effect on growth or cell morphology (Fig. 27; Evangelista et al., 1997). The lethality of *BNI1ΔN* is seen at all temperatures (Fig. 27). Cells expressing the other half of the protein, Bni1p(1-1214), had a very mild cold-sensitive growth defect (Fig. 27). Expression of this protein in *bni1* mutant cells enhanced this growth defect (data not shown).

Toxicity of BNI1ΔN Requires the FH1 Domain

The toxicity of Bni1p(1215-1953) is abrogated by removal of the FH1 domain (Fig. 27). Interestingly, overexpression of the full length protein missing this domain results in a mild cold-sensitive growth defect similar to that seen with Bni1p(1-1214) (Fig. 27).

Figure 26. *sjo1* is allelic to *bni1* and displays most of the same phenotypes as *bni1* mutants. (*A*) To test if *BNI1* rescues the mutant phenotypes of *sjo1*, mating assays, pheromone spot assays (PSA), and mating projection (shmoo) assays were done with Y37 (*sjo1* mutant) transformed with vector (top panels) or with a plasmid bearing *BNI1*, p*BNI1* (p224), obtained by complementation of the mating defect of Y37 with a yeast genomic library (middle panel of PSA and shmoo assays). Mating assays were also performed on wild-type cells (SY2625), Y37 cells and *bni1::URA3* cells (bottom panel). The *bni1::URA3* strain (Y587) was examined for its ability to signal and form shmoos (bottom panels) in comparison to the *sjo1* strain. (*B*) Mating comparisons were made between the following strains: wild-type (SY2625), *bni1 BAR1* (Y285), *bni1 bar1* (Y37), *bni1::URA3 BAR1* (Y483), *bni1::URA3 bar1* (Y587), and *bni1::URA3 sst2* (Y425). (*C*) Growth at 37° C of a wild-type strain (SY2625), Y37 (*bni1* mutant), and a *bni1::URA3* mutant (Y587) streaked onto rich media (left panel). Wild-type cells (SY2625) transformed with vector, and Y37 cells transformed with vector or with p*BNI1* (p224) were grown at 37° C on synthetic medium lacking uracil (right panel).

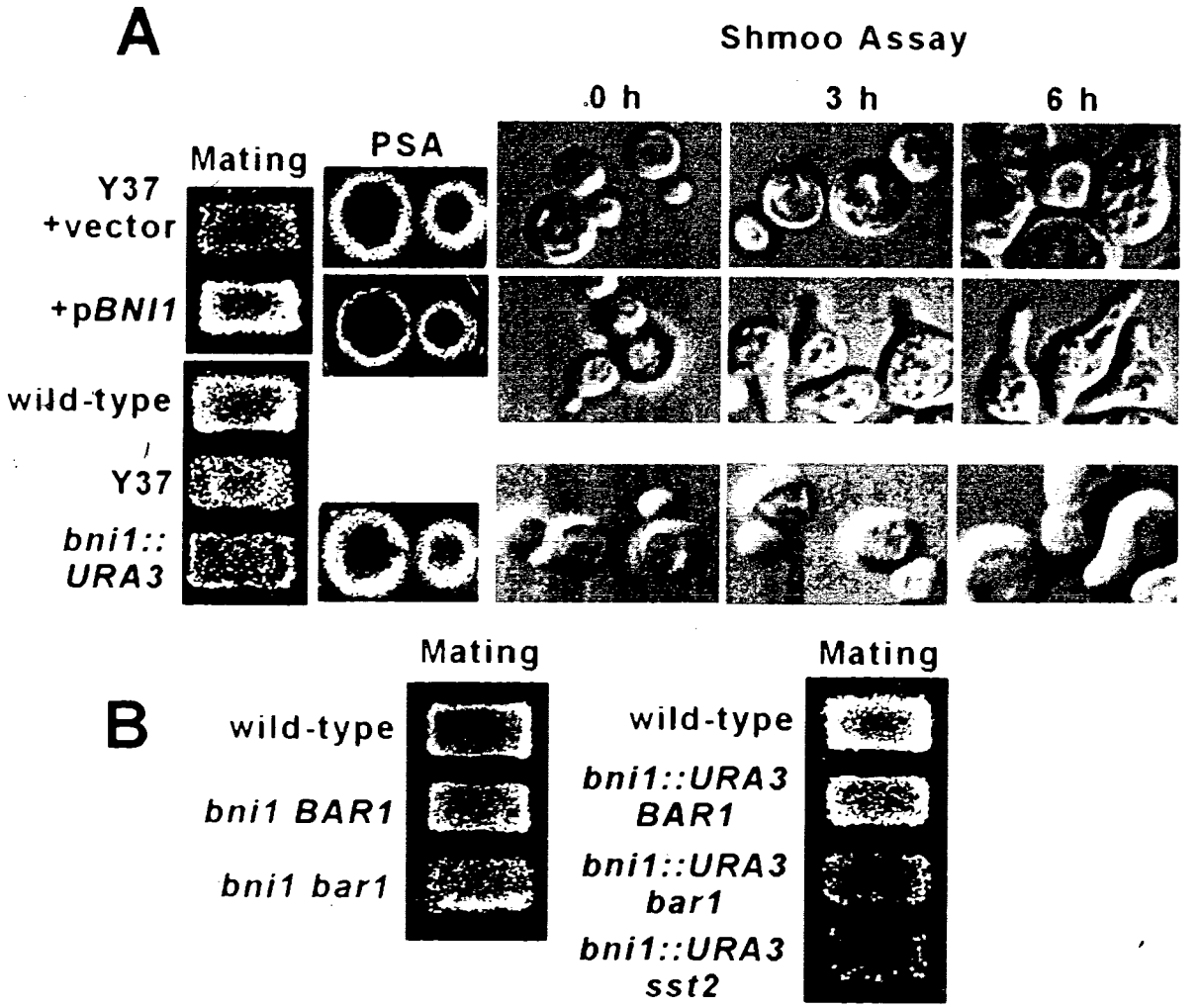

A
Shmoo Assay
0 h
3 h
6 h
Mating
PSA
Y37 +vector
+pBNI1
wild-type
Y37
bni1::URA3
B
Mating
wild-type
bni1 BAR1
bni1 bar1
Mating
wild-type
bni1::URA3 BAR1
bni1::URA3 bar1
bni1::URA3 sst2
C
Y37
wild -type
bni1::URA3
Y37 + vector
wild -type + vector
Y37 + pBNI1

Figure 27. Overproduction of mutant forms of *BNI1* is toxic to cells.

Wild-type cells (SY2625) were transformed with the following *BNI1* constructs: vector (p527), p*GAL1-BNI1* (p915; full length protein), p*GAL1-BNI1ΔFH1* (p1020; in which the conserved polyproline-containing FH1 domain is removed), p*GAL1-BNI1ΔC* (p827; which consists of amino-terminal portion of the protein up to the FH1 domain), p*GAL1-BNI1ΔN* (p1025; in which the amino terminus is deleted up to the FH1 domain), p*GAL1-BNI1ΔNΔFH1* (p1037; in which the amino terminus and FH1 domains are removed). Expression of these constructs is driven by the galactose-inducible *GAL1* promoter. Transformants were grown at the indicated temperatures on synthetic medium lacking uracil with either glucose (left panels) or galactose (right panels) as the carbon source.

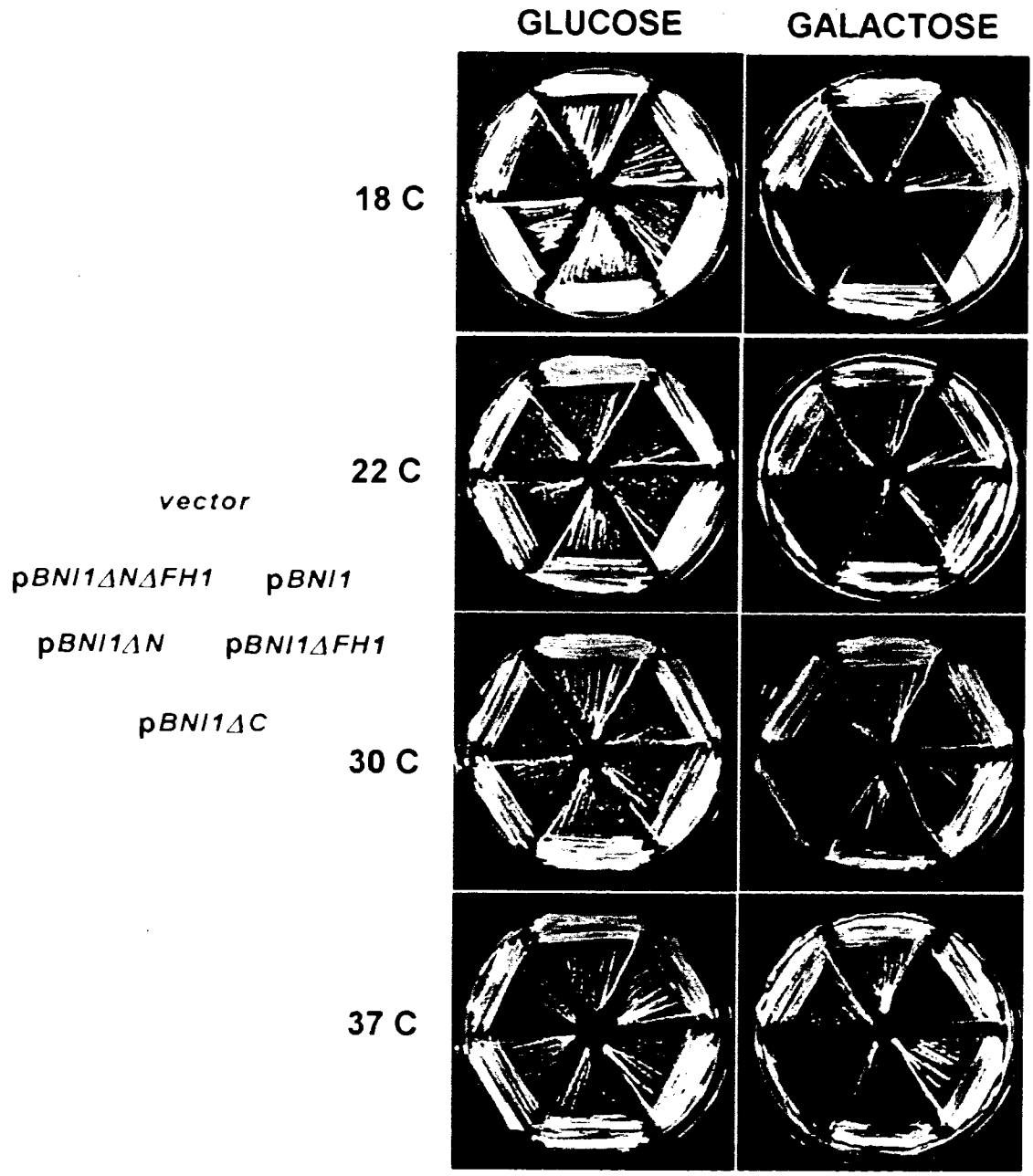
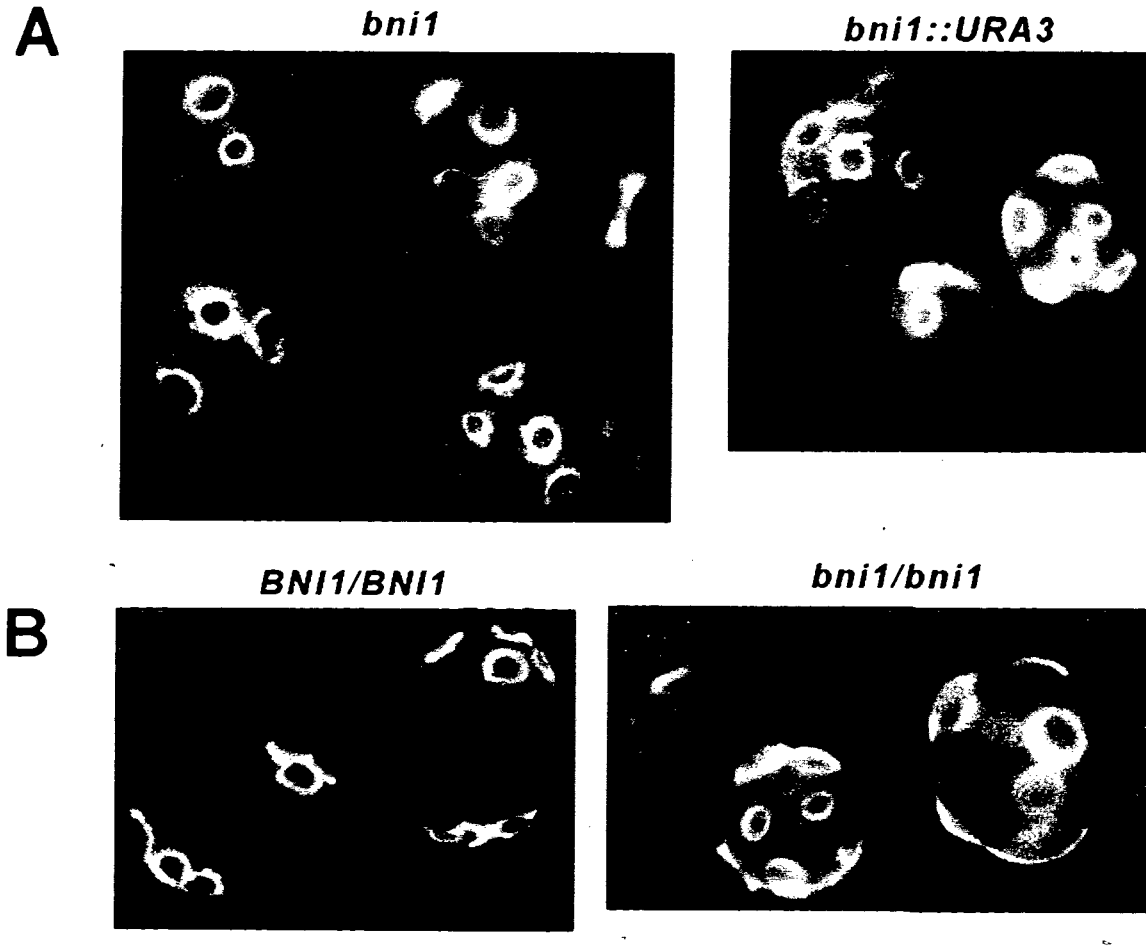

GLUCOSE
GALACTOSE
18 C
22 C
30 C
37 C
vector
pBNI1ΔNΔFH1
pBNI1
pBNI1ΔN
pBNI1ΔFH1
pBNI1ΔC

Figure 28. *BNI1* mutant budding phenotypes and *BUD6* mutant mating defect.

(*A*) The presumed *bni1* mutant, Y37, and *bni1::URA3* cells (Y587) both produce bud necks that can be abnormally wide, as can be seen by calcofluor staining of bud scars. (*B*) Staining of bud scars shows that wild-type *BNI1/BNI1* diploids (Y213) bud at the two poles of the cell but budding is randomized in *bni1/bni1* homozygous diploids (Y1164). (*C*) Mating of *MAT***a** *bud6::LEU2* cells (Y837) to an α-tester strain (Y63) was compared to a *MAT***a** wild-type strain (SY2625) (left panel). Mating to an **a**-tester strain (Y62) was also assayed in the co-isogenic strains of the opposite mating type; *MAT*α wild-type (Y83) and *bud6::LEU2* (Y845) (right panel).


A
bni1
bni1::URA3
B
BNI1/BNI1
bni1/bni1
C
Mating to α-tester
Mating to a-tester
wild-type
bud6::URA3

SFU1 Encodes a Novel Protein

The single clone rescuing the mating defect of Y1063 was obtained and the ends of the insert were sequenced. Several ORFs were found to reside on the genomic fragment. Subclones that complemented the mating defect of Y1063 all contained the ORF YDR027c (Genbank accession no. S50934) which we have called *SFU1* (Shmoo and Fusion defective). This gene encodes a novel protein that shows some homology (22.5% identity and 54.9% similarity over 102 amino acids) to the yeast gene *SSO1* (Genbank accession no. P32867), encoding a syntaxin-related gene involved in exocytosis. *SFU1* also shares some sequence similarity (19% identity and 47% similarity over 92 amino acids) with a human protein, the 3-7 gene product (Genbank accession no. D64159), which is differentially expressed in functionally distinct subclones of a leukemic cell line.

SFU1 is Required for Growth and Pheromone-Induced Morphogenesis

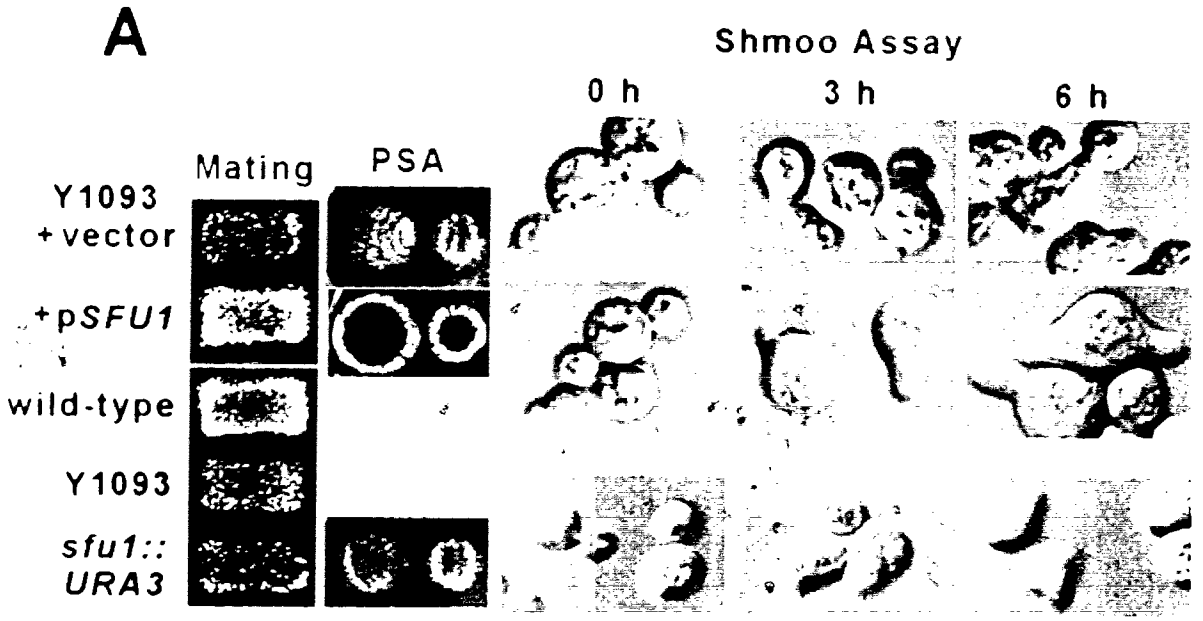
The phenotypes associated with Y1093 were all rescued by *SFU1*, including the G_1 arrest (Fig. 29A), shmoo (Fig. 29A), and growth defects (Fig. 29C). Also, the *sfu1::URA3* mutant had all of the same defects as Y1093, including temperature-sensitive and cold-sensitive growth defects (Fig. 29). Some of the defects of *sfu1::URA3* cells were slightly more severe than in Y1093, suggesting that Y1093 contains a slightly leaky allele of *sfu1*.

sfu1 is Synthetic Sterile with sst2

Like *bni1-10*, *sfu1-1* shows synthetic sterility with *bar1* and *sst2*. Again the mating defect was more pronounced in the more sensitive *sst2* strain (Fig. 29B).

Figure 29. Mutant phenotypes of *sfu1* mutants.

(*A*) To test if *SFU1* rescues the mutant phenotypes of Y1093, mating assays, pheromone spot assays (PSA), and mating projection (shmoo) assays were done with Y1093 transformed with vector (top panels) or with a plasmid bearing *SFU1*, p*SFU1* (p666), obtained by complementation of the mating defect of Y1093 with a yeast genomic library (middle panel of PSA and shmoo assays). Mating assays were also performed on wild-type cells (SY2625), Y1093 cells and *sfu1Δ:URA3* cells (Y892; bottom panel). Y892 was examined for its ability to signal and form shmoos (bottom panels) in comparison to the Y1093. (*B*) Mating comparisons were made between the following strains: wild-type (SY2625), *sfu1 BAR1* (Y881), *sfu1 bar1* (Y1093), *sfu1Δ:URA3 BAR1* (Y961), *sfu1Δ:URA3 bar1* (Y892), and *sfu1Δ:URA3 sst2* (Y1010). (*C*) Growth at 37° C of a wild-type strain (SY2625), Y1093 (*sfu1* mutant), and a *sfu1Δ:URA3* mutant (Y892) streaked onto rich media (left panel). Wild-type cells (SY2625) transformed with vector, and Y1093 cells transformed with vector or with p*SFU1* (p666) were grown at 37° C on synthetic medium lacking uracil (right panel).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Shmoo Assay
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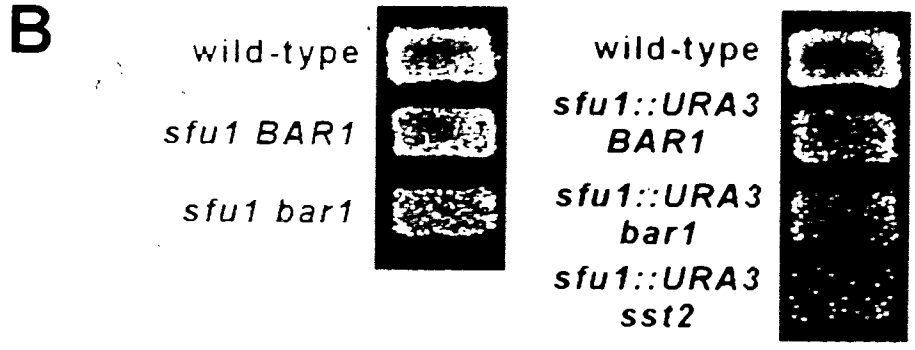
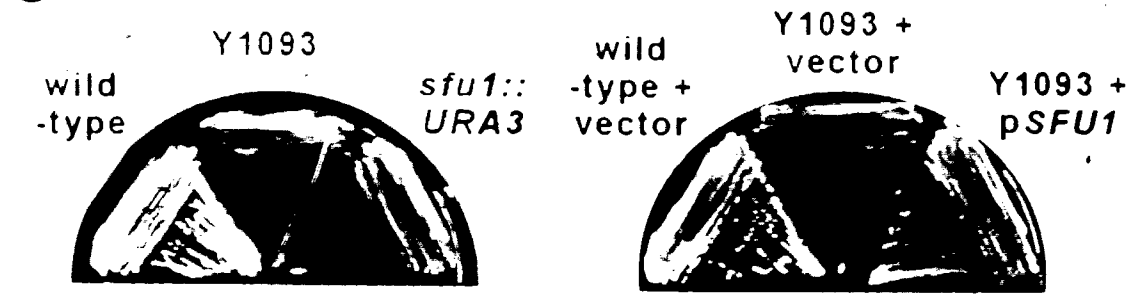
3 h
6 h
Mating
PSA
Y1093 + vector
+pSFU1
wild-type
Y1093
sfu1:: URA3


B
wild-type
sfu1 BAR1
sfu1 bar1
wild-type
sfu1::URA3 BAR1
sfu1::URA3 bar1
sfu1::URA3 sst2


C
Y1093
wild -type
sfu1:: URA3
wild -type + vector
Y1093 + vector
Y1093 + pSFU1

Discussion

The *BNI1* (Bud Neck Interacting) gene was originally isolated as a protein interacting genetically with *CDC12* (H. Fares and J.R. Pringle, unpublished data). The cell separation or cytokinesis defect is consistent with the notion that Bni1p interacts with the septins and aids in this process. Moreover, *bni1* mutants often have large necks that produce large bud scars (Fig. 28). Other formins such as *sepA*, *cdc12*, and *diaphanous* are also implicated in cytokinesis and may similarly interact with septins (Castrillon and Wasserman, 1994; Chang et al., 1997; Marhoul and Adams, 1995).

During normal projection formation cells not only polarize growth to the tip of the mating projection but also concentrate components necessary for cell fusion, such as Fus2p, to this region of the cell surface (Elion et al., 1995). Chemotropic growth of both partners results in contact at the shmoo tips where the fusion machinery is at its greatest concentration. In the absence of a gradient of pheromone, due to high levels of exogenous pheromone or due to mutations in negative regulators of the pheromone response pathway such as *bar1* or *sst2*, cells utilize the previous bud site as a site for projection formation (Chenevert, 1994; Dorer et al., 1997; Valtz et al., 1995). Obviously, if the mating partner is not in this direction the cells will be unable to mate. If by chance the cells do polarize in the same general direction as their partners then they will be able to mate at a reduced efficiency since they are less likely to make contact directly at the shmoo tips. However, if these cells also have a fusion defect, then they will not have enough of the cell fusion components in the area of contact to enable the cells to fuse.

A set of mutants, *spa2*, *fus1*, *fus2*, *fus3*, *pea2*, *rvs161*, *chs5* and *axl1*, all show synthetic sterility with *sst2* and are defective in default mating (T. Favero, unpublished results; Dorer et al., 1997). These mutants also all have cell fusion defects (Dorer et al., 1997). The synthetic sterility of the *axl1*, *fus1* and *fus2* mutants is likely due to their importance in cell fusion (Brizzio et al., 1996; Cid et al., 1995; Dorer et al., 1997). The defects in default mating of *rvs161* and *chs5* are

possibly due to their involvement in vesicular traffic (i.e., they may transport components necessary for cell fusion), while the defects in *pea2*, *spa2*, *bni1* and *sfu1* are likely due to the role of these proteins in polarizing cells and directing secretory vesicles to the shmoo tip. Overproduction of Fus1p, Fus2p or **a**-factor is able to suppress the mating defects of many of these mutants (T. Favero and C. Boone, unpublished observations) including *bni1* (data not shown). This suggests that the mating defects of these mutations are the result of delocalized fusion proteins and that overexpression of these proteins raises their concentration to a level at which they are able to carry out their function, even when the mating projections are misaligned.

BNI1 was also found in a screen for mutants that could not bud in the bipolar pattern (Zahner et al., 1996). Homozygous *bni1/bni1* mutants bud randomly (Fig. 28B). *bni1* mutants were also found to be unable to undergo filamentous growth under starvation conditions, although these mutants could still invade agar (Mosch and Fink, 1997). We also found that *bni1* mutants were not able to hyperpolarize growth under the influence of overexpressed Cln1p (M. Evangelista, K. Blundell, C.J. Chow, N. Adames, C. Boone, unpublished results).

These defects in polarized growth point to a role for Bni1p in coordinating the actin cytoskeleton. *bni1* cells fail to polarize cortical actin patches and form shmoos, and overproduction of *BNI1ΔN* results in the formation of aberrant actin structures over the entire surface of cells which become enlarged and round (Evangelista et al., 1997). Amazingly, an amino-terminally truncated *bni1* mutation was recovered in a screen for proteins that are lethal when overexpressed (Akada et al., 1997). GTP-bound but not GDP-bound Cdc42p, Rho1p, Rho2p, Rho3p and Rho4p all interact with a region in the amino terminus of Bni1p -- a region that is absent in *BNI1ΔN* (Kohno et al., 1996; Evangelista et al., 1997). Therefore, it appears that Bni1p is an immediate target of all of the Rho-related proteins regulating polarized growth in yeast. The mammalian formin p140mDia also interacts with a GTP-bound Rho protein and colocalizes with actin structures in membrane ruffles and phagocytic vesicles in â Rho-dependent manner (Watanabe et al., 1997). It seems likely that Rho proteins interact with all of the formin homologues to regulate the actin cytoskeleton.

What are the downstream effectors for the formins? Cappuccino was found to interact with profilin and mutants in the genes for these proteins have very similar phenotypes (Manseau et al., 1996). We found that excess profilin and tropomyosin suppress the growth defect of overexpressed *BNI1ΔN*, and profilin physically interacts with Bni1p at the proline-rich FH1 domain (Evangelista et al., 1997). Profilin binds to actin monomers and possibly catalyzes the addition of ATP-actin to the barbed ends of actin filaments (Haarer and Brown, 1990). *S. pombe* Cdc12p (Chang et al., 1997) and p140mDia (Watanabe et al., 1997) have since been shown to also bind profilin.

Actin was also shown to bind to the FH1 domain of Bni1p, and an actin allele that displays phenotypes similar to those of *bni1* is lethal in combination with *bni1* (Evangelista et al., 1997). The interaction between actin and Bni1p is likely through profilin rather than a direct interaction. Furthermore, Bud6p interacts with the carboxy terminus of Bni1p and itself interacts with actin (Evangelista et al., 1997). *bud6* mutants also share many of the phenotypes of *bni1* mutants, including cytokinesis defects, random budding in diploids, a shmoo defect and a mating defect (Fig. 28C; Amberg et al., 1997; Zahner et al., 1996; Evangelista et al., 1997). Moreover, Bud6p and Bni1p colocalize to regions of cell surface growth during budding and projection formation (Evangelista et al., 1997). Bud6p is also known to interact with actin (Amberg et al., 1997).

This set of interactions shows that Bni1p is likely a mediator of actin filament assembly controlled by the Rho-related proteins during vegetative growth and mating projection formation. The toxicity of Bni1p(1215-1953) is possibly due to deregulated activity of this protein. The appearance of actin in these cells suggests that deregulated Bni1p(1215-1953) results in the assembly of actin structures over the entire cell instead of at polarized sites of growth.

How Bni1p is regulated remains to be determined. Several possibilities exist. The amino terminus of Bni1p may serve to localize the protein or may regulate its activity or both. The observation that Bni1p is concentrated at sites of active growth implies that Cdc42p or other proteins localize it to these regions. Overproduction of wild-type protein did not produce an

obvious phenotype in cells. This could mean that the target for Bni1p localization is not saturated even when Bni1p is highly expressed. Deletion of the amino terminus could result in delocalization of Bni1p and its activity, resulting in the accumulation of actin over the entire cell.

Alternatively, a high abundance of Bni1p could saturate its target and cause the accumulation of delocalized Bni1p. This would not result in the accumulation of delocalized actin if the amino terminus regulates Bni1p acitivity. Deletion of this region could constitutively activate Bni1p, and overexpression of this protein could saturate the targeting patch and also result in delocalized, activated Bni1p.

Only localization studies using these proteins will resolve the question of what role the amino terminus has in Bni1p activity. In either case, we should observe delocalization of Bni1p(1215-1953), but in the former situation wild-type protein should remain concentrated at the regions of cell surface expansion, implying that the amino terminus is at least required for proper localization. In the latter situation Bni1p should be present over the entire cell, suggesting that the amino terminus is at least required for Bni1p regulation. In either case though, the amino terminus could be necessary for both localization and regulation. Since Cdc42p interacts with the amino terminus of Bni1p, this protein is an obvious candidate for the localizing or regulating activity, but other proteins may also be necessary.

Not surprisingly, the toxic effect of Bni1p(1215-1953) is mediated by the FH1 domain since growth was normal in cells carrying the Bni1p(1215-1953)ΔFH1 protein. Therefore, as suggested by actin localization, the lethality of Bni1p(1215-1953) is due to its activity on actin through profilin. The FH1 domain of Bni1p also interacts with the SH3 domains of Myo3p and Myo5p, yeast's two class I myosins (I. Pot, M. Evangelista and C. Boone, unpublished results). These myosins are involved in the transport of vesicles along actin filaments in the direction of the barbed ends. The interaction of Bni1p with myosins involved in vesicular traffic, and with profilin suggests that Bni1p may facilitate actin polymerization by moving along the growing filament, and could also be involved in vesicular traffic. Alternatively, Bni1p may anchor filaments at the plasma membrane and facilitate polymerization by providing actin monomers to

the barbed (plus end) of filamentous actin through its interaction with profilin and by pushing the growing filament away from the anchor site to permit the introduction of new actin monomers in a ratchet-like mechanism.

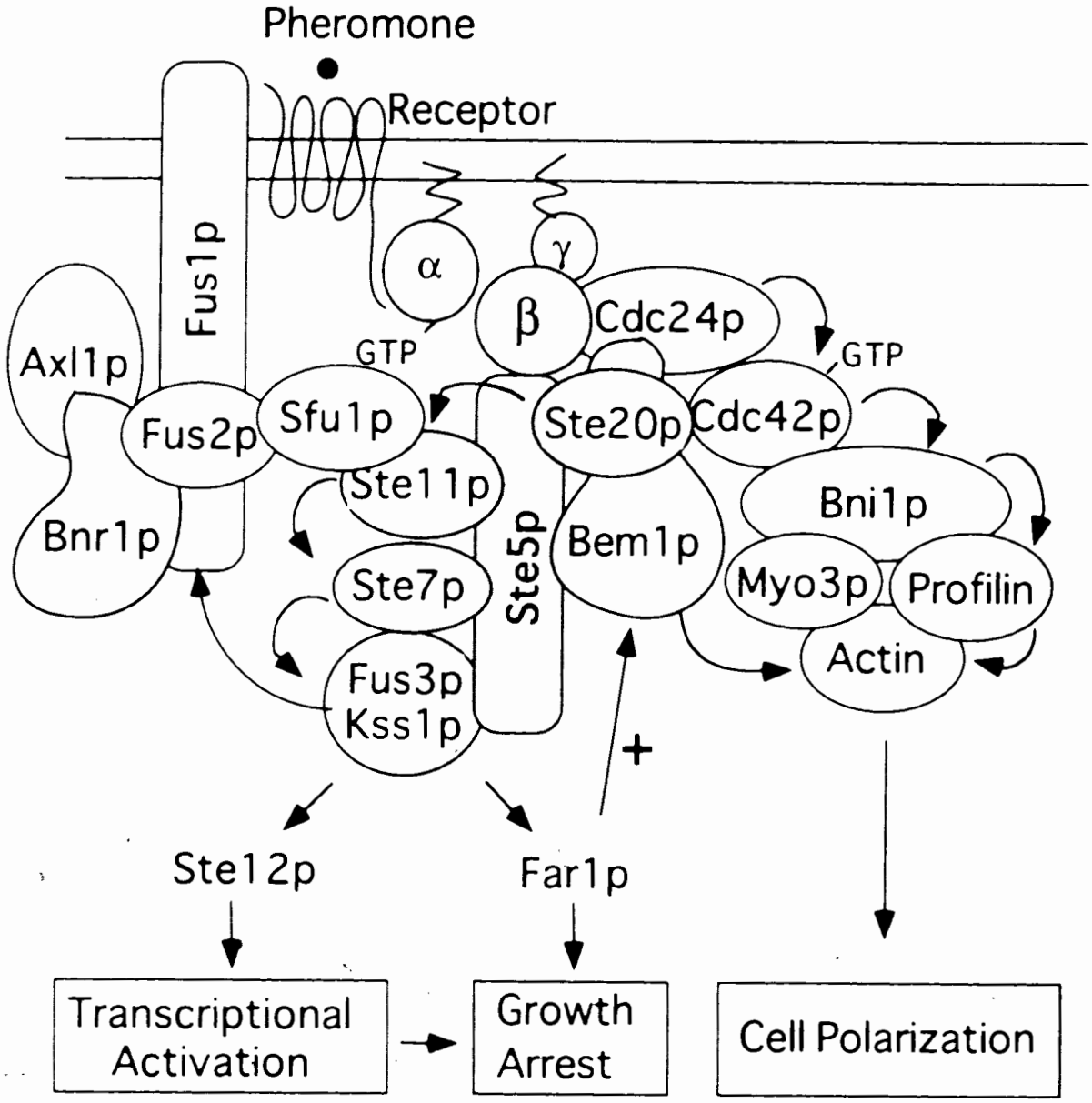
Overexpression of either the amino terminus or full length Bni1p missing the FH1 domain led to cold-sensitive growth defects. A single copy of *BNI1ΔFH1* was unable to rescue the defects of a bni1 mutant (Evangelista et al., 1997), again supporting the conclusion that this domain is crucial to the protein's main function. Cold-sensitivity is a hallmark of defects in polymers such as microfilaments and microtubules. Perhaps these proteins interact with Cdc42p or other proteins that regulate the cytoskeleton, and when overproduced out compete other proteins (including wild-type Bni1p) for binding to Cdc42p. Since these proteins do not have the FH1 domain they could sequester Cdc42p into unproductive complexes, leading to perturbed regulation of growth. The growth defect caused by these proteins is only slightly more severe when they are expressed in *bni1* cells (data not shown), indicating, as one would expect, that other proteins involved in cell growth are also regulated by Cdc42p.

The precise role of profilin, and hence Bni1p, in actin organization has yet to be determined. In a permeabilized cell assay Cdc42p was found to be essential for actin polymerization (Li et al., 1995). This same assay could be used to determine if Bni1p is likewise involved in actin polymerization. Recently, another protein, Bee1p (Las17p), was shown to be involved in actin organization and depletion of this protein reduced actin polymerization in this same assay (Li, 1997). This protein is a homologue of the human Wiskott-Aldrich Syndrome protein (WASP; Symons et al., 1996), and a neuronal homologue (N-WASP; Miki et al., 1996) which are effectors for human Cdc42 and regulate actin (Miki et al., 1996; Symons et al., 1996). Although Bee1p lacks the conserved Cdc42 binding domain of WASP and other proteins (as does Bni1p), it is probable that Cdc42p does bind to and regulate Bee1p. The severity of the polymerization defect of *bee1* mutants was not as great as seen in *cdc42* mutants, suggesting that other proteins regulated by Cdc42p also polymerize actin. Hence, Bni1p could be one of several Cdc42p effectors, each responsible for maintaining specific actin structures.

Recently, another yeast formin, Bnr1p, was shown to be involved in regulating actin. *bni1 bnr1* double mutants are large, round, and multinucleated cells with delocalized cortical actin patches (Imamura et al., 1997). Like Bni1p, Bnr1p interacts with profilin and Rho4p (and possibly also other Rho-related proteins). Therefore, it appears that these two proteins are functionally redundant, accounting for the mild vegetative defects seen in *bni1* cells. Surprisingly, in direct opposition to *bni1* mutants which randomize budding in diploids, *bnr1* mutants randomize budding only in haploids (Imamura et al., 1997). This defect is unique since all mutations that affect bud site selection have either a bipolar defect leading to random budding (bipolar bud site selection genes), an axial defect leading to bipolar budding (axial bud site selection genes), or randomize budding in all cells (general bud site selection genes). A possible explanation for this behaviour was offered in the previous chapter.

The involvement of Sfu1p in polarity establishment should turn out to be equally intriquing. Cells mutated in *SFU1* show many of the same characteristics as *spa2*, *pea2* and *bni1* mutants. All have projection formation defects, are synthetically sterile with *sst2*, and are defective in default mating. However, unlike these other mutants, *sfu1* mutants have growth defects, suggesting that Sfu1p has a prominent role in vegetative growth. *sfu1 bni1* cells are sicker than *sfu1* cells, and *sfu1* is synthetically lethal with *chs3* (chitin synthase III, a protein dependant on *CHS5* for activity). Thus, Sfu1p is likely to be involved in many of the same processes as these other proteins, but seems to have some unique functions not shared by the other proteins. This is further indicated by the severity of the G_1 arrest defect in *sfu1* cells (Fig. 28) and the finding that these cells have a signaling defect (G. Poje and C. Boone, unpublished results). Two hybrid experiments indicate that Sfu1p interacts with Fus2p and Ste11p and weakly with Fus1p (G. Poje and C. Boone, unpublished results). The interaction with Ste11p is consistent with the mutants having a signaling defect while the interaction with Fus2p is consistent with a role in cell fusion during mating. In fact, *sfu1* cells appear to have a fusion defect (G. Poje, M. Moreau and C. Boone, unpublished results), as do *spa2*, *pea2*, and *bni1* mutants (Dorer et al., 1997). Further studies on Sfu1p should provide exciting results.

Figure 30. Model for protein-protein interactions during mating projection formation. Through the heterotrimeric G-protein encoded by *GPA1*, *STE4* and *STE18*, pheromone receptors activate (activations are shown by arrows) the MAP kinase module consisting of Ste20p (PAK), Ste5p, Ste11p (MAPKK kinase), Ste7p (MAPK kinase), and Fus3p/ Kss1p (MAP kinases). This signaling module is shown shaded. Not only do the receptors initiate the signaling cascade leading to activation of transcription (through Ste12p) and G_1 arrest (through Far1p), but they also impart spatial information to this process, thus restricting signaling to the region of the cell experiencing the highest concentrations of pheromone. The polarity establishment proteins, Cdc24p and Cdc42p, and proteins that regulate the cytoskeleton, such as Bni1p, are recruited by this signaling complex. Furthermore, proteins involved in **a**-factor secretion, such as Ste6p and possibly Axl1p, and proteins required for cell fusion, such as Fus1p and Fus2p, are also recruited to this region. The outcome of all this is that the machinery for projection growth and cell fusion are concentrated at the same region of the cell surface, thus facilitating efficient conjugation.


Pheromone
Receptor
Fus1p
α
γ
β
Cdc24p
GTP
GTP
Axl1p
Fus2p
Sfu1p
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Cdc42p
Ste11p
Bni1p
Bnr1p
Ste5p
Bem1p
Ste7p
Myo3p
Profilin
Actin
Fus3p
Kss1p
+
Ste12p
Far1p
Transcriptional Activation
Growth Arrest
Cell Polarization

These results suggest the following model for mating projection formation (Fig. 30). The Gβγ subunits interact with and activate Ste5p and Cdc24p, which in turn activate Ste20p and the MAP kinase cascade described in Fig. 2. The polarity establishment proteins Cdc24p and Cdc42p also interact with Bem1p and Bni1p to promote polarization of the actin cytoskeleton to the region of highest pheromone concentration. The MAPKK kinase Ste11p also interacts with Sfu1p in two-hybrid assays. This interaction possible affects pheromone signaling. Sfu1p also interacts with Fus2p which may be part of a complex with Fus1p, Bnr1p and Axl1p, thus directing both **a**-factor maturation (and likely secretion) and the fusion machinery to the site of polarized growth. Together, these interactions promote growth of a mating projection and concentrate **a**-factor secretion and the cell fusion proteins needed to successfully undergo cell fusion. Far1p might promote the interaction of Bem1p with these proteins and redirect cell polarity to the region of the cell experiencing the highest concentration of pheromone.

CONCLUSION

Rho Rho Rho Your Bud

The set of mutants I obtained in my screen covered a wide array of defects in the conjugation process. Those that have projection formation defects have and will likely prove to be very interesting. Many of the mutants are defective in forming mating projections and many have cell fusion defects (the *ram1* , *bni1*, and *sfu1* mutants). My screen may be predisposed to finding this type of mutant because of the *bar1* and *ram1* mutations in the parental strain. The *bar1* perturbs chemotropic growth of the mating projection, and the very leaky *ram1* allele in the strain would exacerbate the mating defect due to *bar1* and together make the cell fusion process less efficient. The find that Bni1p is an effector of Cdc42p is exciting because Bni1p is one of very few connections known between the Rho-related GTPases and the actin cytoskeleton. How Bni1p activity is regulated by Cdc42p and the other Rho proteins, and how Bni1p regulates the actin cytoskeleton are the next big questions to answer.

It is clear that Rho proteins regulate the actin cytoskeleton and transcription in diverse experimental systems. It is also becoming evident that abnormal interactions between Rho proteins and their effectors are implicated in a number of diseases, including WAS (Derry et al., 1994), Aarskog-Scott syndrome (Olson et al., 1996), AIDS (Lu et al., 1996), and cancer (Michiels et al., 1995). Hence there is a great deal of impetus to resolve the functions of these GTPases and the proteins they regulate and by which they are regulated. The yeast system provides a means to study these proteins with a high degree of efficacy. *S. cerevisiae* has five such GTPases, all involved in the regulation of polarized growth, and at least one of which regulates transcription through a MAP kinase cascade. Uncovering proteins that are required for polarized growth will help resolve the question of how Rho-related GTPases function. The functions of Rho2, Rho3, and Rho4 in budding are completely unknown at present, but perhaps some of the mutants I obtained affect proteins that interact with these GTPases. In any case, there

is still much to discover with respect to the functions of Rho proteins during polarized cell surface growth.

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