A Filamentous Growth Response Mediated by the Yeast Mating Pathway

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ABSTRACT

Haploid cells of the budding yeast *Saccharomyces cerevisiae* respond to mating pheromones by arresting their cell-division cycle in G1 and differentiating into a cell type capable of locating and fusing with mating partners. Yeast cells undergo chemotactic cell surface growth when pheromones are present above a threshold level for morphogenesis; however, the morphogenetic responses of cells to levels of pheromone below this threshold have not been systematically explored. Here we show that *MAT***a** haploid cells exposed to low levels of the α-factor mating pheromone undergo a novel cellular response: cells modulate their division patterns and cell shape, forming colonies composed of filamentous chains of cells. Time-lapse analysis of filament formation shows that its dynamics are distinct from that of pseudohyphal growth; during pheromone-induced filament formation, daughter cells are delayed relative to mother cells with respect to the timing of bud emergence. Filament formation requires the *RSR1*(*BUD1*), *BUD8*, *SLK1/BCK1*, and *SPA2* genes and many elements of the *STE11/STE7* MAP kinase pathway; this response is also independent of *FAR1*, a gene involved in orienting cell polarization during the mating response. We suggest that mating yeast cells undergo a complex response to low levels of pheromone that may enhance the ability of cells to search for mating partners through the modification of cell shape and alteration of cell-division patterns.

THE development of unicellular and multicellular organisms in many cases involves the reception of extracellular signals and subsequent cell differentiation by polarized cell growth and division. Many unicellular organisms possess specialized cell structures, or organelles, such as pseudopods, cilia, and flagella, which allow these creatures to move through different environments to locate nutrients and, in the case of sexual species, mates. Nonmotile unicellular organisms must also accomplish these tasks and thus must possess their own strategies for dispersing progeny cells for these purposes.

In budding yeast, cell polarity is programmed by both internal and external cues (Herskowitz *et al.* 1995; Pringle *et al.* 1995; Drubin and Nelson 1996; Roemer *et al.* 1996a). In response to starvation for specific nutrients, yeast cells are capable of producing filamentous growth forms that have been speculated to represent a mechanism by which progeny cells are transported to more nutrient-rich environments (Gimeno *et al.* 1992; Madden and Snyder 1992). During mating, haploid cells differentiate into specialized cell types that search out and recognize mating partners to accomplish cell fusion. When mating cells are in close proximity and sufficient levels of cell-type-specific peptide pheromones are present, cells of opposite mating types undergo cell-cycle arrest in G1 and form mating projections. These projections are directed along pheromone gradients that are established by their mating partners (Jackson and Hartwell 1990; Segall 1993; Dorer *et al.* 1995). The projections of the different mating partners grow toward one another, eventually leading to cell-cell contact and fusion. Cells that lie at considerable distances from each other presumably also regulate their cell polarity to efficiently find one another and fuse; how this occurs is not well understood.

The basic cellular events and molecular mechanisms of the *Saccharomyces cerevisiae* mating pathway are now beginning to be understood (reviewed in Sprague and Thorner 1992). Haploid *MAT***a** cells mate with cells of the opposite mating type, *MAT*α, through a complex process that involves the production of cell-type-specific peptide pheromones and reception of these pheromones by cells of the opposite mating type. The pheromones are bound by the *STE2* and *STE3* gene products, which are seven transmembrane segment receptors located on the surface of *MAT***a** and *MAT*α cells, respectively. These receptors are coupled to a set of heterotrimeric G proteins and a cytoplasmic mitogen-activated protein (MAP) kinase cascade (Sprague and Thorner 1992). Transduction of the signal by the MAP kinase cascade leads to activation of the transcription factor Ste12p, which in turn promotes the transcription of a set of genes involved in mating-specific functions. These functions include cell-cycle arrest in G1, polarized morphogenesis, agglutination, cell fusion, karyogamy, and adaptation to the pheromone signal.

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Perhaps the best-understood process that occurs in response to pheromone is signal transduction by the MAP kinase cascade. The epistasis relationships and physical contacts between the Ste11, Ste7, and Fus3 and Kss1 kinases (MEKK, MEK, and MAP kinases, respectively) of the cascade and a "scaffolding" protein, Ste5p, which organizes them, have been elucidated by a combination of genetic and biochemical studies (Brill *et al.* 1994; Choi *et al.* 1994; Printen and Sprague 1994; Herskowitz 1995; Pryciak and Huntress 1998). Most surprising, however, has been the finding that many components of the MAP kinase cascade that controls the mating responses, including the Ste12p transcription factor, are also required in both haploid and diploid cells for the transition from the "normal" yeast form of growth to pseudohyphal and growth-invasive forms (Liu *et al.* 1993; Roberts and Fink 1994; Rupp *et al.* 1999). These forms arise when cells are limited for nutrients and bud in a unipolar pattern, spreading through the medium in filamentous arrays (Gimeno *et al.* 1992; Gimeno and Fink 1994; Kron *et al.* 1994; Roberts and Fink 1994). This form of polar growth and unipolar budding has been proposed to be a mechanism by which cells forage for more favorable nutrient-rich environments (Gimeno *et al.* 1992; Kron *et al.* 1994).

In this study we characterize a novel pheromone response that occurs under conditions of low pheromone concentration, below that required for maintaining cell-division arrest. We find that cells exposed to low levels of mating pheromone elongate and bud primarily from their distal ends, resulting in the formation of filamentous arrays. These results provide insight into mechanisms by which mating cells located at some distance from each other effectively find one another and define novel properties of cells that are adapted to the cell-cycle inhibitory effects of mating pheromones.

MATERIALS AND METHODS

Yeast strains and growth conditions: Yeast strains were from the sources indicated in Table 1. Yeast media and strain manipulations were as described in Sherman and Hicks (1986) unless otherwise indicated. Strain Y1490 was derived by sporulating a diploid strain containing an mTn3::lacZ::URA3 insertion at codon 254 of the 603 codon *BUD8* open reading frame. Analysis of a diploid strain homozygous for the *mTn3::lacZ::URA3::bud8* allele indicates that this *bud8* allele is equivalent to a null allele. Yeast strains were grown overnight in rich medium (YPD; Sherman and Hicks 1986) to midlogarithmic phase for all assays. Filamentation assays were conducted by rapidly suspending 10^4 or 10^5 yeast cells in 1.5 ml of YPD 1% top agar and plating in 60-mm petri dishes containing 2.0 ml solidified YPD agar. To establish pheromone gradients, 5 or 10 μg of α-factor was added to 6.5-mm BBL paper discs (Becton Dickinson, San Jose, CA) placed in the centers of the plates, and cell growth and morphology were monitored by microscopy over 48 hr. For experiments examining the responses of cells to uniform concentrations of pheromone, α-factor pheromone was mixed to the appropriate final concentration with 5×10^2 cells in 1% YPD agar by vortexing, followed by plating. Filamentation was quantified by microscopy; regions within the assay plates where colonies displayed filamentous (nonaxial division pattern) responses were located and all colonies within all planes of focus were scored in these microscopic fields. More than 10 regions of each plate were analyzed to derive each value presented. Colonies were scored as filamentous if they displayed nonaxial cell-division patterns. Our measures of filamentation are probably underestimates because filaments extending directly above or below the colonies in the plane of observation are not readily scored. Cells and colonies were visualized and photographed using bright field microscopy at ×40 magnification with a green filter. α-factor pheromone was from Sigma (St. Louis).

Time-lapse microscopy: Time-lapse studies were carried out by suspending cells grown to early log phase at low density in YPD-agar as described previously. Chambers for cell growth and imaging were prepared by placing two layers of Scotch tape ~3 cm apart on a standard microscope slide. The slide and a 20- × 40-mm coverslip were prewarmed to 37° and an aliquot of the agar suspension of cells was pipetted onto the prewarmed slide between the taped regions. The coverslip was placed over the agar and the slide was gently blotted by inversion on absorbent paper. Excess agar was trimmed away with a razor blade and the edges of the coverslip were sealed with valap (a 1:1:1 vaseline, lanolin, paraffin mixture). For these experiments cells were imaged with differential interference contrast optics at ×100 magnification using a minimum level of transmitted light to illuminate the specimen. A time-lapse series of images of the dividing cells was recorded using a Roper/Princeton Instruments Interline 5 mHz Micromax CCD camera.

RESULTS

Cells exposed to low levels of pheromone form filamentous arrays: A screen for genes whose expression is induced upon exposure to mating pheromone identified several genes (*e.g.*, *PHD1*, *DUR1-2*, and *GAP1*) whose products are implicated or likely to participate in pseudohyphal growth (Erdman *et al.* 1998). The observation of common target genes shared by these pathways prompted us to investigate whether morphological similarities also exist between pseudohyphal and mating-differentiated cells.

Haploid *MAT***a** cells embedded in top agar were exposed to discs containing different concentrations of the α-factor mating pheromone, and the cells were examined by microscopy over 48 hr. The cellular and microcolony morphologies varied as a function of distance from the pheromone source. Four classes of cells/colonies were observed. Cells located closest to the pheromone disc (~5 mm or less) arrested as unbudded cells, remained small, and formed multiple pointed projections (usually two within the period of the assay). These projections (~90% of cells) usually emerged from opposite ends of the cell (Figure 1A). At intermediate concentrations of pheromone, located midway through the halo of pheromone-induced growth inhibition (~7.5 mm from the disc), cells arrested cell division, enlarged, and produced single projections many times longer and wider than those formed at the higher pheromone concentrations (Figure 1B). The morphology of these cells

TABLE 1

Strains used in this study

Strain[a]	Genotype	Reference
Y604	*MAT***a** *ura3-52 lys2-801 ade2-101 trp1-901 his3-Δ200*	Gehrung and Snyder (1990)
Y604 (*far1*Δ)	*MAT***a** *ura3-52 far1::URA3*	Dorer *et al.* (1995)
Y602	*MAT***a** *ura3-52 lys2-801 ade2-101 trp1-901 his3-Δ200 spa2::URA3*	Gehrung and Snyder (1990)
Y760	*MAT***a** *ura3-52 lys2-801 ade2-101 trp1-901 his3-Δ200 slk1::URA3*	Costigan *et al.* (1992)
Y1490	*MAT***a** *ura3-52 lys2-801 ade2-101 mTn3::lacZ::URA3::bud8*	This study
E929-6C-0[b]	*MAT***a**	Zhou *et al.* (1993)
E929-6C-14	*MAT***a** *ste4*Δ	
E929-6C-20	*MAT***a** *ste11*Δ	
E929-6C-1	*MAT***a** *ste7*Δ	
E929-6C-34	*MAT***a** *ste5*Δ	
E929-6C-48	*MAT***a** *kss1*Δ	
E929-6C-30	*MAT***a** *fus3*Δ	
E929-6C-50	*MAT***a** *fus3*Δ *kss1*Δ	
E929-6C-6	*MAT***a** *ste12*Δ	
Y799[c]	*MAT***a** (1237-13C background)	
Y477[c]	*MAT***a** *rsr1/bud1*	Madden and Snyder (1992)
Y825[d]	*MAT***a** *leu2::hisG ura3-52* (Σ1278b background)	Madden and Snyder (1992)

[a] All strains are S288c background unless otherwise indicated.
[b] E929-6C-0 through E929-6C-6 are gifts from B. Errede.
[c] Strains 1237-1 (wild type) and 169 (*bud1*) are from Chant and Herskowitz (1991).
[d] Gift from G. Fink laboratory.

strongly resembles that observed for cells actively tracking pheromone gradients (Segall 1993; L. Vallier and M. Snyder, unpublished data). At the periphery of the halo, just inside the zone of growth inhibition by pheromone (9 mm from the disc), a novel type of colony morphology was observed (Figure 1C). Of these growth-inhibited colonies, 72–97% form filamentous arrays in which daughter cells often bud at their distal tip and mother cells bud from either end of the cell (*i.e.*, bipolar budding; Tables 2–4. Cells within the filamentous colonies are often elongated and larger relative to the nearly round shape of vegetatively growing haploid yeast cells; these pheromone-induced morphologies are strikingly similar to cells undergoing haploid invasive growth, a process closely related to pseudohyphal growth (Figure 1C; refer to Roberts and Fink 1994, Figure 2B). As in invasive growth, cells within pheromone-induced filamentous colonies are often uniform in shape and size. In areas where filamentous colonies occur, these colonies contain fewer cells than those outside the halo, and periodic examination of the number and size of cells in these colonies revealed that they undergo cell division at a much slower rate than normal vegetative cells. Within the filamentous zone, the degree of cell-division inhibition correlated with the distance from the pheromone source. The budding pattern and morphological changes in cells within the pheromone-induced filamentous colonies differ from those observed for cells outside the growth inhibition zone (Figure 1D). In the absence of growth inhibition cells are smaller and follow the normal axial budding pattern of haploid cells, in which new cells bud adjacent to the previous site of cytokinesis (Hicks *et al.* 1977; Snyder 1989; Chant and Pringle 1995). The filamentous colony morphology is observed in all laboratory wild-type strains examined thus far (Table 1).

Since many of the properties of pheromone-induced filament formation resemble those of pseudohyphal growth, we used time-lapse microscopy to further investigate the growth dynamics of cells budding in the presence of low levels of pheromone. Figure 2 shows a sequence of frames from one of these experiments. As the sequence shows, bud emergence in mother cells initiates in advance of that of its corresponding daughter cells. These budding kinetics are similar to those seen in vegetative cells dividing in the absence of mating pheromone and are distinct from those observed for pseudohyphal cells or for cells forming filamentous chains because of defects in morphogenesis in which mother and daughter cells bud synchronously (Kron *et al.* 1994; Blacketer *et al.* 1995).

Filamentation can occur when different concentrations of pheromone are present in the disc (Table 4) and when cells are plated in top agar containing uniform concentrations of pheromone. For example, in the presence of isotropic 10 nm α-factor, 90% of the colonies formed are filamentous (N = 200 colonies; 250 cells/ml). This latter observation indicates that the filamentous colony morphology is not due to nutrient deprivation or gradients of nutrients generated in the assay plates [conditions that induce pseudohyphal and haploid invasive growth (Gimeno *et al.* 1992; Roberts

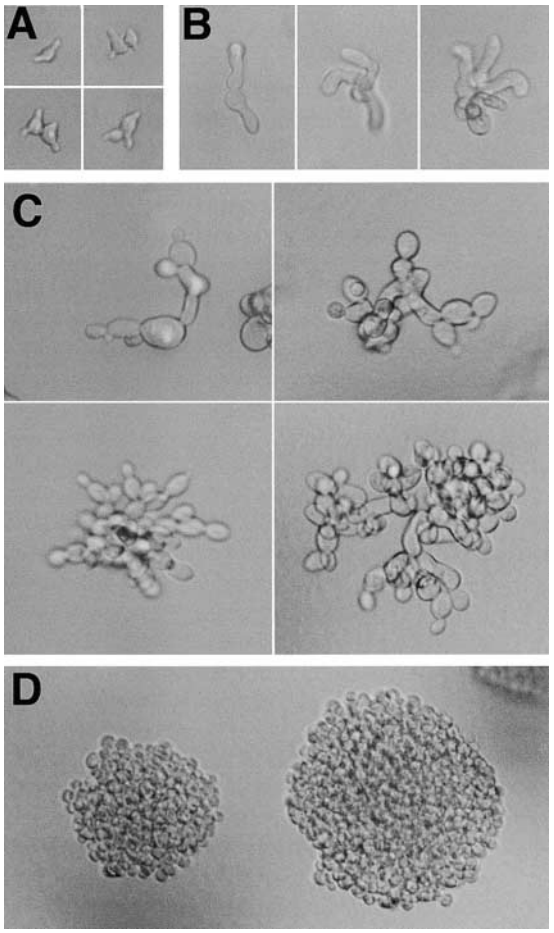

FIGURE 1.—Cell and colony morphology after exposure to mating pheromone. Cells embedded in top agar were spread on a petri dish and exposed to a small filter disc containing 5 or 10 μg/ml α-factor. (A) Cells close to the disc often form multiple projections. (B) Cells in the central region of the halo are enlarged and form long projections. (C) Colonies formed near the periphery of the halo. These colonies are small and often contain linear chains of cells. (D) Cells treated identically, but not exposed to pheromone. Cells outside the growth inhibition zone form colonies that appear identical to these. Note that A and B often contain two or more cells even though cells have arrested cell division. This is probably due to both the fact that time is required for the pheromone to initially diffuse to the cells and the probability that some cells are post-G1 at the time of initial exposure to mating pheromone.

and FINK 1994)]. Consistent with this interpretation, only cells that are exposed to pheromone produce extensive filamentous colonies; filamentous arrays are not observed outside the zone of inhibition of cell division in the disc assays or on plates that lack pheromone. In addition, filament formation can be observed at very early times in the assay in microcolonies of <10 cells, when nutrient depletion is expected to be low, and at different starting cell densities (Table 2; 5×10^2 cells were also tested, but results are not shown).

Filament formation requires an active MAP kinase signaling pathway: To investigate the cellular components necessary for pheromone-induced filamentous growth we first examined whether signal transduction by the mating MAP kinase pathway was necessary for this process. Activation of the mating response pathway requires a G-protein subunit, Ste4p, and a cascade of protein kinases including Ste11p and Ste7p (HERSKOWITZ 1995). Stimulation of these kinases results in activation of two partially redundant MAP kinases, Kss1p and Fus3p, which in turn activate a transcription factor, Ste12p. In pseudohyphal growth, most elements of the Ste11p → Ste12p MAP kinase pathway are required for filamentation; the exceptions are Ste5p, a putative scaffold protein, and the Fus3p MAP kinases (LIU *et al.* 1993; CHOI *et al.* 1994; ROBERTS and FINK 1994). We tested whether *ste4*Δ, *ste11*Δ, *ste7*Δ, *ste5*Δ, *fus3*Δ, *kss1*Δ, *fus3*Δ *kss1*Δ, and *ste12*Δ cells form filaments after exposure to pheromone using the disc assay. As shown in Table 3, *ste4*Δ, *ste11*Δ, *ste7*Δ, *ste5*Δ, *fus3*Δ *kss1*Δ, and *ste12*Δ strains showed markedly reduced levels of filament formation; filaments were rarely observed for most of these strains even at locations of maximal pheromone concentration directly adjacent to the pheromone source. However, filament formation did occur for both *fus3*Δ cells, which fail to undergo substantial pheromone-induced cell-cycle arrest (similar to *far1*Δ cells; see below), and for *kss1*Δ cells, which arrest normally in response to pheromone. Thus, if *FUS3* and *KSS1* directly affect the alteration of budding pattern and cell morphology, they are redundant for this function. These results indicate that activation of the MAP kinase pathway and Ste12p are required for pheromone-induced filament formation. In addition, unlike the case for pseudohyphal growth, *STE4* and *STE5* are required for this process.

Efficient filament formation requires bud site selection machinery: The mechanism by which cells choose successive distal sites for budding under conditions where cell division proceeds in the presence of pheromone was also investigated. Two possible models for the underlying mechanism of this behavior were considered. The presence of pheromone might induce cells to adopt a bipolar budding pattern similar to diploid cells in which daughter cells choose distal sites at a high frequency (HICKS 1977; SNYDER 1989; CHANT and PRINGLE 1995). This pattern would be consistent with that observed for many of the filament-forming colonies (*e.g.*, Figure 1C, top left). Alternatively, pheromone may cause cells to polarize and concentrate growth components at one region of the cells; in combination with loss of the axial bud site selection mechanism, these components might enhance bud site selection at distal sites in the subsequent cell division.

To help distinguish between these mechanisms, we examined pheromone-induced filament formation in *rsr1/bud1*, *bud8*, *slk1/bck1*, and *spa2*Δ mutants. The *RSR1/BUD1* gene is required for bipolar and axial bud site selection during vegetative growth; *rsr1*Δ*/bud1*Δ mutants bud randomly (BENDER and PRINGLE 1989; CHANT

TABLE 2

Colony morphology as a function of distance from a pheromone source

Strain	Distance from disc (mm)	Morphology	% filamentous	*N* (colonies)
WT (Y604)	9.0[a]	Filamentous colonies	97.1	205
		Round/axial colonies	2.9	
WT	7.5	Elongated, arrested	97.3	200
WT	5.0	Multiple projections	98.0	212
*far1*Δ	9.0	Filamentous colonies	4.8	210
		Round/axial colonies	95.2	
*far1*Δ	7.5	Filamentous colonies	78.7	221
		Round/axial colonies	27.1	
*far1*Δ	5.0	Filamentous colonies	97.2	215
		Round/axial colonies	2.8	

Assays were performed at a cell density of 1×10^4 cells/1.5 ml and an α-factor pheromone concentration of 5 μg in the disc.

[a] Beyond 11 mm nearly all (>98%) of the wild-type and *far1*Δ strains were round axial budding colonies.

and Herskowitz 1991), but are unaffected in projection formation and polarization during mating (Valtz *et al.* 1995; data not shown). Mutations in the *BUD8* gene specifically eliminate bud site specification at distal sites but not proximal sites on cells programmed to bud in a bipolar pattern (Zahner *et al.* 1996). Thus, these mutations would not be expected to affect the pheromone-induced formation of filaments if they occur by an enhanced cell polarization mechanism, but should disrupt a mechanism that requires bipolar bud site selection.

Wild-type, *rsr1/bud1*, and *bud8* cells were exposed to filter discs containing mating pheromone as above, and the morphology of colonies just inside the growth inhibition zone was determined. Although most *RSR1/BUD1* colonies form filaments (72–74%), only 20–34% of *rsr1/bud1* colonies in a similar region successfully produce filaments (Figure 3 and Table 4). When filaments containing two or more nonaxial budding cells are scored, 43% of *RSR1/BUD1* filaments have three or more cells that bud successively from the distal region of the cells, with most budding from the distalmost tip of the cell (100 filaments scored). However, only 16% of *rsr1/bud1* filaments have three or more cells that bud from nonaxial regions of the mother cells (100 filaments scored), and for most of these filaments, the daughter cells were not located at the distal poles. Furthermore, whereas 9% of *RSR1/BUD1* filaments are four cells in length, four-cell filaments could not be found in the *rsr1/bud1* filaments (100 filaments scored). As noted for wild-type cells, cell division of *rsr1/bud1* cells was delayed in response to low levels of pheromone and the cells enlarged, indicating that their signaling and growth responses were normal. Moreover, at all cell densities and pheromone concentrations tested the sizes of the halos generated were identical between *RSR1/BUD1* and *rsr1/bud1* cells. Thus, the *RSR1/BUD1* gene is required for effective filament formation during exposure to low levels of mating pheromone, suggesting a requirement for the bipolar machinery. Analysis of *bud8* strains supports this hypothesis; filament formation occurs in only 29% of *bud8* colonies whose growth is inhibited by mating pheromone as compared to 98% filament formation for wild-type colonies under these conditions. Since

TABLE 3

Filament formation in colonies of mating response pathway mutants

Strain	Pheromone (μg)	Cell density (cells/1.5 ml)	% filamentous	*N* (colonies)
WT (E929-6C-0)	5	1×10^4	87.0	108
*ste4*Δ	5	1×10^4	3.8	104
*ste11*Δ	5	1×10^4	12.0	100
*ste7*Δ	5	1×10^4	30.2	106
*ste5*Δ	5	1×10^4	2.9	104
*kss1*Δ	5	1×10^4	92.9	112
*fus3*Δ	5	1×10^4	79.1[a]	110
*fus3*Δ *kss1*Δ	5	1×10^4	3.8	105
*ste12*Δ	5	1×10^4	18.8	101

[a] *fus3*Δ cells, like the *far1*Δ cells described in Table 2, form filaments throughout the growth inhibition zone (0–7 mm from disc).

TABLE 4

Filament formation in colonies of wild-type and *rsr1/bud1* strains

Strain	Pheromone (μg)	Cell density (cells/1.5 ml)	% filamentous	*N* (colonies)
WT (Y799)	None	1×10^5	0.07	300
WT	5	1×10^4	73.6	304
rsr1/bud1	5	1×10^4	19.9	312
WT	10	1×10^5	71.9	310
rsr1/bud1	10	1×10^5	36.0	300

pheromone-induced filamentation is not completely disrupted in *rsr1/bud1* or *bud8* strains, and because wild-type cells can be observed to bud at distal sites of hyperpolarized cells (Figure 1C, top), the enhanced cell polarization mechanism may also contribute in part to filamentous growth.

To investigate the involvement of a cell polarization mechanism in filament formation, we tested the filament formation properties of *spa2* and *slk1/bck1* mutant strains. The *SPA2* and *SLK1/BCK1* genes are required for the pheromone-induced morphogenesis that occurs in response to high levels of mating pheromones; thus they might also be required for a filament-forming polarization mechanism. Analysis of pheromone-induced filament formation in these mutants indicates that these gene products are required for wild-type levels of filament formation (48% filamentous colonies in each case, compared to 93% filament formation by a wild-type strain). In their absence, cells still often choose distal sites for bud emergence; however, the resulting filaments are less extensive and contain fewer elongated cells. Additionally, *slk1/bck1* cells were found to be completely defective in undergoing morphogenesis to form the elongated cells of the class shown in Figure 1B. Collectively, these results indicate requirements for these polarity proteins for maximal levels of pheromone-induced filament formation and show that some aspects of a cell polarization mechanism are likely to characterize this process.

Filament formation does not require *FAR1*: The *FAR1* gene, which is required for pheromone-induced cell-cycle arrest, has been suggested to also play a role in mating projection orientation, either by erasing the axial budding site or by promoting growth toward the pheromone source (Dorer *et al.* 1995; Valtz *et al.* 1995). Recent studies that have identified a complex composed of Far1p, Cdc24p, and free Gβγ-subunits appear to support the latter hypothesis (Butty *et al.* 1998; Nern and Arkowitz 1999). Depending upon the level of pheromone signaling necessary to create cell-cycle inhibition or arrest in G1, *FAR1* might be expected to be required for the transition from an axial to a filamentous budding program. We therefore examined the morphology of *far1*Δ colonies and cells after exposure to mating phermone using the disc assay. As shown in Figure 3 and Table 2, *far1*Δ strains form filaments at similar levels to wild-type cells. The distance from the pheromone source at which *far1*Δ cells form filaments is decreased slightly, probably because these cells are marginally less sensitive to mating pheromone at these

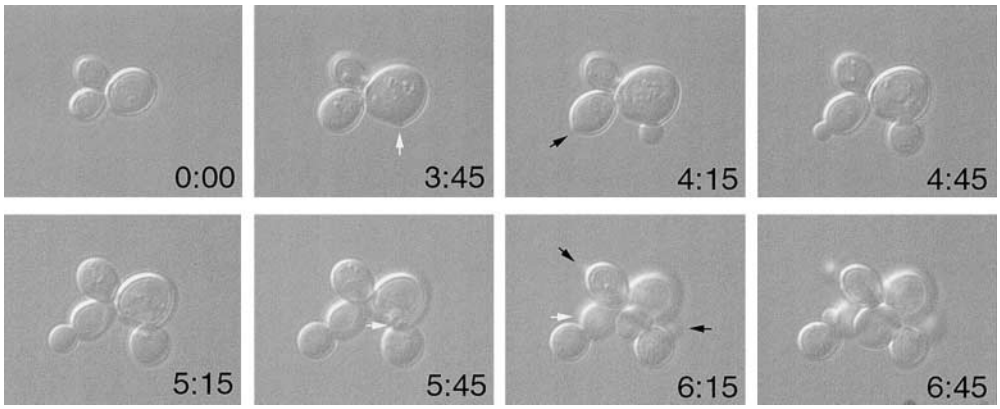

Figure 2.—Time-lapse analysis of cell division in the presence of low levels of α-factor. Cells were resuspended in YPD medium containing 10 nM α-factor and imaged over an 8-hr period. Time after pheromone addition is indicated (hr:min) for each frame. A group of a mother and two daughter cells is shown in which the mother cell is initially budding in the axial pattern characteristic of haploid cells not exposed to mating pheromone. Following exposure to levels of mating pheromone that inhibit but do not prevent cell division, cells can be seen to choose nonaxial sites for bud emergence. The budding patterns of these pheromone-exposed cells are characteristic of a bipolar bud site selection pattern wherein daughter cells have a higher probability than mother cells of choosing distal sites for budding. As indicated by the series of arrowheads indicating the emergence of buds from mother cells (open arrowheads) and corresponding daughter cells (solid arrowheads), pheromone-induced filament formation does not create a mother-daughter synchrony for bud emergence as is observed for pseudohyphal differentiation.

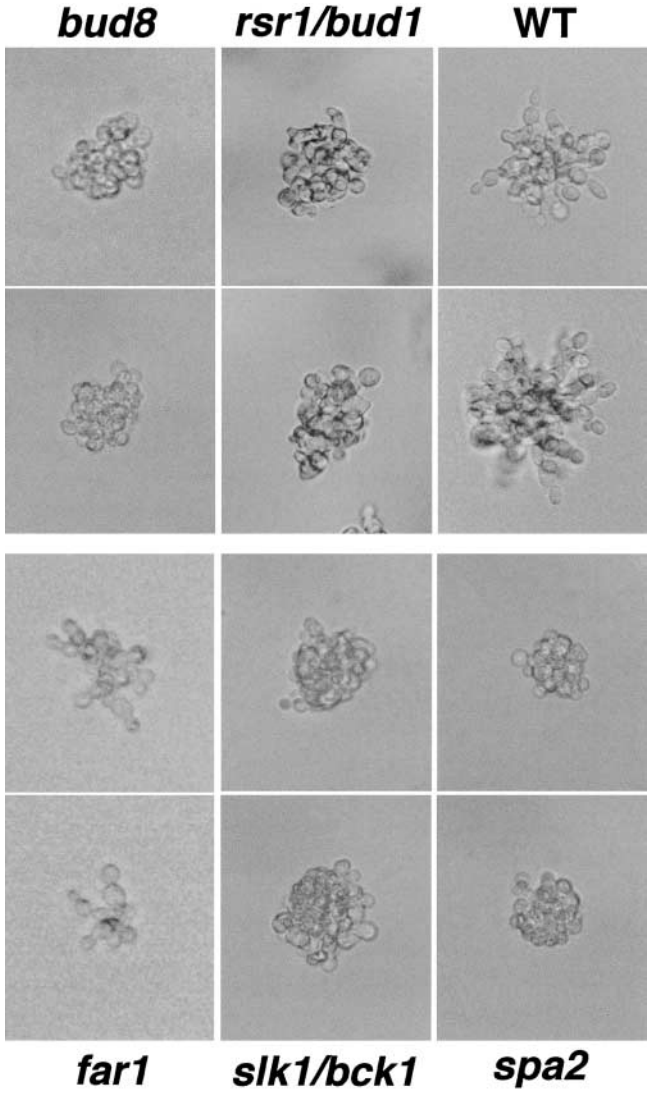

FIGURE 3.—Wild-type (WT), *rsr1/bud1*, *bud8*, *spa2*Δ, *slk1*Δ/*bck1*Δ, and *far1*Δ colonies exposed to low levels of pheromone. Cells were exposed to mating pheromone as described for Figure 1 and colonies near the halo periphery were photographed. In each case cells are partially inhibited for cell division. *rsr1/bud1* and *bud8* mutants are defective in forming pheromone-induced filaments, whereas *spa2* and *slk1*Δ/*bck1*Δ mutants are significantly, but not completely, defective in filament formation. Wild-type and *far1*Δ cells efficiently form the spreading chains of cells that characterize filament formation.

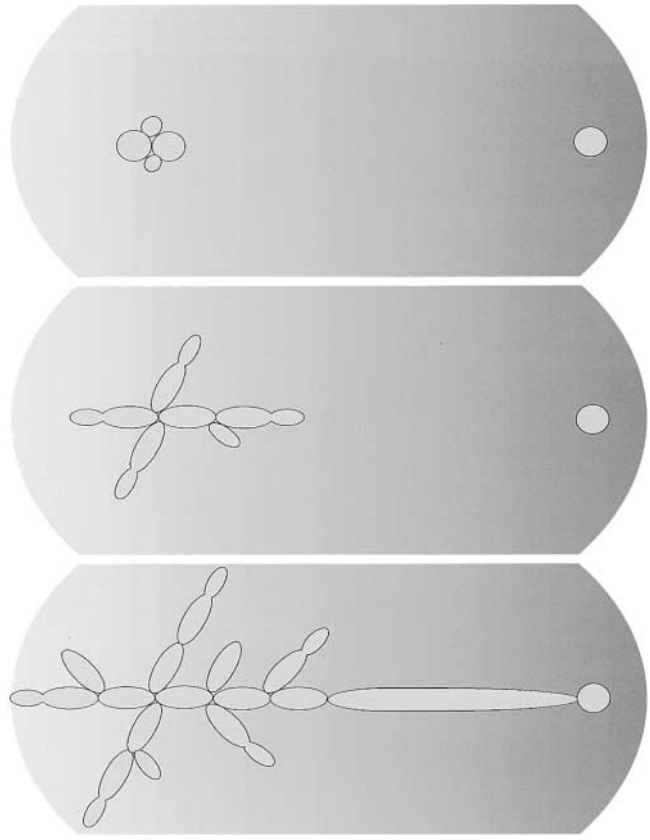

FIGURE 4.—Model for the mating pathway in yeast. Cells exposed to low levels of pheromone (depicted as a gray concentration gradient) alter their budding pattern and growth dynamics and form filamentous chains of cells that may enable more efficient detection of pheromone gradients. As cells approach their mating partners, they encounter higher levels of pheromone, undergo cell-cycle arrest, and elongate toward their partner. For clarity, the responses of only one mating cell type are shown.

levels (see CHANG and HERSKOWITZ 1990, Figure 1). Inspection of cells close to the pheromone source reveals high levels of filamentation; unlike that found for wild-type cells in this region, cells with elongated projections and multiple projections are not observed. This is presumably because *far1*Δ cells do not undergo cell-cycle arrest (CHANG and HERSKOWITZ 1990), although their division rate is slightly inhibited by pheromone. Thus, these results indicate that *FAR1* is not required for filament formation upon exposure to mating pheromone. Furthermore, since *far1*Δ cells do not arrest, these results, taken along with those of the *fus3*Δ cells, indicate that substantial cell-cycle inhibition is not necessary for filament formation. Although this is the case, partial cell-cycle delays, which still occur in the presence of these mutations (CHANG and HERSKOWITZ 1990; S. ERDMAN, unpublished observations), may still be important for the generation of pheromone-induced filaments.

DISCUSSION

The dose-response relationships for mating pheromone-induced cell-cycle inhibition, arrest, and projection formation have been known for some time (MOORE 1983, 1987). These studies, which were carried out primarily in liquid media, did not detect the budding patterns and colony morphology that characterize continuous exposure to different pheromone levels. Our observations, conducted over a 12-hr period, indicate that cells exposed to high levels of mating pheromone remain cell-cycle arrested, producing small mating projections

that emerge in a bipolar pattern. Intermediate concentrations elicit a distinctly different growth response wherein cells choose and maintain a single polarized growth site over a long period of time, in contrast to the periodic bipolar emergence of new projections seen in cells experiencing high levels of mating pheromone. The morphologies of these cells and the levels of pheromone that induce a sustained growth response correspond well to those of cells that are following pheromone gradients in spatial orientation assays (Segall 1993; Valtz *et al.* 1995). Low levels of mating pheromone induce filament formation through distal budding and cell elongation, similar to pseudohyphal and haploid-invasive responses. Additionally, our study demonstrates that cell-cycle progression and morphological responses to pheromones can occur concurrently in wild-type cells over the course of many cell cycles, a circumstance previously observed only in mutants defective in pheromone-mediated cell-cycle arrest (*i.e.*, *fus3* and *far1*; Chang and Herskowitz 1990; Elion *et al.* 1990).

The significance of the distal growth site selection pattern that accompanies different levels of pheromone response has not been addressed previously. We suggest that distal bud site selection in response to low levels of pheromone experienced by mating cells can play a biological role as a search mechanism for locating mating partners and improving the initial detection of pheromone gradients as outlined in Figure 4. Altering bud site selection and cell elongation is expected to promote more efficient spreading of cells through their surroundings and also to expose a greater surface area of each cell to its immediate environment (as compared to an axial division pattern that produces a clustered colony of cells). These properties should also improve the abilities of cells to detect pheromone gradients; in particular, cell elongation generates an enhanced cellular asymmetry that would be expected to aid gradient perception. Recent theoretical modeling of the dynamics of the morphological responses of wild-type and *bar1* α-factor protease-defective cells highlights the critical nature of signaling differences across the cell surface for efficient pheromone-induced morphogenesis (Barkai *et al.* 1998). Such a proposed mechanism would be expected to depend strongly on the local arrangement of cells in regard to signal recognition, again suggesting advantages to an elongated filamentous colony morphology. As cells get closer to potential mates, the levels of pheromone increase, cell-cycle inhibition is greater, and arrested cells elongate toward their mating partners by tracking pheromone gradients analogous to the cells shown in Figure 1B, ultimately fusing to form zygotes (Madden and Snyder 1992; Segall 1993). Thus, different levels of the same pheromone signal can induce three distinct cell differentiation pathways: a filamentous/search response after exposure to very low levels of pheromone, cell elongation and tracking at moderate levels (chemotaxis) and at very high levels, and formation of multiple sequential projections (presumably to contact a nearby partner; Dorer *et al.* 1995).

The mechanism by which cells that are responding to low levels of pheromone no longer bud at axial sites but instead use distal sites is not understood. Haploid cells treated with low levels of mating pheromone for one or less-than-one cell cycle have been shown to exhibit a preference for utilizing distal or other sites for growth (Madden and Snyder 1992; Dorer *et al.* 1995), indicating that this event can occur in a single cell cycle. Loss of axial site utilization presumably results from decreased levels of axial-specific components such as Axl2p after pheromone treatment (Roemer *et al.* 1996b) and perhaps from increased levels of other proteins such as Ste12p targets. Indeed, *FAR1*, which is a Ste12p target, has been shown to be necessary for utilization of nonaxial sites for mating projection formation and polarized growth following exposure to pheromone (Dorer *et al.* 1995; Valtz *et al.* 1995). The ability of *far1*Δ cells to form filaments in response to pheromone, however, indicates that the requirement for Far1p in overriding the axial tag in pheromone-treated cells must be limited to the first, or initial few, polarized growth events (*i.e.*, a mating projection or bud, depending on pheromone levels) that occur in the presence of low levels of pheromone. Alternatively, a threshold for Far1p-dependent axial tag inhibition activity that is higher than that required for cyclin-dependent kinase inhibition by Far1p may exist such that only intermediate levels of pheromone eliminate axial tags. This latter model is attractive in light of recent evidence demonstrating that Far1p can form a complex with Cdc24p and Gβγ-subunits (Butty *et al.* 1998; Nern and Arkowitz 1999). It remains, however, to be explained how this growth site selection activity is specific to axial sites as both low and high levels of pheromone cause cells to form buds and projections, respectively, at bipolar sites.

One possible mechanism to explain the preference for distal sites of bud emergence may involve the G1 cell-cycle delays induced by mating pheromone. These delays may cause cells to lose transient proximal bud site selection "tags" and instead utilize stable tags found at distal sites, as suggested previously for nutrient-starved cells (Madden and Snyder 1992; Fleischer *et al.* 1993; Chant and Pringle 1995). Our results demonstrating a requirement for the *RSR1/BUD1* and *BUD8* gene products for effective filament formation suggest that bipolar bud site selection components collectively might play an important role in filament formation. As many of these components are proteins that have been shown to reside at sites of polarized growth, they represent reasonable candidates for a potential role(s) in the formation of a pheromone-stable tag (Herskowitz *et al.* 1995; Pringle *et al.* 1995; Costigan and Snyder 1996; Drubin and Nelson 1996). It will be interesting to determine the basis for the continuous inhibition or

elimination of axial bud site selection information and the additional requirements for distal bud site selection in cells undergoing filament formation in response to low levels of pheromone.

Our time-lapse and cell-division pattern analyses of the response of cells to low levels of pheromone indicate that these conditions result in maintenance of a distal budding pattern, elongated cell shapes, and cell-cycle delays. Significantly, these events occur through multiple rounds of cell division, suggesting that the pheromone adaptation pathway does not act to block these responses. Analyses of adaptation pathway mutants *bar1/sst1* and *sst2*, which are hypersensitive to pheromone, show that these strains form filaments at appropriately reduced levels of pheromone (data not shown). These studies also indicate that the products of these genes are not required for the alteration of cell-division pattern in response to pheromone. Since "adapted" cells have generally been defined as cells that have become insensitive to pheromone signaling as evidenced by a return to vegetative growth (Sprague and Thorner 1992), the observations presented here suggest that pheromone-adapted cells may possess a number of novel properties that are distinct from those of vegetative cells.

The discovery of filament formation of pheromone-treated cells is consistent with the observation that the mating pathway and the pseudohyphal growth and haploid-invasive growth pathways share many common features. These include their upstream signaling elements (*e.g.*, Ste7p, Ste11p, Ste12p; Liu *et al.* 1993; Roberts and Fink 1994) and the increased expression of several genes that are induced upon activation of this pathway (Erdman *et al.* 1998). Recent genome-wide microarray analyses of gene expression occurring in response to activation of signaling through mating and pseudohyphal/haploid invasive growth cascades have also revealed overlap among the mating and filamentous growth pathways in activating *KSS1*-dependent "filamentous growth" target genes (Roberts *et al.* 2000). Studies of the signaling components required for haploid fruiting (filament formation) of *MAT*α cells of the opportunistic human pathogen *Cryptococcus neoformans* have also revealed a requirement for pheromone signaling components (Gβ-subunits) to carry out filamentous growth via MAP kinase signaling to a STE12 homolog (Wang *et al.* 2000). These observations suggest that the pseudohyphal, haploid invasive, and pheromone-induced filamentous growth pathways are likely to constitute specialized derivatives of a common ancestral morphogenetic pathway that functions in several cellular responses and in many unicellular fungi (Gavrias *et al.* 1996; Mosch *et al.* 1996; Madhani and Fink 1998). However, yeast may be an evolutionary exception in which these functions have become extensively differentiated, because many unicellular eukaryotes (*e.g.*, Tetrahymena, Chlamydomonas, *Schizosaccharomyces pombe*) undergo mating as part of a coordinated response to starvation for certain nutrients (often nitrogen sources).

The modulation of the rate and direction of cell division by specific extracellular signals is a novel general strategy by which cells may differentiate and find potential targets. In both plants and animals, cells respond to extracellular signals through receptors. This signaling can result in changes in cell division and differentiation, thereby producing new cell-cell contacts and pattern formation. The mechanisms by which cell division is modulated in yeast to improve access to and perception of these signals, while of obvious importance to unicellular organisms, may also be of general relevance to developmental processes that occur in multicellular creatures.

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