Actin- and Tubulin-Dependent Functions During *Saccharomyces cerevisiae* Mating Projection Formation

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Several conditional-lethal mutant alleles of the single-copy *Saccharomyces cerevisiae* β -tubulin and actin genes were used to evaluate the roles of microtubules and actin filaments in the pheromone-induced extension of mating projections. Mutants defective in tubulin assembly form projections indistinguishable in appearance from those formed by wild-type cells. However, the tubulin mutants are unable to move their nuclei into the projections and to orient the spindle pole body associated with each nucleus toward the projection tip. Actin mutants are defective in spatial orientation of cell-surface growth required for formation of normal mating projections. Migration of nuclei into mating projections and Spa2p segregation to projection tips are also defective in actin mutants. Studies with *abp1* null mutants showed that the function of the Abp1p actin-binding protein is either not required for projection formation or there are other proteins in yeast with similar functions. Our findings demonstrate that actin is required to restrict cell-surface growth to a defined region for pheromone-induced morphogenesis and suggest that nuclear position and orientation in mating projections depend on direct or indirect interaction of microtubules with actin filaments.

INTRODUCTION

In response to specific intercellular signaling molecules, many cells will arrest their cell cycles and develop a morphology that is adapted for a specific function. For example, during the development of the nervous system, a neuronal progenitor cell will cease to divide and will adopt an elongated morphology to establish electrical connections with other cells. Much more progress has been made in determining how cells transduce the signals that control their fates across the plasma membrane than has been made in determining how these signals cause changes in cell morphology. Studies on yeast provide an opportunity to increase our understanding of how signaling molecules induce changes in cell shape. Like many other cells, yeast will stop dividing and acquire a specialized morphology in response to intercellular signaling molecules (reviewed by Cross *et al.*, 1988).

When haploid *Saccharomyces cerevisiae* cells of the **a** and α mating type are mixed, they undergo a program of events that leads to the formation of **a**/ α diploid cells

(Cross *et al.*, 1988; Herskowitz, 1989). These events include arrest in the G1 phase of the cell cycle, agglutination between cells of the opposite mating type, changes in the expression of certain gene products, selection of an appropriate mating partner (a process termed "courtship"; Jackson and Hartwell, 1990), orientation of growth of the two mating partners toward each other, joining of the cell walls of the mating partners, breakdown of the cell walls between the joined cells, fusion of the plasma membranes of the mating cells, migration of the two nuclei toward each other, and fusion of the nuclei. This program of events is initially triggered by the mating pheromones **a**-factor and α -factor, which are produced by **a** cells and α cells, respectively. The pheromone produced by each cell type acts on the opposite cell type.

The binding of purified pheromone produced by cells of one mating type to receptors on cells of the opposite mating type induces a number of responses normally observed in mating cells. Cell division is arrested in G1, mating genes are induced, a polarized organization of cellular constituents develops, and an elongated projection forms by apical growth of the cell surface (reviewed by Cross *et al.*, 1988). This response has proven to be valuable for genetic dissection of the signal trans-

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Table 1. Yeast strains used

Strain name	Genotype	Source
DDY178	<i>MAT a his4-619 leu2,3-112 act1-4</i>	T. Dunn
DBY1034	<i>MAT a his4-619 lys2-801am ura3-52</i>	Botstein lab
DBY1691	<i>MAT a his4-619 act1-3</i>	Novick and Botstein, 1985
DBY1695	<i>MAT a his4-619 act1-2</i>	Novick and Botstein, 1985
DBY2023	<i>MAT a his4-539am lys2-801am ura3-52 tub2-401</i>	Huffaker <i>et al.</i> , 1988
DBY2055	<i>MAT a his4-619 ura3-52</i>	Botstein lab
DBY2305	<i>MAT a his4-539am lys2-801am ura3-52 tub2-403</i>	Huffaker <i>et al.</i> , 1988
DDY76	<i>MAT a leu2,3-112 lys2-801am ura3-52 Δabp1::URA3</i>	Drubin <i>et al.</i> , 1990
DDY80	<i>MAT a leu2,3-112 lys2-801am ura3-52</i>	Drubin <i>et al.</i> , 1990

duction system that communicates the signal from the outside of the cell to the inside (reviewed by Herskowitz, 1989). Because much is known about the signal transduction system involved in the pheromone response, an opportunity now exists to determine the molecular basis for the interaction of the signaling system with the cellular machinery involved in morphogenesis.

Many, if not all, of the constituents of a yeast cell become arranged in a polarized manner in response to pheromone. As a projection forms and grows, new cell wall is added at the projection tip, and this cell wall appears to have a novel structure (Lipke *et al.*, 1976; Tkacz and MacKay, 1979). Also, several proteins become concentrated at the growing projection tip. These include Spa2p (Gehring and Snyder, 1990), Fus1p (Trueheart *et al.*, 1987), the α -factor receptor (Jackson *et al.*, 1991), and the a-agglutinins (Watzel *et al.*, 1988). The cytoskeleton also becomes polarized (reviewed by Barnes *et al.*, 1990). Filamentous actin accumulates at the growing region of the cell cortex, and actin cables align along the growth axis (Ford and Pringle, 1986; Hasek *et al.*, 1987; Gehring and Snyder, 1990). Microtubules extend from the spindle pole body on the nucleus to a position near or at the projection tip (Rose and Fink, 1987; Gehring and Snyder, 1990; Meluh and Rose, 1990). In addition, most organelles are asymmetrically arranged in cells treated with pheromone (Baba *et al.*, 1989). The nucleus, for example, tends to be located in the mating projection with its spindle pole body oriented toward the projection tip (Byers and Goetsch, 1975; Tkacz and MacKay, 1979; Hasek *et al.*,

1987; Rose and Fink, 1987; Baba *et al.*, 1989; Gehring and Snyder, 1990).

Determination of the significance of the asymmetric distribution of a single cellular constituent to the overall polarity of cell growth requires functional tests. Although some constituents are likely to actively participate in polarization of cell growth, others might become oriented as a consequence, rather than a cause, of polarization of cellular activities. For example, although some plasma membrane proteins might become localized to a particular region of the cell surface to orient growth or to mediate interactions with cells of the opposite mating type, others might become asymmetrically distributed as a consequence of the polar orientation of the secretory pathway without any functional significance to their spatial distribution. One protein asymmetrically distributed on the cell cortex of pheromone-treated cells, Spa2p, has been shown to be important for mating projection formation (Gehring and Snyder, 1990).

Both actin filaments and microtubules have been shown to function in the morphogenesis of various cells that adopt extended morphologies. During mating projection formation in *S. cerevisiae*, actin filaments and microtubules might help to restrict growth to a particular region of the cell cortex and to spread polarity marked on the cell surface to the cytoplasmic compartment of the cell. Studies on mating yeast cells have indicated that microtubules are required for nuclear fusion but not for morphogenesis or cell fusion (Delgado and Conde, 1984; Hasek *et al.*, 1987; Huffaker *et al.*, 1988). We have performed quantitative and qualitative studies, using several mutant alleles of the single actin and β -tubulin genes of *S. cerevisiae*, on the roles of actin and tubulin in pheromone-induced cellular morphogenesis. Our findings show that microtubules are essential for positioning the nucleus in the projection tip but not for morphogenesis. We show that actin is also important for positioning of the nucleus and, in addition, is very important for organizing growth of the cell surface during the formation of mating projections.

MATERIALS AND METHODS

Strains, Media, and Culture Conditions

The yeast strains used in this study are listed in Table 1. All cultures were grown in YEPD medium (1% yeast extract, 2% bacto peptone, and 2% glucose) in rotary-shaking water baths. The pH of media was adjusted to 4.0 with HCl for experiments in which α -factor (Sigma Chemical, St. Louis, MO) was added from 1 mg/ml aqueous stock solutions.

For the initial characterization of changes in actin and tubulin organization during pheromone treatment in wild-type cells (shown in

Figure 1. Microtubule organization in α -factor-treated cells. Log-phase cultures of strain DBY2055, grown at 30°C, were treated for the indicated times with 5 μ g/ml pheromone. Cells were fixed before α -factor treatment (a–c) and after treatment for 1 (d–f), 2 (g–i), or 3 h (j–l). The three columns show, in order, phase optics, DAPI staining of DNA, and anti-tubulin immunofluorescence. Bar is 5 μ m.

Figures 1 and 2), 50–75 μ l of a fresh overnight culture was introduced into 5 ml of YEPD. After 2–3 h at 30°C, pheromone was added to the culture for variable times and the cells were fixed for fluorescence microscopy (see below).

For analysis of tubulin mutants, early log-phase cultures ($1\text{--}2 \times 10^6$ cells/ml) were treated with 0.05 μ g/ml α -factor for 1 h at 30°C. This results in a G1 cell-cycle arrest without inducing mating projections (Moore, 1983). The cells were maintained in 0.05 μ g/ml α -factor and shifted to 11°C for 1 h to disrupt microtubules. Finally, α -factor was supplemented to 5 μ g/ml for 16–18 h at 11°C to induce mating projections. For analysis of actin mutants, early log-phase cultures ($1\text{--}2 \times 10^6$ cells/ml) were treated with 0.05 μ g/ml α -factor for 1 h at 20°C to induce arrest. Next, the cultures were kept in 0.05 μ g/ml α -factor and shifted to the restrictive temperature for 1 h. α -Factor was then supplemented to 5 μ g/ml to induce the morphological response, and the cells were held at the restrictive temperature for 3 h in the continued presence of 5 μ g/ml α -factor.

Fluorescence Microscopy

Immunofluorescent localization of tubulin and actin was as described by Kilmartin and Adams (1984) and Drubin *et al.* (1988). All other fluorescence techniques were as described by Pringle *et al.* (1989). Cells were viewed and photographed using a Zeiss Axioskop or Zeiss Axiophot microscope (Zeiss, Thornwood, NY). Hypersensitized Technical Pan film (Lumicon, Livermore, CA) (Schulze and Kirschner, 1986) was used for all photography. Preparation of affinity-purified anti-yeast actin antisera was described by Drubin *et al.* (1988). Monoclonal anti-yeast tubulin antisera (YOL1/34) (Kilmartin and Adams, 1984) were obtained from Serotech (Indianapolis, Indiana). Affinity-purified Spa2p antisera (Snyder, 1989) were a generous gift of M. Snyder (Yale University). All fluorescent secondary antisera were obtained from Organon Teknica-Cappel (Malvern, PA).

RESULTS

Tubulin and Actin Organization During Mating Projection Formation

Figures 1 and 2 show immunofluorescence localization of tubulin and actin, respectively, in wild-type *MATa* cells treated for up to 3 h with the mating pheromone α -factor at 30°C. During the 3-h time course there is a progressive increase in projection length. The DAPI staining and tubulin immunofluorescence in Figure 1 show that the nucleus is located in the projection and that some of the microtubules emanating from the spindle pole body on the nucleus point to, and often terminate at, the projection tip. This bundle is on the side of the nucleus facing the projection, indicating that the nucleus is oriented with its spindle pole body facing the projection tip. Very often, a long bundle of microtubules extends back from the spindle pole body to the cell body.

Actin is also organized asymmetrically in α -factor-treated cells (Figure 2). Projection tips contain clusters of punctate cortical actin structures. Actin cables in the cytoplasm are aligned along the projection axis. This overall architecture, with cortical actin structures concentrated in actively growing surface regions and cables aligned along the growth axis, is very reminiscent of budding cells (Kilmartin and Adams, 1984; Novick and Botstein, 1985) (Figure 2b).

α -Factor Response in *tub2* Mutants

Two strains carrying different cold-sensitive mutant alleles of *TUB2*, the single β -tubulin gene of *S. cerevisiae* (Huffaker *et al.*, 1988), were used to evaluate the requirement of microtubules for projection formation. In initial experiments using tubulin mutants in the cold, a large reduction in the number of cells that could form projections was observed. Instead, cells with large buds were found to accumulate. This suggested that the inability of tubulin mutants to form projections might be due to a block in mitosis that would prevent cells from reaching G1. Cells only undergo a morphogenetic response to α -factor in G1. This complication was circumvented in subsequent experiments by arresting cells in G1 with a low level of α -factor (0.05 μ g/ml) before induction of projections with elevated α -factor.

As shown in Figure 3, a and b, wild-type cells treated with 0.05 μ g/ml α -factor at 30°C for 1 h and then held at 11°C in 0.05 μ g/ml α -factor for 1 additional h (a procedure used to depolymerize microtubules in the mutant cells before subsequent treatments) are unbudded (~88%) and contain a single microtubule aster characteristic of cells in the G1 cell-cycle phase. Significantly, these low levels of α -factor are not sufficient to induce projection formation (Moore, 1983). Like the wild-type strain, the two *tub2* mutants accumulate in G1 when treated with 0.05 μ g/ml α -factor (Figure 3, d, e, g, and h). However, the cold treatment causes defects in microtubule structure in the mutant cells. The *tub2*-403 cells show some fibers (~81% of cells) or dots (~12%) or no staining at all (~7%). When fibers are seen in *tub2*-403 cells, they emanate from the nucleus but are single elements instead of asters and often appear shorter than in wild-type cells. In *tub2*-401 mutant cells, almost all (~97%) of the cells contain only a single dot of tubulin staining associated with the nucleus. The remaining cells (~3%) showed no tubulin staining.

On treatment of cells that had been arrested in G1 and held for 1 h at 11°C, with 5 μ g/ml α -factor for 16–18 h at 11°C, the wild-type and mutant cells are both able to form short projections of similar length (Figure 3, j–r). Microtubule organization remains basically the same for the mutants (Figure 3, n and q) as it was at the starting time point (Figure 3, e and h), except that fewer *tub2*-403 cells (~24%) have microtubules. The percent of cells with projections is also very similar between the mutant and wild-type cells (Table 2). The slightly reduced percentage of cells with projections for both mutants (72%) when compared with wild-type (83%) is likely to be due to the increased proportion of cells that become trapped in the budded stages of the cell cycle (Table 2), despite the pretreatment with 0.05 μ g/ml α -factor.

For the experiment in Figure 3, the position of the nucleus in the pheromone-treated cells was determined (Figure 4). The nuclei of 97% of the wild-type cells with

Figure 2. Actin organization in α -factor-treated cells. Log-phase cultures of strain DBY2055, grown at 30°C, were treated for the indicated times with 5 μ g/ml pheromone. Cells were fixed before α -factor addition (a and b) and 1 (c and d), 2 (e and f) or 3 h (g and h) after α -factor addition. The two columns show phase contrast optics and anti-actin immunofluorescence, respectively. Bar is 5 μ m.

projections were found to be located near to the projection tips, usually with microtubules running from the nucleus to the projection tip (Figures 3, j–l, and 4). The *tub2-403* cells had a slight reduction in the number of nuclei at the projection tip (Figures 3, m–o, and 4). When

microtubules are present in *tub2-403* mutants, they often appeared to connect the nucleus to the projection tip (Figure 3n). In the *tub2-401* mutants, the nuclei appeared to be randomly distributed in pheromone-treated cells, and no microtubules were observed (Figures 3, p–r, and

Figure 3. G1 arrest and projection formation in wild-type cells and *tub2* mutants. Log-phase cultures were treated with 0.05 $\mu\text{g/ml}$ α -factor for 1 h at 30°C, shifted to 11°C for 1 h in the continued presence of 0.05 $\mu\text{g/ml}$ α -factor, and then treated with 5 $\mu\text{g/ml}$ α -factor for 16–18 h at 11°C. Cells were fixed before (a–i) and after (j–r) the 16- to 18-h incubation. *TUB2* genotypes: a–c and j–l are *TUB2*; d–f and m–o are *tub2-403*; and g–i and p–r are *tub2-401*. The three columns show, in order, phase optics, DAPI staining of DNA, and anti-tubulin immunofluorescence. Bar is 5 μm .

4). In these cells the anti-tubulin staining dot associated with most nuclei appeared to be randomly oriented with respect to the projection tip (Figure 3q).

α -Factor Response in act1 and abp1 Mutants

The ability of three temperature-sensitive actin mutants, which differ in the severity of their defects, and a wild-type control strain to form mating projections at the nonpermissive temperature was evaluated. The mini-

mum nonpermissive temperature (the temperature at which the strains failed to form colonies on YEPD plates) is 35°C for *act1-2* and *act1-3* and 33°C for *act1-4*. Thirty-three degrees, which was used for experiments with *act1-4* cells, was found to be a semipermissive temperature in liquid culture. A similar procedure to that developed for analysis of the cold-sensitive tubulin mutants was used for the temperature-sensitive actin mutants. Cultures were grown in YEPD at 20°C to early log phase ($1\text{--}2 \times 10^6$ cells/ml), at which point α -factor

Figure 3. (Continued)

was added to $0.05 \mu\text{g/ml}$ for 1 h. The cultures in low levels of α -factor were next shifted to the nonpermissive temperature (or semipermissive for *act1-4*) for 1 h to disrupt actin structures and then $5 \mu\text{g/ml}$ α -factor were added for 3 h at the restrictive temperature to induce morphological alterations. Cells incubated at the permissive temperature (20°C) formed projections as well as wild-type cells. Even at the nonpermissive temperature, a defect in the morphological response of the actin mutants to $5 \mu\text{g/ml}$ α -factor was only observed after cells had been preincubated for 1 h at the nonpermissive temperature before addition of $5 \mu\text{g/ml}$ α -

factor. Presumably, this 1-h preincubation is required to render the actin nonfunctional. This pretreatment did not cause cell death (see below).

Cells treated as described above were fixed, and their actin cytoskeletons were visualized by immunofluorescence. Figure 5 shows phase and fluorescence micrographs of mutant cells and wild-type control cells. A population survey in which cells were classified with respect to morphology and actin structure is shown in Figure 6. Each cell was categorized according to three degrees of actin polarity and three degrees of morphological response as described in the legend to Figure 6.

The classification of actin structures refers to the cortical patches and not to the cables because the presence of cables was not required for projection formation. When grown at the semipermissive temperature of 33°C, *act1-4* mutants lacked actin cables but many cells were still able to form long projections (Figures 5d and 6). Those cells that formed projections tended to have polarized cortical actin patches (Figures 5d and 6). After 3 h at 35°C, some cells with large projections could be found to have two clusters of cortical actin: one in the projection tip and one in the cell body (Figure 5b, second cell from left). This most likely occurs when a cell begins to form a second projection. For the cells shown in Figure 5b, actin organization in the three cells on the left would be scored as highly polarized and in the cell on the right would be scored as moderately polarized. By our classification scheme, the three cells in Figures 5, e and f, from left to right, would be classified as follows: 1) moderate size projection and moderate actin polarization, 2) round/oval shape and delocalized actin, and 3) standard size projection and moderately polarized actin. Note that by this classification scheme mutant cells can be classified as having standard-size projections, although their cell bodies are more bloated than wild-type cells (compare cells in Figure 5a with the cell on the right in Figure 5e).

A number of generalities can be made about the ability of the three mutants to assemble a polarized actin cytoskeleton and to form a mating projection. First, the percentage of cells forming projections is reduced for all three mutants (Figure 6), and the projections that do form tend to be significantly shorter than wild-type projections (Figure 5). Second, cells in each mutant strain have a tendency to adopt a bloated morphology. Finally, the ability to polarize cortical actin structures is also affected in each mutant strain, with *act1-2* having the most severe defects and *act1-4* (grown at 33°C) having the most subtle defects. There is a strong correlation between the extent to which the cortical actin cytoskeleton of a cell is polarized and the extent of projection formation (Figure 6). Seventy-seven percent of wild-type cells incubated in 5 µg/ml of α -factor for 3 h at 35°C had lengths > 1.5 times their widths, whereas 84% had highly polarized cortical actin cytoskeletons. At the other extreme, 66% of the *act1-2* mutants were round or oval (they lacked projections) after 3 h at 35°C, and 68% had disorganized cortical actin cytoskeletons. The *act1-3* mutants fell in between these extremes (Figure 6). For *act1-4* cells grown at the semipermissive temperature of 33°C, the percentage of cells with well-developed mating projections was significantly reduced (35 vs. 57% for wild type), but many cells with small projections or lacking projections all together had highly polarized cortical actin (Figure 6). Many wild-type cells grown at 33°C also had highly polarized cortical actin structures but lacked projections or had small projections. It is possible that at 33°C those cells (both wild-

type and mutant) that have polarized cortical actin are beginning to form projections. *act1-3* and *act1-4* cells in general lacked cytoplasmic actin cables, whereas *act1-2* cells often had disorganized cable networks. Although the defects were less severe than in other actin mutants, the *act1-4* mutants also had elevated numbers of cells that had disorganized cytoskeletons and lacked projections. As noted above, many *act1-4* mutants formed large projections despite the fact that the cells lacked cytoplasmic actin cables.

Although actin mutants are defective in projection formation at the nonpermissive temperature, it is important to note that the majority of cells continues to increase in size while remaining round or oval in shape, indicating that these cells are metabolically active for the duration of the experiment. The increase in cell mass is apparent by visual comparison of cells before and after the 3-h incubation and by optical density measurements. In three separate experiments a 40–70% increase in optical density was observed during 3-h incubations at the nonpermissive temperature, with no difference being observed between wild-type and mutant strains.

In the above experiments a subpopulation of cells for each actin mutant strain was able to form projections of fairly normal appearance in response to pheromone at the nonpermissive temperature (see Figure 6). To determine in more detail to what extent these projections resemble the projections of wild-type cells, the position of the nucleus and Spa2p within the cells was determined (Figure 7). Spa2p is a protein that is concentrated at the tips of mating projections (Gehring and Snyder, 1990) (Figure 7). Ninety-seven percent of wild-type cells had their nuclei in the mating projection and 93% showed Spa2p localization to the tip of the mating projection. Spa2p localization and nuclear position were found to be altered in actin mutant cell populations. These effects were greatest in the *act1-2* cells. Only 56% of *act1-2* mutants had nuclei in the projection and only 36% had a spot of Spa2p staining at the projection tip.

Abp1p binds to actin *in vitro* and *in vivo* and has been implicated in morphogenesis because it is located in the cortical actin cytoskeleton and because patterns of cell-surface growth are altered when Abp1p is overproduced (Drubin *et al.*, 1988). This protein is, however, nonessential in mitotic cells (Drubin *et al.*, 1990). *abp1* null mutants were tested for their ability to form projections in response to α -factor. As shown in Figure 8, no defect was observed for projection formation by *abp1* null mutants when compared with an isogenic wild-type control strain.

DISCUSSION

During projection formation in pheromone-treated haploid yeast cells, microtubules and actin filaments, along with many other constituents of the cell, become

Table 2. Projection formation in *tub2* mutants

Strain	TUB2 genotype	Budded (%)	Round/oval unbudded (%)	Projections (%)	Total counted
DBY2055	<i>TUB2</i>	0	17	83	102
DBY2305	<i>tub2-403</i>	11	16	72	110
DBY2023	<i>tub2-401</i>	7	21	72	100

Log-phase cultures were treated with 0.05 $\mu\text{g}/\text{ml}$ α -factor for 1 h at 30°C, shifted to 11°C for 1 h, and then treated with 5 $\mu\text{g}/\text{ml}$ α -factor for 16–18 h at 11°C. Cells were scored as having mating projections if their length was 1.5 times their width. Budded cells may have been underestimated because small buds on the underside of a cell can be missed.

organized asymmetrically along the axis of cell growth. The cytoskeleton has been functionally implicated in polarized cell growth in a number of different organisms. We have in this study addressed the functions of actin filaments and microtubules in pheromone-induced cellular morphogenesis.

Microtubule Cytoskeleton

Both the formation of buds during mitotic cell growth and the formation of projections in response to pheromone involve polarized growth of the cell surface (Drubin, 1991). In both cases the spindle pole body is oriented toward the region of cell-surface growth and microtubules appear to connect the spindle pole body to the growing tip (Byers and Goetsch, 1975; Gehring and Snyder, 1990; Snyder *et al.*, 1991). Similarly, in polarized metazoan cells, microtubule organizing centers often orient in the direction of membrane traffic. This can be seen, for example, in a migrating fibroblast cell (Albrecht-Buehler, 1981) and in a cytotoxic T cell that has selected a target cell for destruction (Kupfer and Singer, 1989). These observations suggest that microtubule-organizing centers and their associated microtubule arrays might play a role in determining the orientation of membrane traffic in a polarized cell. Alternatively, microtubule-organizing centers might become oriented as a consequence, rather than a cause, of polarization of cellular activities.

During the mitotic cell cycle in budding yeast, the spindle pole body and its associated microtubules do not appear to be important for the growth of the cell surface or for the genetically programmed pattern of bud site selection (Huffaker *et al.*, 1988; Jacobs *et al.*, 1988). Instead, microtubules appear to extend to, or near, the incipient bud site after that region has been designated (Snyder *et al.*, 1991). After a bud site is selected, the microtubules become important for moving the nucleus to the mother-daughter neck and for dividing the chromosomes between the mother cell and the daughter cell (Huffaker *et al.*, 1988; Jacobs *et al.*, 1988).

Studies with tubulin mutants and with microtubule inhibitors have shown that during mating, microtubules are important for karyogamy (Delgado and Conde,

1984; Huffaker *et al.*, 1988). Inhibitor studies have also indicated that projection formation and cell fusion can occur in the absence of microtubules (Delgado and Conde, 1984; Hasek *et al.*, 1987). We have performed a quantitative study using two different β -tubulin mutants and have shown that projection formation is indistinguishable in the absence or presence of microtubules. When *tub2-401* mutants were used, no microtubules could be detected and only a single bright anti-tubulin staining spot associated with the nucleus was detected. This is most likely the spindle pole body. We found that the orientation of this region is random with respect to the site of projection formation in *tub2-401* mutants, indicating that polarized cell-surface growth does not require that the spindle pole body be oriented toward the growing surface of the cell.

Although projections form normally in the absence of microtubules, the nucleus does not move to the projection tip. Instead, it tends to remain in the cell body. This suggests that the primary reason that the spindle pole body orients toward the projection tip (presumably through the interactions of extranuclear microtubules with the cell cortex) is to bring the nucleus to the projection tip to facilitate nuclear fusion subsequent to cell fusion. Thus, extranuclear microtubules appear to be important during bud formation and projection formation for positioning the nucleus within the cell. Although microtubules are not required for the formation of projections or for cell fusion, it remains possible that the orientation of the spindle pole body might play a role in courtship. Courtship is the process in which yeast cells discriminate among potential mating partners with a high degree of fidelity (Jackson and Hartwell, 1990; Jackson *et al.*, 1991).

Two β -tubulin mutant alleles were used in our studies. Huffaker *et al.* (1988) found that different β -tubulin alleles differ in the extent to which they are defective in assembly and function. Mutations that affect only cytoplasmic microtubules affect the movement of the nucleus to the bud neck during cell division. The two alleles used in the present study differ in their effects on microtubule assembly and on localization of the nucleus to the projection tip. *tub2-*

401 cells do not have any detectable microtubules, and the cells are defective in nuclear localization. *tub2-403* cells, on the other hand, have microtubules that are detectable as single elements in 24% of the cells incubated in pheromone at low temperatures. Surprisingly, 90% of these *tub2-403* cells were found to have their nuclei located in the projection tips. In the cells where microtubules could be detected, usually only one microtubule element was observed, and it almost invariably connected the nucleus to the projection tip (Figure 3n).

The fact that more *tub2-403* cells were observed to have properly positioned nuclei than were found to have microtubules has several possible explanations. One possibility is that all of the cells with properly positioned nuclei have microtubules that connect the nuclei to the projection tip when the cells are alive, but the microtubules are not efficiently preserved for fluorescence microscopy. Another possibility is that the nuclei become positioned in the projection early in the time course of mating projection formation. When *tub2-403* cells were prearrested in 0.05 $\mu\text{g}/\text{ml}$ α -factor for 1 h at 11°C, 81% had microtubule elements. These microtubules might have mediated the movement of the nucleus to the proximity of the growing surface and then disassembled. If this is the case, then microtubules are not required to maintain the position of the nucleus near the projection tip. Alternatively, transient interactions of dynamically assembling and disassembling microtubules with the projection tip might maintain the correct orientation and position of the nucleus. The observation that almost all of the microtubules seen in *tub2-403* cells, treated with 5 $\mu\text{g}/\text{ml}$ pheromone for 16–18 h at 11°C, seem to connect the spindle pole body to the projection tip could be explained if microtubules are longer lived if they establish connections with the projection tip. The possibility that in wild-type cells microtubules that connect to the projection tip are more stable than other microtubules could be tested by treatment of cells with very high levels of microtubule inhibitors (Cassimeris *et al.*, 1986; Drubin and Kirschner, 1986) and by determining which class of microtubules disappears most rapidly. It is now important to determine whether location of the nucleus to the projection tip is an active process requiring the action of microtubule motors, such as those implicated in karyogamy (Meluh and Rose, 1990), or if localization only requires attachment of extranuclear microtubules to the projec-

Figure 4. Nuclear location in pheromone-treated wild-type cells and β -tubulin mutants at 11°C. Tubulin mutants were treated with pheromone as described in Figure 3. Only cells with a projection were scored. Nuclei were observed by DAPI staining, and their positions were scored as anterior, middle, or projection, depending on the one-third of the cell in which the nucleus was found.

Figure 5. Projection formation in wild-type cells and *act1* mutants. Early log-phase cultures ($1-2 \times 10^6$ cells/ml) were treated with $0.05 \mu\text{g/ml}$ of α -factor for 1 h at 20°C to induce G1 arrest. Next, the cultures were shifted to the restrictive temperature for 1 h in the continued presence of $0.05 \mu\text{g/ml}$ α -factor. α -Factor was then added to $5 \mu\text{g/ml}$ to induce the morphological response, and the cultures were incubated at the restrictive temperature for 3 h. The top image of each pair (a, c, e, g) is phase contrast, and the bottom (b, d, f, h) is anti-actin immunofluorescence. (a and b) Wild-type at 35°C . (c and d) *act1-4* at 33°C . (e and f) *act1-3* at 35°C . (g and h) *act1-2* at 35°C . Bar is 5 μm .

tion tip, in which case the nucleus would move along with the projection tip as it is extended.

Actin Cytoskeleton

Many eukaryotic cells that are enclosed by cell walls grow by apical surface expansion. Thus, yeasts, filamentous fungi, pollen tubes, root hair cells, and certain algae grow by spatially localized secretion and cell-wall synthesis. In these cell types, there is a concentration of actin at actively growing regions of the cell cortex, and there are often actin cables in the cytoplasm that are aligned along the growth axis (reviewed by Harold,

1990). In mitotically growing *S. cerevisiae* cells, actin function has been shown to be required for apical growth resulting in bud formation because the surfaces of actin mutants expand isotropically; cells that are enlarged but unbudded accumulate (Novick and Botstein, 1985). The essential function of actin in polarized growth of the yeast cell surface is not provided by cytoplasmic actin cables because bud formation is not dependent on the presence of cables (Liu and Bretscher, 1989). Organization of cortical actin structures in mitotically growing yeast does correlate well with the ability to grow in a polarized manner (Adams and Pringle, 1984; Novick and Botstein, 1985). However, it must be

noted that buds that are rounder than normal can form when cortical actin localization is compromised (Adams *et al.*, 1991) (see below). Interference with actin function by cytochalasins in fungi and algae also impairs apical growth (reviewed by Harold, 1990) and, additionally, inhibits wall regeneration in fission yeast (Kobori *et al.*, 1989). In total, these observations suggest that cortical actin structures contribute to localized wall growth.

During mating projection formation, actin appears, when viewed by fluorescence microscopy, as patches concentrated at the growing projection tip and as cables aligned along the growth axis of the cell (Ford and Pringle, 1986; Hasek *et al.*, 1987; Gehrung and Snyder, 1990; this study). We tested the ability of actin mutants treated with mating pheromone to grow apically. We found that mutations in the actin gene result in projection formation defects. The cells continue to enlarge but do not do so in the oriented manner characteristic of wild-type cells. As with budding yeast cells, the presence of cytoplasmic actin cables is not required for polarized growth. *act1-4* mutants grown at a semipermissive temperature form normal projections, although no cables can be detected. On the other hand, for three different mutant alleles examined, the severity of the morphogenesis defect correlated very well with the degree of inability to assemble a polarized cortical actin cytoskeleton (Figure 6). For each mutant allele, a population of cells could be identified that had a well-developed projection but had a moderately polarized or delocalized cortical actin cytoskeleton. One possibility is that the cortical actin structures were polarized during projection formation but reorganized before fixation. Another possibility is that, although cortical actin structures are found over the entire cell surface, only those structures at the projection tip are functional. Because actin function is required for projection formation, but cytoplasmic actin cables are clearly not required, it seems very likely that the only other known structure, the actin structures found at the cell cortex, plays the essential role. This conclusion is supported by the strong correlation between cortical actin organization and morphogenesis capability. It may be that the functions of the actin cables and the cortical structures are somewhat overlapping so that patch localization is most important in the absence of cables.

By looking in detail at the small population of actin mutants that formed projections on treatment with pheromone, we could evaluate the importance of actin for establishing aspects of cellular asymmetry other than apical surface growth. As shown in Figure 7, actin mutants are partially defective in localization of the nucleus in the projection and in localization of Spa2p to projection tips. One possible reason for the defect in nuclear localization in actin mutants is that microtubules fail to make a connection with the projection tip. In mitotic cells, nuclear division proceeds in the absence of bud formation (Adams *et al.*, 1990), but the positioning of the nucleus for division appears to depend on interactions of extranuclear microtubules with the cortex (Huffaker *et al.*, 1988). In wild-type cells, the projection tip contains a high density of cortical actin structures. An effect of actin organization on nuclear localization and orientation would establish a hierarchy in which actin must first be organized at the cortex for microtubules to establish contacts at the cell surface. Actin organization is normal in mating projections of cells lacking microtubules (Hasek *et al.*, 1987), indicating that microtubule function is not required for spatial control of actin assembly. It is presently not possible to eliminate the possibility that the defect in nuclear localization in actin mutants is an indirect effect caused by a general lack of organization of proteins at the projection tip, which interferes with the ability of microtubules to establish contacts at the cortex.

Like actin mutants, *spa2* mutants are defective in the morphogenesis of mating projections (Gehrung and Snyder, 1990). It not possible to compare directly the degree of dependence of morphogenesis on Spa2p versus actin because different temperatures and time periods were employed in each study. Furthermore, for Spa2p, it was possible to evaluate projection formation using a null allele completely lacking Spa2p activity. However, because actin is an essential protein, conditional-lethal *act1* alleles must be studied. These might retain partial actin function even at the nonpermissive temperature. For mitotic yeast cells, Snyder *et al.* (1991) concluded that the localization of Spa2p and actin are independent of each other because each can be organized in mitotic cells defective in the function of the other. During mating projection formation, we observed

Figure 6. Classification of morphological responses and cytoskeleton organization in α -factor-treated wild-type cells and actin mutants. Cells are classified according to shape and degree of actin polarization. Cell shape classifications are as follows. Standard size projection: Cells with lengths > 1.5 times the cell width; includes the majority of wild-type cells that respond to pheromone at 33° or 35°C. Moderate size projection: Includes cells having a length of up to 1.5 times the cell width. Cells with very small projections, which belong in this category, were probably undercounted. Round/oval: Includes cells with normal unbudded cell shape and enlarged cells without detectable mating projections. Other: Includes budded cells and rare cells with abnormalities, such as multiple nuclei. Actin polarity classifications are as follows. Highly polarized: >80% of cortical actin patches found concentrated on less than one-fourth of the cell surface; for cells with projections, this class includes those cells exhibiting a response very similar to wild-type. Moderate polarization: Intermediate level of actin polarization; >80% of patches are on the surface of less than one-half of cell, but on more than one-fourth of the cell. Delocalized: Lack of polarization of actin structures; distribution nearly random in appearance. Other: Includes budded cells and cells with rare abnormalities, such as multiple nuclei. All numbers are percentages. The area of each filled region represents the percentage of cells in that class. For each strain at each temperature, ≥ 100 cells were counted.

Figure 7. Nuclear location and Spa2p organization in actin mutants and wild-type cells treated with pheromone for 3 h at 33°C or 35°C. The photographs in the top left show, from top to bottom, Spa2p staining, phase optics, and DAPI staining of DNA in the *act1-4* strain. Note that four of the five cells shown in this field have a region of Spa2p staining at the projection tip. In the bar graphs, the parameters of nuclear location (see Figure 4 legend) and Spa2p staining position were scored. Spa2p staining was scored as occurring at the tip of the projection, as a spot elsewhere in the cell, or as diffuse staining and/or lack of staining. Only cells that had a characteristic mating projection were scored (see text). At least 100 cells were scored for each strain except the *act1-2* strain, in which case only 41 cells were scored because cells with an obvious projection morphology were very rare for this strain.

Figure 8. Projection formation in an *abp1* null mutant. Cells were incubated in 5 $\mu\text{g}/\text{ml}$ α -factor for 3 h at 30°C. Phase optics and anti-actin immunofluorescence microscopy of *abp1* null mutants (a and b) and wild-type cells (c and d). Bar is 5 μm .

a reduction in normal Spa2p localization to projection tips in actin mutants (Figure 7). The differences in the findings reported here compared with those reported by Gehring and Snyder suggest either that actin function contributes to the proper localization of Spa2p during projection formation but not during bud formation or that differences in how the experiments were performed in each study lead to different results. One difference between our study and that of Gehring and

Snyder is that we employed a preshift period at the nonpermissive temperature before beginning to monitor morphogenesis and protein localization. This preshift period was required for the observance of morphogenesis defects (discussed above).

Finally, our findings suggest that actin-binding proteins, through their influence on actin assembly, are likely to play very important roles in controlling the pattern of cell growth during projection formation. However, we found that cells completely lacking the actin-binding protein Abp1p are unimpaired in the ability to form mating projections. This implies that either the function of Abp1p is nonessential for projection formation or that there are proteins in yeast that are functionally redundant with Abp1p. No function of Abp1p has been demonstrated in either mitotically growing yeast (Drubin *et al.*, 1990) or yeast treated with mating factor (this study). The assays presented in this paper will be valuable for testing the roles of other cytoskeletal proteins in pheromone-induced cellular morphogenesis.

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