The life cycle of actin patches in mating yeast

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SUMMARY

Actin patches are core components of the yeast actin cytoskeleton that undergo redistribution during establishment of cell polarity. Using 4D imaging, we observe the life cycle of actin patches in living yeast for the first time. We observe assembly of actin patches at sites of polarized growth, and disassembly of actin patches concomitant with movement away from those sites. The total lifetime of an actin patch is 10.9±4.2 seconds. These findings indicate that actin patches are labile structures, and that the localization of actin patches during establishment of cell polarity occurs by assembly of these structures at sites of polarized cell surface growth. These findings were confirmed and extended by analysis of myosin I proteins and their receptor, verprolin, proteins implicated in actin assembly in yeast. Deletion of type I myosins or their receptor has no effect on the velocity of actin patch movement. However, these mutants show a 65% reduction in number of patch movements and a three-fold increase in patch lifetime. Finally, the actin patch resident proteins Abp1p, fimbrin, and Arp2p show normal association with actin patches in myosin I and verprolin mutants. However, cofilin accumulates in abnormal 'bars' of G-actin in *myo3Δ,myo5Δ* and *vrp1Δ* strains, and Las17p/Bee1p is not associated with actin patches in *vrp1Δ* strains. These findings imply a multi-step process for actin patch assembly. Early events in this process, including assembly of Abp1p, fimbrin and Arp2p with F-actin, can occur throughout the cell and do not require myosin I proteins or their receptor. Later events in this process are myosin I-dependent, and are required for assembly of actin patches at sites of polarized cell surface growth.

Movies available on-line:
http://www.biologists.com/JCS/movies/jcs1990.html

Key words: Polarity, Motor molecule, Cytoskeleton, Actin binding protein, Mating

INTRODUCTION

Polarization of yeast cells occurs at two points during their life cycle: budding and mating. During the mitotic cell cycle, intrinsic factors mark the presumptive bud site. Formation and enlargement of a bud at this site occurs by polarization of the cytoskeleton and all transport processes toward the selected bud site. During mating, an axis of polarization is established in response to extrinsic signals, namely mating pheromone. Binding of pheromone (**a**- or α-factor) to its receptor activates a heterotrimeric G-protein, which in turn triggers a MAP kinase signal transduction pathway (Kurjan, 1992; Kurjan, 1993; Levin and Errede, 1995). Activation of this signaling cascade induces (1) arrest of cells in the G_1 phase of the cell cycle; (2) expression of mating-specific genes; (3) polarization of the actin cytoskeleton; (4) preparation of cell and nuclear fusion machinery; and (5) mating projection formation (Marsh et al., 1991; Sprague and Thorner, 1992).

Polarization of actin cables and actin patches, the two major components of the yeast actin cytoskeleton, is critical for establishment of cell polarity in mating and budding yeast. Actin cables are bundles of actin that align along the axis of polarization. Actin patches are cortical membrane zones invested with F-actin and a host of actin-binding and regulatory proteins including capping proteins (Cap1p and Cap2p), fimbrin (Sac6p), cofilin (Cof1p), coronin (Crn1p), protein kinases (Ark1p and Prk1p), myosin I proteins (Myo3p and Myo5p), a myosin I-receptor (Vrp1p), actin nucleation proteins (the Arp2/3 complex), and actin nucleation regulators (Las17p/Bee1p; for review see Pruyne and Bretscher, 2000). Actin patches are enriched at sites of polarized cell surface growth, that is, in the bud and at the site of cell-cell separation in budding yeast, and in the mating projection of mating yeast.

While it is clear that actin patches must be polarized for establishment of cell polarity in yeast, the mechanism underlying this process is not well understood. There are at least two models for the mechanism of actin patch polarization in yeast. Since actin patches are highly motile structures that move at rates of 490±300 nm/second (Doyle and Botstein, 1996; Waddle et al., 1996), it is possible that actin patches move to sites of polarized cell surface growth. Actin patches are also known to be highly dynamic structures: treatment with latrunculin-A, an agent that binds to G-actin and inhibits actin polymerization, results in loss of patches within 5 minutes of treatment (Ayscough et al., 1997). Therefore it is also possible that actin patch assembly occurs at polarization sites. Alternatively, actin polymerization may be required for patch movement to or retention at those sites.

To distinguish between these models, we studied actin patch movement in pheromone-treated yeast, and examined the role of proteins implicated in actin patch assembly in this process. The type I myosins of *Saccharomyces cerevisiae* are encoded by two homologous, functionally redundant genes, *MYO3* and

Table 1. Yeast strains used in this study

Strain name	Genotype	Reference
HA10-1c	MAT**a**, *ade2-1, his3-11, leu2-3,112, ura 3-1, trp1-1, can1-100*	Goodson et al., 1996
HA31-9c	MAT**a**, *ade2-1, his3-11, leu2-3,112, ura3-1, trp1-1, myo3Δ::HIS3, myo5Δ::TRP1, can1-100*	Goodson et al., 1996
VHA-9c	MAT**a**, *ade2-1, his3-11, leu2-3,112, ura3-1, can1-100, trp1-1, VRP1::VRP1-HA, myo3Δ::HIS3, myo5Δ::TRP1*	This study
VHA-1	MAT**a**, *ade2-1, his3-11, leu2-3,112, ura3-1, can1-100, trp1-1, VRP1::VRP1-HA, myo3Δ::HIS3, myo5Δ::TRP1 bearing the plasmid pMYO5-myc*	Anderson et al., 1998
SC467	MAT**a**/MATα *leu2-3,112/leu2-3,112 ade1/ade1 ura3-52/ura3-52 ile-/ile- MEL1/MEL1*	Vaduva et al., 1997
SC467-1b	MAT**a**, *leu2-3,112, ade1, ura3-52, ile-, MEL1 (spore from tetrad dissection of SC467)*	This study
GVY1	MAT**a**/MATα, *leu2-3,112/leu2-3,112, ade1/ade1 ura3-52/ura3-52, vrp1Δ::LEU2/vrp1Δ:LEU2, MEL1/MEL1, ile-/ile-,*	Vaduva et al., 1997
GVY1-1d	MAT**a**, *leu2-3,112, ade1, ura3-52, vrp1Δ::LEU2, MEL1, ile- (spore from tetrad dissection of GVY1)*	This study

MYO5. Myo5p assembles into punctate, multi-protein structures that are enriched at sites of polarized cell surface growth and co-localize with a subset of actin patches (Goodson et al., 1996). Deletion of both genes (*myo3Δ,myo5Δ*) results in loss of actin cable and patch polarity, and defects in actin-dependent processes including cell wall deposition, secretion and endocytosis (Geli and Riezman, 1996; Goodson et al., 1996). Finally, these proteins interact with the Arp 2/3 complex and an Arp2/3 complex positive regulator, the WASP homologue Las17p/Bee1p. Arp2/3 complex and Las17p/Bee1p localize to actin patches, and are required for actin nucleation and assembly in permeabilized yeast (Lecher and Li, 1997; Evangelista et al., 2000; Lechler et al., 2000).

Localization of Myo5p is dependent upon verprolin (Vrp1p), a proline-rich protein required for actin cytoskeletal polarization (Donnelly et al., 1993). Myo5p patches co-localize with Vrp1p and are depolarized in a *vrp1Δ* mutant. Consistent with this, the SH3 domain of the Myo5p tail interacts with Vrp1p in two-hybrid assays and is required for polarization of Myo5p patches. Finally, Vrp1p co-immunoprecipitates with Myo5p (Anderson et al., 1998). These findings, together with the observation that Vrp1p polarization occurs even in the absence of F-actin (Vaduva et al., 1997), supports the model that Vrp1p is a myosin I receptor (Anderson et al., 1998). Our studies indicate that (1) polarization of actin patches occurs by assembly of these structures at sites of polarized cell surface growth, and (2) type I myosin proteins and verprolin are required for late stages of this assembly process at polarization sites.

MATERIALS AND METHODS

Yeast manipulations and plasmids used in this study

Yeast strains used in this study are listed in Table 1. Yeast manipulations, including tetrad dissection, transformations, and cell culture conditions, were carried out as described (Guthrie and Fink, 1991). The p*MYO5*-myc plasmid used was described in our previous work (Goodson et al., 1996). pTD40 is a plasmid that contains *ABP1* fused to the gene encoding green fluorescent protein (p*ABP1*-GFP; Doyle and Botstein, 1996). The p*BEE1*-myc plasmid was constructed by inserting the 3.0-kb *Pvu*II fragment of pTL6 (Lechler and Li, 1997) into the *Pvu*II site of pRS426.

The COOH terminus of Sac6p was tagged with GFP (S65T), using PCR-based insertion into the chromosomal copy of *ARC15* (Longtine et al., 1998). PCR fragments for integration by homologous recombination were first amplified from plasmids pFA6a-13Myc-*TRP1* and pFA6a-GFP(S65T)-kanMX6, with forward primer, 5′AATTATTACTTTTATCGCTTCGTTAATGACTTTGAACAAACG-GATCCCCGGGTTAATTAA3′ and reverse primer, 5′CTGAGTAGAA-AACAGGTTACGAAAGTTGTTTGTTGGCTCAGAATTCGAGCTC-GTTTAAAC3′. The GFP-tagged yeast strain was characterized for correct integration of the tagging cassette at the *SAC6* locus using PCR and western blot analysis.

Previous studies showed that addition of GFP to the C-terminus of Sac6p has no effect on function, and that the Sac6p-GFP fusion protein labels all actin patches within cells (Doyle and Botstein, 1996). The Sac6p-GFP produced for these studies showed the same properties: it labels all actin patches and has no obvious effect on yeast growth rates, actin organization or actin function when expressed at wild-type levels in lieu of wild-type *SAC6*.

Fixation and immunofluorescence

The fixation and immunofluorescence methods used are variations of the methods described previously (Pringle et al., 1991). Indirect immunofluorescence labeling of Myo5p was carried out as described previously (Goodson et al., 1996). Epitope-tagged Vrp1p and Las17p/Bee1p were detected as described (Anderson et al., 1998; Li, 1997, respectively). Actin-binding proteins (Abp1p, Sac6p and Cof1p) were visualized using rabbit polyclonal antibodies (Ayscough et al., 1997) and FITC-coupled goat anti-rabbit antibodies (Kirkegaard and Perry Laboratories, Gaithersburg, MD). The actin cytoskeleton was visualized using either Alexa Fluor™ 594 phalloidin (Molecular Probes, Eugene, OR) or a mouse monoclonal antibody (C4D6) raised against actin. Visualization of cofilin, Arp2p and actin by indirect immunofluorescence required the cold methanol/acetone step described by Pringle et al. (Pringle et al., 1991). Stained spheroplasts were mounted on microscope slides using mounting solution (1 μg/ml *p*-phenylenediamine, 90% (w/v) glycerol, and 1× PBS) containing the DNA-binding dye 4′,6-diamidino-2-phenylindole (DAPI; Williamson and Fennell, 1975) at a final concentration of 1 μg/ml. Coverslips were then sealed onto glass microscope slides with clear nail polish. Slides were stored at –20°C in the dark prior to viewing.

Microscopy

A growth chamber was prepared using a modification of methods described previously (Koning et al., 1993). The middle well of a 3-well Teflon-coated microscope slide was filled with synthetic complete media without uracil, containing 4× adenine (8 mg/ml; SC-Ura + 4× Ade), 3% (w/v) high melting point agarose (Gibco BRL, Grand Island, NY), and α-factor (10 μM, Research Genetics, Huntsville, AL). A coverslip was placed over the filled well, and uniform force was applied to the coverslip to compress the agar into the well. The coverslip and excess agar were removed from the well. Cells expressing pTD40 were grown in SC-Ura + 4× Ade to a concentration of 5×10^6 cells/ml and then incubated with α-factor (10 μM) at 25°C for 1 hour. Cells were then collected by centrifugation and resuspended in SC-Ura + 4× Ade + 10 μM α-factor to a concentration of 2×10^8 cells/ml. 6×10^5 cells were spread across the surface of the solidified agar and the chamber was covered with a coverslip.

Cells were viewed with a Zeiss Axioplan II microscope (Oberkochen, Germany) using a Plan-Apochromat 100×, 1.4 NA objective lens. Images were acquired using a Hamamatsu Orca 1 cooled CCD camera

(Bridgewater, NJ). Light output from the 100 W mercury arc lamp was controlled using a Uniblitz D122 shutter driver (Vincent Associates, Rochester, NY). Image enhancement and analysis were performed on a Macintosh PowerMac 9600 computer (Cupertino, CA) and a Compaq Presario 5690 Computer (Houston, TX) using IPLab 3.0 (Scanalytics, Fairfax, VA), Adobe Photoshop 4.1 and Premiere 4.21 (Adobe Systems Inc., San Jose, CA) and NIH Image 1.61.

Velocity measurements of Abp1p-GFP labeled actin patches were performed by determining the change in position of each patch in the plane of focus as a function of time. To do so, 100 images of pheromone-treated cells expressing Abp1p-GFP were acquired in a single focal plane using 445 millisecond exposures and no pause between exposures. Only those patches that persisted in the plane of focus and moved in a linear fashion for ≥3 consecutive frames were considered for measurements.

The number of Abp1p-GFP labeled actin patches per cell, and fluorescence output from Alexa-phalloidin labeled F-actin, were determined using 3D reconstructions of living or fixed and stained yeast. The direction of movement and life span of Abp1p-GFP labeled actin patches were determined using 4D imaging. Optical sectioning for 3D and 4D imaging was carried out using a piezoelectric focus motor mounted on the objective lens of the microscope (Polytech PI, Auburn, MA). Each z-series was subjected to digital deconvolution using Inovision (Raleigh, NC) software on a Silicon Graphics O_2 computer (MountainView, CA).

For 3D reconstruction of fixed cells, optical sections were obtained at 300 nm z-intervals through the entire cell with an exposure time of 500 milliseconds per section. For 4D imaging, we obtained five 500 nm steps with exposure times of 100 milliseconds/frame. Using these parameters, we obtained 60 consecutive z-series in 65 seconds, or 1.08 seconds per z-series. These cells were imaged from the top down, with the first of the z-axis steps (z-depth 0) above the cell and therefore not used for further examination. The second through fifth steps (z-depths 1-4) sectioned cells from the plasma membrane to approximately the center of the cell.

To determine Abp1p-GFP labeled actin patch life span, we examined only those patches that appeared and disappeared in the third or fourth z-step (z-depths 2-3). As a result we were always able to exclude the possibility of z-axis movements by analyzing adjacent z-steps. In order for a patch to traverse two different z-steps, it would need to be moving at a velocity greater than 500 nm/0.2 second or 2.5 μm/second, which is 5-6 times faster than has been measured.

RESULTS

The assembly, disassembly and polarization of actin patches in polarized yeast

Abp1p is an actin binding protein with some sequence similarity to cofilin and is found exclusively in actin patches in vegetative yeast (Drubin et al., 1988). Expression of the fluorescent protein Abp1p-GFP in yeast has no obvious effect on cell growth, mating efficiency, or actin cytoskeletal organization and has been used as a marker for actin patch movements previously (Doyle and Botstein, 1996; Belmont and Drubin, 1998). To assess actin patch motility, cells expressing Abp1p-GFP were incubated for 1 hour in α-factor and visualized at various stages of shmoo formation. Under these incubation conditions, it is possible to detect cells at 4 stages of shmoo formation: (1) non-polarized G_1-arrested cells; (2) cells bearing an asymmetric distribution of actin patches, but no obvious change in morphology; (3) cells with small mating projections; and (4) cells with fully formed mating projections. Abp1p-GFP-labeled actin patches localize to the shmoo cortex and co-localize with actin patches at all stages of shmoo formation (data not shown).

4D imaging (time-lapse imaging combined with 3D reconstruction) was used to examine the life events of actin patches in wild-type yeast shmoos expressing Abp1p-GFP. An example of this analysis is illustrated in Fig. 1A (and see movie). The appearance of an Abp1p-GFP labeled actin patch within the mating projection is noted by the arrow in a. The fluorescence intensity of the patch increases over the next 4

Table 2. Abp1p-GFP patch velocities and directionality in wild-type cells during establishment and maintenance of cell polarity

	Velocity ± SE (nm/sec)	*n*	Direction (% Total)			# of Movements (patches/section)
			Anterograde	Retrograde	Lateral	
Stage 1	419.76 ± 11.73	202	n/a	n/a	n/a	3.7
Stage 2	395.81 ± 23.26	76	28.9	57.9	13.2	3.3
Stage 3	321.50 ± 13.13	89	29.2	61.8	9.0	4.7
Stage 4	373.79 ± 17.36	124	29.8	60.5	9.7	4.4

Wild-type cells expressing pABP1-GFP were pheromone treated, and patch movements were determined by time-lapse fluorescence imaging as described in Materials and Methods. Cells were visualized at various stages of mating projection formation. Illustrations indicate representative cell shape and organization of actin structures. Velocities of Abp1p-GFP patches were determined for patches that moved in a linear manner and remained in the plane of focus for 3 or more consecutive frames. Standard error (SE) was determined by dividing the standard deviation by the square root of the sample size (*n*). Directionality was determined in polarized cells by assessing the net direction of movement of each Abp1p-GFP patch that showed linear movement.

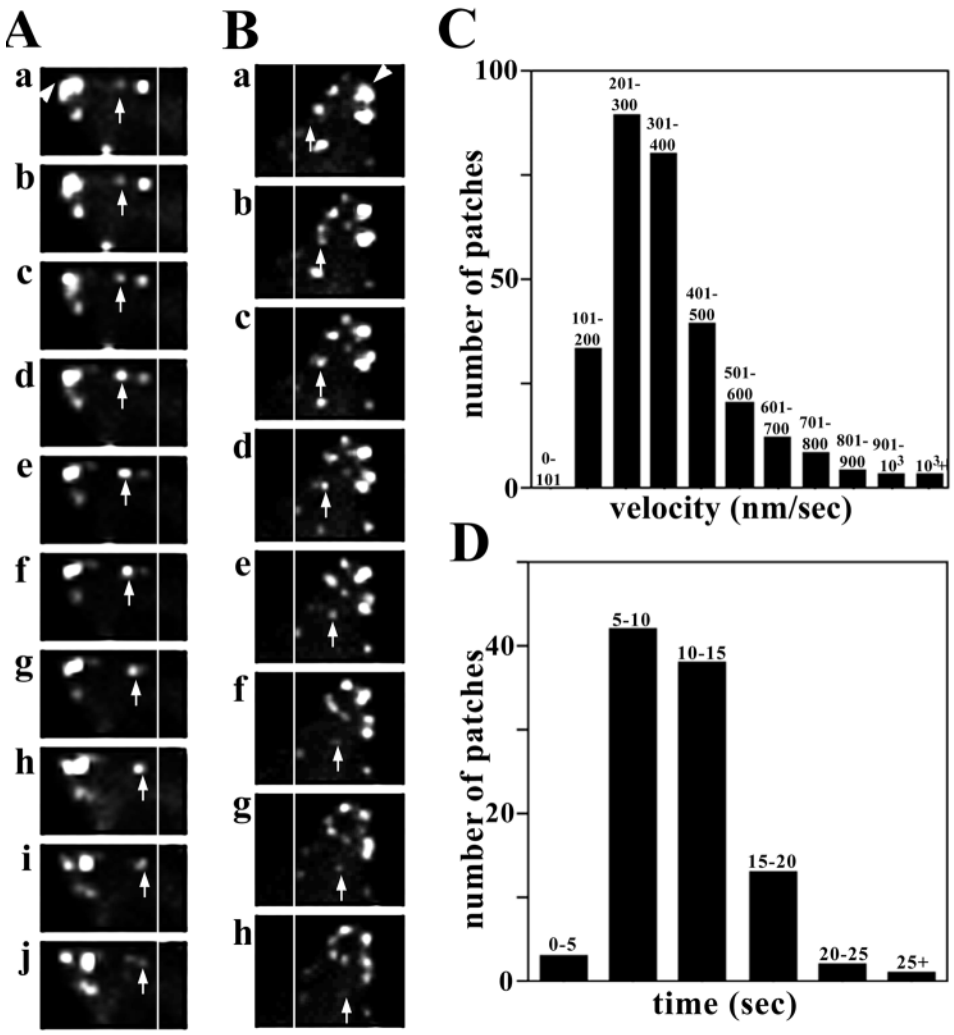

Fig. 1. Abp1p-GFP patch assembly, movements and disassembly in wild-type, pheromone-treated cells. Wild-type cells expressing pAbp1p-GFP were pheromone-treated and optically sectioned into 5 images with a step size of 500 nm. Sectioning was repeated until 60 z-series were obtained. Each image was acquired with a 100 millisecond exposure. Each z-series was deconvolved and rendered into a single two-dimensional projection. (A) The figure shows still frames from a 4D movie at 1 second intervals. The arrowhead points to the mating projection tip. The arrow points to a single patch through a cycle of assembly within the mating projection tip (a-e) and disassembly concomitant with retrograde movement (h-i). Bar, 0.25 μm. (B) The figure shows consecutive still frames from 4D imaging at 1 second intervals. The arrow points to another example of assembly within the mating projection tip (a-c) and disassembly concomitant with retrograde movement (e-h). The arrowhead points to the mating projection tip. Bar, 0.25 μm. (C) The frequency distribution of actin patch movement velocities. Velocities were measured in pheromone-treated yeast, as described in Materials and Methods. (D) The frequency distribution of actin patch lifespan. The total lifespan of actin patches, from assembly to disassembly, was determined in pheromone-treated yeast as described in Materials and Methods.

frames (b-e) and remains constant for 2 frames (f-g). Since 2D projections include optical sections above and below the plane of focus of each patch evaluated, and patches that might have moved into or out of the examined z-planes were excluded from analysis, it is clear that changes in fluorescence intensity of Abp1p-GFP labeled actin patches are due to assembly and disassembly of Abp1p, and not to movement in the z-axis.

Movement and disassembly of an actin patch is shown in Fig. 1A, g-i. Movement of this actin patch is linear and directed away from the site of polarized cell surface growth. The average velocity of patch movement in shmooing yeast is 386.6±176.9 nm/second (Table 2), a velocity similar to that described for actin patch movements in vegetative yeast (Doyle and Botstein, 1996; Waddle et al., 1996; Barral et al., 2000). The majority of Abp1p-GFP-labeled patch movements were between 200 and 400 nm/second although some faster movements were observed (Fig. 1C).

Evaluation of 100 patches in wild-type cells revealed that Abp1p-GFP-labeled patches persist for 10.9±4.2 second. The distribution of individual patch lifespans suggest that all actin patches are short-lived and the persistence of patches beyond 20 seconds is a rare event (Fig. 1D). This patch lifespan is consistent with a previous report in which F-actin turnover in cortical patches occurs within 10 seconds upon treatment with LAT-A (Karpova et al., 1998a).

The pattern of actin patch movement in polarized yeast

Time-lapse imaging revealed that most Abp1p-GFP-labeled actin patches move in a non-linear fashion. These non-linear movements are oscillatory and do not result in a net change in position over the interval studied. In contrast, some Abp1p-GFP-labeled actin patches display linear movement (Fig. 1B). Patch directionality was assessed only for those patches that remained in the plane of focus and moved in a linear fashion for 3 or more consecutive frames. Patch movements with a net displacement toward or away from the shmoo tip were defined as anterograde or retrograde, respectively. Movements perpendicular to the long axis of the shmoo were defined as lateral movements (Table 2).

We find that the majority of the movements detected are retrograde: 60.2% of the Abp1p-GFP-labeled actin patches moved in a retrograde fashion (Table 2). Similarly, 69.3% of Sac6p-GFP-labeled actin patch movements were in the retrograde direction (data not shown). Therefore, using two independent in vivo actin patch markers, we find that approximately two-thirds of actin patch movements are directed away from sites of polarization. 29.4% of patch movements

were in the anterograde direction. The remaining patches (10.4%) showed lateral movement. Next, we measured the distances that Abp1p-GFP-labeled actin patches traveled in each of these three directions. Patches displayed a net displacement of 764.7±420.0 nm in the anterograde direction as compared to 616.2±286.9 and 692.9±331.1 nm in the retrograde and lateral directions, respectively. Finally, we observe a decrease in the intensity of Abp1p-GFP fluorescence in the actin patch concomitant with its movement away from the mating projection. In summary, anterograde, retrograde and lateral movements all show the same average displacement, but the majority (60-70%) of actin patch movements are retrograde. These findings are consistent with the model that actin patch polarity is established, not by movement of actin patches to polarity sites, but by assembly of actin patches at those sites.

To confirm that these movements are true indicators of actin patch life events that are not artifacts of the use of Abp1p-GFP for visualization, we repeated this analysis using a Sac6-GFP fusion protein. Sac6, or yeast fimbrin, localizes to both actin patches and actin cables and has been used previously to label actin patches (Adams et al., 1989; Doyle and Botstein, 1996). Sac6p-GFP-labeled actin patches display the same pattern of assembly in sites of polarized cell surface growth, and disassembly concomitant with movement away from those sites. Moreover, the life span of Sac6p-GFP labeled actin patches, 11.2±2.8 seconds, is similar to that of Abp1p-GFP labeled actin patches. Since more patches are leaving than entering the shmoo tip, new patches must be formed there in order to maintain the asymmetric steady-state distribution that is observed. These data suggest that (1) actin patches turn over at a high rate, (2) actin patch assembly occurs at the site of polarized cell surface growth, and (3) actin patch disassembly occurs concomitant with their movement away from the polarization site. Moreover, our data support a model in which polarization of actin patches occurs by assembly at sites of polarized growth, and not by movement of pre-existing actin patches to those sites (Table 2).

Deletion of myosin I proteins or verprolin produces partial defects in actin patch polarization

Myosin I proteins and their receptor, verprolin, are required for polarization of the actin cytoskeleton in budding yeast. We find that these proteins are also required for polarization of the actin cytoskeleton in mating yeast. In wild-type cells, actin patches accumulate in the shmoo tip (Fig. 2A and C, arrows), while actin cables align along the axis of polarization (Fig. 2A and C, arrowheads). This pattern of actin cytoskeletal polarization is compromised in myosin I and verprolin mutants. Here, actin is defined as polarized if ≤10% of the total cellular actin patches are localized outside the mating projection. Using this criterion, 62.4% of pheromone-treated wild-type cells show actin polarization. In contrast, actin polarization in response to mating pheromone occurs in only 4.8% and 14.4% of verprolin and myosin I mutants, respectively. Approximately two-thirds of *myo3Δ,myo5Δ* and *vrp1Δ* mutants show completely depolarized actin, i.e., uniform distribution of actin patches throughout the cell. The remaining 20% show partial actin polarization (Fig. 2B and D). Regulation of actin cable polarization appears to be governed by an alternative mechanism since *myo3Δ,myo5Δ* and *vrp1Δ* mutants do not show defects in actin cable orientation. The observed defects in actin patch polarity are not due to slow growth or poor physiology of these cells, since depolarization of the actin cytoskeleton is a phenotype specific to mutations associated with the actin cytoskeleton (Karpova et al., 1998b; Fig. 2).

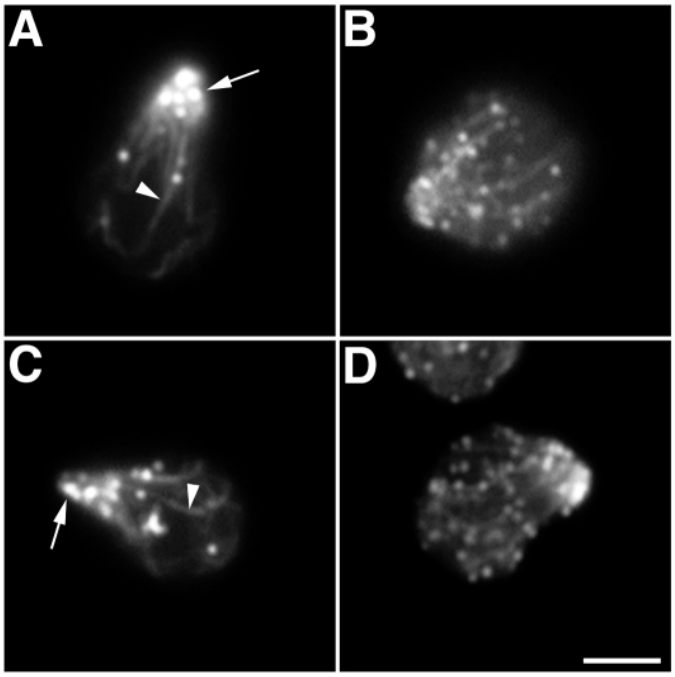

Fig. 2. Actin is disorganized in pheromone-treated *myo3Δ,myo5Δ* and *vrp1Δ* cells. Mid-log phase yeast were pheromone-treated, fixed and stained for actin using Alexa-phalloidin as described in Materials and Methods. (A) *MYO3,MYO5* (HA10-1c) cells; (B) *myo3Δ,myo5Δ* (HA31-9c) cells; (C) *VRP1* (SC467-1b) cells; and D: *vrp1Δ* (GVY1-1d) Arrows indicate actin patches; arrowheads indicate actin cables. Bar, 3 μm.

Actin patch composition is defective in myosin I and verprolin mutants

Our data support a role for myosin I proteins and their receptor in actin patch polarization. In light of our finding that actin patch polarization occurs largely by assembly at sites of polarized cell growth, we examined the role of myosin I proteins and verprolin in assembly of actin patches. To do so, we studied the effect of mutation of these proteins on association of proteins with actin patches. Specifically, we studied localization of Abp1p; a subunit of the Arp2/3 complex (Arp2p); an Arp2/3 complex-activating protein (Las17p/Bee1p); cofilin (Cof1p); and fimbrin (Sac6p). Abp1p, cofilin, Arp2p, and Las17/Bee1p localize exclusively to actin patches in vegetative and mating yeast (Drubin et al., 1988; Moon et al., 1993; Moreau et al., 1996; Winter et al., 1997; Li, 1997; Lechler and Li, 1997). In contrast, fimbrin is found within both actin patches and actin cables (Adams et al., 1989).

In contrast, we find that myosin I proteins and their receptor are required for assembly of cofilin into actin patches. In wild-type shmoos, we detect two types of cofilin-containing punctate structures: (1) brightly stained structures that co-localize with actin patches in 90% of shmoos examined, and (2) weakly stained punctate structures that are distributed throughout the cytoplasm (Fig. 3A and B, E and F). Deletion of both myosin I genes alters this pattern of cofilin localization. *myo3Δ,myo5Δ* cells contain weakly stained cofilin-containing

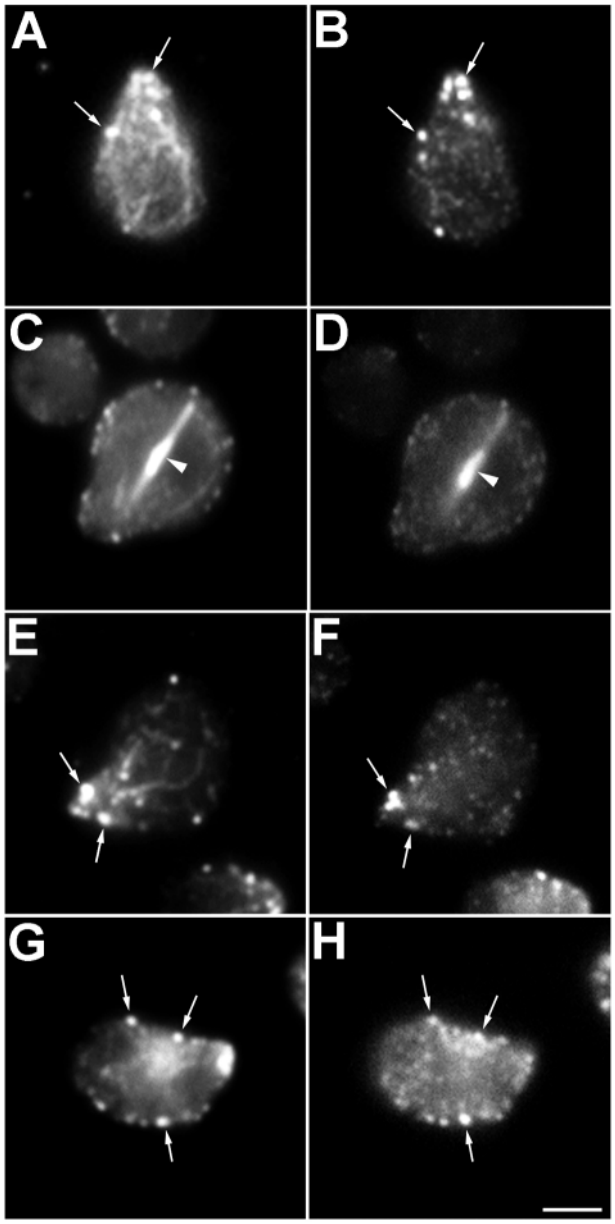

Fig. 3. Cofilin is mislocalized in *myo3Δ,myo5Δ* and *vrp1Δ* cells. Cells were incubated in the presence of mating pheromone for 2.5 hours and fixed with paraformaldehyde, converted to spheroplasts and labeled using indirect immunofluorescence. Indirect immunofluorescence of individual actin patch components was compared to actin fluorescence. Actin (A,C,E,G) and Cof1p (B,D,F,H) were localized using monoclonal and polyclonal antibodies, respectively. Wild-type control cells (HA10-1c; A and B) and SC467-1b (E and F) display co-localization of Cof1p with actin patches. *myo3Δ,myo5Δ* cells (HA31-9c; C and D) show mislocalization of cofilin into bar-like structures that contain G-actin. *vrp1Δ* (GVY1-1d; G and H) show co-localization of some cofilin with depolarized actin patches and diffuse cofilin staining throughout the cytoplasm. Arrows indicate regions of co-localization between cofilin patches and actin patches. Arrowheads indicate G-actin- and cofilin-rich bars. Bar, 2 μm.

particles; some of these particles align with weakly stained actin-containing particles. Strongly stained cofilin particles are not detected in myosin I mutants. Instead, 35% of the shmoos examined contain abnormal cofilin-containing bar structures (Fig. 3). These cofilin bars co-localize with bars of G-actin that are detected with anti-actin antibodies, but not with fluorochrome-coupled phalloidin.

Cofilin localization is also defective in *vrp1Δ* shmoos. Like wild-type cells, *vrp1Δ* cells contain brightly stained cofilin-containing particles that co-localize with actin patches, and weakly stained cofilin particles. In addition, all *vrp1Δ* shmoos examined showed diffuse cofilin staining throughout the cytoplasm. Finally, 13% of *vrp1Δ* shmoos show abnormal cofilin- and G-actin-containing bar structures (Fig. 3).

There is a precedent for G-actin- and cofilin-containing bars in yeast (Novick and Botstein, 1985; Drubin et al., 1993; Winter et al., 1999). Work from other laboratories indicates that (1) overexpression of the actin patch-associated protein kinases Prk1p and Ark1p results in the accumulation of G-actin bars; and (2) deletion of these kinases results in the accumulation of enlarged actin clumps that contain multiple actin patch components (Cope et al., 1999; Zeng and Cai, 1999). Consistent with this, Winter et al. found that cofilin localizes to bar-like structures in cells deleted for Arp2p, a subunit of the Arp2/3 complex (Winter et al., 1999). These findings, that mutations in proteins implicated in actin patch nucleation or assembly result in abnormal G-actin- and cofilin-containing bars, support the idea that these abnormal bar structures are indicative of actin patch assembly defects. The presence of similar structures in myosin I and verprolin mutants suggests that: (1) myosin I proteins are required for actin patch assembly; and (2) assembly of actin patches at sites of polarized cell surface growth is dependent upon verprolin binding and localization of myosin I proteins to these sites.

We find that Las17p/Bee1p, another protein implicated in patch assembly, is not associated with actin patches in verprolin mutants (data not shown). Previous studies show that Las17p/Bee1p localizes to actin patches, activates the actin nucleation activity of the Arp2/3 complex in vitro, and is required for actin patch integrity and actin assembly in permeabilized yeast (Li, 1997; Lechler and Li, 1997). In addition, recent 2-hybrid assays, co-immunoprecipitation, and in vitro binding data support direct interactions between the Arp2/3 complex, Las17p/Bee1p, and Myo5p (Evangelista et al., 2000; Lechler et al., 2000). We confirmed previous data showing that Las17p/Bee1p co-localizes with actin patches in wild-type cells. Further, we find that Las17p/Bee1p co-localizes with actin patches in myosin I double deletion cells. However, deletion of the myosin I protein receptor, verprolin, produces delocalization of Las17p/Bee1p to the cytosol in 36% of shmoos (Fig. 4).

Finally, we find that some proteins are recruited to actin patches in the absence of myosin I proteins. Abp1p, Arp2p, and fimbrin localize to actin patches in pheromone-treated wild-type cells, and to F-actin-containing punctate structures presumed to be actin patches in *myo3Δ,myo5Δ* mutants and *vrp1Δ* mutants (Fig. 4). Co-localization of these proteins with actin patches is not influenced by the polarization state of actin patches: co-localization is observed in pheromone-treated cells with polarized or depolarized actin patches (Fig. 4).

Actin patch number is defective in myosin I and verprolin mutants

Actin patches are more numerous in both *myo3Δ,myo5Δ* and *vrp1Δ* cells than in wild-type cells (Fig. 2; Table 3). Optical

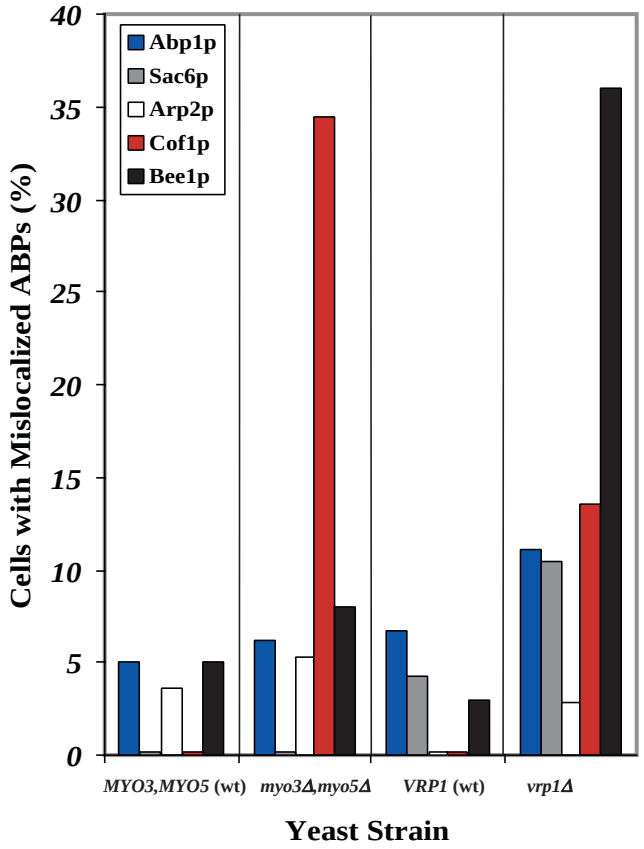

Fig. 4. Not all actin patch proteins are properly localized to actin patches in mutant pheromone-treated cells. Pheromone treatment and staining were carried out as described for Fig. 3. Localization of the actin binding proteins (ABPs) Abp1p, Sac6p, Arp2p, Las17p/Bee1p-myc and cofilin was determined by indirect immunofluorescence. Alexa-phalloidin was used to visualize F-actin. The number of cells with no actin binding protein co-localization with actin patches is represented graphically.

sectioning, digital deconvolution and 3D reconstruction of shmoos stained for F-actin reveals a 2-fold increase in the number of actin patches in *myo3Δ,myo5Δ* and *vrp1Δ* cells compared to wild-type cells. This increase in the number of actin patches is not accompanied by an increase in the amount of F-actin in these mutant cells. Measurements of the total Alexa-phalloidin fluorescence through the entire cell reveal that wild-type and mutant cells contain the same amount of F-actin. Thus, yeast myosin I and verprolin proteins suppress the formation of actin patches and increase the incorporation of actin into each patch. In light of our finding that myosin I proteins are required for association of some proteins with actin patches, it is possible that the actin patches detected in these mutants are intermediates in the actin patch assembly that accumulate due to a block in the assembly pathway.

Actin patch motility and stability are defective in myosin I and verprolin mutants

Finally, we studied the effect of myosin I or verprolin mutation on actin patch assembly, movement and disassembly. We observe assembly, movement, and disassembly of actin patches in myosin I and verprolin mutants. However, there are fundamental differences in actin patch behavior in *myo3Δ,myo5Δ* cells and *vrp1Δ* cells as compared to wild-type cells. First, we find that actin patches in myosin I or verprolin deletion mutants assemble throughout the cell, not primarily at sites of polarized cell surface growth. Second, we find that these mutations have no effect on the velocity of movement, but do produce a 65-75% decrease in the frequency of movement (Table 3). Finally, we analyzed the life span of actin patches in the mutant strains. We find that the average patch life span is 31.5±10.3 seconds in the *myo3Δ,myo5Δ* cells and 26.9±8.4 seconds in *vrp1Δ* shmoos (*n*=100 patches for each strain). Lifespans were obtained for patches located throughout the shmoo. No significant differences in patch duration were observed when comparing those patches within the shmoo tip to others located throughout the cell body (HA31-9c: 33.67±7.79 within shmoo tips (*n*=12) vs 31.27±10.54 (*n*=88) in cell body; GVY1-1d: 28.01±8.46 within shmoo tips (*n*=21) vs 26.55±8.35 in cell body (*n*=79)). These data suggest that yeast myosin I and verprolin proteins not only suppress the formation of actin patches, but more specifically, suppress the formation of actin patches outside the zone of polarized cell growth. Furthermore, these proteins reduce actin patch life spans and increase the frequency of actin patch movements.

DISCUSSION

Yeast cell polarity is initiated by the choice of a site on the cell surface towards which growth is directed. During vegetative growth, this site is determined by the site of a previous cell division; during mating, this site is determined by mating pheromone secreted by a potential mating partner. In both cases, the cytoskeleton and cellular transport events are polarized toward the selected bud site or shmoo tip.

We find that the myosin I protein Myo5p is present in punctate structures at polarization sites in mating and vegetative yeast. Myo5p particles co-localize with some, but not all, actin patches within these polarity sites. Since Myo5p mutations that mislocalize the protein also result in defects in actin organization, Myo5p localization is critical for its function in control of actin organization. Here, we studied actin

Table 3. Actin patch dynamics in wild-type cells and cells bearing a deletion in myosin I protein or verprolin

	MYO3,MYO5	*myo3Δ,myo5Δ*	*VRP1*	*vrp1Δ*
Actin patch number	15.4±2.4	30.4±4.4	14.9±1.9	29.6±3.1
No. of actin patch movements	4.13	1.46	3.31	0.86
F-actin content (whole cell)	10.4±2.7	13.6±2.7	9.4±3.1	12.3±2.4
Velocity (nm/second)	363.48±10.60	333.84±18.04	342.21±7.11	384.95±13.09
Life span	10.9±4.2	31.5±10.3	10.4±7.3	26.9±8.4

Mid-log phase yeast were pheromone-treated, fixed, stained, and visualized as described in Fig. 1. Actin patches were counted in projections obtained after deconvolution and compression of optical z-series. Whole cell actin fluorescence was measured by integrating the total Alexa-phalloidin fluorescence output from the same deconvolved z-steps of 3D images. All other measurements were made as described in Table 2. *n*=100 patches for all measurements.

patch assembly, movement and disassembly in living yeast for the first time, and investigated the role of myosin I proteins and their receptor, verprolin, in these processes.

The life events of actin patches during establishment and maintenance of cell polarity

Using Abp1p-GFP to label actin patches for 4-dimensional imaging, we observe four stages in the life of an actin patch: (1) assembly at the site of polarized cell surface growth, (2) retention at polarity sites, (3) movement away from the polarization site (retrograde movement), and (4) disassembly. Although actin patches are enriched within the mating projection, 70% of movements are directed away from that region. The average time from actin patch assembly to disassembly is 10.9±4.2 seconds (n=100). Similar results were obtained using Sac6p-GFP as a marker for actin patches.

These are the first studies to document actin patch assembly and disassembly in living yeast. In addition, they support a mechanism for polarization of actin patches in which actin patches are enriched at sites of polarization not by movement to those sites, but by assembly at these sites. Our findings are consistent with previous studies which have detected actin nucleation activity as well as proteins required for nucleation at sites of polarized growth (Li et al., 1995; Moreau et al., 1996; Winter et al., 1997; Mullins et al., 1998). Moreover, since Waddle et al. observed bidirectional movement of Cap2p-GFP-labeled actin patches in vegetative yeast, it is possible that this mechanism of actin patch assembly at polarization sites also occurs in other instances of polarity establishment in the yeast life cycle (Waddle et al., 1996).

Role of myosin I proteins in control of actin patch assembly

Phenotypic analysis of myosin I deletion mutants suggests a role for these proteins in actin patch assembly. We detect accumulation of abnormal actin patches in myosin I or verprolin deletion mutants. These structures resemble actin patches: that is, they contain F-actin and proteins ordinarily found in actin patches including Sac6p, Abp1p, and Arp2p. However, there are fundamental differences in the F-actin-containing patches in myosin I or verprolin mutants compared to those detected in wild-type cells. These abnormal actin patches have a lower F-actin content, lower frequency of motility, and longer lifespan compared to actin patches in wild-type cells. Moreover, assembly of these abnormal actin patches in myosin I or verprolin mutants is not restricted to the site of polarized cell surface growth. Finally, cofilin and Las17p/Bee1p, resident proteins in actin patches of wild-type cells, are mislocalized and do not associate normally with actin patches in these mutants.

There are many ways to interpret the abnormal actin-containing patches that accumulate in myosin I or verprolin deletion mutants. Our interpretation draws from the reasoning used to order events in pathways including those in central metabolism; that is, that a block in a step in the pathway would lead to accumulation of intermediates in the pathway that are produced upstream from the blocked site. We find that patches containing Abp1p, Sac6p and F-actin accumulate in myosin I deletion mutants. These patches are (1) more numerous than actin patches in wild-type cells, (2) contain lower F-actin content than actin patches in wild-type cells, and (3) do not contain other proteins found in wild-type actin patches. We interpreted these patches as intermediates in the actin patch assembly pathway that accumulate when later steps in the pathway, i.e. myosin I-mediated steps, are blocked.

Taken together, these studies suggest that actin patch assembly is a multi-step process consisting of myosin I-dependent and -independent events. Assembly of Sac6p, Abp1p, and Arp2p with F-actin into an actin patch assembly intermediate does not require myosin I proteins. In contrast, late stages of assembly, which include association of cofilin and Las17p/Bee1p with actin patches, are dependent upon myosin I proteins and their receptor. The early and late stages of actin patch assembly normally occur at sites of polarized cell surface growth, but can occur outside of polarization sites.

Our results are consistent with findings from two other groups. Evangelista et al. find that (1) the acidic tail region of myosin I protein interacts with the yeast homologue of the Wiskcott-Aldrich syndrome protein (WASP), Las17p/Bee1p, and (2) the SH3 domain in the myosin I protein tail interacts with two different members of the Arp2/3 complex, Arc40p and Arc19p (Evangelista et al., 2000). Thus, myosin I proteins have the capacity to associate with actin-nucleating proteins that are implicated in actin patch assembly. Similarly, others have found that type I myosins can stimulate actin nucleation at sites of polarized growth in permeabilized yeast (Lechler et al., 2000). Finally, Winter et al. found that mutation of the Arp3p subunit of the Arp 2/3 complex results in defects in actin patch integrity and motility. This supports roles for myosin I proteins, Arp2/3 complex and Las17p/Bee1p in actin patch assembly, and indicates that actin patch movement requires normal assembly (Winter et al., 1999).

Yet to be determined is the mechanism of myosin I action in actin patch assembly. Since myosin I proteins and verprolin can bind actin and other ligands, including the subunits of the Arp2/3 complex, it is possible that these proteins deliver essential components to actin patches. Alternatively, since actin nucleation is required for actin patch assembly, it is possible that repeated rounds of F-actin binding and dissociation by the myosin I motor domain are required to stabilize new actin polymers and deliver these filaments to sites of actin patch assembly. Future studies are directed toward identifying (1) other proteins that contribute to myosin I and verprolin particle assembly and targeting, and (2) other patch proteins dependent upon myosin I for their localization.

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