Movement of Cortical Actin Patches in Yeast

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Abstract. In yeast, actin forms patches associated with the plasma membrane. Patch distribution correlates with polarized growth during the cell cycle and in response to external stimuli. Using green fluorescent protein fused to capping protein to image actin patches in living cells, we find that patches move rapidly and over long distances. Even patches in clusters, such as at the incipient bud site, show movement. Patches move independently of one another and generally over small distances in a local area, but they can also move larger distances, including through the mother-bud neck. Changes in patch polarization occur quickly through the cell cycle. These observations provide important new parameters for a molecular analysis of the regulation and function of actin.

In yeast, actin functions in polarized secretion and growth (6, 37). Actin filaments in yeast are found in cortical patches associated with invaginations of the plasma membrane and in cables running through the cytoplasm (26). The polarization of patches and the orientation of cables correlates with polarized cell growth (1). The distribution of both patches and cables changes dramatically over time during the cell cycle and in response to external stimuli (10). Cell cycle regulators control the redistribution processes (20), and several actin-binding proteins are necessary for patch polarization and cable formation (6, 37).

Actin is essential for viability (31). Mutants lacking patches have never been observed, but mutants without cables are viable. Also, mutations in genes encoding other protein components of patches are lethal (2, 25). Therefore, patches may be the location of the essential function of actin. Temperature shift experiments indicate that actin is important for secretion (27) and endocytosis (19). Together, these observations suggest that the location of patch polarization specifies the location of polarized growth and cell wall remodelling.

Therefore, we wish to understand how the location of cortical actin patches in a living yeast cell is controlled. In particular, we want to determine whether patches change their distribution by moving about or by disassembly at one place with re-assembly elsewhere. Also, we want to understand how rapidly the patches change between polarized and depolarized states. These pieces of information are important for a molecular understanding of how actin function is controlled by cell cycle and other regulators.

An analysis of actin patch dynamics is now possible in yeast with the advent of green fluorescent protein (GFP)[1] technology (33). We constructed a GFP derivative of the β subunit of capping protein (Cap2p) because capping protein is found only at cortical actin patches (3). GFP-Cap2p localized to patches and was able to function like Cap2p, rescuing a null mutant.

We found that the distribution of patches changed on a time scale of seconds, much faster than landmark events through the cell cycle. Patches moved, at rapid rates and over long distances, even through the mother/bud neck. Even polarized patches, found in clusters at incipient bud sites, in the bud and at cell division sites, showed movement. Also, the transitions between polarized and depolarized patch distributions were remarkably rapid.

Materials and Methods

DNA Manipulations: GFP-Cap2p Fusion Protein

Wild-type (wt) GFP was fused to the NH_2 terminus of Cap2p by placing a PCR-amplified cassette of the GFP coding region (from plasmid TU no. 65, kindly provided by Martin Chalfie, Columbia University, NY) into a synthetic BamHI site 10 nt 5′ to the initiating ATG of *CAP2* (pBJ 321, a CEN TRP1 plasmid derived from pRS 314 [reference 32]). The wt GFP in the resulting plasmid (pBJ 643) was then converted to the Ser65Thr variant (14) by replacement of a NcoI-HpaI restriction fragment from pRSET-S65T (pBJ 645), kindly provided by R. Heim and R.Y. Tsien (HHMI, University of California, San Diego). All experiments here were performed with the S65T variant (pBJ 646).

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1. *Abbreviations used in this paper*: 2-D, two-dimensional; GFP, green fluorescent protein; wt, wild-type.

Yeast Strain Construction and Characterization

Transformation and growth of yeast was by standard methods (16). To minimize autofluorescence, *ADE2* strains were used, and strains were grown in SD medium.

The GFP-CAP2 fusion gene was placed into *cap2Δ* cells either by integration at the CAP2 locus or on a CEN-based expression plasmid under control of the *CAP2* promoter, pBJ 646. In *CAP2* strains, the GFP-Cap2p fusion protein accumulated at levels too low for imaging. Studies here were done with a diploid (YJC 1265) carrying two integrated copies of the GFP-Cap2p expression construct and one on a CEN plasmid. The genotype of that strain is MAT**a**/α *ura3/ura3 trp1/trp1 leu2/leu2 GFP-CAP2/GFP-CAP2* (pBJ 646: CEN *TRP1 GFP-CAP2*).

The ability of GFP-Cap2p to restore viability to *sac6 cap2Δ* double mutants was tested in a plasmid shuffle assay. A *sac6 cap2Δ* strain, covered by a *CAP2 URA3* plasmid, (YJC 456, MATα *cap2::HIS3 sac6::LEU2 ade2 his3 leu2 ura3 trp1* [pBJ 198 URA3 CAP2]) was transformed with a CEN *TRP1* plasmid expressing GFP-Cap2p (pBJ 643 above). This transformant was viable on medium containing 5-FOA, which counter-selected against the *CAP2 URA3* plasmid. The number of transformants viable on 5-FOA was 60 of 60 for the GFP-Cap2p plasmid and 0 of 60 for a control plasmid. Therefore, GFP-Cap2p was able to replace Cap2p completely in this test.

myo1 (*myo1Δ::LEU2*, strain SBY3), *myo4* (*myo4Δ::URA3*, strain U5-2A), and *smy1* (*smy1Δ-2::LEU2*, strain SLY57) mutants were obtained from Susan Brown (University of Michigan, Ann Arbor, MI). *myo3* and *myo5* were obtained as a double mutant (*myo3Δ::HIS3 myo5Δ::TRP1*, strain 9c) from Liza Pon (Columbia University, NY). *tpm2* (*tpm2Δ::LEU2*, strain ABY418) and a *tpm1* disruption plasmid (YIp352) were obtained from Anthony Bretscher (Cornell University, Ithaca, NY). *myo2* (*myo2-66*, strain JP7a) was obtained from Richard Singer (Dalhousie University, Halifax, Nova Scotia). *vps33* (*vps33Δ::HIS3*, strain LBY317) was obtained from Stephanie Rieder and Scott Emr (University of California, San Diego, CA). The GFP-CAP2 integration was introduced into these strains by crosses and tetrad dissection. For each mutant, three or four GFP-CAP2 segregants were tested—two mutant and one or two wt segregants.

GFP Fluorescence Microscopy

Sample preparation was important to minimize background fluorescence, immobilize the cells, and prevent drying. A drop of molten 2% agarose in either distilled water or a simplified (nonfluorescent) synthetic medium (KH_2PO_4 0.9 g/l, K_2HPO_4 0.23 g/l, $MgSO_4$ 0.5 g/l, $(NH_4)_2SO_4$ 3.5 g /L, glucose 20 g/L, CSM-Trp media supplement (BIO101, La Jolla, CA)) was placed in a well of a teflon coated glass slide (no. 10-1189-A; Cell-Line Associates, Inc., Newfield, NJ) and immediately flattened by covering with an uncoated glass slide. The upper glass slide was removed leaving behind a circular, flattened agarose pad. Using a razor blade, the pad was trimmed into a square that matched the dimensions of the well. 1–2 μl of cells was placed in the center of the pad and then covered with a no. 2 glass coverslip that had a thin (~0.5 mm) line of Vaseline at the outer edges. Slight pressure was applied to the coverslip to disperse the cell droplet over the surface of the agarose pad and make a seal.

Living cells were imaged at room temperature, 22–24°C, on an Olympus Bmax-60F microscope equipped with a 1.35 NA 100× UPlanApo objective and a U-MWIBA interference filter set (Olympus, Lake Success, NY). Photobleaching was minimized by closing the excitation shutter between image collections and reducing the illumination intensity with neutral density filters. For most experiments, an Olympus magnification changer increased the magnification twofold. The microscope image was collected and processed with a DAGE ISIT-68 camera, RC68 controller, and DSP-2000 processor. Custom macros (available on request from J.A. Waddle) and NIH Image 1.58 (written by Wayne Rasband at the National Institutes of Health and is available by anonymous ftp at zippy.nimh.nih.gov) coordinated a stage and shutter controller (MAC2000; Ludl Electronic Products Ltd., Hawthorne, NY), a framegrabber (AG-5; Scion Corporation, Frederick, MD) and a Power Macintosh 8100/80.

Long-term experiments were limited by gradual loss of the GFP signal due to photobleaching. Cells remained viable and growing during long experiments. Cell left in the experimental chamber after an experiment proceeded to divide many times and form microcolonies. Based on these criteria and the morphology of the cells and their actin cytoskeleton, we saw no evidence for cell damage caused by the experiment.

Two different acquisition strategies were used. First, rapid acquisition at a single focal plane for a short time (minutes) was used to monitor cells at sub-second acquisition rates to determine the rate, direction, and range of patch movements. Second slow, time-lapse, acquisition of 10 focal planes 0.5 μm apart for a long time (hours) with projection of all focal planes onto a single two-dimensional (2-D) image was used to determine the extent of polarization of patches over the cell cycle. Patch movements are best appreciated by viewing the movies, which are available at our world wide web site (http://www.cooper lab.wustl.edu/).

For cell permeabilization, cells were subjected to a freeze/thaw procedure as described (17). Loss of viability after permeabilization was documented by plating a portion of the sample on YPD plates.

Phalloidin Staining

Fixation and rhodamine phalloidin staining were as described (3) except that the concentration of rhodamine-phalloidin was lowered by a factor of 20, to minimize crossover of the rhodamine fluorescence into the GFP channel. Images were collected with an Optronics 470-DEI-T video CCD camera (Optronics Inc., Goleta, CA) on the video microscope system above. To compare the location of phalloidin-stained actin patches with those of GFP-labeled CP sites, images from the red and green emission channels were merged with NIH Image.

Calculation of Velocity

Instantaneous speeds of patch movement were calculated for two groups of patches. Group 1 contained patches that remained visible for >2 s and showed obvious movement. Group 2 contained patches that remained visible for >4 s regardless of obvious movement. Group 1 is biased in favor of moving patches and Group 2 in favor of nonmoving patches. The (x, y) positions of the selected patches at 0.2 s (Group 1) or 0.4 s (Group 2) time intervals were collected using NIH Image. Speed was calculated as distance divided by time at 0.4-s intervals for each. The distance between adjacent pixels was 80 nm; therefore, the smallest possible speed greater than 0 was 0.2 μm/s.

Cell Volume Measurements

Major and minor axes of mother and bud were measured using NIH Image and individual frames of time-lapse movies. Each frame was a projection of multiple focal planes onto a single 2-D image, permitting visualization of the cell borders. Cell volumes were calculated from the axes assuming that mother and bud were prolate spheroids.

Results

The GFP-Cap2p Fusion Protein Can Substitute for Cap2p In Vivo

To determine the validity of using GFP-tagged capping protein as a marker for cortical actin patches in living cells, we tested the ability of GFP-Cap2p to function in place of Cap2p. First, we found that GFP-Cap2p localized to actin cortical patches, the same location as found for capping protein in immunofluorescence studies (3). Cells carrying GFP-Cap2p were fixed and stained with rhodamine-phalloidin. Nearly all patches stained with rhodamine-phalloidin were also fluorescent with GFP-Cap2p, and vice-versa (Fig. 1). The only difference in the distribution of GFP-Cap2p compared to previous results is that an actin-containing structure at the neck late in the cell cycle contained GFP-Cap2p (Fig. 1, *arrow*) but did not stain with antibodies to capping protein (3). Because GFP alone shows a diffuse localization through the cytoplasm (data not shown; reference 33) and because negative immunofluorescence results due to poor penetration of supramolecular structures by antibodies have been found in studies of other proteins (18), we suspect that the GFP-Cap2p localization is correct.

Second, GFP-Cap2p rescued the phenotype of *cap2* null mutants by several criteria. The GFP-CAP2 gene fully rescued the synthetic lethality of *cap2Δ* with *sac6* (2) by a

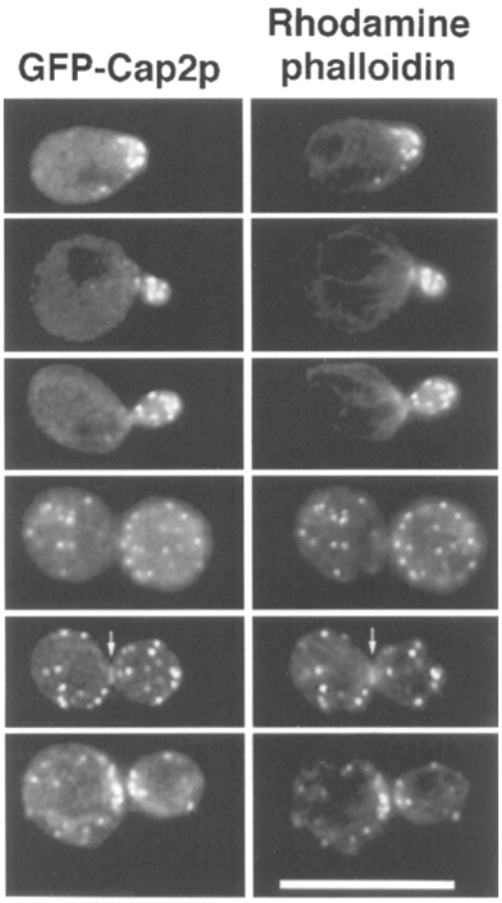


Figure 1. Localization of GFP-Cap2p at cortical actin patches. Double fluorescence microscopy of six representative cells at different stages of the cell cycle are shown. Cells carrying GFP-Cap2p, which is detected in the fluorescein channel, were fixed and stained with rhodamine-phalloidin. Nearly all cortical actin patches contain GFP-Cap2p, and GFP-Cap2p is located at patches. The diffuse cell background in the GFP channel is largely autofluorescence, based on images of cells lacking GFP. However, free GFP-Cap2p may also contribute to the diffuse pattern. The arrow points to an actin-containing structure at the cell division site that contains GFP-Cap2p and stains with rhodamine-phalloidin. This structure did not stain with antibodies to capping protein in previous work (3). Bar, 10 μm.

quantitative plasmid shuffle assay (data not shown). GFP-Cap2p corrected the defective actin distribution of the *cap2Δ* mutant (4), restoring wt cable formation and patch distribution (Fig. 1). Finally, the presence of GFP-Cap2p stabilized the level of the Cap1p subunit, which falls to very low levels in a *cap2* mutant (4) (data not shown).

Finally, because overexpression of GFP-Cap2p might cause abnormal localization or function, we determined that the level of GFP-Cap2p in several strains lacking endogenous Cap2p, including the one used for video microscopy experiments, was about one-third that of Cap2p in a wt strain (data not shown).

Short Term Imaging of Cortical Actin Patches in Living Cells

To monitor changes in the distribution of cortical patches over time, we initially collected images at 15-s intervals at all Z-axis planes. For each timepoint, all the Z-axis planes were projected onto a single 2-D image to visualize all the patches in one cell. Unexpectedly, the distribution of patches changed dramatically and rapidly (Fig. 2). Nearly every patch changed its position at each timepoint. An extreme example was one medium-budded cell in which the number of patches in the mother changed greatly during one 15-s time interval (Fig. 2 *A*, *arrow*). Also surprising is the observation that the position of polarized patches, such as ones at the incipient bud site (Fig. 2 *B*, *arrow*) changes dramatically over each 15-s time interval.

To investigate the basis for the rapid redistribution of patches, we collected images at rapid acquisition rates at a single Z-axis plane of focus. In these movies, patches were observed to move laterally, rapidly and, occasionally, over great distances. Seven representative sequences extracted

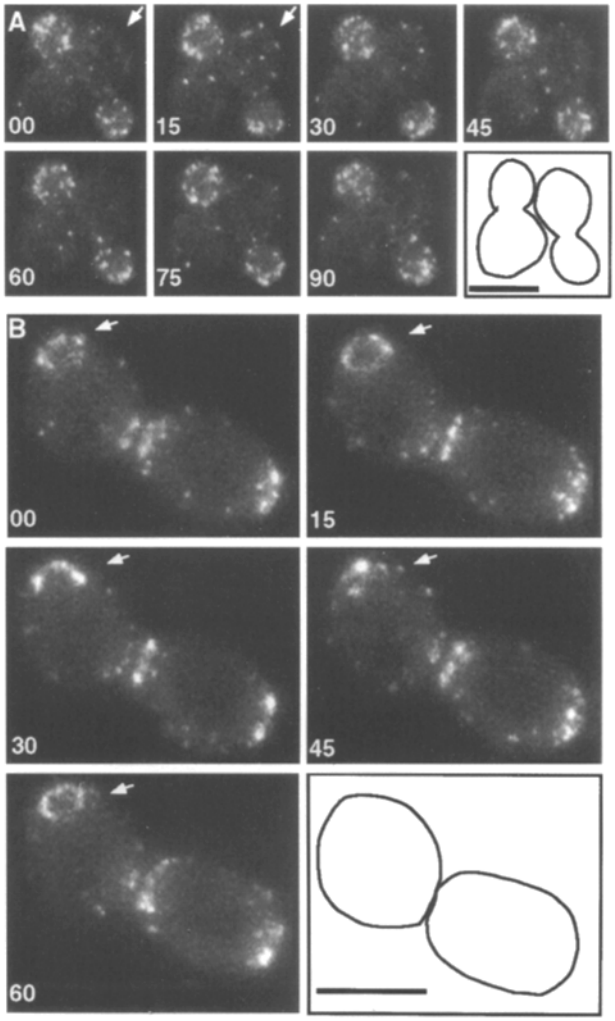


Figure 2. Actin patch distribution in three cells at 15-s intervals. Each image is a 2-D projection of images collected at all Z-axis planes. Note how much the patch distribution changes from one timepoint to the next. *A* shows two medium-budded cells and includes one striking example (*arrow*) where the mother cell at t = 0 s has many fewer patches than at 15 s. *B* shows a mother and large bud near cell division and includes an en face view of an incipient bud site (*arrow*) showing that even highly polarized patches change their distribution rapidly. The insets show schematics of the cell boundaries for reference. Bar, 5 μm.

from movies are shown in Fig. 3, along with tracks of patch movements. Each row highlights the movement of one patch shown in yellow.

A review of >50 single focal plane movies revealed that essentially all patches showed some degree of movement within a few seconds. We found no evidence for flow phenomena in which nearby patches moved together; instead, patches moved independently of each other. Generally, patches moved relatively short distances (~0.5 μm) and frequently changed directions. Occasionally, we observed longer excursions, one example of which is shown in row *3* of Fig. 3. Also, patches were observed to move through the mother / bud neck (Fig. 3, row *2*). Movement through the neck is far easier to appreciate from the movies than from a selection of still frames.

Patch movements were confined to near the plasma membrane. In movies where the plane of focus was the equator of the cell, patches did not cross the central cytoplasm. We observed occasional short movements perpendicular to the plane of the membrane, directed inward to the cell center. However, such patches generally promptly reversed their direction and returned to the periphery. Since the vacuole occupies a fair amount of the cytoplasm in the cell, we considered that patches may be unable to cross the central cytoplasm simply because the vacuole blocks their path. Therefore, we tested patch movement in a *vps33* null mutant, which lack any identifiable vacuolar structures (5). In the absence of a vacuole, patch movements were still confined to the periphery of the cell, near the plasma membrane.

The degree of patch movement observed here is sufficient to account for the redistribution of patches. Another hypothesis is that whole patches disassemble and reassemble at different locations over time. We observed that patches often appeared and disappeared in movies focused on a single plane at the equator of the cell. These events could be caused by either vertical movement of the patches, in and out of the plane of focus, or to wholesale assembly and disassembly. To distinguish between these two possibilities, we examined a plane of focus at the upper surface of the cell, which gives an en face view of patch movement. Here, patches translocated across the cell surface and only appeared or disappeared at the edges, where the cell surface curved out of view. These observations indicate that patch appearance and disappearance in a single focal plane can be explained by vertical movement in and out of the plane of focus and not to wholesale assembly and disassembly.

The negative conclusion about patch assembly and disassembly as a mechanism for patch redistribution is limited by our inability to track every patch at all times and provide a life history of each patch. Two unsuccessful approaches were attempted to track patches in three dimensions. First, we visualized a patch by eye and adjusted the focus manually to keep the patch in focus. Second, we collected a complete Z-axis series of focal planes rapidly,

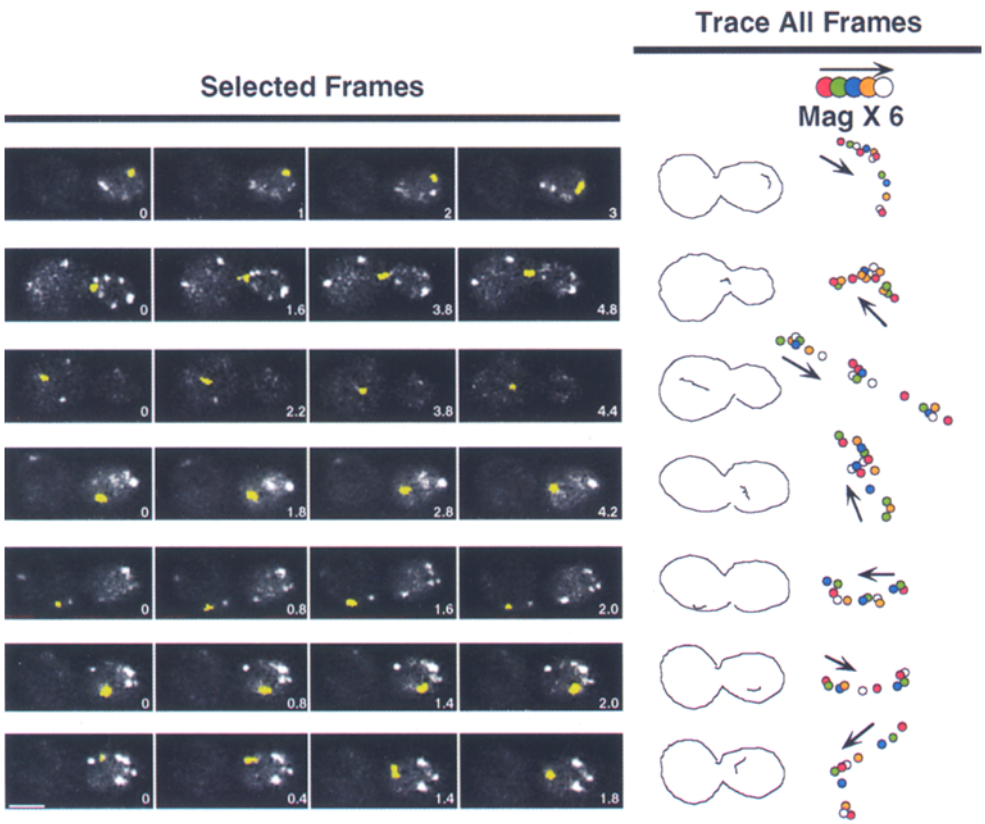


Figure 3. Movement of cortical actin patches. Movies were made at a single plane of focus with frames collected at 0.2-s intervals. Each row illustrates the movement of one patch. On the left are four selected frames from the movie; the moving patch is colored yellow. On the right is the path of the highlighted patch, tracked in every frame. Under *Trace All Frames* the path of the highlighted patch is drawn, first as a black line inside an outline of the cell at the same magnification as the micrographs on the left. Under *Mag X 6* the individual successive positions at each frame are shown, colored in the sequence red-green-blue-orange-white, at a sixfold increased magnification. Since the time interval between frames is 0.2 s, the interval between red dots is 1 s. In this illustration, the colored dots are placed on top of each other as time proceeds, which means that if a patch does not move, then that dot is covered over and masked by a subsequent dot. Therefore, when colors are missing from the sequence red-green-blue-orange-white, the patch has not moved or has returned to a previous position. Of course, the movies illustrate this movement better. Bar, 3 μm.

moving through the entire cell in one second. In each case, the speed, change in direction, and crowding of patches precluded unambiguous tracking. Because of these technical limitations, we cannot rule out the possibility that patch disassembly and reassembly accounts for some redistribution within cells.

Rate of Cortical Actin Patch Movement

To determine the speed of patch movement and to determine whether different classes of movement could be identified based on speed, we tracked the (x, y) coordinates of patches in rapid acquisition movies at a single focal plane (Fig. 4). First, we tracked only those patches that remained in focus for >2 s and that showed obvious movement, such as those in Fig. 3. Eleven patches were tracked at 0.2-s intervals, and instantaneous speed was calculated at 0.4-s intervals. The mean was 0.49 μm/s, the median was 0.28 μm/s, the standard deviation was 0.30 μm/s, the range was 0–1.7 μm/s, and $N = 183$. Because this data set was biased in favor of moving patches, we subsequently tracked 30 patches, requiring only that they be visible in the same focal plane for >4 s, a bias that favors nonmoving patches. The mean was 0.31 μm/s, the median was 0.28 μm/s, the standard deviation was 0.28 μm/s, the range was 0–1.9 μm/s, and $N = 392$. The range of patch speeds is within the range of myosin-mediated movement of actin filaments (0.05–4 μm/s) (38) as well as speeds of the microtubule motors kinesin and dynein (0.2–8 μm/s) (35). For both data sets, the distribution of patch speeds shows only a single mode or peak (Fig. 4). Therefore, these data offer no evidence for different classes of patches or patch movements.

Clustered Actin Patches Show Movement

Actin patches become clustered at several times and places through the cell cycle, presumably mediating cell wall growth in buds and shmoo tips, and cell wall remodelling at the cell division site. The mechanism of actin patch clustering is an important unresolved question. To gain insight into the dynamics of patch clusters, we examined single-focal-plane movies of patches clustered at incipient bud sites, small buds, shmoo tips, and cell division sites. Surprisingly, clustered patches showed a remarkable degree of movement at all four locations. Two examples of movement of patches within a cluster are shown in Fig. 5. Nearly all patches contained in clusters moved. Patches were generally restricted in their range of movement and often remained in or near the cluster. Occasionally patches showed large excursions in and out of the cluster. A quantitative analysis of patch speed in clusters was precluded by the inability to resolve and track crowded patches. Nevertheless, in a qualitative comparison, based on viewing the movies, the speed of patches in clusters was similar to that of unclustered patches.

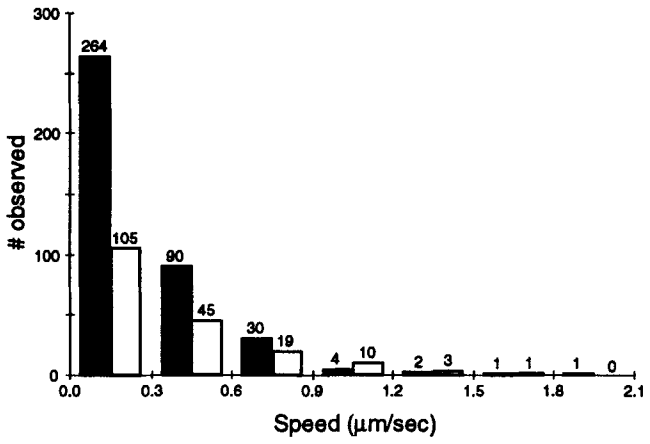


Figure 4. Histogram of instantaneous speeds of patches. Solid bars represent the data from the set biased in favor of nonmoving patches, and open bars represent the set biased in favor of moving patches.

Studies on the Mechanism of Patch Movement

To investigate the mechanism of patch movement, we first tested whether ATP was necessary for movement. Cellular ATP levels were lowered by permeabilizing cells with a freeze/thaw procedure that introduces small holes in the plasma membrane and cell wall (21). Permeabilization caused patches to stop completely as soon as the sample was viewed after freezing (data not shown). These results are most consistent with an ATP-dependent process, such as a motor or actin polymerization, driving patch movement. However, we do not feel that these results rule out movement driven by thermal energy. The effect of loss of cellular ATP may be to increase the viscoelastic properties of the medium in which the patches move.

Because patches contain actin filaments, myosin is an obvious candidate to power patch movement. If so, a myosin mutant might show no patch movement. To test this hypothesis, we qualitatively assessed patch movement in short-term movies of strains carrying loss-of-function mutations in each of the five known myosin heavy chain genes, *myo1* through *myo5*. *myo1* is the only yeast gene in the conventional myosin family (myosin II) (30, 34), *myo2* and *myo4* are in the dilute family (myosin V) (13, 15), and *myo3* and *myo5* are in the myosin I family (reference 12 and Goodson, H.V., B.L. Anderson, H.M. Warrick, L.A. Pon, and J.A. Spudich, manuscript submitted for publication). *MYO1, MYO3, MYO4,* and *MYO5* are not essential for viability; therefore, we tested complete gene disruptions. *MYO2* is essential; therefore, we tested a conditional allele, *myo2-66,* at room temperature, where cells are viable but grow poorly. Patches still moved in all the single mutants, and the speed of movement was qualitatively similar in all the *myo* mutants and wt controls. We also tested the *myo3 myo5* double mutant, to eliminate both members of the myosin-I family, and found patch movement at normal speeds. *myo2-66* can be suppressed by overexpression of a kinesin heavy chain gene, *SMY1* (22), and Myo2p and Smy1p co-localized in cells (23); therefore, we also tested an *smy1* null mutant. Again, patches showed movement at normal speeds. A quantitative analysis of patch movement has not been performed with these mutants. We plan to do a quantitative analysis after developing a better method for tracking patches.

Therefore, none of the these five myosins is the sole motor for patch movement. However, additional myosins or non-myosin actin motors may exist. Since tropomyosin

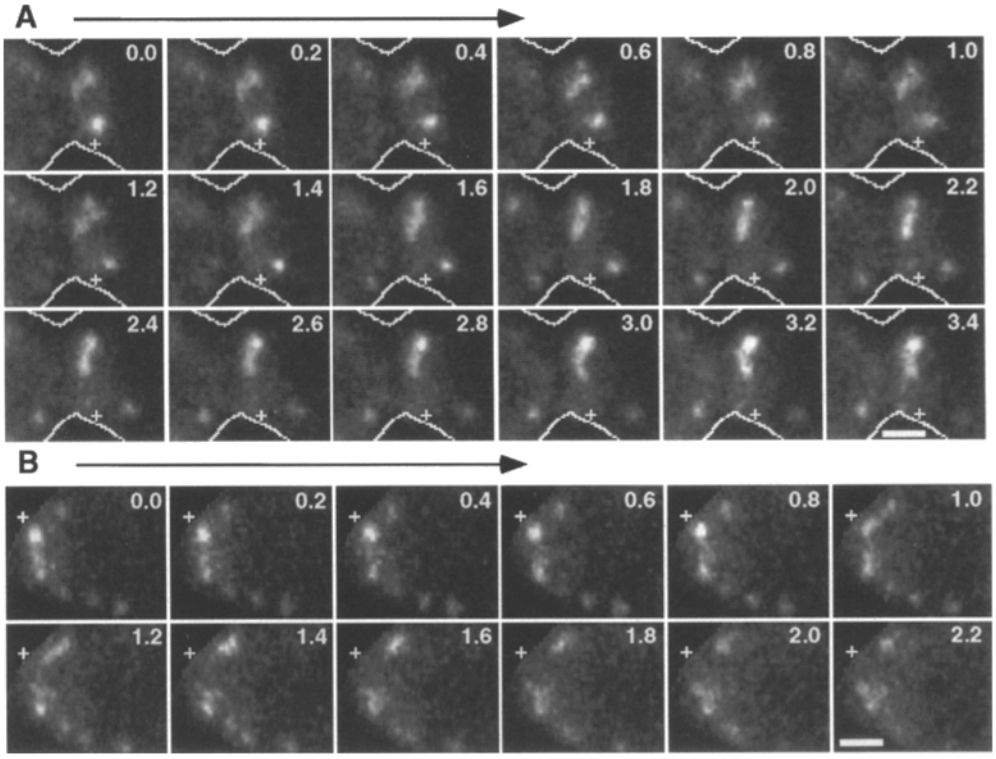


Figure 5. Two examples of movement of clustered patches. Sequential frames from rapid acquisition movies at a single focal plane are shown in order left to right, top to bottom. The time interval between frames is 0.2 s. *A* shows the movement of patch associated with a cell division site, and *B* shows a patch at an incipient bud site. The moving patch in each panel is indicated by a nearby + sign in the first frame. The + sign remains at the same position, to serve as a landmark for observing how the patch moves away. Both examples are movement out of the cluster, but movement into the cluster was also seen. Also, many shorter movements and patches appearing and disappearing can be seen in the movies and by careful inspection of these images. A schematic of the boundaries of the cell in *A* are shown for perspective. Bar, 1 μm.

regulates the interaction of myosin with actin, we hypothesized that tropomyosin mutants may show no patch movement. Tropomyosin in yeast is encoded by two known genes, *TPM1* and *TPM2* (11, 24). Single null mutants in *tpm1* or *tpm2* showed actin patch movement at wt speeds, again based on qualitative observations of short-term movies.

Long Term Observations of Actin Patch Distribution

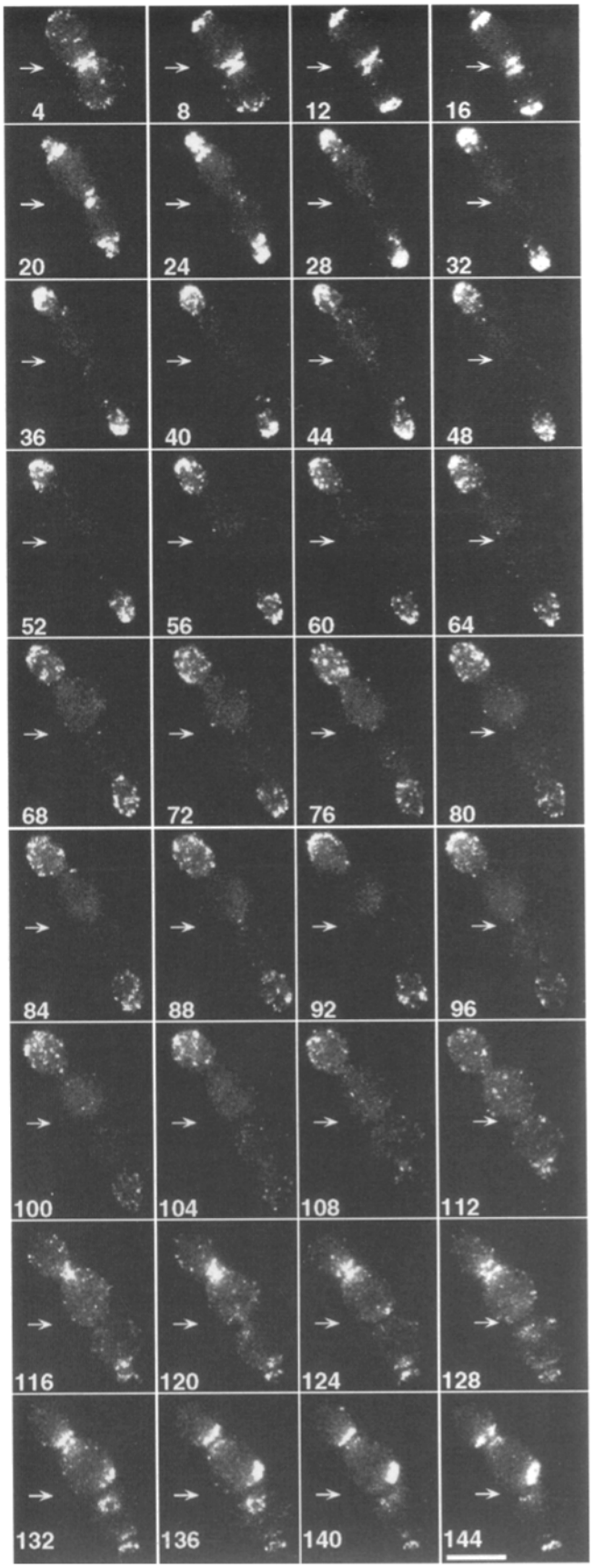
Previous analyses of patch distribution in fixed cells indicates that patches undergo stereotyped changes in distribution through the cell cycle. To determine how rapidly such changes occur, we made time-lapse movies at all focal planes and visualized all patches during more than a complete cell cycle. The distribution of the patches in living cells is consistent with results from previous fixed-cell analyses, but the ability to follow a single cell over time has provided novel findings.

A representative example to illustrate these findings is shown by the mother cell in the upper left corner of Figure 6. At the beginning of the cell cycle, actin patches cluster at the site of the incipient bud (Fig. 6, 12-min timepoint). This new cluster of patches forms at a time when the patch cluster at the cell division site still exists (*arrow*), a feature seen more often in rapidly dividing cells. Next, patches fill the very small bud as it enlarges (24 min). As the bud grows, patches cluster at the bud tip (40–56 min). Later, the bud tip cluster disperses but patches remain in the bud (72–88 min). Occasionally, the bud tip cluster reforms, as illustrated in this cell (92–96 min). Eventually, patches completely depolarize and disperse throughout the mother and bud (112 min). Finally, patches cluster on both sides of the cell division site (116–128 min). Patches then move from the cell division site to the next incipient bud site (132–144 min).

Three novel findings emerge from analysis of 10 cells monitored by movies similar to those in Fig. 6. First, the transitions between different clustered or polarized states are rapid. The time lapse interval in these experiments was 4 min. In all of the cells, each transition between polarized and depolarized states occurred over one to two time intervals, i.e., ≤4–8 min. Second, actin patch clusters are so dynamic that they sometimes disperse completely and reform. This phenomenon is seen most often with the cluster at the tip of medium-sized buds, as illustrated in Fig. 6 (72–96 min). The dynamic nature of the patch cluster at the cell division site is also apparent in Fig. 2 *B*, even though the cluster does not completely disperse at any time. Third, cells can form a new cluster while a previous cluster still exists. In rapidly growing cells, patches cluster at the incipient bud when the cluster at the cell division site still exists, as illustrated twice in Fig. 6 (12 min, 132 min). The coexistence of clusters indicates that regulation of the number of clusters is probably not a feature of cell cycle regulation and supports the view that the mechanism of clustering is controlled locally.

Correlation of Patch Polarization with Polarized Growth

The ability to visualize actin patches through the cell cycle allowed us to test the hypothesis that patch polarization is necessary for polarized growth by asking whether these two phenomena were correlated in time. We observed actin patches and measured mother and bud size in individ-



ual cells through the cell cycle (Fig. 7). In each frame of a time-lapse movie, we measured the length and width of the mother and bud and calculated their volume. Bud growth, defined as increase in volume over time, correlated with patch polarization. Bud volume increased linearly with time when patches were polarized to the bud and stopped when patches were depolarized in the mother and bud. This correlation supports the hypothesis that polarized growth requires patch polarization. However, patches are present in both mother and bud late in the cell cycle when growth ceases; therefore, the mere presence of patches is not sufficient for growth. Instead, the data indicate that clustering, or polarization, of patches is necessary for growth to occur.

Discussion

We report here the first observations of cortical actin patches in living yeast cells. Two findings are important because of their implications for understanding actin function and regulation. Actin patches move at a remarkable rate, and the polarized distribution of patches changes rapidly relative to the length of the cell cycle.

We conclude that patch movement is sufficient to account for the redistribution of patches because of the speed of the patches, the distances they traverse, and the independence of their movement relative to each other. Specifically, a given patch can traverse the entire length of a cell in a few seconds. Therefore, rapid changes in patch distribution through the cell cycle, which can occur in under 5 min, can be accounted for by patch movement.

An alternative mechanism to account for patch redistribution is the wholesale disassembly and reassembly of patches. We found no evidence for the existence of this process. However, the strength of this negative conclusion is limited by our current inability to track every patch at all times and thereby provide a life history of each patch. Because patches must be created as a consequence of cell growth, and we have not observed patch creation, we suspect that patch formation occurs continuously through the cell cycle, either by the de novo assembly of a patch or by the fission and growth of existing patches.

Model for Polarization of Cortical Actin Patches

The mechanism for specifying the position of actin patches is unknown. Understanding this mechanism is important

Figure 6. Actin patch distribution in two cells at 4 min intervals through the cell cycle. Each image is a 2-D projection of images collected at all Z-axis planes. The arrow at t = 4 min marks the cell division site between mother and daughter. This arrow remains at the same position in later panels to serve as a landmark. Both mother and daughter bud in a bipolar pattern, and at the end, the original mother is about to bud again. Note how rapidly changes in patch polarization occur, often within one 4-min interval. Also, note that in some cases, one cell contains clustered patches in two places at the same time. At t = 16 min and t = 132 min, some cells have clusters at both the cell division site and the incipient bud site. Also note that clusters can disperse and reform, such as at the bud tip in the upper left from t = 32 min through t = 96 min. Bar, 5 μm.

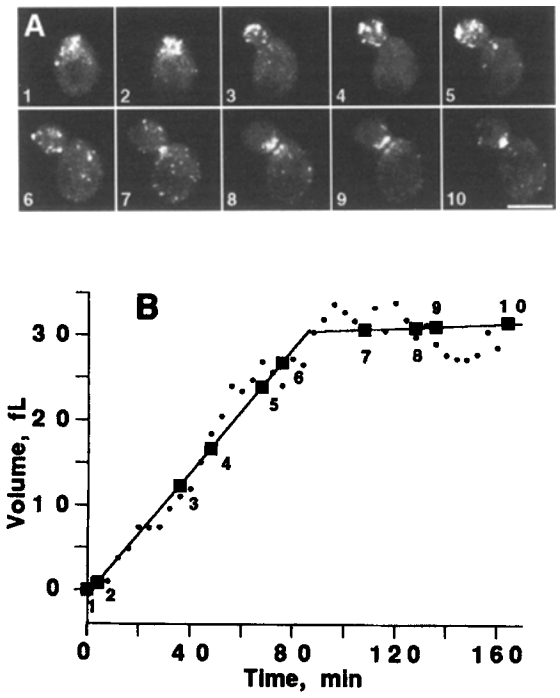


Figure 7. Correlation of growth with patch polarization in the bud. Data for one representative cell are shown; very similar results were found for two other cells with the same duration of the budding cycle. *A* shows patch localization over time. Each image is a 2-D projection of images collected at all Z-axis planes. *B* plots the volume of the bud (femtoliters) vs time (min). The numbered points on the graph correspond to the numbered sequential images in *A*. Between images 6 and 7, patch polarization in the bud ends and bud growth stops. In the three cells analyzed, the volume of the mother cell remained nearly constant, and the bud volume was 30–50% of the volume of the mother at cell division. Bar, 5 μm.

because previous studies indicate that cell cycle regulators specify the position of actin patches during the cell cycle, actin patches mediate polarized secretion and growth, and, therefore, the position of actin patches specifies the location of polarized growth.

The discovery of patch movement suggests that one likely model for causing patches to cluster is to create molecular links between patches and a location in the cell, such as the incipient bud site during G1 or the cell division site during M. The site could be marked initially by site-specific proteins such as Bud3p and Bud4p for haploid bud site selection (8). Subsequently, the site-specific proteins would interact with a general set of actin regulatory proteins, including Cdc24p and Cdc42p (7), to create links between patches and the specific sites.

While the model of molecular links is appealing in its simplicity, the degree of patch movement observed here places strong constraints on the model and even raises questions as to its validity. First, patches are constantly moving, even when clustered. Therefore, the molecular links must be either short-lived or very flexible. Second, patches rapidly convert between polarized and depolarized states. Once the link mechanism is activated, what brings the patches together? Perhaps patches move at random, and the rate and extent of their movement is sufficient to bring them close to the cluster site that contains links. Qualitative observations of the movement support this view. However, the notion that patches are directed to move toward the cluster site is not excluded by our observations. Resolution of this question will require improved tracking of patches and a quantitative analysis of the pattern of the movement.

As an alternative to a molecular link model, patches may cluster because the mechanism that drives their movement is inhibited at the cluster site. Patches would move at random, as in the molecular link model, but then move more slowly near a specific site. Our qualitative observations do not reveal a dramatic difference in the movement of patches in and out of clusters; however, a small difference may be sufficient to achieve clustering.

Mechanism of Patch Movement

How do the patches move? A simple and compelling hypothesis is that motor molecules drive patch movement. Because patches contain actin filaments, myosin is an obvious choice for this motor. Yeast contain five myosin heavy chain genes in families I, II, and V (9, 12). Myo2p, a class V myosin, is located at the bud tip and the mother/bud neck and does not colocalize with actin patches there (23). The location of other yeast myosins has not been described.

Mutations in any of the known myosin heavy chain gene, the tropomyosin genes, or the myosin-interacting kinesin heavy chain gene *SMY1,* did not stop patches from moving. However, additional myosin genes may yet be uncovered, and non-myosin actin-based motors may exist. Therefore, the details of this model still deserve serious consideration.

Because patches sit at the interface between the plasma membrane and the cytoplasm (26), the motor could be located either on the membrane or in the cytoplasm. Biochemical properties of some myosin families support the possibility of either location. For example, myosin-I is a good candidate for a membrane-associated motor because its tail contains a lipid binding site (29). The myosin would also need to be held stationary in the membrane by other interactions. Similarly, myosins in the cytoplasm could drive patch movement. Tails of some myosin-I's have non–ATP-dependent actin-binding sites (29), so these myosins can cause sliding of two actin filaments in opposite directions. The cytoplasm contains actin filaments bundled as cables and perhaps as single filaments as well. Cytoplasmic actin filaments could serve as stationary footing due to their cross-linking into a network. Because patches are found at the ends of cables in favorable views (26), perhaps cables are attached to patches, and cables move patches. Patches probably do not slide along cables because we do not observe patch movements through the central cytoplasm, where cables are located.

Because myosin moves toward the barbed end of actin filaments, myosin-based patch movement might require all the filaments in a patch to be oriented in the same direction. Additional ultrastructural information about the filaments of a patch would be highly valuable for future analysis of the molecular mechanism of patch movement. By immunoelectron microscopy, patches are associated with

invaginations of the plasma membrane, and favorable views show a helical array of filaments surrounding the invagination (26). At this point, we have little or no knowledge about the length and polarity of the actin filaments, how they are oriented with respect to one another, and how they are oriented with respect to the direction of patch movement. This information will be crucial to the development of molecular models for this movement.

Another possible source of energy for patch movement is thermal energy, with patches undergoing 2-D diffusion along the membrane. We cannot exclude this possibility, but think it unlikely for several reasons. First, the patches seem too large to diffuse at the rates observed, especially if they are associated with membrane invaginations. Second, occasionally the tracks of patches are relatively long and linear.

A different model for patch movement is treadmilling, in which patch components are simultaneously added on one side of the patch and lost on the opposite side. The addition and loss occur at equal rates, so that the net number of components in the patch does not change. Components in the patch interact with something stationary, again either on the membrane or in the adjacent cytoplasm. The observation of patch movement in this study provides no information on the existence or extent of treadmilling, which would require a pulse-labelling experiment to mark the position of a region of the patch. In animal cells, photobleaching actin showed that treadmilling does occur in the cell cortex, where a dense meshwork of actin filaments is attached to the plasma membrane (36). A potential pulse experiment in yeast is the addition of rhodamine-actin to patches in permeabilized cells (21). The addition of rhodamine-actin should occur on only one side of a patch if treadmilling exists, which may require electron microscopy for sufficient resolution. The rate of patch movement places an important constraint on treadmilling models. Components must add and leave at rates consistent with the speed of patch movement. For actin filaments, movements of 0.1–0.6 μm/s parallel to the filament axis would require subunit dissociation rate constants of 40–200 /s, which is substantially higher than those observed at the ends of actin filaments in vitro (<10 /s) (28). On the other hand, entire filaments might be added or lost, in processes for which these in vitro rates do not apply.

Our observations of the movement of cortical actin patches in living yeast cells provide important new parameters to evaluate molecular models for how actin functions and is regulated in vivo. Now the mechanisms by which patches move and cluster can be investigated in live cells manipulated by molecular genetic and pharmacologic methods.

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