Calmodulin Concentrates at Regions of Cell Growth in *Saccharomyces cerevisiae*

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Abstract. Calmodulin was localized in *Saccharomyces cerevisiae* by indirect immunofluorescence using affinity-purified polyclonal antibodies. Calmodulin displays an asymmetric distribution that changes during the cell cycle. In unbudded cells, calmodulin concentrates at the presumptive site of bud formation ~10 min before bud emergence. In small budded cells, calmodulin accumulates throughout the bud. As the bud grows, calmodulin concentrates at the tip, then disperses, and finally concentrates in the neck region before cytokinesis. An identical staining pattern is observed when wild-type calmodulin is replaced with mutant forms of calmodulin impaired in binding Ca^{2+}. Thus, the localization of calmodulin does not depend on its ability to bind Ca^{2+} with a high affinity. Double labeling of yeast cells with affinity-purified anti-calmodulin antibody and rhodamine-conjugated phalloidin indicates that calmodulin and actin concentrate in overlapping regions during the cell cycle. Furthermore, disrupting calmodulin function using a temperature-sensitive calmodulin mutant delocalizes actin, and *actl-4* mutants contain a random calmodulin distribution. Thus, calmodulin and actin distributions are interdependent. Finally, calmodulin localizes to the shmoo tip in cells treated with α-factor. This distribution, at sites of cell growth, implicates calmodulin in polarized cell growth in yeast.

CALMODULIN is a small, highly conserved, Ca^{2+}-binding protein present in all eukaryotic cells. It has been characterized as the Ca^{2+}-dependent regulator of cyclic nucleotide metabolism, muscle contraction, glycogen breakdown, and neurotransmitter release (for review see Cohen and Klee, 1988). Calmodulin is also required for cell proliferation in *Saccharomyces cerevisiae* (Davis et al., 1986), *Schizosaccharomyces pombe* (Takeda and Yamamoto, 1987), and *Aspergillus nidulans* (Rasmussen et al., 1990).

The role of calmodulin in cellular proliferation is not known. Several studies suggest that calmodulin interacts with microfilaments. Calmodulin localizes with actin based stress filaments in 3T3 cells (Luby-Phelps et al., 1985; Welsh et al., 1978). This association with actin is probably not direct since binding between F-actin and calmodulin has not been detected in a gel overlay assay or by sedimenting F-actin in the presence of calmodulin (Piazza and Wallace, 1985). In red blood cells, calmodulin regulates shape through an interaction with spectrin–protein 4.1-actin complexes. Calmodulin enhances the destabilizing effect of Ca^{2+} on red blood cell membranes (Takakuwa et al., 1990) possibly through a conformational change of an apocalmodulin–protein 4.1 complex that occurs when calmodulin binds Ca^{2+} (Tanaka et al., 1991). Furthermore, calmodulin associates with actin-binding proteins, including caldesmon (Sobue et al., 1981) and myosin I from brush border microvilli (Howe and Mooseker, 1983).

Information about actin function in *S. cerevisiae* is accumulating rapidly. The distribution of the actin cytoskeleton reflects the asymmetry of yeast cell growth (Adams and Kilmartin, 1984; Adams and Pringle, 1984). An unbudded yeast cell initiates growth from a particular site on the cell surface and grows in one direction due to the localized fusion of vesicles with the cell membrane (Sloat et al., 1981). Actin cables are aligned towards the bud in the mother cell, and actin cortical patches accumulate at the bud tip. Characterization of temperature-sensitive actin mutants confirmed that actin is essential in polarized cell growth (Novick and Botstein, 1985). Under nonpermissive conditions, actin mutants arrest as unbudded cells and enlarge uniformly rather than directing material to the bud. Furthermore, at least 10 different genes important for actin cytoskeletal function have been identified in yeast (Drubin, 1990) including actin-binding proteins similar to fimbrin (Adams et al., 1991), tropomyosin (Liu and Bretscher, 1989), profilin (Magdolen et al., 1988) conventional myosin (Watts et al., 1987), and unconventional myosin (Johnston et al., 1991).

In addition to interacting with microfilaments, calmodulin in higher eukaryotes is associated with microtubules. In plant and vertebrate cells, calmodulin localizes to kinetochore microtubules (Vantard et al., 1985; Welsh et al., 1979). Consistent with the localization of calmodulin, expression of calmodulin anti-sense RNA in C127 cells results in a delay in metaphase of mitosis (Rasmussen and Means, 1989). The mechanism of calmodulin function in mitosis is unclear.

S. cerevisiae provides a simple system for analyzing the

function of calmodulin in cell proliferation. We previously demonstrated that the requirement for calmodulin in cellular proliferation does not depend on its ability to bind Ca^{2+} (Geiser et al., 1991). Mutant calmodulins with inactivated Ca^{2+}-binding loops substitute for wild-type calmodulin without affecting cell growth. The accompanying paper reports the characterization of a temperature-sensitive calmodulin mutant (Davis, 1992). Characterization of the phenotype of this mutant suggests calmodulin is involved in bud growth, cytokinesis, and chromosome segregation. In this report, we localized calmodulin by indirect immunofluorescence. The calmodulin distribution resembles that of proteins involved in polarized cell growth and overlaps actin throughout the cell cycle. To determine the role of calmodulin in establishing and maintaining cell polarity we analyzed calmodulin and actin distributions in temperature-sensitive calmodulin and actin mutants.

Materials and Methods

Strains and Plasmids

The strains used in this study are listed in Table I. Strains KWY231 and KWY474 are derived from S288C and were kindly provided to us by Ken Wertman (University of California, Berkeley, CA). All other strains are derivatives of W303 (Wallis et al., 1989). Strain TDY66 was constructed from strain TDY62-3D by a single step gene disruption (Rothstein, 1983) of the *SST1* gene. The *sst1::LEU2* construct was a gift of V. MacKay (Zymogenetics, Seattle, WA). The genotype of strains showing supersensitivity to α-factor was confirmed by Southern blot analysis.

Plasmids pJG19 and pJG26 contain *CEN4*, *URA3*, *ARS1*, fl origin, and the mutated yeast calmodulin gene, encoding 3E→V[1] or 3D→A, respectively. Plasmid pJG60 is similar except that it contains a vertebrate calmodulin cDNA in place of the yeast calmodulin gene (Geiser et al., 1991).

Antibody Purification and Immunoblot Analysis

Antibodies were raised in rabbits as previously described (Geiser et al., 1991) and affinity purified as follows. A fraction enriched in IgG was prepared by passing 1.0 ml of serum over a 1.0 ml protein-A Sepharose column. After washing the column with 50 to 100 ml of TBS (150 mM NaCl, 20 mM Tris-HCl, pH 7.5), the bound antibodies were eluted with 0.1 M glycine, pH 2.5, and then immediately neutralized with 1.0 M Tris-HCl, pH 8.0. Fractions containing protein were pooled and dialyzed against TBS. The dialyzed sample (~5.0 ml) was then incubated for 1 h at room temperature with 1.0 ml CaM-Sepharose (10 mg yeast calmodulin coupled to 1.0 ml CNBr-activated Sepharose). After incubation, the CaM-Sepharose column was washed with 10 to 20 ml of TBS. The antibodies were then eluted with 0.1 M glycine, pH 2.5, and immediately neutralized using 1.0 M Tris-HCl, pH 8.0. Fractions containing protein were pooled, dialyzed against TBS and concentrated using a centricon-3 microconcentrator (Amicon Corp., Danvers, MA).

Immunoblot analysis was performed as described (Geiser et al., 1991) except that the blot was incubated with affinity-purified anti-calmodulin antibody for 1 h. Whole cell yeast extracts were made by TCA precipitation (Wright et al., 1989). SDS-PAGE was performed as described (Davis et al., 1986).

Indirect Immunofluorescence

Cells were grown in 10 ml YPD (Geiser et al., 1991) at 30°C. At 100 Klett units (~3.5×10^7 cells/ml), formaldehyde was added to a final concentration of 3.7%. After fixation for a minimum of 1 h at room temperature, the cells were washed once with PBS (Pringle et al., 1989), and then once with SP (1.2 M sorbitol, 100 mM potassium phosphate buffer, pH 6.5). Cells were permeabilized by incubation for 30 to 60 min at 37°C in 1.0 ml SP containing 5 μg/ml zymolyase 100T (ICN-Biomedicals Inc., Costa Mesa, CA) and 3 μl β-mercaptoethanol. After incubation, the culture was washed three times with PBS, resuspended in 100 μl PBSB (PBS containing 1% BSA) and stored at 4°C for up to one week. Cells were then labeled either in solution or when mounted to polylysine-coated slides (Pringle et al., 1989). Mounted cells were labeled for 1 h with a 1:200 dilution of affinity-purified anti-calmodulin antibody as described (Pringle et al., 1989). For labeling in solution, permeabilized cells (5 μl) were incubated for 2 h in 3.0 ml PBSB containing affinity-purified anti-calmodulin antibody (1:500). Cells were then washed twice with PBS and then incubated for 1 h at room temperature with fluorescein-conjugated goat anti-rabbit (1:200 in 1.0 ml PBSB) secondary antibodies (Boehringer Mannheim Biochemicals, Indianapolis, IN). Cells were pelleted in a microfuge (3,000 rpm for 3 min) and then incubated with rhodamine-conjugated phalloidin (Molecular Probes Inc., Eugene, OR) diluted 1:10 in PBSB containing 0.1 μg/ml 4',6-diamidino-2-phenylindole (DAPI). After 15–30 min in the dark, cells were washed five times with PBS, resuspended in 50 μl Citifluor glycerol (Ted Pella Inc., Redding, CA) and put on a slide that had previously been coated with 1.0 mg/ml polylysine (Sigma Chemical Co., St. Louis, MO). When actin was not stained, the incubation with rhodamine-conjugated phalloidin was omitted and the first wash contained 0.1 μg/ml DAPI. Cells containing mutant forms of calmodulin (3D→A, 3E→V, or cmd1-1p) were only faintly stained after a 2-h incubation with affinity-purified anti-calmodulin antibody. Thus, yeast strains containing mutant forms of calmodulin were incubated for 12 h with anti-calmodulin antibody (see Figs. 5, 6, and 7). The mutant yeast strains stain less well than a wild-type strain because (*a*) the mutant proteins are less abundant than wild-type calmodulin (Geiser et al., 1991; and T. N.

Table I. Strains

Strain	Genotype	Reference
CRY1	*MATa ade2-1oc can1-100 his3-11,15 leu2-3,112 trp1-1 ura3-1*	Robert Fuller
CRY2	*MATα ade2-1oc can1-100 his3-11,15 leu2-3,112 trp1-1 ura3-1*	Robert Fuller
JGY46	*MATa/MATα ade2-1oc/ade2-1oc can1-100/can1-100 his3-11,15/his3-11,15 leu2-3,112/leu2-3,112 trp1-1/trp1-1 ura3-1/ura3-1*	Geiser et al., 1991
JGY44-2A	*MATa ade2-1oc can1-100 cmd1-1 his3-11,15 leu2-3,112 trp1-1 ura3-1*	Davis, 1992
TDY55-5D	*MATa ade2-1oc ade3Δ can1-100 cmd1Δ::TRP1 his3-11,15 leu2-3,112 lys2Δ::HIS3 trp1-1 ura3-1*	Geiser et al., 1991 and Davis, 1992
TDY62-2D	*Mata ade2-1oc can1-100 cmd1-1 his3-11,15 leu2-3,112 lys2Δ::HIS3 trp1-1 ura3-1*	Davis, 1992
TDY66	same as TDY62-2D except *sst1::LEU2*	This study
TDY72	TDY66 ×CRY2	This study
TDY72-5D	*Mata ade2-1oc can1-100 his3-11,15 leu2-3,112 sst1::LEU2 trp1-1 ura3-1*	This study
KWY231	*Mata/Matα act1-4/act1-4 ade2-101/ADE2 his3Δ200/his3Δ200 leu2-3,112/LEU2 LYS2/lys2-80 ura3-52/ura3-52*	Ken Wertman
KWY474	*Mata/Matα ACT1::HIS3/ACT1::HIS3 ADE2/ade2 ade4/ADE4 can1-1/can1-1 leu2-3, 112/leu2-3,112 his3Δ200/his3Δ200 tub2-201/tub2-201 ura3-52/ura3-52*	Ken Wertman

Davis, unpublished observation) and (*b*) the affinity-purified anti-calmodulin antibody has a lower affinity for the mutant proteins than for the wild-type protein as judged by immunoblot analysis (data not shown). Stained cells were viewed with an Axioplan fluorescent microscope (Carl Zeiss, Thornwood, NY) and photographed using TMAX 400 professional film (Eastman Kodak Co., Rochester, NY). Cells were printed using different exposure times to show optimum detail. The intensity difference between cells in different prints is not a measure of differences in calmodulin concentrations.

Isolation of Unbudded Cells

Strain CRY1 was grown to ~400 Klett units in YPD. 4 ml of cells were pelleted, resuspended in 0.5 ml YPD, sonicated for 5 s, and then loaded on a 37 ml 12–18% gradient of ficoll (Sigma Chemical Co.) dissolved in YP. Cells were sedimented in an HB-4 swinging bucket rotor in a high speed centrifuge (RC-2B; Sorvall Instruments, Wilmington, DE) for 5 min at 750 rpm (100 *g*). Fractions (0.5 ml) were collected and analyzed by phase contrast microscopy. Fractions 8 and 9 contained >95% unbudded cells. Cells in fractions 8 and 9 were washed, resuspended in 5.0 ml YPD, and incubated at 30°C until ~30% of the culture was budded. The cells were then fixed and prepared for indirect immunofluorescence as described above.

Synchronization Using α-Factor

TDY72-5D was grown in 20 ml YPD at 30°C. At 20 Klett units (7×10^6 cells/ml), α-factor was added to a final concentration of 30 ng/ml. Greater than 95% of the cells had formed a shmoo by 1.5 h. Cells were harvested by centrifugation after 3.0 h, resuspended in 30 ml fresh YPD, and incubated with shaking at 30° C. Aliquots (2.0 ml) were removed and fixed every 5 min for the first 30 min and then every 20 min. The fraction of unbudded and budded cells was determined by phase contrast microscopy. Cells were prepared for indirect immunofluorescence as described above. Over 200 cells were counted for each time point. Three separate experiments gave similar results.

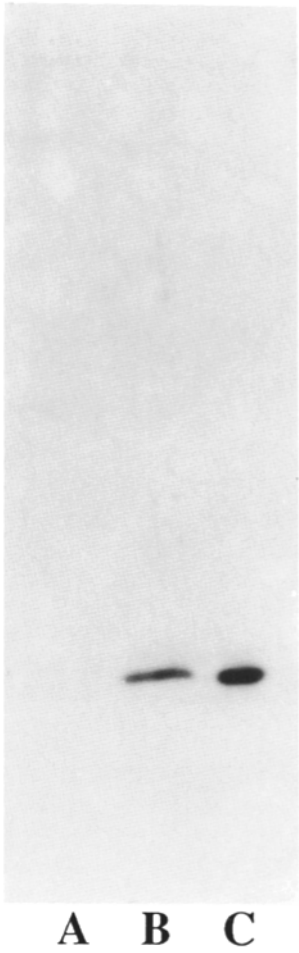

Figure 1. Immunoblot of total yeast proteins probed with affinity-purified anti-calmodulin antibody. Lane *A* contains an aliquot (50 μg) of a crude yeast extract from strain TDY55-5D (*cmd1Δ::TRP1*) carrying pJG60. pJG60 contains a cDNA encoding vertebrate calmodulin under the control of the *CMD1* promoter (see Materials and Methods). Lane *B* contains an aliquot (50 μg) of protein from yeast strain CRY1 (*CMD1*). Lane *C* serves as a positive control and contains 60 ng yeast calmodulin purified from *E. coli* as described (Brockerhoff and Davis, 1992).

Results

Specificity of Anti-Calmodulin Antibodies

Rabbit polyclonal antibodies were made against yeast calmodulin expressed in *E. coli* (Geiser et al., 1991) and the antibody was affinity-purified as described in Materials and Methods. On an immunoblot, the affinity-purified anti-calmodulin antibody reacted primarily with wild-type calmodulin in a crude yeast extract (Fig. 1, lane *B*). The antibody did not interact with any protein with the same mobility as calmodulin in a yeast extract containing vertebrate instead of yeast calmodulin (Fig. 1, lane *A*). Thus, although vertebrate calmodulin can functionally replace yeast calmodulin in yeast cells (Davis and Thorner, 1989; Ohya and Anraku, 1989; Persechini et al., 1991), these two calmodulins do not share the epitopes recognized by the antibody used in this study. High molecular weight bands were barely detectable in the extract containing yeast calmodulin and the extract containing vertebrate calmodulin (Fig. 1, lanes *A* and *B*).

The immunoblot analysis suggested that the affinity-purified antibody identified, albeit weakly, several proteins in addition to calmodulin. However, the results of two other experiments convinced us that the immunofluorescent signal was specific to calmodulin. First, if the affinity-purified antibody was preincubated with a three-fold molar excess of yeast calmodulin prepared from *E. coli* for 30 min before labeling yeast cells, no immunofluorescent staining was visible (data not shown). This result indicated that yeast calmodulin, free of any other yeast protein, was sufficient to compete away the immunofluorescent signal seen in yeast. However, this result did not establish whether the signal was due to an antigenically related protein.

To verify that the immunofluorescent signal was due solely to the presence of calmodulin, we stained yeast cells containing vertebrate instead of yeast calmodulin with the affinity-purified anti-yeast calmodulin antibody. As shown above, the affinity-purified anti-yeast calmodulin antibody did not interact with vertebrate calmodulin. Thus, if all the signal seen in yeast cells is due to calmodulin, cells containing vertebrate instead of yeast calmodulin should not be stained. When cells containing vertebrate calmodulin were stained with affinity-purified anti-yeast calmodulin antibodies, no staining over background was detected (Fig. 2 *A*). DAPI staining (Fig. 2 *B*) aided in determining the location of the cells. In contrast, cells containing wild-type yeast calmodulin were brightly stained by affinity-purified anti-calmodulin antibody and had a distinct staining pattern (Fig. 2 *C* and Fig. 3). From these results, we concluded that our affinity-purified anti-calmodulin antibody specifically localizes yeast calmodulin.

Immunofluorescent Localization of Calmodulin

Calmodulin was localized in an asynchronously growing

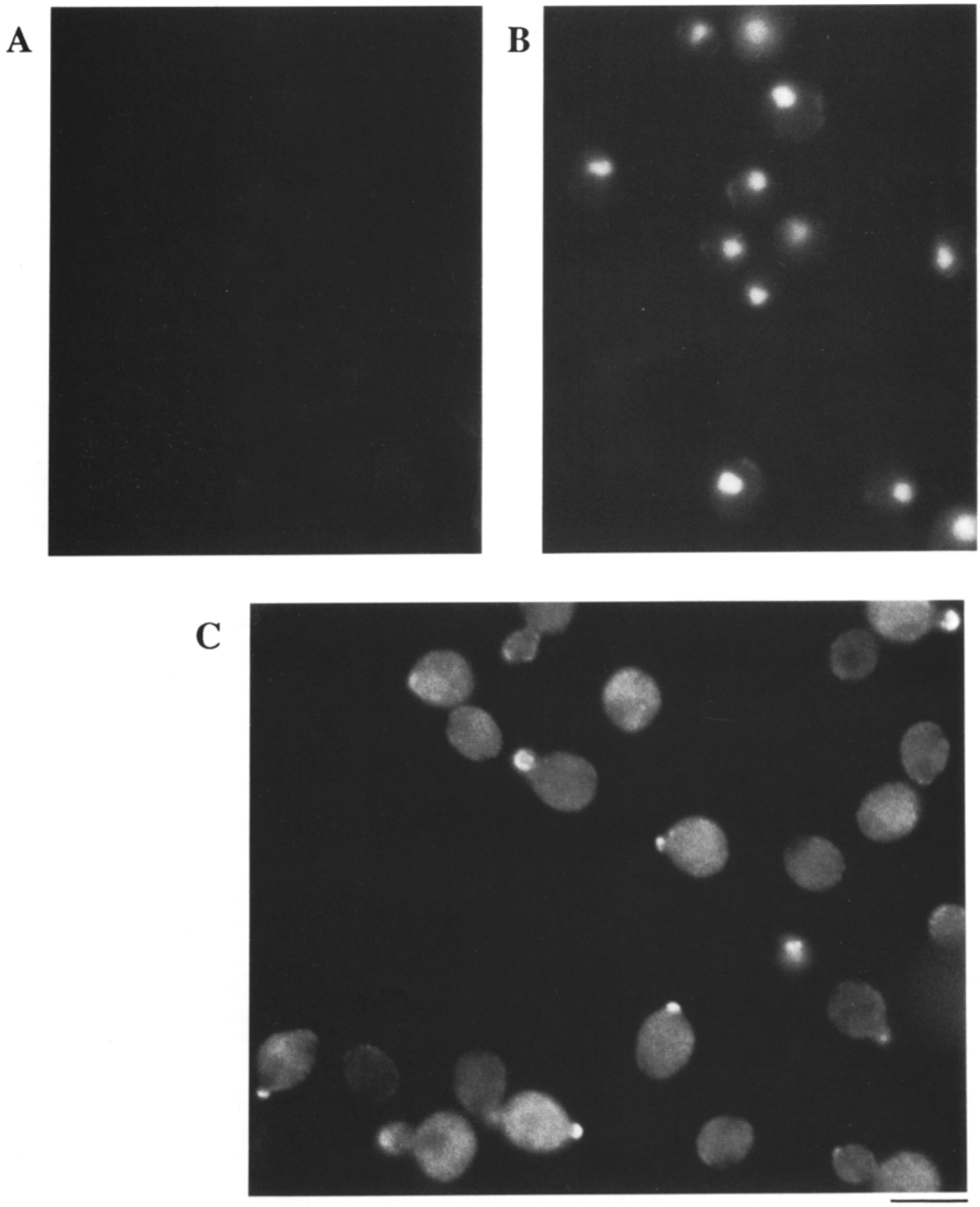

Figure 2. Anti-yeast calmodulin staining of yeast cells expressing vertebrate calmodulin or wild-type yeast calmodulin. Indirect immunofluorescence was done as described in Materials and Methods. Cells were simultaneously stained with DAPI to see the nuclear DNA. (*A*) Strain TDY55-5D (*cmd1Δ::TRP1*) containing pJG60 (see Fig. 1) stained with affinity-purified anti-yeast calmodulin antibody. (*B*) DAPI stain of the same cells as in *A*. (*C*) Strain CRY1 (*CMD1*) stained with affinity-purified anti-yeast calmodulin antibody. Bar, 6 μm.

culture by indirect immunofluorescence as described in Materials and Methods. The calmodulin distribution was asymmetric and changed as cells progressed through the cell cycle (Fig. 3). In 70% of 322 unbudded cells, calmodulin concentrated in a patch (Fig. 3 *A*). 25% did not have a visible patch of calmodulin. The remaining 5% had two patches. In small budded cells calmodulin concentrated throughout the bud (Fig. 3 *B*). As the bud grew, calmodulin accumulated at the

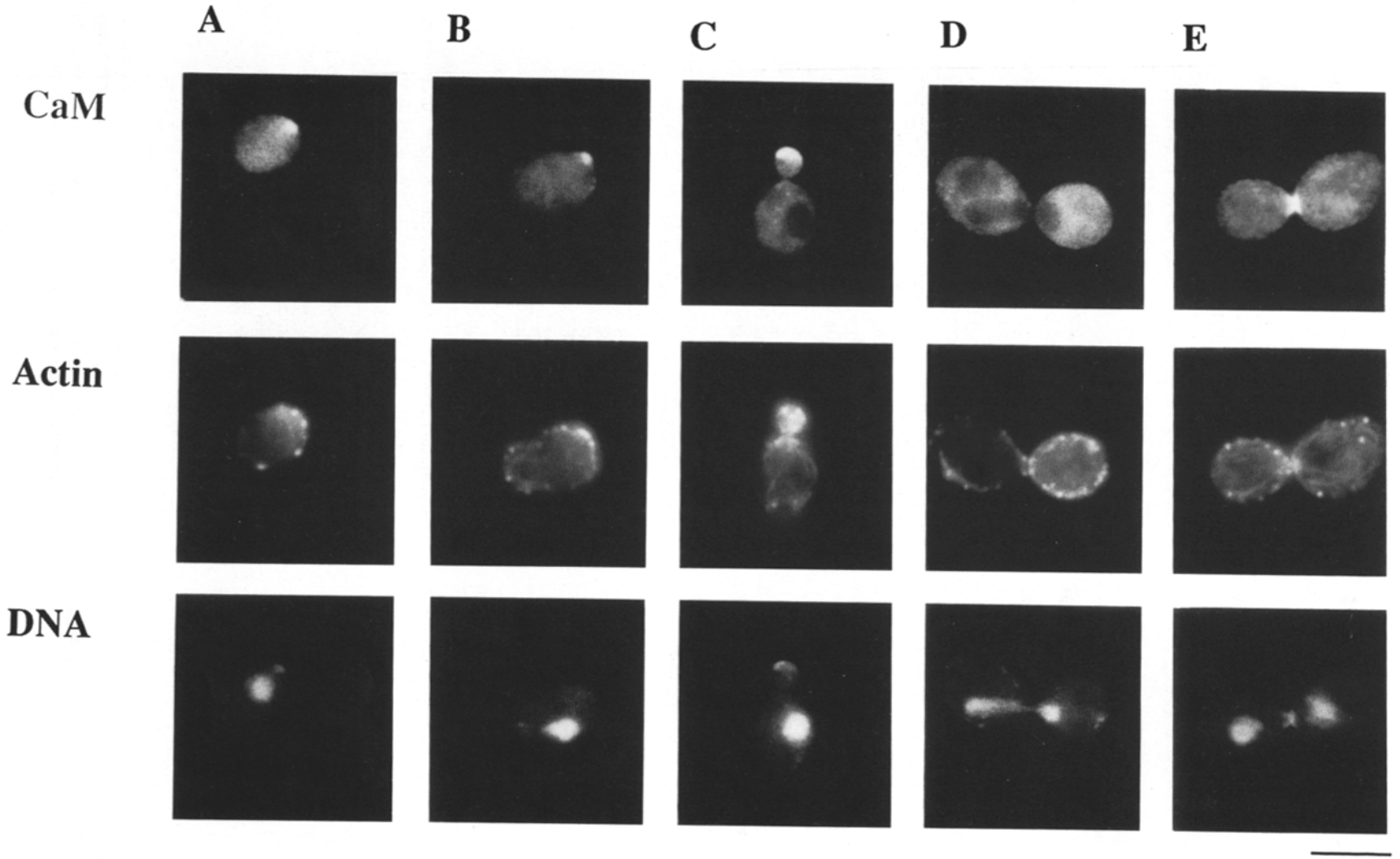

Figure 3. Localization of calmodulin in an asynchronous wild-type culture. Strain JGY46 was stained with affinity-purified anti-calmodulin antibody as described in Materials and Methods. Cells were simultaneously stained with rhodamine-conjugated phalloidin (*middle*) which binds F-actin, and DAPI (*bottom*) to observe the position of the nuclear DNA. Some FITC fluorescence is visible when viewing for DAPI fluorescence. (*A*) unbudded yeast cell; (*B*) small budded yeast cell; (*C*) medium budded yeast cell; (*D*) large budded cell in mitosis (see DAPI); and (*E*) large budded cell during cytokinesis. *CaM*, calmodulin. Bar, 6 μm.

tip (Fig. 3 *C*), dispersed (Fig. 3 *D*), and then finally concentrated in the neck region between mother and daughter cells (Fig. 3 *E*) at cytokinesis. Some cells, which appeared to have completed cytokinesis but not cell separation, had faint patches directly facing each other. The calmodulin distribution during mitosis was diffuse and calmodulin did not appear to concentrate in the nucleus (Fig. 3 *D*) at this time. In many cells, at all stages of the cell cycle, calmodulin appeared less abundant in the nucleus than in other regions of the cell. These results indicate that calmodulin concentrates at sites of cell growth during vegetative growth, namely the bud tip and the neck region in cytokinesis.

Yeast cells also exhibit polarized growth during shmoo formation. To determine whether calmodulin concentrates at the shmoo tip during shmoo formation, we localized calmodulin in cells treated with α-factor. Cells treated with α-factor stained brightly for calmodulin. A patch similar to that observed in unbudded cells in an asynchronous culture was present at the shmoo tip (Fig. 4).

Calmodulin Localization in Synchronized Cells

Calmodulin was localized in a synchronous culture to determine when its distribution becomes polarized in unbudded cells and to determine the percent of budded cells with an asymmetric distribution. Yeast cells were synchronized in G1 by treatment with the mating pheromone α-factor. After release from α-factor arrest, aliquots were taken at various times for 1.5 generations, fixed, counted to determine percent unbudded and budded cells, and then prepared for indirect immunofluorescence.

After release from α-factor, ~10 min before bud emergence, calmodulin concentrated in a patch. Once budded, >95% of the small and medium budded cells had calmodulin throughout the bud or at the bud tip. 50 min after bud formation, 82% of cells were large budded. The majority of large budded cells were either in mitosis and had nuclear DNA stretched between mother cell and bud, or had completed mitosis and had separated nuclei. Of the cells in mitosis, 48 out of 50 had a diffuse calmodulin staining pattern. Of 102 cells with calmodulin in the neck, all had separated nuclei. These results are in agreement with those obtained with an asynchronous culture and indicate that calmodulin polarization occurs before bud emergence.

Calmodulin and Actin Localization

Like calmodulin, actin concentrates in regions of cell growth (Adams and Kilmartin, 1984; Adams and Pringle, 1984). To directly compare the actin and calmodulin distributions we double labeled cells with affinity-purified anti-calmodulin antibody and rhodamine-conjugated phalloidin. Actin cortical patches overlapped with regions containing a high concentration of calmodulin during the cell cycle (Fig. 3). However, the distributions of calmodulin and actin patches were not identical. In some cells calmodulin was concentrated on some of the cortical patches. In general, the region containing actin cortical patches was larger than the region containing calmodulin.

To understand more about the relationship between calmodulin and actin we analyzed the timing of polarization of these two proteins. Small unbudded cells were isolated by velocity sedimentation (see Materials and Methods) and grown until ~30% of the culture was budded. The cells were then fixed, stained with both rhodamine-conjugated phalloidin, and affinity-purified anti-calmodulin antibody and counted to determine the number of unbudded cells that contained a polarized distribution of both calmodulin and actin, just actin, just calmodulin, or neither. If calmodulin polarizes before actin, we should identify cells containing only calmodulin patches. On the other hand, if actin concentrates first, some cells should contain a polarized actin distribution but no calmodulin patches. Of 120 unbudded cells, 35 contained a polarized distribution of both calmodulin and actin and nine had an asymmetric actin distribution but no calmodulin patch. The remaining cells had a nonpolarized distribution of both calmodulin and actin. Thus, the patch of calmodulin in unbudded cells probably forms at the presumptive bud site after actin accumulates at this site.

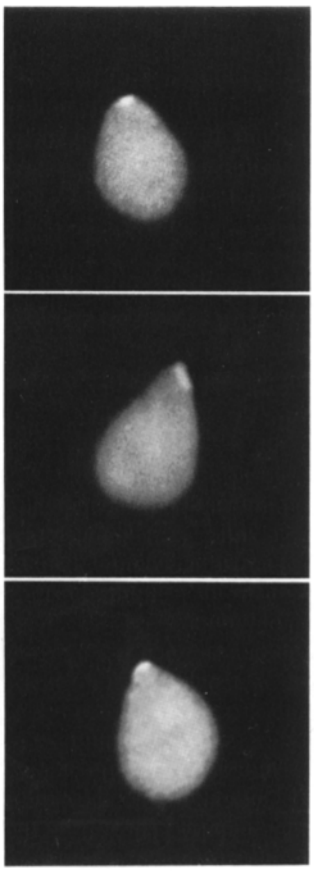

Figure 4. Calmodulin localization in cells treated with α-factor. TDY72-5D was grown in 7 ml YPD to 20 Klett units (7 × 10^6 cells/ml), and then α-factor was added to a final concentration of 30 ng/ml. The cells were then prepared for indirect immunofluorescence as described in Materials and Methods except 15 μg/ml zymolyase instead of 5 μg/ml was used to permeabilize cells. Bar, 9 μm.

Localization of Calmodulin and Actin in Mutant Strains

The distributions of calmodulin and actin overlap during the cell cycle. To determine how disruption of calmodulin function may affect its distribution and the distribution of actin we localized calmodulin and actin in a temperature-sensitive calmodulin mutant *cmdl-1*. For comparison, we analyzed the distribution of calmodulin and actin in an isogenic wild-type strain treated identically. The cells were analyzed after incubation at the nonpermissive temperature (36°C) for 75 min. The percent of budded (75%) and unbudded cells was the

same in both cultures. Calmodulin localization in wild-type cells (n = 408) grown at 36°C was indistinguishable from that seen in the same wild-type strain grown at 21° or 30°C; 94% of the wild-type cells stained with affinity-purified anti-calmodulin antibodies and 72% of these had a polarized calmodulin distribution.

After incubation at the nonpermissive temperature, the cells containing *cmdl-1* differed from the wild-type culture in two respects. First, only 67% of the mutant cells (n = 448) grown at 36°C stained for calmodulin. Second, of those cells that stained, only 30% had a polarized calmodulin distribution. In 70% of the stained cells, calmodulin appeared diffusely distributed throughout the mother cell and bud (Fig. 5, *B* and *C*). The bud often stained more faintly than the mother cell. In contrast to wild-type cells, a diffuse distribution of calmodulin was observed at stages of the cell cycle other than G1 and mitosis as judged by the position of DAPI staining material and by the bud size. Furthermore, while 85% of wild-type cells showed a polarized actin distribution (Fig. 5 *A*), only 30% of the stained *cmdl-1* cells showed a polarized actin distribution. In many *cmdl-1* cells, the disrupted actin cytoskeleton was characterized by mother cells containing few if any actin cables and containing many more cortical dots than usual along the cell periphery (Fig. 5 *C*). Other mutant cells contained a diffuse actin distribution (Fig. 5 *B*). Thus calmodulin appears necessary for maintaining and/or establishing a polarized actin distribution. Calmodulin and actin localization in *cmdl-1* containing cells grown at permissive temperature was indistinguishable from that seen in wild-type cells (data not shown).

Next, we analyzed whether disrupting actin function affected the calmodulin distribution. We examined the calmodulin and actin distributions in a strain containing the temperature-sensitive actin mutation *actl-4* and in a congenic wild-type strain. Under nonpermissive conditions, large unbudded cells accumulate in a strain containing *actl-4* (Dunn and Shortle, 1990). The results described above indicate that the distribution of calmodulin is diffuse in many unbudded cells. To ensure that the observed difference in calmodulin polarization between *actl-4* and a wild-type strain was not just due to the increase in unbudded cells in the mutant culture, we separately compared mutant and wild-type unbudded and budded cells. After 75 min at the restrictive temperature, the actin distribution was polarized in the wild-type strain and most (79%) of the cells stained with affinity-purified anti-calmodulin antibodies (Fig. 6 *A*). Of the stained cells, 65% of the budded cells (n = 105) and 42% of the unbudded cells (n = 146), contained a polarized calmodulin distribution. We noticed that fewer unbudded cells contained a polarized calmodulin distribution in this wild-type strain than in the wild-type strains described above. This difference may be because the strain containing the *actl-4* mutation and the corresponding wild-type strain are in a different genetic background than all other strains used in this study (see Materials and Methods).

In contrast to the congenic control strain, actin was not

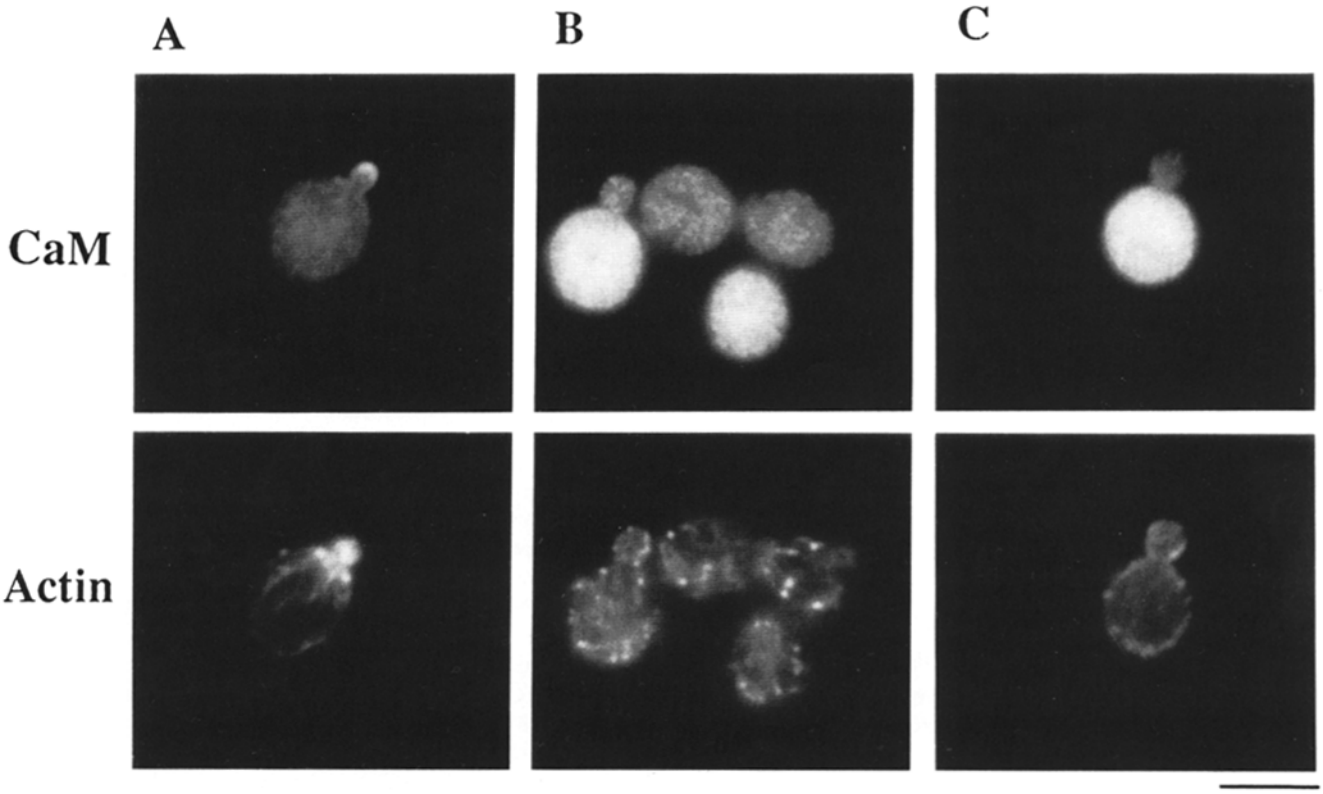

Figure 5. Immunofluorescent localization of actin and calmodulin in a calmodulin mutant. Log phase cultures (6×10^6 cells/ml) of strain CRY1 (*CMDI*) and strain JGY44-2A (*cmdl-1*) grown in YPD were shifted from 21° to 36°C for 75 min. Cells were then fixed and prepared for indirect immunofluorescence as described in Materials and Methods. Greater than 60% of the mutant cells can recover and form colonies if returned to the permissive temperature after 75 min. (*A*) CRY1; (*B* and *C*) JGY44-2A. *CaM*, calmodulin. Bar, 5 μm.

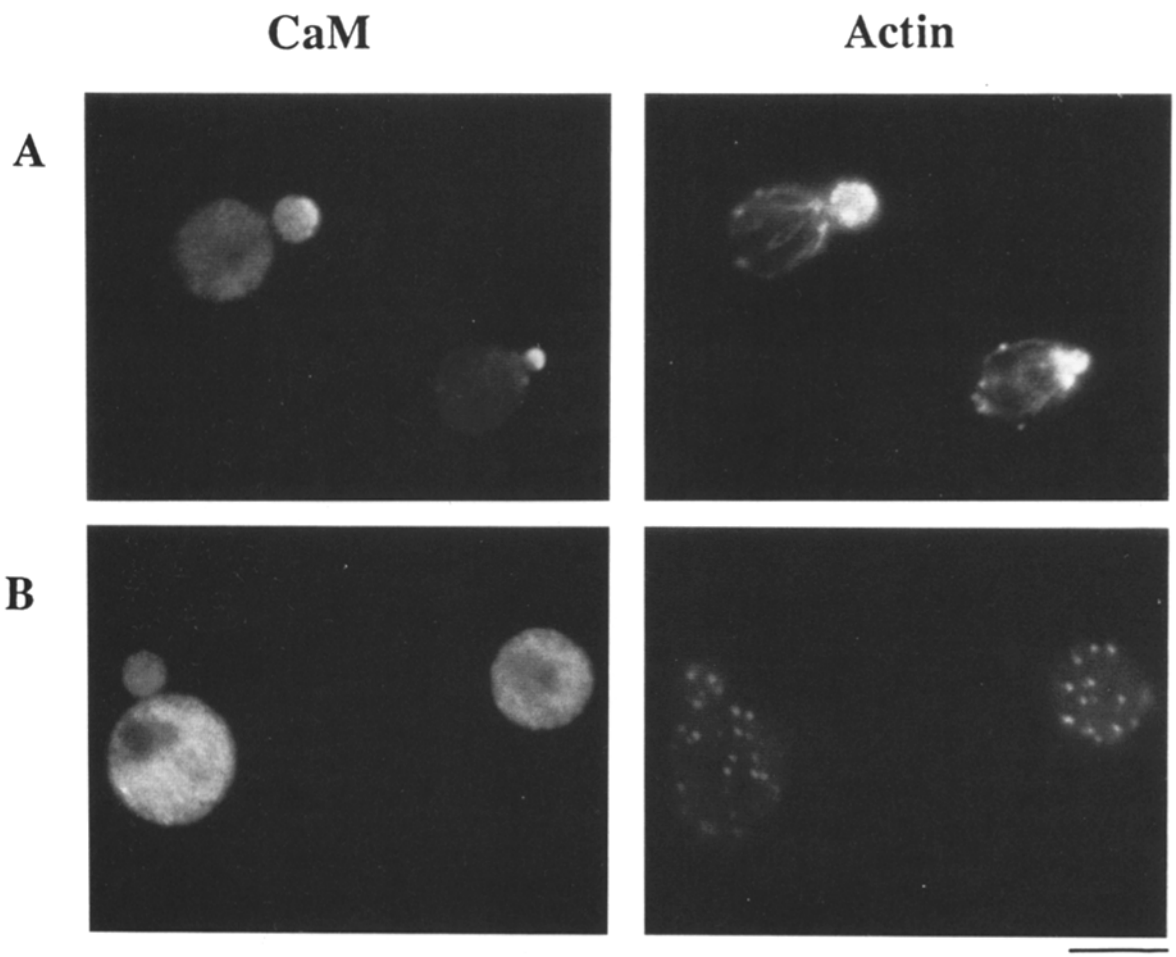

Figure 6. Immunofluorescent localization of actin and calmodulin in an actin mutant. Log phase cultures (6×10^6 cells/ml) of strain KWY474 (*ACT1*) and strain KWY231 (*act1-4*) grown in YPD were shifted from 21° to 36°C for 75 min. Cells were then fixed and prepared for indirect immunofluorescence as described in Materials and Methods. (*A*) KWY474; (*B*) KWY231; *CaM*, calmodulin. Bar, 12 μm.

polarized in the *act1-4* containing strain after incubation under nonpermissive conditions (Fig. 6 *B*). Furthermore, only 30% of the cells containing the *act1-4* mutation stained with anti-calmodulin antibodies. Of the stained unbudded cells (n = 101), only 5% had a polarized distribution of calmodulin. Of the stained budded cells (n = 100), none had a polarized calmodulin distribution (Fig. 6 *B*). Thus calmodulin and actin have a reciprocal effect on establishing and/or maintaining the distribution of each other. However, disrupting actin function has a more dramatic effect on the distribution of calmodulin than disrupting calmodulin has on the polarization of actin.

3D→A and 3E→V Mutant Calmodulins Have the Same Localization As Wild-type Calmodulin

Mutant forms of calmodulin defective in binding Ca^{2+} can support the growth of yeast cells (Geiser et al., 1991). We localized mutant calmodulins, 3D→A and 3E→V, to determine whether the ability to bind Ca^{2+} affects the distribution of calmodulin. Cells containing 3E→V and 3D→A were very similar to wild-type cells with respect to calmodulin localization (compare Fig. 7 and Fig. 3). Calmodulin concentrated at the presumptive bud site, at the bud tip, and in the neck region at cytokinesis in the mutant cells as in wild-type cells. Furthermore, similar to a congenic wild-type culture, 70% of the cells containing 3E→V or 3D→A had a polar calmodulin distribution.

Discussion

Localization of calmodulin by indirect immunofluorescence revealed that calmodulin concentrates at sites of cell growth. Calmodulin and actin concentrate in overlapping regions during the cell cycle. In unbudded cells, calmodulin concentrates in a patch ~10 min before bud emergence shortly after actin. The timing of patch formation and the fact that actin concentrates in the same region indicates that calmodulin is accumulating at the nascent bud site. Calmodulin concentrates throughout the bud in small budded cells and remains in the tip as the bud grows. During mitosis, calmodulin is dispersed throughout the cell and bud, and then moves to the neck during cytokinesis. In cells treated with α-factor, calmodulin concentrates at the shmoo tip. This distribution implicates calmodulin in polarized cell growth in *S. cerevisiae.*

The actin network in yeast cells is required for proper localization of material to the bud. Mutations in actin lead to

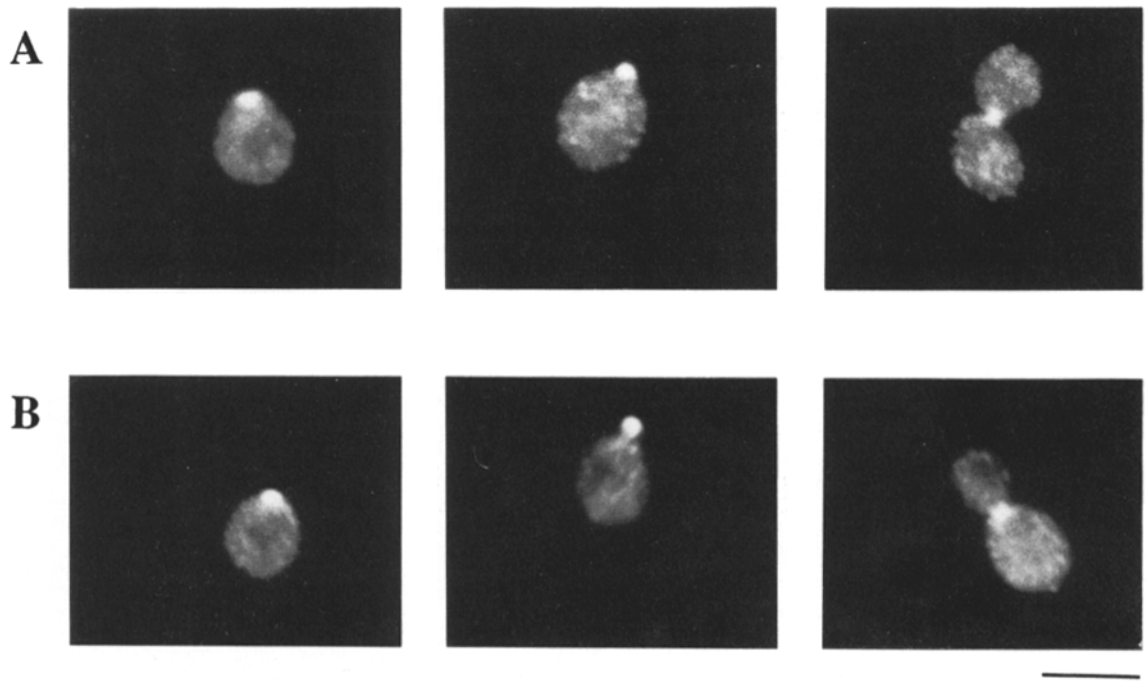

Figure 7. Immunofluorescent localization of mutant calmodulins impaired in binding Ca^{2+}. An asynchronous culture of strain TDY55-5D containing (*A*) pJG19 (3E→V), or (*B*) pJG26 (3D→A), was fixed and prepared for indirect immunofluorescence as described in Materials and Methods except that cells containing pJG19 were incubated with anti-calmodulin antibody diluted 1:1,000. Bar, 4 μm.

a disruption of the actin cytoskeleton and a subsequent defect in polarized cell growth (Novick and Botstein, 1985). To determine the role of calmodulin in polarized cell growth and the relationship between calmodulin and actin in this process, we analyzed the dependence of these molecules on each other for proper localization. We found that the polarized distributions of actin and calmodulin are interdependent: a mutation in actin disperses calmodulin, and conversely, disruption of calmodulin function disorganizes the actin network in most cells. From this result, a strain containing a temperature-sensitive form of calmodulin is predicted to be defective in polarized cell growth under nonpermissive conditions. However, as presented in the accompanying paper, the *cmdl-1* mutant exhibits only a mild defect in bud growth and dies as it proceeds through mitosis. Unlike mutations in actin which severely disrupt the cytoskeleton and completely inhibit bud growth, mutations in calmodulin apparently only partially disrupt the actin cytoskeleton. Thus, calmodulin may facilitate but not be essential for bud growth. Alternatively, the calmodulin function in bud growth may be incompletely inactivated in the *cmdl-1* mutant whereas the function in mitosis is completely abolished.

Mutations in actin-binding proteins also reorganize the actin cytoskeleton but do not prevent bud growth. Yeast strains deleted for the gene encoding the yeast homolog to fimbrin, *SAC6*, are viable at 23°C although they contain few actin cables and the cortical patches are not limited to the bud (Adams et al., 1991). Disruption of the gene encoding tropomyosin leads to a loss of actin cables but does not prevent bud growth (Liu and Bretscher, 1989). Calmodulin shares characteristics with actin-binding proteins, but unlike the distributions of actin and actin-binding proteins, calmodulin, and actin distributions overlap but are mostly not coincident. This suggests that the interaction between calmodulin and actin is indirect. Consistent with this possibility, a direct interaction between mammalian calmodulin and actin has not been detected although diverse assays have been used (Luby-Phelps et al., 1985; Piazza and Wallace, 1985).

The distribution of Spa2p and calmodulin in yeast cells is strikingly similar (Gehrung and Snyder, 1990; Snyder, 1989; Snyder et al., 1991) suggesting that these two proteins may interact. However, Spa2p and calmodulin appear to play significantly different roles in the polarization of yeast cells. Neither actin nor Spa2p are necessary for maintaining the localization of the other. Actin mutants properly localize Spa2p (Snyder et al., 1991) and the majority of cells in a culture of a *spa2* mutant have a normal distribution of actin (Gehrung and Snyder, 1990). Thus, although Spa2p and calmodulin share a similar distribution, current knowledge of their functions suggests that they are not components of the same complex.

Chitin (Hayashibe and Katohda, 1973) and the components of the 10-nm filaments, Cdc3p and Cdc12p, accumulate at the nascent bud site shortly before bud emergence as does calmodulin (Haarer and Pringle, 1987; Kim et al., 1991). Distinct from calmodulin, chitin, and the components of the 10-nm filaments remain at the neck between the mother and the bud during the cell cycle. The 10-nm filaments are assembled early in the cell cycle but are not required until cytokinesis (Haarer and Pringle, 1987; Kim et al., 1991). The temperature-sensitive calmodulin mutant displays a severe defect in cytokinesis if shifted to the nonpermissive temperature from early G1 but not if shifted to the nonpermissive temperature from later G1 or G2 (Davis,

1992) suggesting that calmodulin is required for the arrangement of the 10-nm filaments. In the absence of calmodulin in early G1, the 10-nm filaments may either not form or assemble incorrectly resulting in an inhibition of cytokinesis.

Results of a genetic analysis suggest that an unconventional myosin encoded by the *MYO2* gene acts as the molecular motor to transport secretory vesicles along actin cables to the site of bud development. Under nonpermissive conditions, a strain containing *myo2-66* arrests as a large unbudded cell with an abnormal actin distribution and an accumulation of secretory vesicles (Johnston et al., 1991). The unconventional myosin is a likely target for calmodulin action since it has six putative calmodulin-binding sites. Furthermore, a related myosin from bovine brain, P_{190}, has been shown to bind calmodulin in a Ca^{2+}-independent manner (Johnston et al., 1991; Larson et al., 1988). In yeast, mutant calmodulins in which the Ca^{2+}-binding sites have been inactivated show a distribution very similar to wild-type calmodulin. Thus, Ca^{2+}-binding is not required for a polarized distribution of calmodulin. One model is that calmodulin binds directly to Myo2p in a Ca^{2+}-independent manner and facilitates bud growth.

The characterization of the temperature-sensitive mutant reported in the accompanying paper and the immunolocalization reported here highlight different aspects of calmodulin function. The temperature-sensitive mutant shows a severe defect in chromosome segregation and cannot survive even a single mitosis at the nonpermissive temperature (Davis, 1992). The concentration of calmodulin at sites of cell growth shown in this paper does not suggest a mitotic function. In fact, calmodulin appears to be less abundant in the nucleus than in the cytoplasm. We have thought of two possible explanations for these seemingly contradictory results. One is that calmodulin performs an unknown cytoplasmic function that affects chromosome segregation. Alternatively, the mitotic function may require calmodulin in the nucleus at concentrations below our level of detection. In higher eukaryotic organisms, calmodulin localizes on kinetochore microtubules (Vantard et al., 1985; Welsh et al., 1979). Since yeast have only one kinetochore microtubule per chromosome, as opposed to many in higher eukaryotes (Peterson and Ris, 1976), a similar distribution in yeast might be difficult to detect.

In conclusion, calmodulin localizes at sites of cell growth and is involved in the process of polarized growth in yeast. Calmodulin depends on actin for proper localization and disruption of calmodulin function delocalizes actin. Calmodulin may be interacting with the unconventional myosin, Myo2p, recently identified. Future studies will continue to examine the relationships between the proteins involved in polarized cell growth.

We thank J. R. Geiser for critical reading of the manuscript. We thank Dr. J. B. Hurley for darkroom equipment and advice on darkroom procedures.

This work was supported by grants to T. N. Davis from the National Institutes of Health (GM40506) and the Searle Scholars Program/The Chicago Community Trust (88-G-116).

Received for publication 6 February 1992 and in revised form 1 May 1992.

References

Adams, A. E. M., and J. V. Kilmartin. 1984. Structural rearrangements of tubulin and actin during the cell cycle of the yeast *Saccharomyces*. *J. Cell Biol.* 98:922–933.

Adams, A. E. M., and J. R. Pringle. 1984. Relationship of actin and tubulin distribution to bud growth in wild-type and morphogenetic-mutant *Saccharomyces cerevisiae*. *J. Cell Biol.* 98:934–945.

Adams, A. E. M., D. Botstein, and D. G. Drubin. 1991. Requirement of yeast fimbrin for actin organization and morphogenesis *in vivo*. *Nature (Lond.)*. 354:404–408.

Brockerhoff, S. E., and T. N. Davis. 1992. Structural analysis of wild-type and mutant yeast calmodulins by limited proteolysis and electrospray ionization mass spectrometry. *Prot. Sci.* 1:504–516.

Cohen, P., and C. B. Klee. 1988. Calmodulin. Molecular Aspects of Cellular Regulation. Elsevier, New York. 371 pp.

Davis, T. N. 1992. A temperature-sensitive calmodulin mutant loses viability during mitosis. *J. Cell Biol.* 118:607–617.

Davis, T. N., and J. Thorner. 1989. Vertebrate and yeast calmodulin, despite significant sequence divergence, are functionally interchangeable. *Proc. Natl. Acad. Sci. USA.* 86:7909–7913.

Davis, T. N., M. S. Urdea, F. R. Masiarz, and J. Thorner. 1986. Isolation of the yeast calmodulin gene: calmodulin is an essential protein. *Cell.* 47: 423–431.

Drubin, D. G. 1990. Actin and actin-binding proteins in yeast. *Cell Motil. Cytoskeleton.* 15:7–11.

Dunn, T. M., and D. Shortle. 1990. Null alleles of SAC7 suppress temperature-sensitive actin mutations in *Saccharomyces cerevisiae*. *Mol. Cell Biol.* 10:2308–2314.

Gehrung, S., and M. Snyder. 1990. The SPA2 gene of *Saccharomyces cerevisiae* is important for pheromone-induced morphogenesis and efficient mating. *J. Cell Biol.* 111:1451–1464.

Geiser, J. R., D. van Tuinen, S. E. Brockerhoff, M. M. Neff, and T. N. Davis. 1991. Can calmodulin function without binding calcium? *Cell.* 65:949–959.

Haarer, B., and J. R. Pringle. 1987. Immunofluorescence localization of the *Saccharomyces cerevisiae CDC12* gene product to the vicinity of the 10 nm filaments in the mother-bud neck. *Mol. Cell Biol.* 7:3678–3687.

Hayashibe, M., and S. Katohda. 1973. Initiation of budding and chitin ring. *J. Gen. Appl. Microbiol.* 19:23–39.

Howe, C. L., and M. S. Mooseker. 1983. Characterization of the 110-kDa actin-, calmodulin-, and membrane-binding protein from microvilli of intestinal epithelial cells. *J. Cell Biol.* 97:974–985.

Johnston, G. C., J. A. Prendergast, and R. A. Singer. 1991. The *Saccharomyces cerevisiae MYO2* gene encodes an essential myosin for vectorial transport of vesicles. *J. Cell Biol.* 113:539–551.

Kim, H. B., B. K. Haarer, and J. R. Pringle. 1991. Cellular morphogenesis in the *Saccharomyces cerevisiae* cell cycle: localization of the CDC3 gene product and the timing of events at the budding site. *J. Cell Biol.* 112:535–544.

Larson, R. E., D. E. Pitta, and J. A. Ferro. 1988. A novel 190 kDa calmodulin-binding protein associated with brain actomyosin. *Brazilian J. Med. Biol. Res.* 21:213–217.

Liu, H., and A. Bretscher. 1989. Disruption of the tropomyosin gene in yeast results in the disappearance of actin cables from the cytoskeleton. *Cell.* 57:233–242.

Liu, H., and A. Bretscher. 1989. Purification of tropomyosin from *Saccharomyces cerevisiae* and identification of related proteins in *Schizosaccharomyces* and *Physarum*. *Proc. Natl. Acad. Sci. USA.* 86:90–93.

Luby-Phelps, K., F. Lanni, and D. L. Taylor. 1985. Behavior of a fluorescent analogue of calmodulin in living 3T3 cells. *J. Cell Biol.* 101:1245–1256.

Magdolen, V., U. Oechsner, G. Muller, and W. Bandlow. 1988. The intron-containing gene for yeast profilin (PFY1) encodes a vital function. *Mol. Cell Biol.* 8:5108–5115.

Novick, P., and D. Botstein. 1985. Phenotypic analysis of temperature-sensitive yeast actin mutants. *Cell.* 40:405–416.

Ohya, Y., and Y. Anraku. 1989. Functional expression of chicken calmodulin in yeast. *Biochem. Biophys. Res. Commun.* 158:541–547.

Persechini, A., R. H. Kretsinger, and T. N. Davis. 1991. Calmodulins with deletions in the central helix functionally replace the native protein in yeast cells. *Proc. Natl. Acad. Sci. USA.* 88:449–452.

Peterson, J. B., and H. Ris. 1976. Electron-microscopic study of the spindle and chromosome movement in the yeast *Saccharomyces cerevisiae*. *J. Cell. Sci.* 22:219–242.

Piazza, G. A., and R. W. Wallace. 1985. Calmodulin accelerates the rate of F-actin polymerization. *Proc. Natl. Acad. Sci. USA.* 82:1683–1684.

Pringle, J. R., R. A. Preston, A. E. M. Adams, T. Stearns, D. G. Drubin, B. K. Haarer, and E. W. Jones. 1989. Fluorescence microscopy methods for yeast. *Meth. Cell Biol.* 31:357–435.

Rasmussen, C. D., and A. R. Means. 1989. Calmodulin is required for cell-cycle progression during G1 and mitosis. *EMBO (Eur. Mol. Biol. Organ.) J.* 8:73–82.

Rasmussen, C. D., R. L. Means, K. P. Lu, G. S. May, and A. R. Means. 1990. Characterization and expression of the unique calmodulin gene of Aspergillus nidulans. *J. Biol. Chem.* 265:13767–13775.

Rothstein, R. J. 1983. One-step gene disruption in yeast. *Methods Enzymol.* 101:202–211.

Sloat, B. F., A. Adams, and J. R. Pringle. 1981. Roles of the CDC24 gene product in cellular morphogenesis during the *Saccharomyces cerevisiae* cell

cycle. *J. Cell. Biol.* 89:395–405.
Snyder, M. 1989. The SPA2 protein of yeast localizes to sites of cell growth. *J. Cell Biol.* 108:1419–1429.
Snyder, M., S. Gehrung, and B. D. Page. 1991. Studies concerning the temporal and genetic control of cell polarity in *Saccharomyces cerevisiae*. *J. Cell Biol.* 114:515–532.
Sobue, K., Y. Muramoto, M. Fujita, and S. Kakiuchi. 1981. Purification of a calmodulin-binding protein from chicken gizzard that interacts with F-actin. *Proc. Natl. Acad. Sci. USA.* 78:5652–5655.
Takakuwa, Y., T. Ishibashi, and N. Mohandas. 1990. Regulation of red cell membrane deformability and stability by skeletal protein network. *Biorheology.* 27:357–365.
Takeda, T., and M. Yamamoto. 1987. Analysis and *in vivo* disruption of the gene coding for calmodulin in *Schizosaccharomyces pombe*. *Proc. Natl. Acad. Sci. USA.* 84:3580–3584.
Tanaka, T., K. Kadowaki, E. Lazarides, and K. Sobue. 1991. Ca2(+)-dependent regulation of spectrin/actin interaction by calmodulin and protein 4.1. *J. Biol. Chem.* 266:1134–1140.
Vantard, M., A. M. Lambert, J. DeMey, P. Picquot, and L. J. Van Eldik. 1985. Characterization and immunocytochemical distribution of calmodulin in higher plant endosperm cells: localization in the mitotic apparatus. *J. Cell Biol.* 101:488–499.
Wallis, J. W., G. Chrebet, G. Brodsky, M. Rolfe, and R. Rothstein. 1989. A hyper-recombination mutation in *S. cerevisiae* identifies a novel eukaryotic topoisomerase. *Cell.* 58:409–419.
Watts, F. Z., G. Shiels, and E. Orr. 1987. The yeast MYO1 gene encoding a myosin-like protein is required for cell division. *EMBO (Eur. Mol. Biol. Organ.) J.* 6:3499–3505.
Welsh, M. J., J. R. Dedman, B. R. Brinkley, and A. R. Means. 1978. Calcium-dependent regulator protein: localization in the mitotic apparatus of eukaryotic cells. *Proc. Natl. Acad. Sci. USA.* 75:1867–1871.
Welsh, M. J., J. R. Dedman, B. R. Brinkley, and A. R. Means. 1979. Tubulin and calmodulin. *J. Cell Biol.* 81:624–634.
Wright, A. P. H., M. Bruns, and B. S. Hartley. 1989. Extraction and rapid inactivation of proteins from *Saccharomyces cerevisiae* by trichloroacetic acid precipitation. *Yeast.* 5:51–53.