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# Calmodulin localizes to the spindle pole body of *Schizosaccharomyces pombe* and performs an essential function in chromosome segregation

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## SUMMARY

**The essential calmodulin genes in both *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe* were precisely replaced with genes encoding fusions between calmodulin and the green fluorescent protein (GFP). In living budding yeast the GFP-calmodulin fusion protein (GFP-Cmd1p) localized simultaneously to sites of cell growth and to the spindle pole body (SPB), the yeast analog of the centrosome. Having demonstrated proper localization of GFP-calmodulin in budding yeast, we examined the localization of a fusion between GFP and calmodulin (GFP-Cam1p) in fission yeast, where calmodulin had not been localized by any method. We find GFP-Cam1p also localizes both to sites of polarized cell growth and to the fission yeast SPB. The localization of calmodulin to the SPB by GFP fusion was confirmed by indirect immunofluorescence. Antiserum to *S. pombe* calmodulin labeled the ends of the mitotic spindle stained with anti-tubulin antiserum. This pattern was identical to that seen using antiserum to Sad1p, a known SPB component. We then characterized the defects in a temperature-sensitive *S. pombe* calmodulin mutant. Mutant *cam1-E14* cells synchronized in S phase completed DNA synthesis, but lost viability during transit of mitosis. Severe defects in chromosome segregation, including hypercondensation, fragmentation, and unequal allocation of chromosomal material were observed. Immunofluorescence analysis of tubulin revealed a population of cells containing either broken or mislocalized mitotic spindles, which were never observed in wild-type cells. Taken together with the subcellular localization of calmodulin, the observed spindle and chromosome segregation defects suggest that calmodulin performs an essential role during mitosis at the fission yeast SPB.**

Key words: Calmodulin, Mitosis, Centrosome, GFP, Yeast

## INTRODUCTION

Calmodulin is a small eukaryotic protein that reversibly binds $Ca^{2+}$. Study of calmodulin in genetically tractable organisms has yielded many insights into calmodulin function. Calmodulin is essential for proliferation in fungi, including the yeasts *Saccharomyces cerevisiae* (Davis et al., 1986), and *Schizosaccharomyces pombe* (Takeda and Yamamoto, 1987), as well as the filamentous fungi *Aspergillus nidulans* (Rasmussen et al., 1990). The function of calmodulin has been extensively studied in *S. cerevisiae* with appreciable work also performed in *A. nidulans*. Although both species are fungi, the relative importance of the $Ca^{2+}$-dependent functions for calmodulin differ markedly.

In *S. cerevisiae*, the $Ca^{2+}$-binding function of calmodulin is dispensable for cell growth and division. Mutant calmodulins in which the $Ca^{2+}$-binding sites are inactivated support growth (Geiser et al., 1991). Furthermore, neither the $Ca^{2+}$-calmodulin-dependent protein kinase nor the $Ca^{2+}$-calmodulin-dependent phosphatase calcineurin are required for growth (Cyert et al., 1991; Cyert and Thorner, 1992; Moser et al., 1996; Ohya et al., 1991; Pausch et al., 1991). However, $Ca^{2+}$-calmodulin and the $Ca^{2+}$-calmodulin dependent enzymes are required for survival in mating pheromone and for maintaining ion homeostasis (Cunningham and Fink, 1996; Moser et al., 1996; Pozos et al., 1996). Thus, the conservation of the $Ca^{2+}$-binding sites in calmodulin throughout evolution is readily explained.

Diverse techniques have identified two essential $Ca^{2+}$-independent functions for calmodulin during the *S. cerevisiae* cell cycle. First, calmodulin is required to bind Spc110p, a coiled-coil protein that connects the inner and central layers of the spindle pole body (SPB), the yeast mitotic microtubule organizing center (Geiser et al., 1993; Kilmartin et al., 1993; Stirling et al., 1994). Calmodulin localizes to the SPB and this localization is dependent on the calmodulin-binding site in Spc110p (Spang et al., 1996). This interaction is essential for proper assembly of spindle pole components because mutations in the C-terminal calmodulin-binding site of Spc110p result in the formation of large electron-dense structures that appear to nucleate microtubules (Sundberg et al., 1996). The presence of calmodulin at the SPB is also required for proper function of the spindle during mitosis (Davis, 1992; Kilmartin and Goh, 1996; Stirling et al., 1996; Sun et al., 1992a; Sundberg et al., 1996).

Calmodulin is also important for polarized growth in

*S. cerevisiae* (Davis, 1992; Ohya and Botstein, 1994). The polarized growth function of budding yeast calmodulin is mediated by interaction with an unconventional type V myosin, Myo2p (Brockerhoff et al., 1994). Calmodulin binds in a $Ca^{2+}$-independent manner in vitro to a region of Myo2p containing IQ sites (Brockerhoff et al., 1994). This binding is believed to be responsible for the co-localization of calmodulin and Myo2p throughout the cell cycle (Brockerhoff et al., 1992, 1994; Lillie and Brown, 1994; Sun et al., 1992b).

In striking contrast to *S. cerevisiae*, *A. nidulans* requires $Ca^{2+}$-calmodulin for cell cycle progression. *A. nidulans* calmodulins mutated in the $Ca^{2+}$-binding sites do not support growth and division. Calmodulin appears to function at two points in the *A. nidulans* cell cycle (Rasmussen et al., 1992). Deletion of the gene encoding the calcineurin A subunit causes *A. nidulans* to arrest early in the cell cycle, suggesting an essential role for $Ca^{2+}$-calmodulin at the $G_1$ to S phase transition (Rasmussen et al., 1994). Later in the cell cycle, depletion of calmodulin blocks the ability of *A. nidulans* to progress from a *nimT* block between $G_2$ and M (Lu et al., 1993). The unique gene encoding *A. nidulans* $Ca^{2+}$-calmodulin-dependent protein kinase is also essential (Means, 1994). Thus, *A. nidulans* requires $Ca^{2+}$-calmodulin during cell cycle progression to activate both calcineurin and $Ca^{2+}$-calmodulin-dependent protein kinase. If calmodulin functions at the SPB or at sites of polarized growth and whether these putative functions are $Ca^{2+}$-dependent in *A. nidulans* is not known.

*S. pombe* requires at least one intact $Ca^{2+}$-binding site for growth (Moser et al., 1995). Thus, like *A. nidulans*, *S. pombe* appears to require $Ca^{2+}$-calmodulin for some aspect of cellular proliferation. The importance of $Ca^{2+}$-binding to calmodulin in fission yeast might suggest that $Ca^{2+}$-dependent calmodulin targets that are essential in *A. nidulans* might also be essential in *S. pombe*. However, the only $Ca^{2+}$-dependent calmodulin binding protein found so far in *S. pombe*, calcineurin, is not essential for growth although over-expression of calcineurin does affect SPB and nuclear positioning (Yoshida et al., 1994). Essential $Ca^{2+}$-calmodulin dependent enzymes may yet be found in *S. pombe*. Alternatively, the essential $Ca^{2+}$-independent SPB and polarized growth functions of calmodulin found in *S. cerevisiae* might also exist in *S. pombe*, but they may be $Ca^{2+}$-dependent. As a starting point in assessing the essential roles of calmodulin in *S. pombe*, the subcellular localization of calmodulin was determined and compared to that in *S. cerevisiae*. We also characterized the defects conferred by temperature-sensitive mutations in *S. pombe* calmodulin. These cytological and genetic methods suggest that calmodulin functions in *S. pombe* strongly resemble those previously identified in *S. cerevisiae*, thus suggesting universal functions for calmodulin.

## MATERIALS AND METHODS

### Media

*S. cerevisiae* rich medium was YPD and minimal medium was SD (Sherman et al., 1986) supplemented as described (Geiser et al., 1991). Uracil auxotrophs were isolated on SD complete medium containing 1 mg/ml 5-fluoro-orotic acid (5-FOA) (Toronto Research).

*S. pombe* rich media were YE (Gutz et al., 1972) and minimal medium (EMM2) (Mitchison, 1970) supplemented with 100 μg/ml adenine, leucine and uracil. Either 5-FOA or G418 (Gibco BRL) were added to YE agar at mg/ml and 100 μg/ml, respectively.

### Plasmids

#### *S. pombe*

Plasmid pZA59 containing GFP engineered with a C-terminal Gly-Ala spacer and N- and C-terminal *Bsp*HI restriction sites that can be inserted upstream of a gene containing an *Nco*I site at the initiating ATG was constructed by PCR amplification using plasmid TU#65 (Chalfie et al., 1994) as a template with primers 5′GGTCATGAG-TAAAGGAGAAGAAC3′ and 5′CGTACCTACTTGATATGTT TCCGCGGCCGCGACCACGACCACGACCGCGGTAGTACTT TATCC3′. The PCR product was cloned using the pGEM T-Vector (Promega). Plasmid pZA66 encoding S65T-GFP (Heim et al., 1995) lacking an internal *Nco*I site was constructed by oligo-mediated mutagenesis (Kunkel et al., 1987) with pZA59 as a template using primer: 5′GGAAAACTACCTGTTCCTTGGCCAACACTTGTCAC-TACTTTCACTTATGGTGTTCAATGC3′. Plasmid pZA61 (*ars1, LEU2, GFP::cam1*$^+$), plasmid pZA72 (*ars1, LEU2, S65T-GFP::cam1*$^+$) and plasmid pZA73 (*ars1, LEU2, S65T-GFP::cam1-E14*) containing N-terminal fusions of GFP to *S. pombe cam1* were constructed by ligating the 747 bp *Bsp*HI fragments from either plasmid pZA59 or pZA66 into the unique *Nco*I site at the initiating ATG of *cam1*$^+$ in plasmids pIRT2/CAM1ΔINT (*ars1, LEU2, cam1*$^+$*Δintron*) (Moser et al., 1995) and pZA70 (*ars1, LEU2, S65T-GFP::cam1-E14Δintron*).

#### *S. cerevisiae*

The 747 bp *Bsp*HI fragment from pZA66 containing S65T-GFP was ligated in frame to the *CMD1* gene at the initiating ATG at the unique *Nco*I sites in plasmid pJG7 (Geiser et al., 1991), and plasmids pHS9 and pHS24 (Geiser et al., 1993); resulting in plasmid pMM135 (*CEN, URA3* and *S65T-GFP::CMD1)*, plasmid pMM137 (*S65T-GFP::cmd1-6*), and plasmid pMM138 (*S65T-GFP::cmd1-1*), respectively.

### Strains

#### *S. pombe*

The genotype of wild-type strain MP5-1C is *h*$^-$, *ade6-M216, cam1*$^+$, *leu1-32, ura4-D18*; that of MP33-6B is *h*$^-$, *ade6-M210, cam1*$^+$, *leu1-32, ura4-D18*. Calmodulin mutant strains MP64 (*h*$^-$, *ade1-D25, ade6-M210, cam-E14, leu1-32, ura4-D18*), MP76 (*h*$^-$, *ade1-D25, ade6-M210, GFP::cam1*$^+$, *leu1-32, ura4-D18*) and MFP7 (*h*$^-$, *ade1-D25, ade6-M210, S65T-GFP::cam1*$^+$, *leu1-32, ura4-D18*) were constructed as follows. Strain MP24-6C (*h*$^-$, *ade1-D25, ade6-M210, cam1Δ::ura4*$^+$, *leu1-32, ura4-D18*) containing plasmid pADE1/VCAM encoding G418 resistance, *ade1*$^+$, and vertebrate calmodulin (Moser et al., 1995) was transformed with plasmid pZA10 (*cam-E14*) (Moser et al., 1995), plasmid pZA61 (*GFP::cam1*$^+$), or plasmid pZA72 (*LEU2, S65T-GFP::cam1*$^+$) and leucine prototrophs were selected. Leu$^+$ transformants that had lost the pADE1/VCAM plasmid were identified as those unable to produce red colony pigment and sensitive to G418. Strains dependent solely on *LEU2* plasmid encoded mutant calmodulin were first grown on YE medium and then colonies resistant to 5-FOA were selected. Resistant colonies had undergone recombination between the plasmid encoded *cam1* sequences and the chromosomal *cam1Δ::ura4*$^+$ locus. Strains resistant to 5-FOA were screened for uracil and leucine auxotrophy to isolate integrant strains that had undergone replacement and then lost the plasmids. Proper integration was confirmed by Southern blot analysis. The identity of the *cam1-E14* mutations introduced into strain MP64 were further checked by PCR and DNA sequence analysis. In an attempt to localize Cam1-E14p within cells, a fusion of S65T-GFP to *cam1-E14* carried on plasmid pZA73 was introduced into MP24-6C. We were unable to isolate FOA resistant strains that were leucine auxotrophs. Southern blot analysis showed that the FOA resistant, leucine prototroph strains we did isolate contained multiple copies of the *GFP::cam1-E14* plasmid integrated at the *cam1* locus. The GFP-calmodulin in these cells was overexpressed at levels 15 times higher than the wild-type GFP fusion, as measured by FACS (data not shown).

As a consequence, GFP-Cam1-E14p fusion localized uniformly throughout the cell at both permissive and non-permissive temperature, as did plasmid encoded fusion protein. Fusion of GFP to the temperature-sensitive calmodulin encoded by *cam1-E14* is a lethal mutation that can only be rescued by overexpression.

### *S. cerevisiae*

Strain MMY28 (*MAT***a**; *ade2-1oc*; *ade3Δ-100*; *can1-100*; *S65T-GFP::CMD1*, *his3-11,15*, *leu2,3-112*; *lys2Δ::HIS3*; *trp1-1*; *ura3-1*) was constructed as follows. Strain TDY55-5D (*MAT***a**; *ade2-1oc*; *ade3Δ-100*; *can1-100*; *cmd1Δ::TRP1*; *his3-11,15*; *leu2,3-112*; *lys2Δ::HIS3*; *trp1-1*; *ura3-1*) (Geiser et al., 1991) containing a *cmd1-1*, *URA3* plasmid pTD79 (Davis, 1992) was transformed with a gel purified 2.3 kb *Sal*I/*Bam*HI fragment from plasmid pMM135. After selecting for growth at 37°C, colonies resistant to 5-FOA were selected. Strains resistant to 5-FOA were screened for uracil and tryptophan auxotrophy, and considered to be integrants of *S65T-GFP::CMD1*. Proper integration in MMY28 was confirmed by Southern blot, PCR, and DNA sequence analysis. Plasmids pMM135, pMM137, and pMM138 were introduced into strain TDY55-5D containing plasmid pTD56 (*2μ*, *ADE3*, *LYS2*, *CMD1*) (Geiser et al., 1991). Only pMM135 and pMM137 encoding GFP-Cmd1p and GFP-Cmd1-6p could replace the Cmd1p from plasmid pTD56, forming white sectoring colonies from which $Lys^-$, $Ura^+$ strains incapable of red pigment formation could be isolated. Plasmid pMM138 does not allow white sector formation, indicating that the GFP-Cmd1-1p fusion cannot replace the *CMD1* encoded wild-type protein. Thus, fusion of GFP to a temperature-sensitive budding yeast calmodulin is also a lethal mutation.

### Microscopy

Unless noted otherwise, cells were viewed using a Zeiss Axioplan microscope with a ×100 1.4 NA objective and an optovar set at 1.25. Filter set for GFP was the XF14 (425DF45, 475DCLPO2, 535DF55) (Omega Optical). Images were captured using an Imagepoint cooled CCD video camera (Photometrics) fitted to the microscope in conjunction with IP Lab software (Signal Analytics). Images were prepared for publication with Photoshop 4.0 (Adobe Systems, Inc.).

### Preparation of cells for indirect immunofluorescence

Cells were grown in YE liquid medium at 30°C and then fixed, washed, digested, and stained using the combined aldehyde method (Hagan and Hyams, 1988) with the following modifications. Phosphate-buffered saline (PBS) (Harlow and Lane, 1988), PBS supplemented with 1% bovine serum albumin and 0.05% sodium azide (PBS/BSA), and 1.1 M sorbitol in 0.1 M sodium phosphate buffer, pH 6.5 (SP) were substituted for PEM, PEMS and PEMBAL buffers, respectively. Digestions were performed in 100 μl SP containing 0.5 mg/ml Zymolyase-100T (ICN) and 10 mg/ml mutanase (Novo Nordisk BioChem) for 30 minutes at 30°C. Cells were simultaneously incubated overnight at room temperature on a rotating platform in primary antisera: purified rat anti-tubulin monoclonal antibody YOL1/34 (1:50) (Kilmartin et al., 1982) and rabbit anti-Cam1p polyclonal antiserum (1:50) (Moser et al., 1995), or affinity-purified rabbit anti-Sad1p antibodies (1:25) (Hagan and Yanagida, 1995) in 100 μl PBS/BSA. Following three washes in PBS/BSA, cells were resuspended in 100 μl PBS/BSA containing secondary antibody conjugates: fluorescein isothiocyanate-labeled rabbit anti-rat IgG (1:50) (Boehringer/Mannheim), Oregon Green 488-labeled goat anti-rat IgG (1:50) (Molecular Probes), and rhodamine isothiocyanate-labeled goat anti-rabbit IgG (1:50) (Boehringer/Mannheim). Following incubations in secondary antibody for at least 4 hours at room temperature on a rotating platform, cells were washed three times in PBS/BSA. DNA was stained with 100 ng/ml di-amidino phenyl indole (DAPI) (Sigma Chemical Co.), and cells were then mounted onto polylysine-coated 10-well slides (Cel-Line Associates, Inc.) in Citifluor Glycerol (Ted Pella).

### Characterization of the temperature-sensitive *S. pombe* calmodulin mutant

Strains MP5-1C and MP64 were cultured in YE liquid medium at a permissive temperature of 30°C to a logarithmic phase of growth. Hydroxyurea was added to the cultures at a final concentration of 11 mM. After 4 hours the drug was removed by filtration, the cells were washed and transferred to fresh medium at the nonpermissive temperature of 37°C. At 20 minute intervals, samples were removed and titered for viability in triplicate or fixed as described above.

## RESULTS

### Localization of calmodulin using calmodulin fusions to green fluorescent protein (GFP)

#### *S. cerevisiae*

Indirect immunofluorescence previously showed that calmodulin localizes to two different regions in the budding yeast cell. First calmodulin localizes to sites of cell growth and overlaps the location of actin patches in buds (Brockerhoff and Davis, 1992; Sun et al., 1992b). Secondly, calmodulin localizes to the spindle pole body (Geiser et al., 1993; Stirling et al., 1994). The presence of calmodulin at both these sites has been confirmed by immunoelectron microscopy (Spang et al., 1996; Sundberg et al., 1996) (H. Sundberg and T. N. Davis, unpublished results). Using the previous techniques, calmodulin was not seen at both subcellular locations at the same time, because fixation procedures that preserved the sites of cell growth did not allow visualization at the SPB and vice versa. Therefore, we extended our experiments in *S. cerevisiae* to living cells by visualizing the location of a fusion between S65T-GFP and the N terminus of budding yeast calmodulin. A gene encoding the GFP-Cmd1p fusion protein expressed under control of the *CMD1* promoter and terminator was integrated in place of the wild-type *CMD1* gene. The fluorescent GFP-Cmd1p in living cells shows a pattern consistent with a location at both the SPB and at areas of polarized growth. Unbudded cells and cells with small buds each contain one dot of fluorescence as expected for either a single SPB or for two SPBs in the side-by-side configuration. These cells also have a bright patch of fluorescence at the nascent bud site or the small bud (Fig. 1A). Cells with a medium bud contain two small fluorescent dots near the neck region of the cell as expected for a protein at the poles of the short spindle (Fig. 1B). In addition patches of fluorescence appear in the bud similar to the pattern seen for the actin capping protein, Cap2p (Waddle et al., 1996). In a large budded cell, both mother and daughter contain a dot of GFP-Cmd1p presumably corresponding to their single SPB (Fig. 1C). In addition a bright ring appears at the neck (Fig. 1C). Importantly, the $Ca^{2+}$-binding mutant GFP-Cmd1-6p fusion had a pattern of localization identical to that of the wild-type fusion protein (Fig. 1D-F). This is the first demonstration that a mutant calmodulin defective in binding $Ca^{2+}$ localizes to the SPB in addition to sites of cell growth.

#### *S. pombe*

The localization of calmodulin in *S. pombe* cells was determined using fusions of *S. pombe* calmodulin to GFP. A gene encoding a fusion of wild-type GFP to the N terminus of wild-type *S. pombe* calmodulin expressed under the control of the *cam1*$^+$ promoter and terminator was integrated in place of the *cam1*$^+$ gene. Because we were examining living cells, the behavior of the GFP-Cam1p in single cells could be observed over a period of time. GFP-calmodulin displayed two distinct patterns of localization. First, small fluorescent dots behaved exactly as previously demonstrated for both the budding and fission yeast SPBs

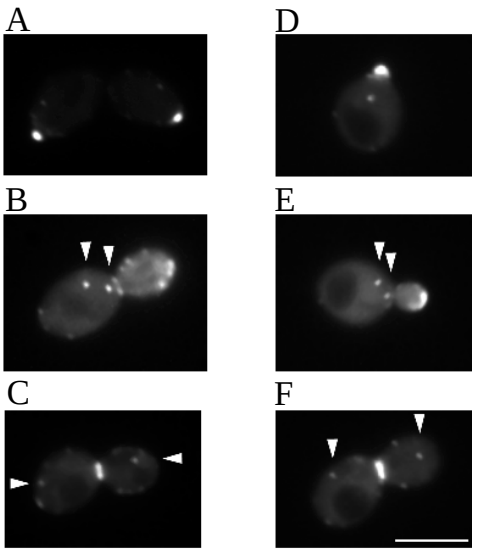


**Fig. 1.** Localization of GFP-Cmd1p to the SPB of *S. cerevisiae*. An equal volume of a culture of strain MMY28 (*GFP::CMD1*) (A-C) or strain TDY55-5D containing plasmid pMM137 (*GFP::cmd1-6*) (D-F) grown in YPD medium at 21°C was mixed with supplemented SD (SDC) medium containing 1% low-melting-temperature agarose on a microscope slide and visualized as described in Materials and Methods. GFP-calmodulin localization to the SPB (arrowheads). Bar, 5 μm.

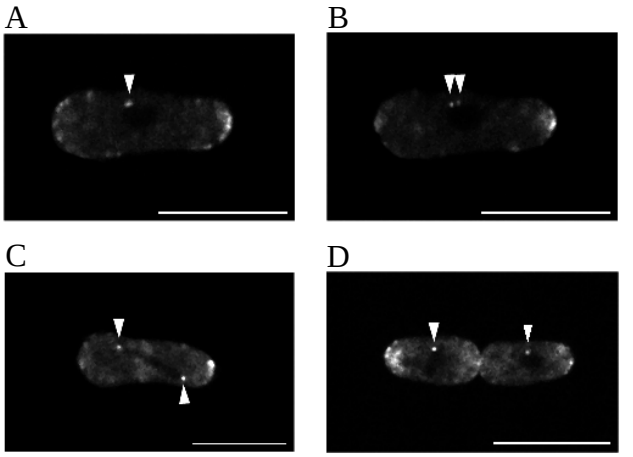


**Fig. 2.** Localization of GFP-Cam1p to the SPB of *S. pombe*. An equal volume of a culture of strain MP76 grown in YE medium at 21°C was mixed with EMM2 medium containing 1% low-melting-temperature agarose on a microscope slide. Cells were visualized with a MRC600 laser scanning confocal microscope (Bio-Rad) on 10% power with a ×60 objective. GFP-calmodulin localization to the SPB (arrowheads). A and B are images of the same cell taken 1 minute apart. Note that the focal plane optimal for visualization of the SPB results in poor visualization of the cortical cytoskeleton. A QuickTime movie of the GFP-Cam1p behavior at the SPB can be viewed at http://www.cityscape.co.uk/users/ag64/. Bars, 5 μm.

(Byers, 1981; Hagan and Hyams, 1988; McCully and Robinow, 1971) (Fig. 2A-D). In cells early in the cell cycle as judged by their small size, a single spot of fluorescence was observed at the edge of the nucleus (Fig. 2A). The dot of fluorescence elongated and eventually divided (Fig. 2B). Later the separation between the dots increased (Fig. 2C) with one spot of the pair eventually being distributed to each new daughter cell following mitosis and cytokinesis (Fig. 2D). Notably, calmodulin was not seen at the additional MTOCs that nucleate microtubules near the septum in *S. pombe* cells following mitosis.

We observed a second pattern of fluorescence indicating that GFP-Cam1p localized at regions of the fission yeast cell undergoing polarized growth (Fig. 3A-D). In cells undergoing septation and cytokinesis, bright fluorescence was found on both sides of the growing septum dividing the cell (Fig. 3A). In a newly divided cell immediately following cytokinesis, fluorescence was seen to move from the septum to the opposite or 'old' end of the cell (Fig. 3B,C). The old end corresponds to the former end of the mother cell and is in opposition to the end directly created by septation. *S. pombe* cells are known to only grow from the old end just after division (Mitchison and Nurse, 1985). Later the fluorescence redistributed to both ends of the lengthening cell (Fig. 3D). This bipolar localization coincides with an event in the *S. pombe* cell cycle known as NETO, or new end take off, the time when both ends of the cell begin to grow (Mitchison and Nurse, 1985). A strain containing an integrated fusion of S65T-GFP to *cam1*$^+$ (MFP7) was found to have a fluorescence pattern that was identical to that observed with the fusion to wild-type GFP except that the fluorescence was much easier to detect (data not shown).

### Indirect immunofluorescence of *S. pombe* calmodulin

Indirect immunofluorescence using antiserum to fission yeast calmodulin gave a pattern very similar to that seen with GFP-Cam1p. The growing ends and the septum as well as what appeared to be the SPB all were stained by the antiserum. The presence of fission yeast calmodulin at the SPB was confirmed by comparing the localization of calmodulin to that of Sad1p. Sad1p is a recognized component of the *S. pombe* SPB and remains permanently associated with the SPB throughout the cell cycle (Hagan and Hyams, 1988). Attempts were made to localize Sad1p in cells containing GFP-calmodulin fusions, but the combined aldehyde fixation procedures necessary for tubulin immunofluorescence were found to eliminate GFP fluorescence. Instead we found that the localization of calmod-

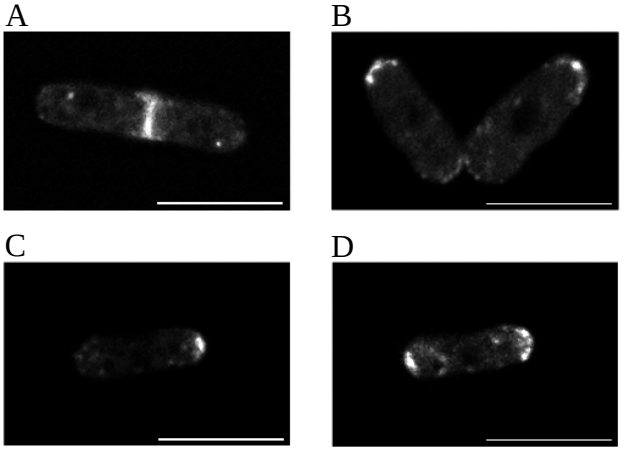


**Fig. 3.** Localization of GFP-Cam1p to sites of polarized growth in *S. pombe*. Cells were visualized as described for Fig. 2. Images C and D are of the same cell taken 9 minutes apart. Note that the focal plane optimal for visualization of the cortical cytoskeleton results in poor visualization of the SPB. Bars, 5 μm.

ulin, tubulin and DNA matched that of Sad1p, tubulin and DNA in cells processed in parallel (Fig. 4). Tubulin staining, representing the elongating spindle, was observed between the two dots of calmodulin staining. Sad1p dots were found to be similarly localized to the ends of the spindle. The localization of Sad1p, in relation to tubulin and DNA, is identical to that previously described (Hagan and Yanagida, 1995).

### Phenotypic analysis of a temperature-sensitive *S. pombe* calmodulin mutant

When the $Ca^{2+}$-binding-site mutant *cam1-E14* allele is carried on a plasmid it does not confer temperature-sensitivity (Moser et al., 1995). However, when *cam1-E14* is integrated as a single copy in place of the wild-type *cam1*$^+$ gene, fission yeast cannot form colonies at temperatures above 32°C. Calmodulin mutant and wild-type fission yeast were arrested in S phase with hydroxyurea at a temperature permissive for growth and then released from arrest and shifted to the restrictive temperature. The wild-type culture completed S phase and proceeded normally through mitosis and then cytokinesis. The septation index peaked 80 minutes after the shift to 37°C and the number of viable cells began to increase 100 minutes after the shift (Fig. 5). In contrast, 80 minutes after the shift to 37°C cells in the mutant culture began to lose viability (Fig. 5). Cytokinesis in the mutant culture was delayed and asynchronous although most of the cells did eventually form a septum.

The calmodulin localization and cell-cycle timing studies suggested that mitotic defects might underlie the loss of viability in the calmodulin mutant strain. Therefore, an analysis of DNA and spindle morphology was performed on synchronized cultures of both the wild-type and calmodulin mutant following incubation at non-permissive temperature. DNA-specific staining with DAPI in control cells revealed the chromatin as a brightly staining crescent with a faintly stained nucleolus completing the sphere of the nucleus. As the cells progressed through mitosis, the nucleus divided and the chromatin segregated to either end of the dividing cell. Two discrete nuclei formed, followed by septation.

Analysis of microtubule distribution by indirect immunofluorescence revealed the disappearance of cytoplasmic microtubules concomitant with formation of a mitotic spindle in cells undergoing mitotic transit. As previously described (Hagan and Hyams, 1988), early short spindles aligned nearly perpendicular to the length of the cell and spanned the centrally-located chromosomal DNA mass (Fig. 6A). Later, spindle alignment became more parallel with the long axis of the elongating cell. The spindle midpoint corresponded with the midpoint of the cell. As well, chromatin segregating along medium to full-length spindles was always found near the two endpoints of the spindle (Fig. 6B). At full spindle extension, the newly segregated chromosomal masses were observed at the two ends of the cell (Fig. 6C). Following mitosis, spindle microtubules disappeared and post-anaphase arrays of cytoplasmic microtubules became apparent (Fig. 6D). Cytokinesis resulted in two daughter cells each containing an equal mass of segregated chromosomal DNA.

The *cam1-E14* mutant cells exhibited several abnormal phenotypes. When the culture first began to lose viability (80 minutes), the chromosomal DNA appeared strikingly condensed in 20% of the cells ($n$=200). Metaphase plates with three pairs of discrete chromosomes could often be observed (Fig. 7A). The DNA also frequently appeared to be broken or fragmented by improper distribution along the spindle (Fig. 7B). For comparison, only 2.5% of the wild-type cells contained abnormal DNA at that time point. Anti-tubulin immunofluorescence staining of mutant spindles was more

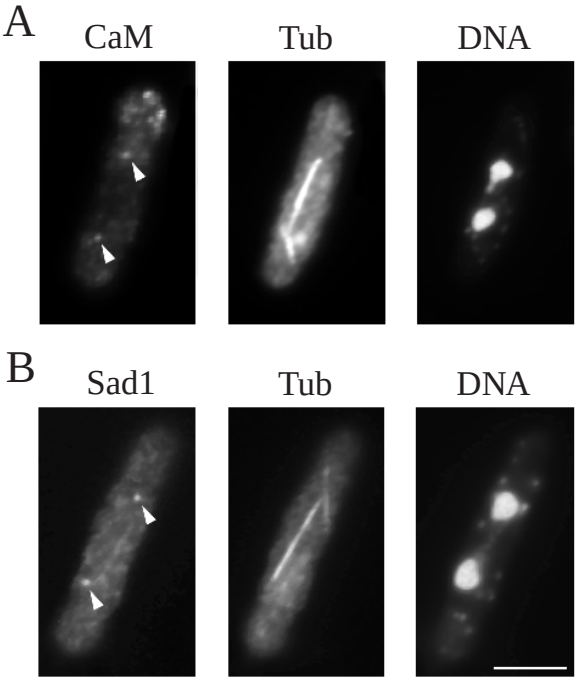


**Fig. 4.** Localization of calmodulin/tubulin and Sad1p/tubulin at the *S. pombe* SPB. A culture of *S. pombe* strain MP5-1C (*cam1*$^+$) grown at 30°C to logarithmic phase in YE supplemented with adenine, leucine and uracil was prepared for immunofluorescence as described in Materials and Methods. Cam1p or Sad1p localization to the SPB (arrowheads). Bar, 5 μm.

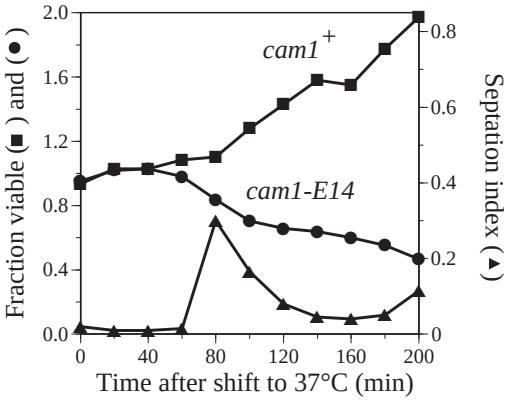


**Fig. 5.** Viability of wild-type and calmodulin mutant cells following shift to nonpermissive temperature. Cultures of strain MP33-6B (*cam1*$^+$) (■) and strain MP64 (*cam1-E14*) (●) were arrested by treatment with hydroxyurea at 30°C, washed and released at 37°C as described in Materials and Methods. At the times shown, samples were removed and either titered or fixed in 3.7% formaldehyde. The degree of synchrony attained was comparable to that attained previously (Enoch et al., 1992). The septation index (▲) is the fraction of cells with a septum. Only the wild-type septation index is shown for clarity.

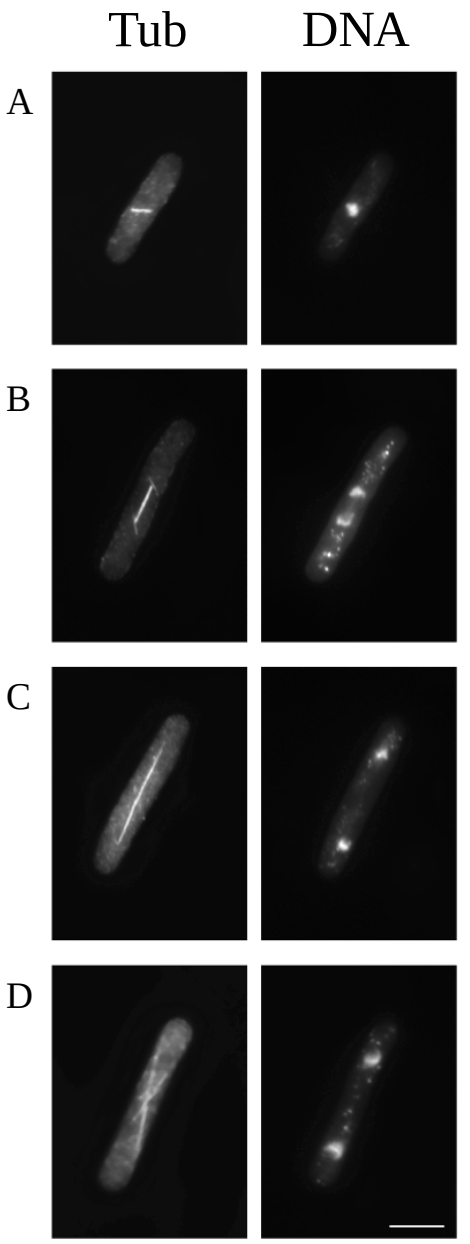


**Fig. 6.** Tubulin and DNA morphology in synchronized wild-type cells. Cultures of strain MP5-1C (*cam1*$^{+}$) synchronized with hydroxyurea were incubated at 37°C , fixed, and prepared for immunofluorescence as described in Materials and Methods. (A) Short spindle; (B) medium spindle; (C) full-length spindle; (D) cytoplasmic microtubules. Bar, 5 μm.

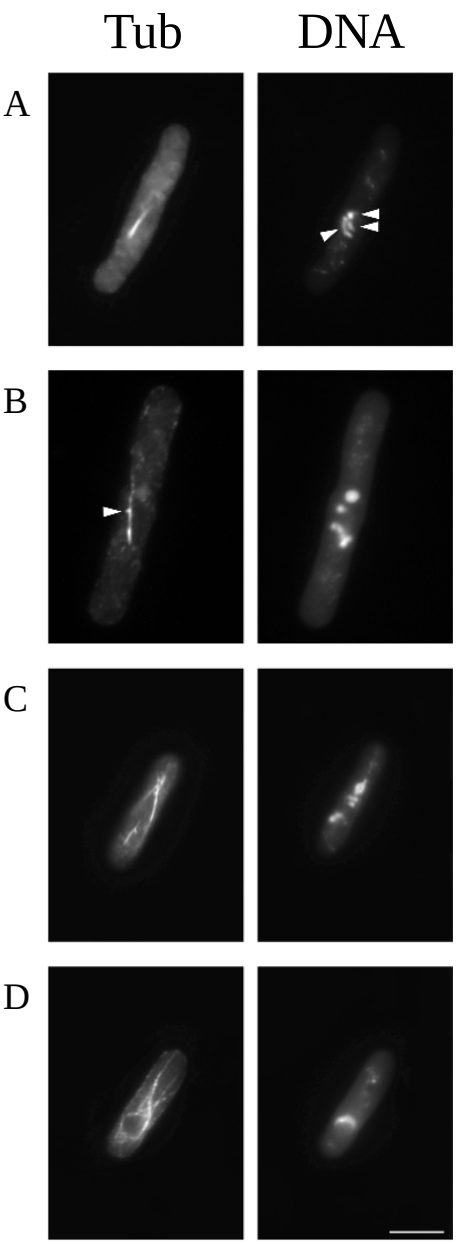


**Fig. 7.** Tubulin and DNA morphology in synchronized calmodulin mutant cells. Cultures of strain MP64 (*cam1-E14*) synchronized with hydroxyurea were incubated at 37°C , fixed, and prepared for immunofluorescence as described in Materials and Methods. (A) Hyper-condensed chromosomes (arrowheads); (B) fragmented chromatin and spindle with abnormal spur (arrowhead); (C) broken spindle; (D) mislocalized chromatin and cytoplasmic microtubules. Bar, 5 μm.

diffuse and less uniform than wild type (Fig. 7B). Perhaps the most striking class of cells was those that appeared to have broken mitotic spindles (Fig. 7C).

At later times, mutant cells appeared with chromosomes that were missegregated. Some cells divided with two nuclei on one side of the septum while other cells divided with only one DAPI-staining mass that often appeared abnormally large. Cytoplasmic microtubules of normal morphology were found assembled in association with the mislocalized chromatin (Fig. 7D). At 240 minutes after the shift, 27% of the mutant cells contained mislocalized DNA and 11% contained no apparent nuclear DNA.

## DISCUSSION

We demonstrate that calmodulin localizes to the SPB in *S. pombe*. Antiserum to calmodulin appears at the end of the spindle in a pattern identical to Sad1p, a molecular marker whose localization has been previously defined with respect to

SPB position (Hagan and Yanagida, 1995). A GFP-Cam1p fusion protein allowed examination of the SPB in living cells and demonstrated that in living cells the SPB went through the stages previously observed in fixed isolates (Hagan and Hyams, 1988; McCully and Robinow, 1971; Tanaka and Kanbe, 1986). Unlike a GFP fusion to Dis1p (Nabeshima et al., 1995), GFP-Cam1p is the first molecular marker for SPBs that can be seen in living *S. pombe* cells throughout the cell cycle. This integrated marker should allow monitoring of SPB behavior in mutant fission yeast cells.

Localization of calmodulin using both a green fluorescent protein fusion and indirect immunofluorescence also indicates an association between *S. pombe* calmodulin and areas of polarized growth. GFP-Cam1p was observed in the region of the septum as it formed, then at only one end of newly formed cells. Later, fluorescence was seen at both ends of the growing cell. These behaviors are similar to those of known components of the polarized fission yeast actin cytoskeleton (Marks et al., 1986).

The SPB localization and the polarized localization of GFP-Cam1p can easily be differentiated using two criteria. First, the SPB fluorescence is best seen in a focal plane that bisects the center of the cell while the polarized localization is more clearly seen when the focus is in a plane that is level with the surface of the cell. The second criterion can only be applied to live cells. The spot of SPB fluorescence remains relatively constant in location for several minutes. During this time interval, the fluorescence associated with cortical cytoskeleton can be seen to undergo extensive remodeling, as has been previously reported in *S. cerevisiae* (Waddle et al., 1996).

Subcellular localization of calmodulin to the SPB and the cortical cytoskeleton suggests roles for *S. pombe* calmodulin in mitosis and in polarized cell growth, respectively. Analysis of the restrictive-temperature phenotype of a temperature-sensitive calmodulin mutant further substantiates a mitotic function for *S. pombe* calmodulin and indicates a role for calmodulin in facilitating chromosome segregation. At the restrictive temperature, *S. pombe* cells containing a temperature-sensitive $Ca^{2+}$-binding-site calmodulin mutant begin to lose viability after DNA replication, at a time when wild-type cells are completing mitosis and initiating cytokinesis. Examination of the DNA of the mutant cell reveals abnormal chromatin and spindle morphologies that are consistent with a failure in chromosome segregation. First, at the time when the cells begin to lose viability, the chromatin appears to condense and fragment. The chromatin may appear as distinct chromosomes like that found in cold-sensitive fission yeast *nda3* mutants when incubated at the non-permissive temperature (Hiraoka et al., 1984). The *nda3*$^+$ gene encodes β-tubulin, an essential component of the mitotic spindle apparatus. Consistent with a presumed defect in spindle assembly, mutant cells were found that appear to contain broken spindles. However, while the tubulin defect in the *nda3* mutant is detected by a spindle checkpoint causing a reversible mitotic arrest at the restrictive temperature, the defect in the *cam1-E14* mutant cells does not cause arrest. The *cam1-E14* calmodulin mutant continues to divide, resulting in cells with DNA mislocalized on only one side of the septum and a significant number of cells containing no nuclear DNA. This observation suggests several, as yet untested hypotheses. Calmodulin may have a role in a mitotic checkpoint function. Alternatively, the calmodulin defect may simply not be detected by the checkpoint mechanism. Furthermore, the fact that the mutant cells can form a septum suggests that any defect the *cam1-E14* mutant may have in polarized growth is not severe enough to manifest itself before the lethal effects of mitotic failure.

In *S. cerevisiae*, GFP-calmodulin fusions are easily seen at the SPB and at sites of cell growth in living cells. This is the first demonstration of the presence of calmodulin at both structures at the same time. Previous methods employing fixation showed calmodulin at only one place or the other (Brockerhoff et al., 1992; Geiser et al., 1993; Stirling et al., 1994; Sun et al., 1992b; Sundberg et al., 1996). Furthermore, although the *cmd1-6* $Ca^{2+}$-binding calmodulin mutant is viable (Geiser et al., 1991), immunofluorescence methods have not been able to detect the Cmd1-6p protein product at the SPB. The GFP-Cmd1-6p fusion provides the first cytological evidence that the interaction between calmodulin and the budding yeast SPB is $Ca^{2+}$-independent. GFP-calmodulin serves as an excellent integrated marker for the SPB allowing the observation of SPB behavior in living cells. Our results are similar to those observed previously examining the location of Nuf2p-GFP, a protein that associates with the microtubules near the SPB (Kahana et al., 1995; Osborne et al., 1994).

Calmodulin appears to perform a similar mitotic function in both fission and budding yeasts. The subcellular localization pattern of calmodulin in both *S. cerevisiae* and *S. pombe* is quite similar. Calmodulin can be found at the SPB and at sites of cell growth associated with the polarized cortical cytoskeleton. The mitotic phenotypes of temperature-sensitive calmodulin mutants from both species are also related. Like the *cam1-E14* mutant, the *S. cerevisiae cmd1-1* mutant loses viability at non-permissive temperature during mitosis and accumulates cells with broken spindles and fragmented DNA (Davis, 1992). Calmodulin in *S. cerevisiae* is known to function in mitosis by direct interaction with the SPB component Spc110p. Therefore, we propose that calmodulin also performs an essential mitotic function via an interaction at the *S. pombe* SPB. Yet, unlike in *S. cerevisiae* (Geiser et al., 1991), in *S. pombe* at least one wild-type $Ca^{2+}$-binding-site in calmodulin is required to maintain viability (Moser et al., 1995). Thus, the essential interaction between *S. pombe* calmodulin and the SPB may be a $Ca^{2+}$-dependent process. However, any determination of the $Ca^{2+}$-dependence of the interaction between calmodulin and centrosome components from *S. pombe* as well as from other species awaits their isolation and characterization.

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