# Purification, Characterization, and Immunofluorescence Localization of *Saccharomyces cerevisiae* Capping Protein

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***Abstract.*** Capping protein binds the barbed ends of actin filaments and nucleates actin filament assembly in vitro. We purified capping protein from *Saccharomyces cervisiae.* One of the two subunits is the product of the *CAP2* gene, which we previously identified as the gene encoding the $\beta$ subunit of capping protein based on its sequence similarity to capping protein $\beta$ subunits in chicken and *Dictyostelium* (Amatruda, J. F., J. F. Cannon, K. Tatchell, C. Hug, and J. A. Cooper. 1990. *Nature (Lond.).* 344:352–354). Yeast capping protein has activity in critical concentration and low-shear viscometry assays consistent with barbed-end capping activity. Like chicken capping protein, yeast capping protein is inhibited by $PIP_2$. By immunofluorescence microscopy yeast capping protein colocalizes with cortical actin spots at the site of bud emergence and at the tips of growing buds and shmoos. In contrast, capping protein does not colocalize with actin cables or with actin rings at the site of cytokinesis.

CAPPING protein is an actin-binding protein that binds the barbed end of actin filaments and nucleates actin polymerization in vitro. The protein is a heterodimer with subunits of 28–36 kD and is further characterized by $Ca^{++}$ independence and by a lack of filament-severing activity (5, 10). In striated muscle, capping protein (CapZ) is located at the Z-line (8) and in epithelial cells, capping protein is found in cell junctions where actin filaments are associated with the plasma membrane (30a). The in vitro activity and the localizations suggest that capping protein may capture filaments by their barbed ends or nucleate new filaments to effect the proper orientation of the actin cytoskeleton in cells.

Capping protein appears to be ubiquitous in eukaryotes: it has been found in protozoans, fungi, slime molds, and vertebrates (2, 3, 7, 16, 18). The yeast *Saccharomyces cerevisiae* is an attractive system in which to study the in vivo role of capping protein, both because the yeast actin cytoskeleton is relatively simple and because yeast molecular genetic techniques offer a powerful approach to some of the questions.

The intracellular distribution of yeast actin is asymmetric and changes in a defined way during the cell cycle (1, 20). Early in the cell cycle, spots or patches of actin localize to the site from which the bud will emerge; later these spots are localized toward the tips of growing buds. Spots are also found at the tips of cells undergoing morphogenesis before mating (shmoos) and are thought to correlate with sites of new cell wall growth. In contrast to the bud, mother cells contain relatively few spots and instead have actin cables. Shortly before the cells divide, these cables disappear, approximately equal numbers of spots are present on both cells, and a single or double ring of actin is often seen at the bud neck. The hypothesis that the actin cytoskeleton is required for maintaining the normal polarity of secretion during the budding cycle (1) is supported by the phenotype of cells bearing temperature-sensitive alleles of actin at the restrictive temperature (24).

Previously we reported the identification, sequencing, and disruption of *CAP2*, the gene encoding the $\beta$ subunit of capping protein in *S. cerevisiae* (2). *cap2* null mutants are viable but grow more slowly than wild type and exhibit altered morphology. The mutants have alterations in the actin cytoskeleton, including the loss of actin cables.

*CAP2* was presumed to be the gene for the $\beta$ subunit of capping protein because its predicted protein sequence is similar to that for cDNAs encoding capping protein $\beta$ from chicken (6) and *Dictyostelium* (16). In other systems, capping protein is an $\alpha/\beta$ heterodimer that binds the barbed ends of actin filaments. In this report we have purified the *CAP2* gene product and find that the protein is part of a heterodimer that binds actin in a manner characteristic of capping protein. In addition, antibodies against the purified protein have been used to localize capping protein. We find it associated with some but not all actin-containing structures.

## Materials and Methods

### Materials and Supplies

Unless otherwise noted, chemicals were from Sigma Chemical Co. (St. Louis, MO). Solvents and supplies were from Fisher Scientific Co. (St. Louis, MO).

### Yeast Strains

Yeast strains used were YJC091 (MAT$\alpha$ *trp1 pep4-3 prb1 prc1*) = BJ405 (E. W. Jones, Carnegie-Mellon University, Pittsburgh, PA); YJC093

(MATa, *ade2-1 his3-11,15 leu2-3,112 trp1-1 ura3-1*) = W303-1A (R. Rothstein, Columbia University, New York); YJC169 (MATa/MATα *ade2-1/ade2-1 his3-11,15/his3-11,15 leu2-3,112/leu2-3,112 trp1-1/trp1-1 ura3-1/ura3-1*), a W303 diploid; YJC163 (MATa/MATα *ade2-1/ade2-1 his3-11,15/his3-11,15 leu2-3,112/leu2-3,112 trp1-1/trp1-1 ura3-1/ura3-1 cap1-1::TRP1 cap2-1::HIS3*); and YJC283 (MATa/MATα *cdc24-4/cdc24-4*) = JPT19-HO5 (J. Pringle, University of North Carolina, Chapel Hill, NC; see Ref. 1).

## Purification of S. Cerevisiae Capping Protein

The protease-deficient *S. cerevisiae* strain YJC91 (*pra, prb, pep4*) was grown to an $OD_{600}$ of 20–25 in 10 *l* YPD (31). Attempts to purify capping protein from commercial yeast bricks proved unsatisfactory, owing to high levels of proteolysis. Cells (typically 300 g wet weight) were harvested, washed once in DEAE buffer (10 mM Tris, pH 8.0, 125 mM NaCl, 1 mM EDTA, 0.1 mM DTT, 1.5 mM $NaN_3$), and resuspended in an equal volume of DEAE buffer plus protease inhibitors (0.1 mM benzamidine, 1 mM PMSF, 1 μg/ml pepstatin A, 1 μg/ml leupeptin, 1 μg/ml aprotinin). Cells were disrupted in several batches using 0.5-mm glass beads in a bead-beater (Bio-Spec, Bartlesville, OK) with five 1-min cycles separated by 2-min cooling periods. The bead-beater was jacketed in ice, and all manipulations were performed at 4°C. Cell lysis was monitored by phase microscopy and was >90% complete. After disruption, the beads were washed with an equal volume of DEAE buffer and the wash was combined with the lysate.

The cell lysate was spun at 25,000 *g* for 40 min to remove glass beads and undisrupted cells. (We had previously ascertained, by immunoblotting with an anti-CAP2p antibody, that capping protein remains in the supernatant under these conditions). The supernatant (~27 g protein) was combined with 660 ml DE-53 resin (Whatman; preequilibrated with DEAE buffer) and the resulting slurry loaded into a 5 × 50 cm column containing 300 ml DE-53 resin. The column was eluted with a 3.6 liter linear gradient from 125 to 400 mM NaCl, and 20-ml fractions were collected. Fractions containing yeast capping protein (typically 180–210 mM NaCl) were identified with a low-shear viscometric assay (see below) and pooled. A 25–65% (wt/vol) $(NH_4)_2SO_4$ fraction was prepared, suspended in 7 ml buffer S (10 mM Tris, pH 8, 150 mM NaCl, 0.1 mM DTT, 1.5 mM $NaN_3$), and dialyzed 45 min vs. 2 liter buffer S. The dialyzed protein sample was clarified and loaded onto a 2.5 × 94 cm column of Sephacryl S-200 (Pharmacia Fine Chemicals, Piscataway, NJ) in Buffer S. 8-ml fractions were collected and the active fractions were pooled and applied to a mono Q HR16/10 column (Pharmacia Fine Chemicals) equilibrated with 10 mM Tris, pH 8, 0.1 mM DTT, 1.5 mM $NaN_3$, and 225 mM NaCl. The protein was eluted with a 240-ml linear gradient to 275 mM NaCl in the same buffer. 6-ml fractions were collected and the active fractions (typically at 240–255 mM NaCl) were pooled and applied to a 2.5 × 6 cm hydroxylapatite column (Bio-Gel HT, Bio-Rad Laboratories, Cambridge, MA) equilibrated in 10 mM $KPO_4$, pH 7, 100 mM NaCl, 0.1 mM DTT, and 1.5 mM $NaN_3$. Protein was eluted with a 400-ml linear gradient to 100 mM $KPO_4$ and 3-ml fractions were collected. Capping protein eluted as a single peak at ~45 mM $KPO_4$.

The extinction coefficient at 280 nm is 0.96 ml $mg^{-1}$ as determined using the interference optics system of a Beckman Model E ultracentrifuge (4). The molar extinction coefficient at 280 nm is $6.08 \times 10^4$ $M^{-1}$ $cm^{-1}$, based on the molecular weight of 63,333 predicted by the DNA sequences of the capping protein α and β subunits (ref. 2 and Amatruda, J. F., D. J. Gattermeir, and J. A. Cooper, manuscript submitted for publication).

Capping protein can be stored with an equal volume of glycerol at −80°C without loss of activity.

## Generation of Peptide $NH_2$-terminal Amino Acid Sequence

The α and β subunits of capping protein were separated by reverse-phase chromatography on a $C_4$ column with a 28–60% acetonitrile gradient in 0.1% TFA. The α and β subunits eluted at ~50 and 55% acetonitrile, respectively.

The capping protein β subunit was digested to completion with endoproteinase Lys-C (Boehringer-Mannheim Biochemicals, Indianapolis, IN) according to the manufacturer's instructions. The resulting peptides were purified on a $C_{18}$ column (Phenomenex, Torrance, CA) with a 0–75% acetonitrile gradient in 0.1% TFA. Peak fractions were pooled and further purified by rechromatographing under the same conditions. A purified peptide was sequenced by the Protein Chemistry Facility at Washington University as described (6).

## Low-Shear Viscometry Assay

Capping protein activity was monitored using falling-ball viscometry (25). 75 μl 12 μM chicken skeletal muscle F-actin (prepared as described [17]) in MKEI (2 mM $MgCl_2$, 100 mM KCl, 1 mM EGTA, 20 mM imidazole, pH 7.0) was added to 100 μl sample and incubated 30 min in a glass capillary tube before apparent viscosity was determined. For experiments in which the effect of $PIP_2$ on capping protein was tested, a one-tenth volume of 120 μM sonicated $PIP_2$ was added at the same time as capping protein.

## Determination of the Critical Concentration for Actin Polymerization

The critical concentration for actin polymerization in the presence or absence of yeast capping protein was determined as described (9). Briefly, 0, 20, or 200 nM capping protein was incubated at 23°C with varying concentrations of chicken actin in MKEI (2 mM $MgCl_2$, 100 mM KCl, 1 mM EGTA, 20 mM imidazole, pH 7.0) plus 1 mg/ml BSA. 12.5% of the actin monomers were pyrene labeled. Pyrene fluorescence was measured after 12 h. The values had not changed when measured after 12 additional h.

## Electrophoresis and Blotting

SDS-PAGE was performed according to Laemmli (22). For immunoblotting, proteins were transferred to nitrocellulose (35). After transfer the membrane was blocked in TTBS/2% fish gelatin for 20 min at 25°C. Antibody incubations were 1 h and were followed by four 5-min washes in TTBS. The primary antibody was at 0.1 μg/ml; the secondary was a 1:5,000 dilution of alkaline phosphatase–conjugated, affinity-isolated goat anti-rabbit IgG (Tago; Burlingame, CA), and the color was developed as described (12).

## Preparation of trpE-CAP2 Fusion Protein

A 1.8-kB BamHI fragment containing the *CAP2* coding sequence (2) was subcloned into the BamHI site of pATH-1 (36), and recombinant plasmid was transformed into *E. coli* RR1. Overnight cultures were grown in M9 medium (29) supplemented with tryptophan and ampicillin at 30°C, diluted 10-fold into 100 ml M9 with ampicillin lacking tryptophan and grown for 1 h at 30°C. Indole-acrylic acid was added to 20 μg/ml and growth continued 2 h at 30°C. Cells were harvested and resuspended in 5 ml 10 mM sodium phosphate, pH 7.2, 1% 2-mercaptoethanol, 1% SDS, 6 M urea. After addition of 1.5 ml 4× SDS-PAGE sample buffer (4% SDS, 20% glycerol, 0.2 M Tris, pH 7.0, 4 mM EDTA, 0.16 M DTT, 0.2 mg/ml Bromphenol Blue, 0.2 mg/ml Pyronin Y) and boiling, 4 ml of sample was loaded on a 3.0-mm, 8% SDS-PAGE preparative gel. A gel strip was cut from the side and Coomassie stained to identify the position of the trpE-CAP2 polypeptide. The corresponding position on the prep gel was cut out, equilibrated in water for 15 min, diced, Dounce homogenized, and lyophilized.

## Preparation of Antibodies

To prepare antibodies recognizing the *CAP2* gene product, dried gel fragments containing the trpE-CAP2 fusion protein were resuspended in PBS homogenized with an equal volume of Freund's adjuvant. Rabbits were immunized with four subcutaneous injections of 300 μg each. To affinity purify anti-trpE-CAP2 antibodies, trpE-CAP2 fusion protein was electrophoresed on a 10% preparative polyacrylamide gel and blotted to nitrocellulose, and the strip of nitrocellulose containing the fusion protein was excised from the blot. Antisera were incubated with these nitrocellulose strips, the strips were washed, and specific antibodies were eluted by incubating the strips 2 × 1 min with 1 ml 100 mM glycine, pH 2.8. The fractions were collected and neutralized by the addition of 50 μl 1 M Tris-Cl, pH 8.5. Antibodies were pooled and stored at 4 or −20°C after addition of an equal volume of glycerol.

To prepare an antiserum recognizing both subunits of yeast capping protein, rabbits were injected in both popliteal lymph nodes with 50 μg purified yeast capping protein, followed by subcutaneous boosts of 100 and 250 μg. Anti-capping protein antibodies were affinity purified by passing the antisera through a 0.5-ml Sepharose yeast-capping protein column, made by covalently coupling 250 μg purified yeast capping protein to CNBr-activated Sepharose (Pharmacia Fine Chemicals) according to the manufacturer's instructions. After washing with TTBS to remove nonspecifically bound protein, antibodies were eluted with 20 mM Tris, 4 mM $MgCl_2$, pH 6.8, and immediately desalted on a 2.5 × 15 cm Sephadex G-50 column

equilibrated in 10 mM Tris, pH 8, 100 mM NaCl, and 1.5 mM $NaN_3$. Fractions containing antibody were pooled and stored at 4 or −20°C after addition of an equal volume of glycerol.

### *Immunofluorescence*

We used modifications of several published protocols (26, 27). The diploid *S. cerevisiae* strain YJC163 was fixed as described (26) and briefly sonicated. Approximately $10^7$ cells were digested 45 min at 37°C in 1 ml of 100 mM KPi, pH 7, 1 M sorbitol, 25 mM 2-mercaptoethanol to which 10 U zymolyase (ICN) had been added. Digestion was monitored by phase microscopy. Cells were washed twice in 100 mM KPi, 1 M sorbitol and resuspended in 200 μl of the same buffer. Cells were allowed to settle for 5 min on an 18-mm square coverslip coated with 1 mg/ml polylysine which was subsequently washed and incubated 20 min with blocking solution (10 mM Tris, pH 7.5, 150 mM NaCl, 0.01% Tween-20, 1% nonfat dry milk, 10 mg/ml BSA, 1% fish gelatin).

All antibody incubations were carried out for 1 h at room temperature in blocking solution. Affinity-purified rabbit anti-yeast capping protein antibodies were absorbed against a strain lacking capping protein (YJC169) as described (28). To visualize yeast capping protein we used a "sandwich" procedure as follows (coverslips were washed 10 times with blocking solution between each step). The first incubation was a 1:10 dilution of the affinity-purified, absorbed anti-capping protein antibodies. This was followed by a 1-μg/ml solution of goat anti-rabbit IgG, and finally 1 μg/ml DTAF-conjugated donkey anti-goat IgG (Chemicon, Temecula, CA). Additional intermediary antibodies increased the brightness of the staining, but this was usually not necessary.

To costain capping protein and actin, after the final antibody incubation coverslips were washed and incubated 20–30 min with a 3.3 μM solution of rhodamine-conjugated phalloidin (Molecular Probes, Inc., Eugene, OR) in PBS. After this incubation was complete, the coverslips were washed five times in PBS and mounted in PBS containing 50% glycerol and 1 mg/ml *p*-phenylenediamine. Cells were photographed on TMax film using a Zeiss Axiovert microscope equipped for epifluorescence microscopy.

To stain tubulin, cells were fixed and zymolyase treated as described above, and then incubated with mAb 4A1 (anti-*Drosophila* tubulin, generously provided by Dr. L. S. B. Goldstein, Harvard University, Cambridge, MA) followed by a 1:200 dilution of Rhodamine-conjugated goat anti-mouse IgG (Boehringer-Mannheim Biochemicals). Coverslips were mounted and photographed as described above.

### *Alpha-factor Treatment*

Haploid MAT*a* strain YJC093 was grown to a density of $10^7$ cells/ml in YPD, diluted 10-fold into fresh YPD medium containing 1 μM synthetic α-factor (Sigma Chemical Co.), and growth was continued at 30°C (34). Shmoo formation was monitored in the microscope, and after ~3 h the culture was fixed and processed for microscopy as described below.

## *Results*

### *Purification and Characterization of S. cerevisiae Capping Protein*

Having identified a gene, *CAP2*, encoding the β subunit of yeast capping protein on the basis of sequence similarity to the β subunit of chicken and *Dictyostelium* capping protein (2), we purified the *CAP2* protein. As assays during the purification we used immunoblots with antibodies against the *CAP2* gene product and the inhibition of the low-shear viscosity of actin filaments, which has been used previously to purify capping protein from other sources (5, 10). The CAP2 protein and the activity copurified during the procedure. Fig. 1 shows material through the stages of the purification. The protocol, described in detail under Materials and Methods, yields 1 mg capping protein from 300 g (wet weight) yeast in 3 d. The purified protein migrates on SDS-polyacrylamide gels as two subunits of relative molecular mass 33.7 and 32.1 kD. Capping protein from other organisms is a heterodimer with subunits of approximately these values (5, 9, 19, 30). The upper 33.7-kD subunit is recognized on an immunoblot by an antibody raised against a *trpE-CAP2* fusion protein; therefore, it is the *CAP2* gene product (β subunit) (see Fig. 3). In chicken and *Dictyostelium*, the β subunit has a lower mass and a faster migration in SDS-PAGE. $NH_2$-terminal amino acid sequencing of a proteolytic peptide of the purified β subunit yielded the sequence NQSDHSNW, matching residues 148–155 of the predicted amino acid sequence of the *CAP2* gene product (2).

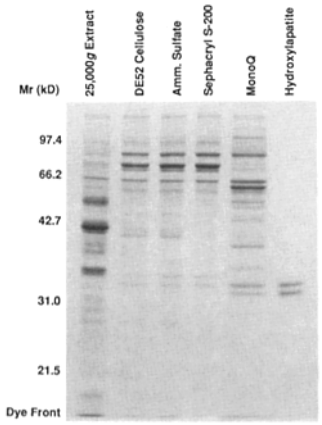


*Figure 1.* Purification of *S. cerevisiae* capping protein. 9% SDS–polyacrylamide gel of samples from successive stages of the purification. (Lane *1*) 25,000-*g* extract; (lane *2*) pool from DE52 cellulose column; (lane *3*) ammonium sulfate $P_{25\text{-}65}$; (lane *4*) pool from S-200 gel filtration column; (lane *5*) pool from MonoQ column; (lane *6*) pool from hydroxylapatite column. 5 μg were loaded into all lanes except lane 6, which contains 0.5 μg. Relative molecular masses of size standards are indicated on the left.

The activity of yeast capping protein was compared to that of chicken muscle capping protein (CapZ, [5]) using chicken skeletal muscle actin as a substrate in the low-shear viscometry assay (Fig. 2). In this experiment yeast capping protein had 42% of the activity of chicken capping protein. Yeast capping protein also increased the critical concentration of chicken actin polymerization (Table I). The critical concentration effect is comparable to that of chicken capping protein, consistent with the result of the low-shear viscometry assay.

The binding of chicken CapZ to actin is inhibited by anionic phospholipids including $PIP_2$ (17). Yeast capping protein is also inhibited by $PIP_2$. The apparent viscosity of 3.5 μM F-actin was 9.7 centipoise (cp); in the presence of 190 nM capping protein the apparent viscosity was reduced to 1.2 cp. However, in the presence of capping protein and 12 μM $PIP_2$ the apparent viscosity was 10.3 cp, demonstrating that the viscosity-lowering activity of capping protein is in-

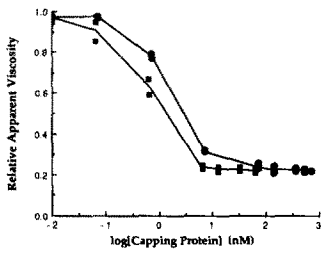


*Figure 2.* Relative activity of chicken and yeast capping proteins. Activity was measured using falling-ball viscometry with chicken skeletal muscle actin as the substrate. Apparent viscosity (normalized to control values for each curve) is plotted versus the concentration of capping protein in the capillary tube. (*squares*) Chicken capping protein; (*circles*) yeast capping protein. Two separate experiments were performed. Single curves have been estimated for the chicken capping protein and yeast capping protein data points, respectively.

*Table I. Effect of Purified Yeast Capping Protein on the Critical Concentration for Actin Polymerization*

| Capping Protein | Critical concentration | |
|---|---|---|
| | Experiment 1 | Experiment 2 |
| *nM* | *μM* | *μM* |
| 0 | 0.35 | 0.35 |
| 1.2 | ND | 0.35 |
| 12.0 | 0.82 | 0.92 |
| 120.0 | 0.96 | 1.00 |

The results of two separate experiments are shown. The concentration of capping protein given is the final concentration in the reaction mixture.

hibited by $PIP_2$. $PIP_2$ alone had no effect on the apparent viscosity of the actin solution.

Capping protein is ~0.1% of total protein in a 25,000-*g* supernatant of a whole cell yeast extract, based on quantitative immunoblots using purified yeast capping protein as a standard. The purification protocol therefore recovers ~4% of the capping protein in this supernatant. *Acanthamoeba* capping protein was also found to be ~0.1% of total cell protein (9).

### *Immunofluorescence Localization of Capping Protein in Yeast*

Antibodies prepared and affinity-purified against denatured protein did not stain cells well. Therefore, to localize capping protein we prepared a polyclonal antiserum against the purified native protein and affinity-purified antibodies on a column of native protein. These antibodies specifically recognize the two subunits of yeast capping protein on immunoblots of total yeast extracts. IgGs purified from the preimmune serum recognized no bands on the immunoblot (Fig. 3).

Capping protein is found at the incipient bud site (Fig. 4). Actin spots are clustered at one pole of the cell; in favorable views these spots are seen to form a ring. Cables are infrequently seen at this stage. Capping protein colocalizes with the actin spots both at and away from the budding site.

During bud growth actin and capping protein colocalize in spots at the tip of the growing bud (Fig. 5). These cells also have actin cables in the mother, and capping protein does not colocalize with the actin cables (Fig. 6). The cells in Figs. 5 and 6 are from the same experiment. The difference between the images is the printing of the negative. In mother cells, capping protein is present in cortical actin spots and in a uniform, fine, punctate distribution that does not coincide with the actin cables. The nucleus, detected by DIC microscopy, is not stained. The staining pattern observed is specific, because no staining was detected in strains bearing capping protein null alleles, and because preimmune IgG did not stain cells.

At a late stage in the cell cycle, when the size of the bud approaches that of the mother, actin assembles at the bud neck where new cell wall growth will separate the two cells. Capping protein is not found in this actin-containing structure (Fig. 7). 95 of 100 such cells scored showed no capping protein staining at the neck. The other five were similar to the bottom cell in Fig. 7, in which some capping protein localizes to the bud neck (fig. 7, *arrow*). In these cells the capping protein staining does not precisely match the pattern of actin staining, and we suggest that some of the actin- and capping protein–containing spots are near the neck by chance.

Haploid *S. cerevisiae* cells, when exposed to pheromone from a cell of the opposite mating type, arrest in G1 and form a projection; this is the site where the two cells will fuse during conjugation (11). Capping protein and actin are present in spots clustered at the tip of the projection (Fig. 8; ref. 13). Capping protein did not colocalize with actin cables in pheromone-treated cells. However, cables are present only rarely in such cells, which limits the strength of this conclusion.

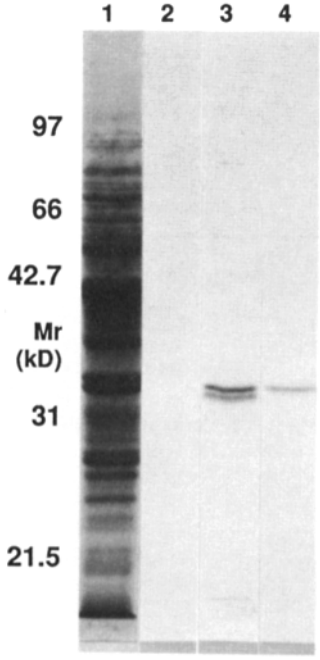


*Figure 3.* Specificity of anti-capping protein antiserum. Immunoblot of total protein extract from wild-type strain YJC163 (~5 μg protein per lane). (Lane *1*) Total protein stained with Amido black; (lane *2*) preimmune IgG; (lane *3*) affinity-purified anti-capping protein antibodies; (lane *4*) affinity-purified anti-trpE-CAP2 antibodies.

### *Actin and Capping Protein Distribution after Release from Stationary Phase*

In Fig. 4 we identified the ring of spots with capping protein and actin as the site of incipient bud emergence. Another possibility is that this actin is the residue of the cytokinesis actin ring. We do not think the capping-protein rings are the remnants of previous cytokinesis rings, given the finding that capping protein is not a component of the cytokinesis ring (Fig. 7).

To answer this question more directly, we observed the appearance of capping protein/actin rings on cells emerging from stationary phase (Table II). In this experiment, a *cdc24* strain was grown to stationary phase and released to the permissive temperature. At stationary phase (0 h time point) only 4% of cells were budded, and only 3% of unbudded cells had capping protein/actin clusters. As cells were released from stationary phase (23°C, 1, 2, and 3 h time points) a progressively higher percentage of the cells budded, and this increase was paralleled by an increase in the percentage of cells bearing actin/capping protein rings, up to 44%. During the same time period, fewer than 20% of the cells advanced to late stages in the cell cycle, as judged by examination of the mitotic spindle (Table II, spindle stain-

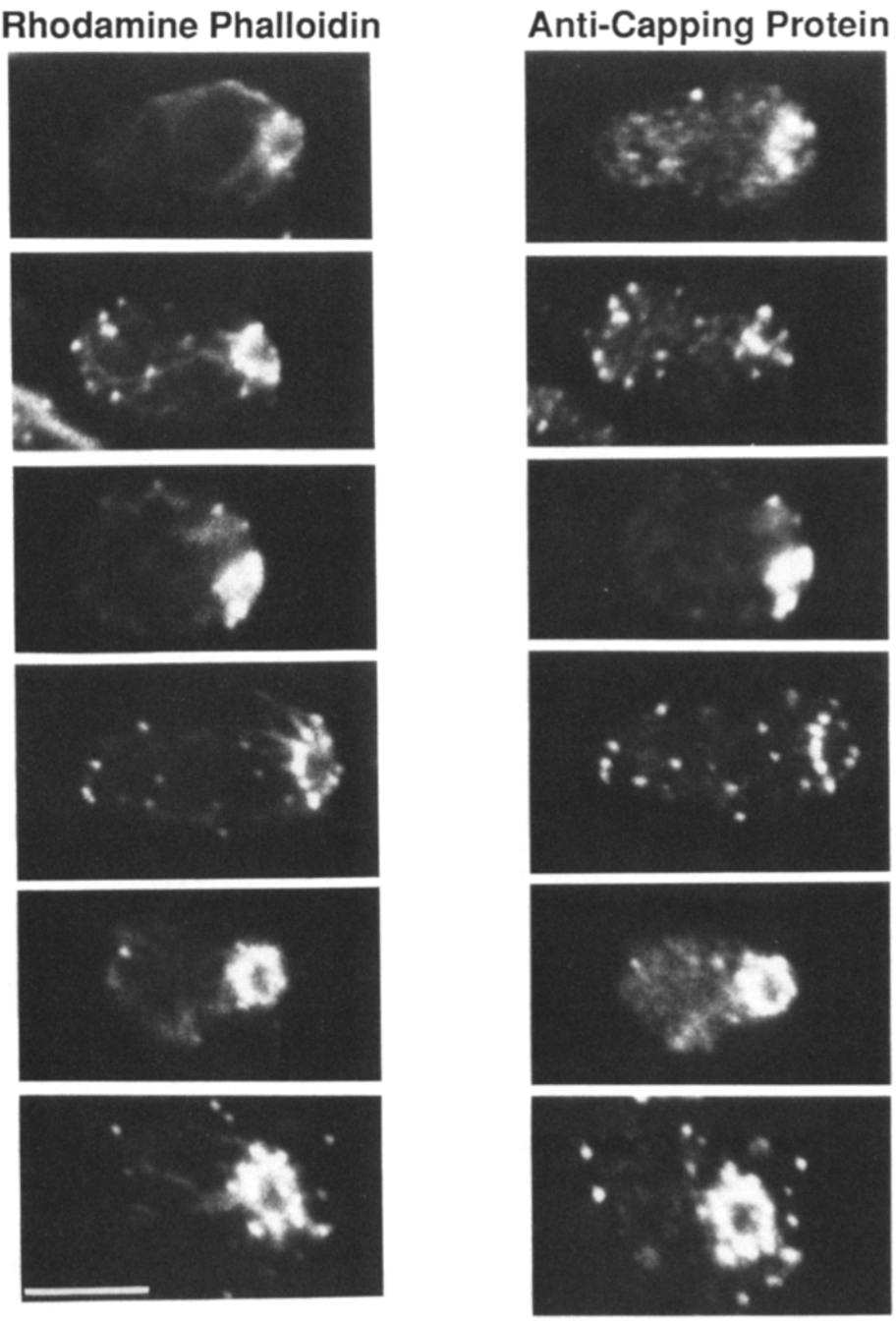


*Figure 4.* Capping protein and actin localization in unbudded cells. Diploid strain YJC163 was fixed and stained with anti-capping protein antibodies and with rhodamine-phalloidin as described in Materials and Methods. Bar, 5 $\mu$m.

ing). Therefore, it is likely that actin/capping protein clusters are sites of new bud emergence, rather than remnants of the cytokinesis ring.

Because capping protein binds barbed ends and nucleates filament assembly in vitro, we asked whether capping protein precedes actin in the bud site rings. At no time point did we see rings containing only capping protein or only actin. Thus, within the limited temporal resolution of our experiment, neither protein precedes the other at the bud site.

### *Actin and Capping Protein Distribution in cdc24 Cells at the Restrictive Temperature*

Cells bearing mutant alleles of *CDC24* continue growth, DNA synthesis, and nuclear division at the restrictive temperature, but fail to bud or to deposit actin and other components in a polarized manner at the bud site (1, 32, 33). We asked whether capping protein appears at the bud site in these mutants at the restrictive temperature of 36°C. At the restrictive temperature these cells failed to bud even though the spindles began to elongate (Table II). During this time, neither capping protein nor actin clustered into a ring at the bud site.

## *Discussion*

### *Purification of S. cerevisiae Capping Protein*

In our previous work we identified a gene, *CAP2*, with protein sequence similarity to the $\beta$ subunit of capping protein.

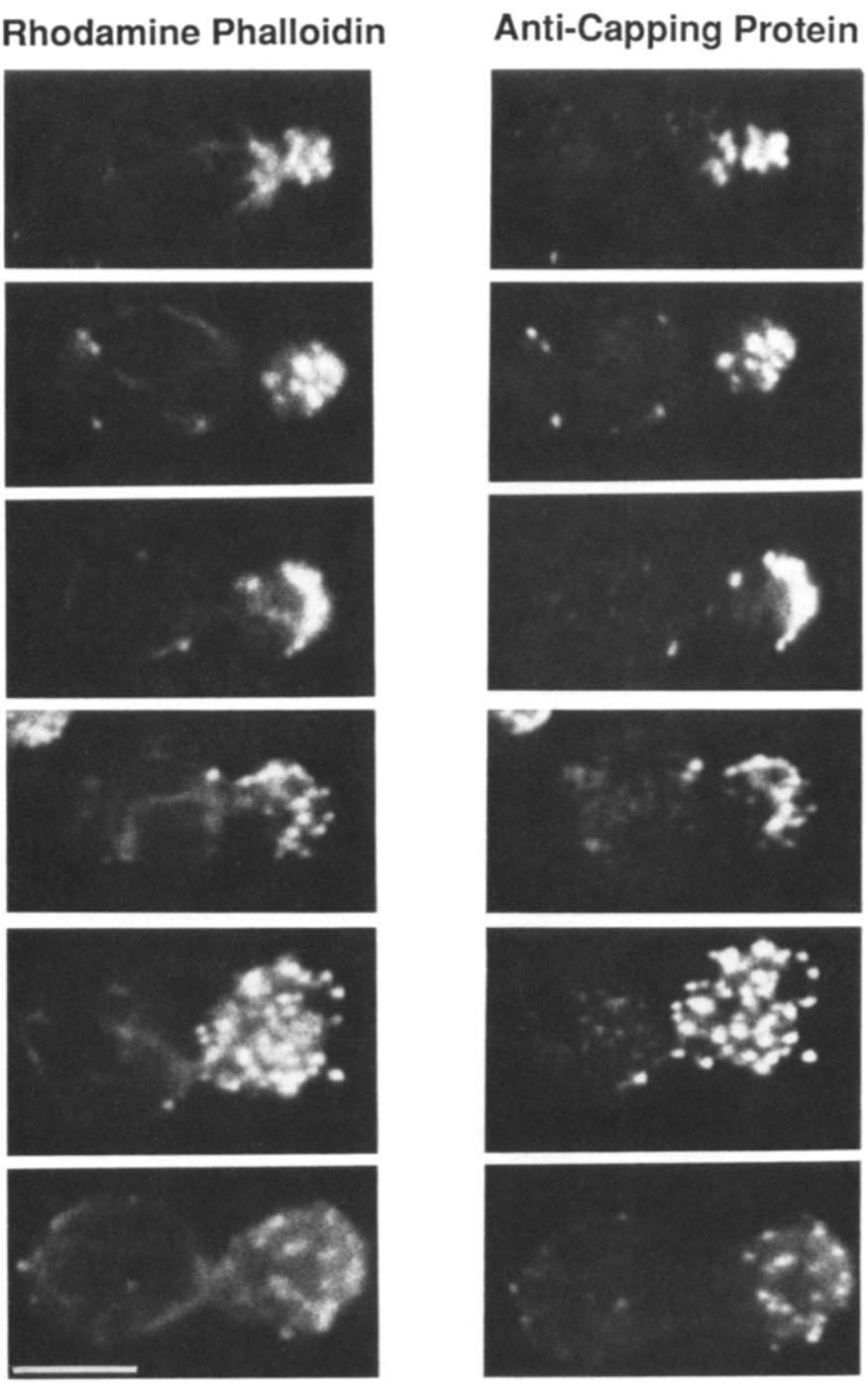


*Figure 5.* Capping protein and actin localization in budding cells: Spots. This figure is taken from the same experiment as Fig. 4. The figures are printed to display the cortical spots. Bar, 5 $\mu$m.

To confirm that CAP2p is the yeast homologue of capping protein $\beta$, we purified the *CAP2* protein. The purified protein is recognized on immunoblots by an antibody specific for a trpE-CAP2 fusion protein, yields a proteolytic peptide with an amino acid sequence matching that predicted by the *CAP2* nucleotide sequence, and migrates on SDS-polyacrylamide gels at a relative molecular mass close to that predicted by the *CAP2* nucleotide sequence.

CAP2p shares the following characteristics of capping protein $\beta$ from other organisms (3, 5, 7, 10, 17, 19, 30). (*a*) CAP2p copurifies with another protein of similar size (the $\alpha$ subunit); the two subunits are not immunologically cross-reactive. (*b*) The heterodimer decreases the low-shear viscosity of actin filaments and increases the critical concentration for actin polymerization in a manner consistent with capping barbed ends. (*c*) Its activity is not $Ca^{++}$ dependent and is inhibited by $PIP_2$.

### *Immunofluorescence Localization of Yeast Capping Protein*

Our results show that yeast capping protein colocalizes with yeast actin spots in sites of new cell wall growth—including the site of bud emergence, the tip of growing buds, and the shmoo tip—but not with actin cables or with the actin ring associated with cytokinesis.

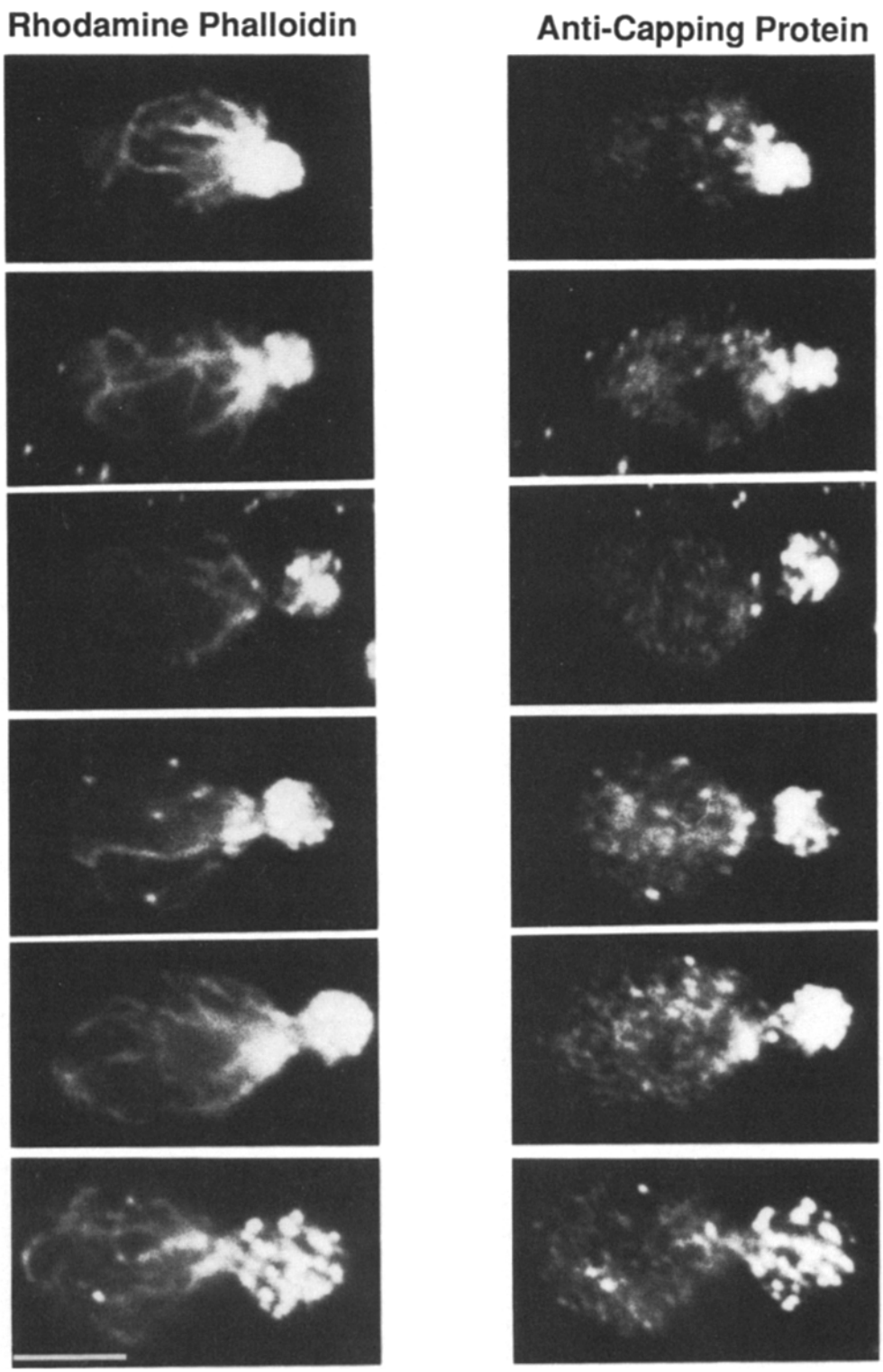


*Figure 6.* Capping protein and actin localization in budding cells: cables. This figure is taken from the same experiment as Fig. 4. The figures are printed to display the actin cables. The second cell from the top is the same as the first cell from the top in Fig. 5. Bar, 5 $\mu$m.

### *Spots*

The ultrastructure of the spots, including the polarity, length, and number of actin filaments in each spot, is unknown. The staining with anti-capping protein antibodies suggests that the spots contain filament barbed ends, because capping protein binds barbed ends in vitro. The localization of capping protein to these spots in yeast is interesting, because in chicken epithelial cells capping protein is located at sites where actin filaments encounter the membrane (30a). Our results thus provide support for the hypothesis (1) that the spots are sites where actin filaments interact with the plasma membrane.

What is the function of capping protein in the spots? On the basis of its in vitro activity and localization to the Z line of striated muscle, capping protein is predicted to bind filament barbed ends in vivo. Capping protein may localize in the spots before actin does, and subsequently capture pre-

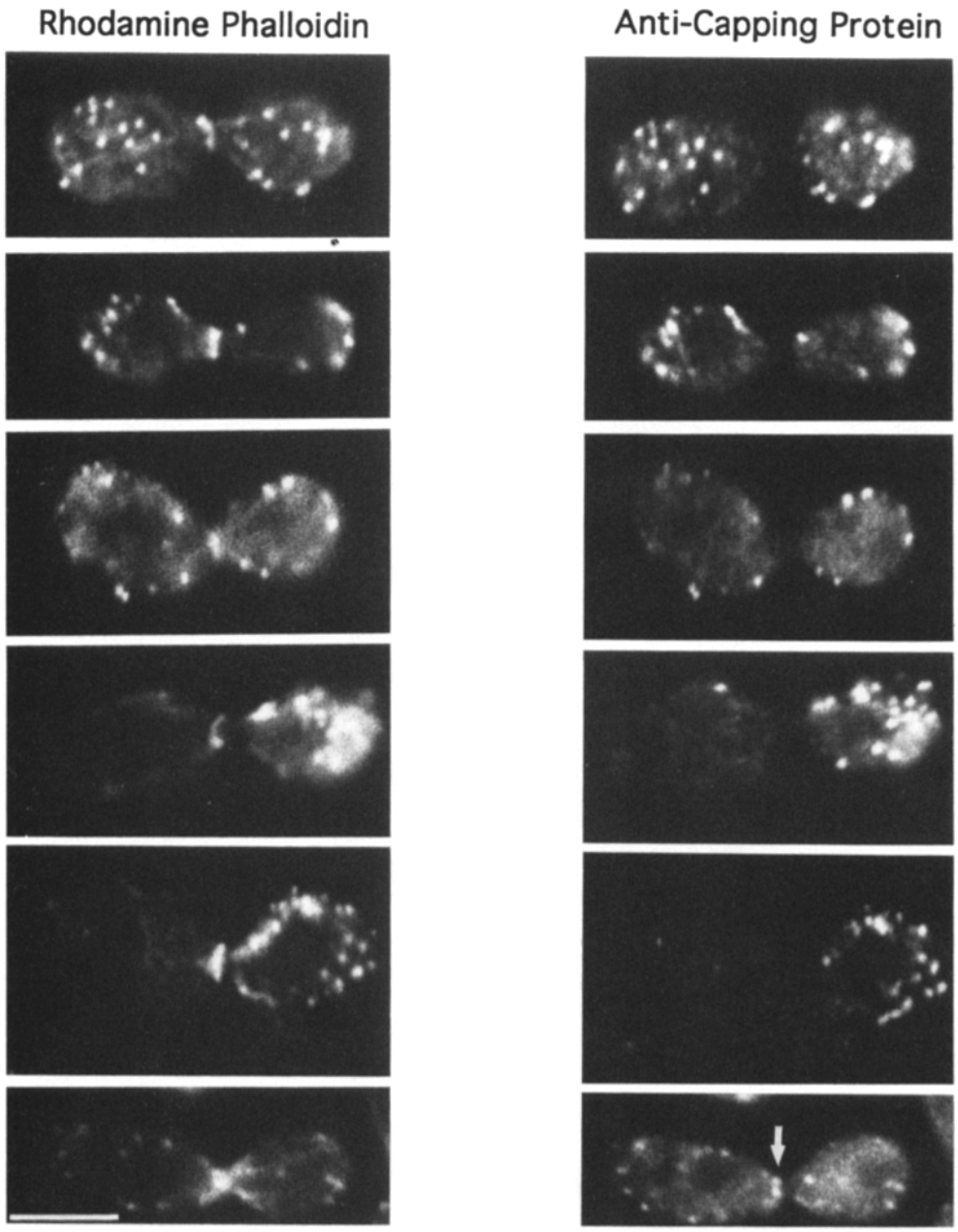


*Figure 7.* Capping protein and actin localization in large budded cells. This figure is taken from the same experiment as Fig. 4. (*Arrow*) Capping protein present at the bud neck. Bar, 5 μm.

formed filaments or nucleate the assembly of new ones. Alternatively, capping protein already present on a filament could be bound by another component of the spots and so orient the filaments in the spot. A third possibility is that capping protein is carried passively on the actin filaments, which are directed into the spots by some other means.

A related question concerns the organization of the spots into larger structures such as the prebudding ring, and what role capping protein might play in these processes. We considered the possibility that capping protein, with its potential for orienting actin filaments, might act at the same level as the product of the *CDC24* gene, which is known to be essential for assembly of the bud site complex (1). However, our experiment in which a *cdc24* strain was released from stationary phase to the restrictive temperature shows that capping protein does not localize to the bud site when *CDC24* is not functional. Therefore, *CDC24* acts upstream of capping protein in the assembly of the actin cytoskeleton associated with the bud site.

Spots in the bud and shmoo are presumed to mediate remodeling and/or growth, but the mother cells also contain actin spots and grow very little, if all all. We find that spots in all these places contain capping protein in addition to actin. The extent to which spots in the bud, shmoo, and mother are structurally and functionally similar is not well understood.

### *Cables*

Capping protein is not present in the actin cables, which are presumed to be bundles of actin filaments because they stain with fluorescent phalloidin and anti-actin antibodies and be-

*Table II. Release of cdc24-4 Cells from Stationary Phase*

| | Time | Budded | Spindle staining dot/short/long | Unbudded cells % + actin/YCP clusters |
|---|---|---|---|---|
| | *h* | % | | |
| – | 0 | 4 | ND | 3 |
| 23°C | 1 | 11 | 61/39/0 | 12 |
| 23°C | 2 | 39 | 49/50/1 | 18 |
| 23°C | 3 | 58 | 60/22/18 | 44 |
| 36°C | 1 | 2 | 69/30/1 | 1 |
| 36°C | 2 | 2 | 57/43/0 | 1 |
| 36°C | 3 | 1 | 33/67/0 | 0 |

Strain YJC283 was grown to stationary phase in YPD ($OD_{600}$ ~60) and diluted to $OD_{600}$ = 0.5 in prewarmed medium at 23 or 36°C. At 0, 1, 2, and 3 h, aliquots were removed, fixed, and processed for microscopy as described. At each time point, the percentage of budded cells was recorded, 100 cells were scored for tubulin staining, and 100 unbudded cells were scored for the presence of actin/yeast capping protein clusters. For tubulin staining, the first number represents cells with a single spot of tubulin fluoresence, the second number those with a short spindle, and the third those with a long spindle. (This experiment is adapted from one described in Kim, H. B., S. R. Ketcham, B. K. Haarer, and J. R. Pringle, manuscript submitted for publication.) Tubulin staining was poor at the 0 time point, perhaps owing to changes in the cell wall in stationary cultures.

cause bundles of ~10-nm filaments have been observed by EM (1, 23). The length and polarity of these filaments is not known. One hypothesis is that single filaments run the entire length of the cables. In this case one might see capping protein at one or both ends of the cables, depending on the polarity of the filaments. We looked carefully for capping protein staining at the ends of cables and did not see it. However, the sensitivity and resolution of fluorescence microscopy clearly limits the strength of this conclusion. We cannot be certain that we could detect a small number of capping protein molecules at the end of a cable, especially because at the bud neck the cables seem to converge and are superimposed on the brightly staining spots. Another alternative is that cables contain relatively short filaments, whose ends are distributed through the length of the cable, not just the ends. We cannot exclude this possibility, especially since the cytoplasm has a diffuse capping protein stain which might obscure cable staining. The apparent absence of capping protein from the cables is intriguing because when the $\beta$ subunit of capping protein is deleted from yeast the cables disappear (2).

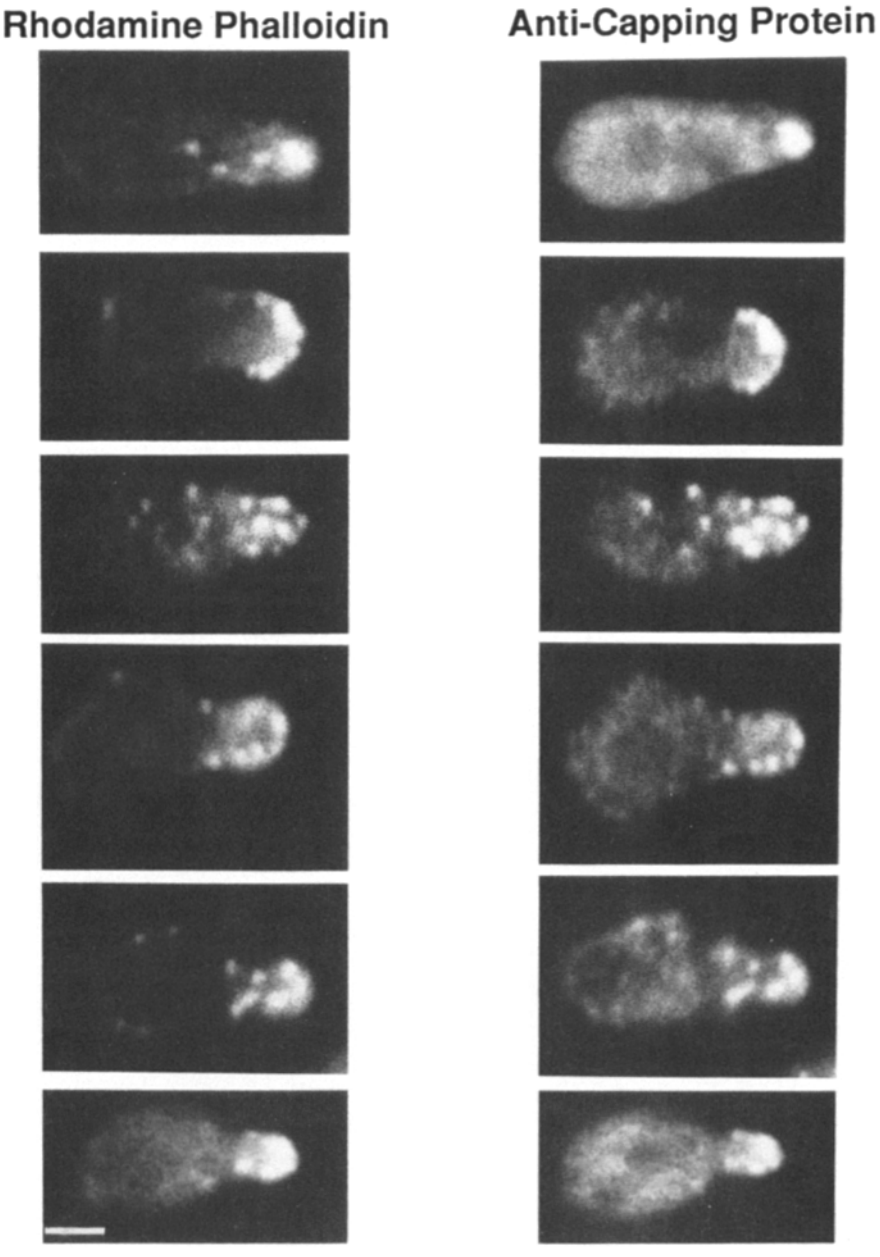


*Figure 8.* Actin and capping protein localization in shmooing cells. Haploid MATa cells (YJC093) were treated with $\alpha$-factor. Bar, 2 $\mu$m.

## *Cytokinesis Rings*

At the time of cytokinesis a single or double ring of actin is seen at the bud neck. This structure is not identical to the actin ring found at the site of bud emergence, because we have shown that it does not contain capping protein whereas the bud site ring does. Further evidence for the nonidentity of the two rings is that an actin ring is not a permanent feature of the bud neck. Although remnants of the bud site ring can still be seen in many cells with small buds, it is absent from cells with medium-sized buds (Fig. 5 and ref. 20). While certain components such as a chitin ring and the products of the CDC3, CDC11, and CDC12 genes have been shown to assemble at the prebudding site and to persist through cytokinesis (14, 15, 21) our data indicate that actin filaments do not simply remain in this complex through the budding cycle. This finding argues against the hypothesis that the actin cytoskeleton of the bud site directs the formation of the actin cytoskeleton of the next, adjacent bud site.

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