Yeast Actin-binding Proteins: Evidence for a Role in Morphogenesis

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Abstract. Three yeast actin-binding proteins were identified using yeast actin filaments as an affinity matrix. One protein appears to be a yeast myosin heavy chain; it is dissociated from actin filaments by ATP, it is similar in size (200 kD) to other myosins, and antibodies directed against *Dictyostelium* myosin heavy chain bind to it. Immunofluorescence experiments show that a second actin-binding protein (67 kD) colocalizes in vivo with both cytoplasmic actin cables and cortical actin patches, the only identifiable actin structures in yeast. The cortical actin patches are concentrated at growing surfaces of the yeast cell where they might play a role in membrane and cell wall insertion, and the third actin-binding protein (85 kD) is only detected in association with these structures. This 85-kD protein is therefore a candidate for a determinant of growth sites. The in vivo role of this protein was tested by overproduction; this overproduction causes a reorganization of the actin cytoskeleton which in turn dramatically affects the budding pattern and spatial growth organization of the yeast cell.

SACCHAROMYCES CEREVISIAE cells grow asymmetrically; new cells form as buds on mother cells. Thus, a growing yeast cell faces several problems of spatial organization. First, a bud site must be chosen on the surface of the unbudded yeast cell. Once this growth axis is established, transport of organelles and materials synthesized in the mother cell must be directed along the axis to the growing bud, and growth must be restricted to the bud. The actin cytoskeleton has been implicated, by its organization and by mutant phenotypes, as a key determinant of axis formation and growth organization in yeast.

The disposition of actin within the yeast cell reflects the asymmetry of growth. When visualized by fluorescence microscopy (Kilmartin and Adams, 1984; Adams and Pringle, 1984), the actin in mother cells is seen as filamentous cables, while the actin in buds appears as cortical patches. Replacement of the single yeast actin gene (Ng and Abelson, 1980; Gallwitz and Seidel, 1980) with a mutant actin gene (Shortle et al., 1984) results in loss of normal actin organization, and in multiple bud morphogenesis defects (Novick and Botstein, 1985). At nonpermissive temperatures, 70% of cells carrying the *act1-1* mutation accumulate in an unbudded state. At the same time, secretion, normally targeted to the growing bud, is defective, and vesicles accumulate. Furthermore, chitin, which in wild-type cells is deposited in a ring at the base of the bud, becomes aberrantly localized.

The site of bud emergence in *Saccharomyces cerevisiae* cells is not randomly chosen. In haploid yeast, buds emerge adjacent to previous bud sites, while in diploid yeast, buds emerge from one of two opposite poles (see Sloat et al., 1981, and references cited therein). Actin might play a role in determining the bud emergence site. Early in the cell cycle cortical actin patches form a ring on the cortex of the unbudded yeast cell. This ring is an indicator of the precise site of bud emergence. The ring of actin patches also directly underlies the ring of chitin that forms around the bud neck (Kilmartin and Adams, 1984).

Actin organization in yeast suggests how the actin cytoskeleton might function in morphogenesis (Kilmartin and Adams, 1984; Adams and Pringle, 1984). Actin cables generally are aligned along the axis of bud growth. These actin cables might mediate transport of membrane-bound vesicles from mother cells to growing buds. As mentioned above, vesicles accumulate in actin mutants. Actin-mediated organelle transport has been observed in other organisms (Kachar and Reese, 1988; Kohno and Shimmen, 1988; Adams and Pollard, 1986). In addition, cortical actin patches are concentrated in regions actively involved in cell surface growth in yeasts (Adams and Pringle, 1984; Kilmartin and Adams, 1984; Marks et al., 1986). In *Saccharomyces cerevisiae* these cortical patches are concentrated in the bud, which grows by insertion of plasma membrane and cell wall material at its tip (Field and Schekman, 1980; Tkacz and Lampen, 1972; Cabib, 1975), and the patches rearrange to a different region of surface growth, the region of septum formation, at cytokinesis. Thus, the cortical actin structures are well placed to be involved in membrane and cell wall insertion at growth sites.

The spatial organization of the yeast actin cytoskeleton is intimately associated with the spatial organization of yeast

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growth. The yeast actin polypeptide, though, is not likely to contain all of the information that specifies the spatial organization and timing of actin filament assembly. First, yeast actin and animal muscle actins are ~85% identical in amino acid sequence, yet yeast actin is quite different in organization and function from animal muscle actin. Furthermore, the single yeast actin polypeptide assembles into two different structures in yeast: cortical structures and cytoplasmic cables. We therefore suppose that the organization of the yeast actin cytoskeleton is largely specified by actin-binding proteins. Yeast actin-binding proteins could, by virtue of their ability to organize the actin cytoskeleton, actually organize the spatial pattern of yeast growth.

Further understanding of how the actin cytoskeleton functions to organize the spatial pattern of yeast cell growth requires identification of the yeast proteins that interact with actin. Recently a method using phalloidin-stabilized actin filament affinity chromatography to identify actin-binding proteins was described by Miller, K. G., and B. M. Alberts (manuscript submitted for publication). We report here the use of this method to identify three yeast actin-binding proteins by virtue of their affinity for yeast actin filaments. One 200-kD protein appears to be a yeast myosin, a type of protein already known to interact with actin in vivo. Immunofluorescence experiments using affinity-purified antisera demonstrate that the two other actin-binding proteins interact with actin in yeast cells. A 67-kD yeast actin-binding protein associates in vivo with both actin cables and cortical actin patches. An 85-kD yeast actin-binding protein is a candidate for a protein that can organize actin at growing yeast surfaces, and as a result, can determine the yeast growth pattern; it interacts exclusively with the cortical actin structures found in actively growing regions of yeast cells. Overexpression of the gene encoding this 85-kD protein results in rearrangement of the actin cytoskeleton, an altered spatial growth pattern, and failure to choose the proper site of bud emergence.

Table I. Yeast Strains Used

Strain	Genotype	Source
DBY877	*MATα his4-619*	This laboratory
DBY1696	*MATa ade2-1 his4-619 act1-2* *MATα + + act1-2*	Novick and Botstein, 1985
DBY1840	*MATa ura3-52 his4-619 sac1**	This laboratory
DBY1843	*MATa ura3-52 his4-619* (pRB393)‡	This laboratory
DBY2055	*MATa ura3-52 his4-619*	This laboratory
DBY4866	*MATa ura3-52 his4-619* *MATα + +*	This work
DBY5178	*MATa ura3-52 his4-619* (pRB307)	This work
DBY5179	*MATa ura3-52 his4-619* (pRB1201)	This work
DBY5181	*MATa ura3-52 his4-619 leu2-3.112* *MATα ura3-52 + +* (pRB307)	This work

* The *SAC1* gene was identified as a suppressor of a mutant *ACT1* allele. This *SAC1* allele has a 2.6-kb Bam HI–Bam HI fragment deleted. This work will be described by Novick et al., 1988.

‡ pRB393 contains the *SAC1* gene (Novick et al., 1988) cloned on YEp24 (Botstein et al., 1979).

Materials and Methods

Yeast Strains and Genetic Techniques

The yeast strains used in this study are listed in Table I. The strain DBY877 was used for most biochemical work. For the particular experiment shown in Fig. 1, DBY877-derived strains with altered *SAC1* (*SAC1* was isolated as a suppressor of an actin mutant; Novick et al., 1988) gene dosage were used (see Table I), however, direct comparisons showed that identical results are obtained when DBY877 is used (not shown).

Standard genetic techniques of crossing, sporulation, and tetrad analysis were as described by Sherman et al. (1974). YEPD medium contains 1% yeast extract, 2% bactopeptone, and 2% glucose. Synthetic medium, plus required supplements, was prepared as described by Sherman et al. (1974). Solid media contain 2% agar.

Isolation and Analysis of Yeast Actin-binding Proteins

Yeast cells were disrupted using a Bead-Beater homogenizer (Biospec Products, Bartlesville, OK). All cell lysates were prepared in buffer supplemented immediately before lysis with a 10^{-3} dilution of aqueous protease inhibitor cocktail (1,000× = 0.5 mg/ml of the following dissolved in water: antipain, leupeptin, pepstatin A, chymostatin, and aprotinin) and a 10^{-2} dilution of ethanol protease inhibitor cocktail (100× = an ethanol solution containing 0.1 M phenylmethylsulfonyl fluoride, 1 mM benzamidine HCl, and 0.1 mg/ml phenanthroline). Concentrated protease inhibitor cocktails were stored at −20°C. For column buffers, the aqueous cocktail was used at 0.1× and the ethanol cocktail was omitted. To maximize phenylmethylsulfonyl fluoride action, dithiothreitol was not added to lysates until 15–30 min after cell lysis.

Yeast actin for affinity columns was prepared by the Zechel (1980) DNase 1 affinity-purification procedure that was originally applied for yeast by Kilmartin and Adams (1984). The actin isolated in this manner appeared homogeneous when visualized on Coomassie Blue-stained SDS gels as was seen by Kilmartin and Adams (1984), except for a very faint 70-kD polypeptide occasionally seen on overloaded gels. DNaseI was obtained from Boehringer Mannheim Biochemicals (Indianapolis, IN). For large scale preparations actin was isolated from fresh wet blocks of commercial Red Star baker's yeast (Universal Foods, Randolf, MA).

0.5-ml columns containing 0.4 mg of phalloidin-stabilized yeast actin filaments, or 0.4 mg of BSA, were constructed by coupling a solution of 2 mg/ml F-actin with 5 μg/ml phalloidin or a solution of 3 mg/ml BSA (a higher BSA concentration was used because the coupling efficiency was lower) in coupling buffer (50 mM Hepes, pH 7.5, 0.2 mM ATP, 0.2 mM $CaCl_2$, 0.1 M KCl, 6.0 mM $MgCl_2$) to a 1:1 mixture of Affi-Gel 10 (Bio-Rad Laboratories, Richmond, CA) and Sepharose CL-6B (an inert resin that improved column flow properties; Pharmacia, Inc., Piscataway, NJ). The construction and use of F-actin columns was as described at length by Miller et al. (1985) who have shown that these columns can be used to isolate many well-characterized actin-binding proteins from lysates prepared from a variety of cell types. Initially, the yeast actin filaments, formed in vitro for use on affinity columns, were shown by electron microscopy to be of the correct size (7 nm diam) and structure. Subsequently, actin filament formation and phalloidin stabilization were monitored before column construction by fluorescence microscopy visualization of filaments labeled with a molar excess of rhodamine phalloidin (Molecular Probes, Inc., Eugene, OR).

To identify yeast actin-binding proteins, yeast for each column were grown in 1 liter YEPD to ~5×10^6 cells per milliliter. The cells were collected by centrifugation and washed twice in wash buffer (5 mM Hepes, pH 7.5, 1 mM Na_3 EDTA, 20 mM KCl). The cells from each 1-liter culture were disrupted in 40 ml of lysis buffer (5 mM Hepes, pH 7.5, 1 mM Na_3 EDTA, 20 mM KCl, 0.1% NP-40, 0.5 mM dithiothreitol), minus dithiothreitol and supplemented with protease inhibitors (see above). Disruption involved three 1-min homogenizations in an ice-jacketed Bead-Beater homogenizer (Biospec Products), allowing several minutes between homogenizations for cooling. After 15–30 min on ice, the lysate was made 0.5 mM in dithiothreitol.

After sedimentation at 60,000 *g* for 60 min, lysates were loaded in parallel over actin filament and BSA columns at ~3 ml/h. The columns were washed with 10–20-column volumes of lysis buffer, then eluted stepwise at 6 ml/h with lysis buffer containing first 0.5 mM ATP, 3 mM $MgCl_2$, then 0.1 M KCl, and finally, 0.5 M KCl. 10-column volumes were pooled for each elution condition.

To analyze eluted proteins, one-fourth volume 50% trichloroacetic acid, 2 mg/ml sodium deoxycholate, was added to each pooled aliquot. The

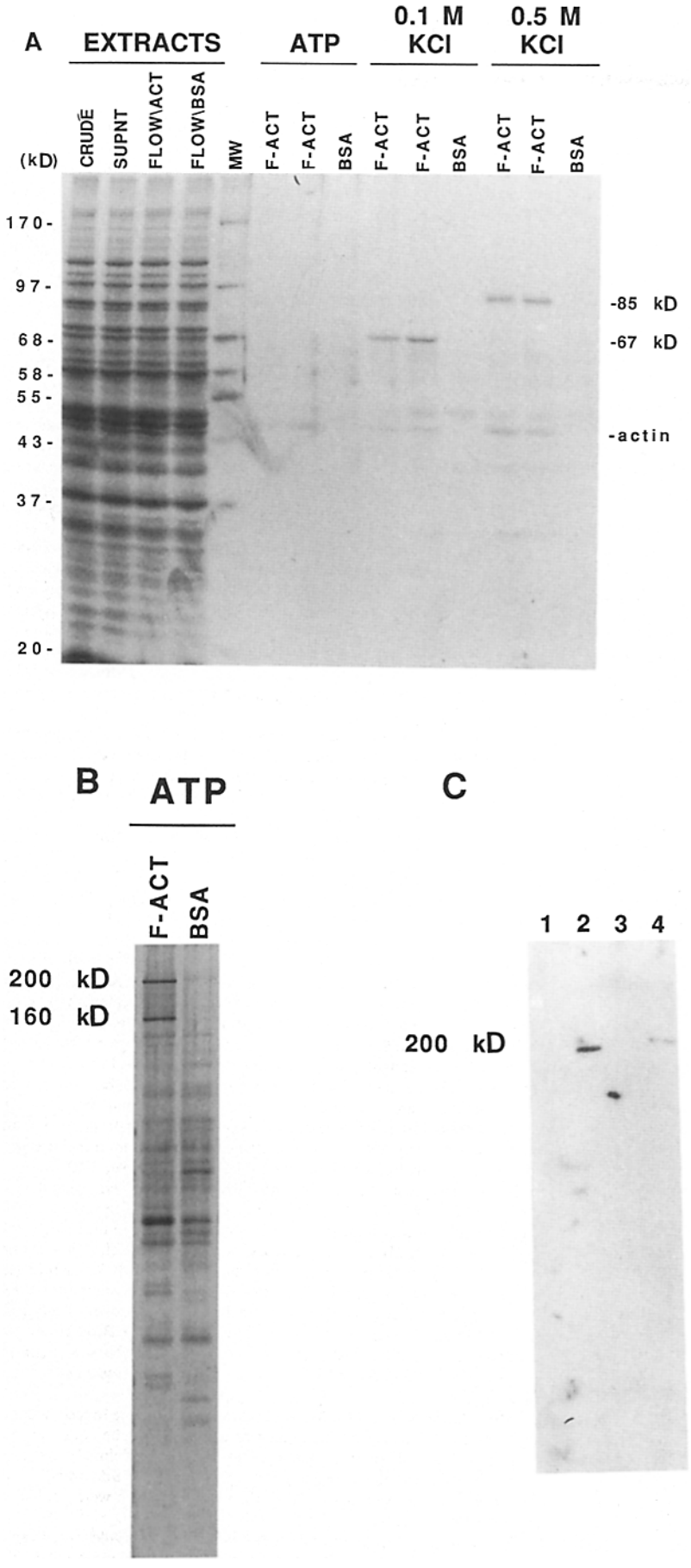

Figure 1. Identification of yeast actin-binding proteins. (*A*) Coomassie Blue–stained SDS–8.5% polyacrylamide gel showing protein fractions from an actin-binding protein preparation. The extracts in the first four lanes are from strain DBY1843. The proteins dissociated by ATP, 0.1 M KCl, or 0.5 M KCl, from two F-actin and one BSA control column, were (in order from left to right for each elution condition) from strains DBY1840, DBY-1843, and DBY1843 again. All three columns were loaded and eluted in parallel. (*B*) Silver-stained SDS–8.5% polyacrylamide gel showing DBY1843 proteins dissociated from F-actin and BSA control columns by ATP. Aliquots from the same column fractions were used for the gel in *A*. (*C*) Autoradiograph of an immunoblot from an SDS–6% polyacrylamide gel probed with a 1:1,000 dilution of anti–*Dictyostelium* myosin antiserum, followed by ^{125}I-labeled protein A. (Lane *1*) Approximately 10 μg of total yeast cell extract. (Lane *2*) ATP-dissociated protein fraction from an F-actin column. This fraction contains ~50 ng of the 200-kD actin-binding protein. (Lane *3*) ATP-dissociated protein fraction from a BSA control column run in parallel with the F-actin column used to generate the protein in lane *2* (the spot in lane *3* is an artifact on the blot, not an immunoreactive band). (Lane *4*) 100 ng of chicken muscle myosin (a gift from Dr. Daniel Kiehart, Harvard University, Cambridge, MA).

Table II. Effects of 85-kD Actin-binding Protein Overproduction on Bud Scar Pattern

		Number of cells with bipolar scars*				
		Bud scar number				
Strain	Feature	2	3	4	5 or more	Average
						%
DBY5178	Haploid control	2/29 (7)‡	2/22 (9)	1/12 (8)	1/8 (13)	(8)
DBY5179	Elevated ABP 85	7/29 (25)	15/37 (41)	11/21 (52)	6/9 (66)	(41)
DBY5181	Diploid control	16/34 (47)	39/44 (89)	22/23 (96)	7/7 (100)	(78)

* Cells in random fields were scored as bipolar if a scar was present that was not directly adjacent to any other scars. Only cells with two or more scars were scored. Focusing up and down with the microscope was important because adjacent scars often were in different focal planes.
‡ Number of cells with scars scored as bipolar/total number of cells scored (% of cells with bipolar scars).

resulting precipitate was washed once with 10% trichloroacetic acid, and twice with acetone. Each precipitate was dissolved in SDS-polyacrylamide gel sample buffer, and one-fourth of each sample was loaded onto an 8.5% polyacrylamide gel (Laemmli, 1970). Proteins were visualized by Coomassie Blue staining or using a silver staining kit (Accurate Chemical & Scientific Corp., Westbury, NY).

Preparation of Antisera

To obtain sufficient amounts of actin-binding proteins to generate antisera, 200 ml lysates were prepared from 6-liter haploid yeast cultures grown to 2×10^7 cells per milliliter. The lysates were passed first through a 10-ml column containing 10 mg BSA to remove nonspecific proteins, then over a 2-ml column containing 1.5 mg of yeast actin filaments. Actin-binding proteins were collected as above, but no concentration step was necessary to observe eluted proteins on Coomasie Blue-stained gels.

Yeast actin and the 67- and 85-kD actin-binding proteins were excised from preparative SDS-polyacrylamide gels and the gel slices were equilibrated in PBS (0.13 M NaCl, 2 mM KCl, 8 mM Na_2HPO_4, 2 mM KH_2PO_4, pH 7.2). The slices were crushed, mixed with PBS, then 2 vol of Freund's complete (first two injections) or incomplete (all subsequent injections) adjuvant were added, and the samples were homogenized. Rabbits were immunized with about 50 μg of actin, or 10–20 μg of the actin-binding proteins, and were boosted with similar amounts of protein at days 20 and 35. At day 47 rabbits were test bled. Antisera were tested by immunoblotting, using nitrocellulose strips that contained total yeast cell lysates, or column fractions enriched for actin-binding proteins, that had been transferred from SDS-polyacrylamide gels (Burnette, 1981). One out of three rabbits immunized with yeast actin, and two out of two rabbits immunized with either the 67- or the 85-kD actin-binding proteins tested positive. Antisera were affinity purified and stored as described by Pfeffer et al. (1983), and specificity was evaluated by immunoblotting (see Fig. 2). The *Dictyostelium* myosin antiserum (Berlot et al., 1985) was a generous gift of Kent Matlack and Catherine Berlot (Stanford University, Stanford, CA).

Immunofluorescence and Calcofluor Staining

Yeast were grown to early log phase: generally 2×10^6 cells per milliliter. Fixation and immunofluorescence procedures were adapted from Kilmartin and Adams (1984). Best results were obtained when cells were grown in synthetic medium and fixed by adding 0.67 ml of 37% formaldehyde directly to a 5-ml culture. After 60 min at room temperature, the cells were washed twice with 2 ml 1.2 M sorbitol, 0.1 M potassium phosphate, pH 7.5. To remove cell walls, the fixed cells were resuspended in 0.5 ml of the same buffer, then 1 μl β-mercaptoethanol and 10 μl of 1 mg/ml zymolyase 60,000, were added. After 30 min at 37°C, 15 μl of the cell suspension was transferred to a polylysine-coated well on a multiwell slide. After 60 s, the suspension was removed by aspiration, and the slide was immersed in −20°C methanol for 6 min, and −20°C acetone for 30 s. (This step was essential for actin immunofluorescence, both using fixed yeast cells and actin filaments assembled from pure actin in vitro.) After air drying the slide briefly, the cells were rehydrated with PBS containing 1 mg/ml BSA (PBS-BSA). All antisera were diluted in PBS-BSA. Antibody incubations were 60-min each, followed by five PBS-BSA washes. Slides were mounted in 90% glycerol containing *p*-phenylenediamine as described by Kilmartin and Adams (1984).

The affinity-purified antisera against yeast actin, and the 67- and 85-kD actin-binding proteins were used for immunofluorescence at 1:50, 1:40, and 1:40 dilutions, respectively. The affinity-purified rat anti-yeast actin antiserum (a generous gift of Dr. John Kilmartin, Medical Research Council Laboratory of Molecular Biology, Cambridge, England) was used in double-label experiments at 1:30. Fluorescein- and rhodamine-conjugated anti-heavy and -light chain secondary antisera were obtained from Organon Teknika-Cappel (Malvern, PA).

For double-label experiments it is essential that the fluorochrome-conjugated secondary antisera show absolute species specificity for the primary antisera used in the experiment. In control experiments, the rhodamine-conjugated anti-rat antiserum showed no reactivity with rabbit primary antisera. However, the fluorescein-conjugated anti-rabbit antiserum reacted with rat primary antisera. To remove the rat-reactive component, the antiserum was absorbd against resin containing immobilized rat IgG (Organon Teknika-Cappel). Control experiments demonstrated that the rat-reactive component was entirely removed.

To label chitin, fixed and washed cells were incubated in 0.1% calcofluor (fluorescent brightener 28; Sigma Chemical Co., St. Louis, MO) in water for 10 min, then washed, and resuspended in water.

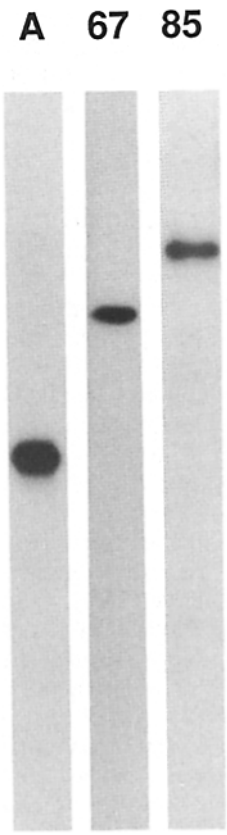

Figure 2. Specificity of affinity-purified antisera. Anti-yeast actin (*A*), anti-67-kD actin-binding protein, and anti-85-kD actin-binding protein antisera were used at 1:200, 1:50, and 1:100, respectively, to probe immunoblots containing ~5 μg each of total yeast cell lysates.

Results

Identification of Yeast Actin-binding Proteins

Columns constructed from phalloidin-stabilized rabbit muscle actin filaments have been used previously to isolate both well-known and previously unidentified actin-binding proteins from both mammalian and nonmammalian cell lysates (Miller, K. G., and B. M. Alberts, manuscript submitted for publication). In the present study we used yeast actin filaments to identify yeast actin-binding proteins. Yeast cell lysates, prepared in a low ionic strength buffer that favors disassembly of actin filaments, were passed over columns containing yeast actin filaments stabilized with phalloidin, and actin-binding proteins were dissociated first with Mg^{++}-ATP, and then with steps of 0.1 M KCl and 0.5 M KCl. At each step, a specific protein was found: Mg^{++}-ATP, 0.1 M KCl, and 0.5 M KCl eluted proteins of 200, 67, and 85 kD, respectively.

For the experiment shown in Fig. 1 *A*, independent lysates from two different yeast strains were passed in parallel over two actin filament columns and one BSA control column. The first four gel lanes show cell lysate fractions for comparison with lanes containing affinity-purified actin-binding proteins, and the fifth lane contains molecular mass standards. The next three lanes show that little protein is detected with Coomassie Blue when Mg^{++}-ATP is used to dissociate proteins from the two yeast F-actin columns and the BSA control column, except for some actin that leaches off the actin columns (actin leaches off the columns continuously, beginning with the washes preceding the application of extracts). However, silver staining reveals many proteins eluted by Mg^{++}-ATP (Fig. 1 *B*). Proteins of 200 and 160 kD elute from actin columns but not from BSA control columns. Other differences between proteins eluted from actin and BSA columns seen in Fig. 1 *B* were not found to be reproducible. The presence of the 160-kD protein in the ATP eluate was somewhat variable; this protein could be a proteolytic product of the 200-kD protein.

The 200-kD protein has the size of a myosin heavy chain and, characteristic of myosins, is dissociated from actin filaments by ATP. Significantly, a *Dictyostelium* myosin heavy chain antibody binds to this protein in immunoblotting experiments (Fig. 1 *C*), suggesting that it is a yeast myosin heavy chain. No light chain candidates were identified.

The remaining six lanes in Fig. 1 *A* show proteins of 67 and 85 kD that are reproducibly dissociated from F-actin, but not BSA columns, by 0.1 M KCl and 0.5 M KCl, respectively (higher salt concentrations do not result in the release of additional actin-binding proteins, nor does boiling the resin in SDS). These two proteins do not correspond to any major proteins present in the cell lysate fractions; in fact, we believe each represents ~0.01% of soluble protein (data not shown), so that they are each enriched in one affinity-purification step ~5,000-fold to ~50% purity. Immunoblotting experiments using the affinity-purified antisera described in the next section, indicated that passage of cell lysates over yeast actin columns results in near quantitative binding of the 85-kD actin-binding protein, and ~50% binding of the 67-kD actin-binding protein (not shown). When rabbit actin filaments were used as an affinity matrix instead of yeast actin filaments, the 85- and the 200-kD actin-binding proteins were isolated, but the 67-kD actin-binding protein was not (not shown), despite the fact that rabbit muscle actin is 85% identical in amino acid sequence to yeast actin.

In Vivo Localization of Yeast Actin-binding Proteins

The 85- and the 67-kD actin-binding proteins were localized in vivo by indirect immunofluorescence microscopy. To do this, polyclonal antisera were raised against these actin-binding proteins and yeast actin, and the resulting antisera were affinity purified. The specificity of these affinity-purified antisera, when used to probe immunoblot strips containing total yeast cell lysates, is shown in Fig. 2. For the immunofluorecence experiments described below, it is important to note that the antisera against the actin-binding proteins do not cross react with actin. These affinity-purified antisera were used to look for cross-reacting proteins in lysates prepared from *Dictyostelium*, *Acanthamoeba*, and a variety of vertebrate cells. The only proteins detected in these experiments were polypeptides of ~124 and 129 kD in *Dictyostelium* and *Acanthamoeba*, respectively, detected with the antiserum against the 85-kD yeast actin-binding protein (not shown). Subsequent experiments showed that this antiserum does not react with either of two known actin-binding proteins of similar electrophoretic mobility found in *Dictyostelium* and *Acanthamoeba*; no reaction was observed with *Acanthamoeba* myosin I isotypes A and B (Côté et al., 1985; kindly provided by Tom Lynch, National Institutes of Health) or with the 120-kD *Dictyostelium* actin-binding protein (Condeelis et al., 1984; kindly provided by Anne Bresnick, Albert Einstein College of Medicine, NY).

Fig. 3 *A* shows yeast actin organization visualized by indirect immunofluorescence microscopy using the affinity-purified yeast actin antiserum. As reported previously, actin cables, aligned along the yeast cell growth axis, and cortical actin patches, concentrated at growth sites (particularly in buds), are observed. When the affinity-purified antiserum against the 67-kD protein is used for indirect immunofluorescence microscopy (Fig. 3 *B*) actin-like cables, aligned along the growth axis, and cortical patches, concentrated in buds, are seen. As shown in Fig. 3 *C*, the affinity-purified 85-kD actin-binding protein antiserum detects actin-like cortical structures in growing bud regions of yeast cells, but even very long photographic exposures reveal no actin-like cable structures, despite the fact that the 85-kD protein immunofluorescence is brighter than the 67-kD protein immunofluorescence (the mother cells are not visible in Fig. 3 *C*, the staining is restricted largely to buds). The immunofluorescent images in Fig. 3, *A–C* suggest that both the 85- and the 67-kD proteins associate with actin in vivo. Further support for this conclusion comes from the two experiments discussed below.

A double-label immunofluorescence experiment is shown in Fig. 4. The left panels (Fig. 4, *A* and *C*) show anti–actin immunofluorescence using an affinity-purified rat antiserum (Kilmartin and Adams, 1984) and rhodamine-labeled secondary antiserum. The right panels show anti–67-kD actin-binding protein (Fig. 4 *B*) and anti–85-kD actin-binding protein (Fig. 4 *D*) immunofluorescence of the same cells shown in the corresponding left panels, using the affinity-purified rabbit antisera characterized in Fig. 2, and a fluorescein-labeled secondary antiserum. Control experiments (see Materials and Methods) demonstrated the specificity of the fluorescein and rhodamine images for the appropriate primary

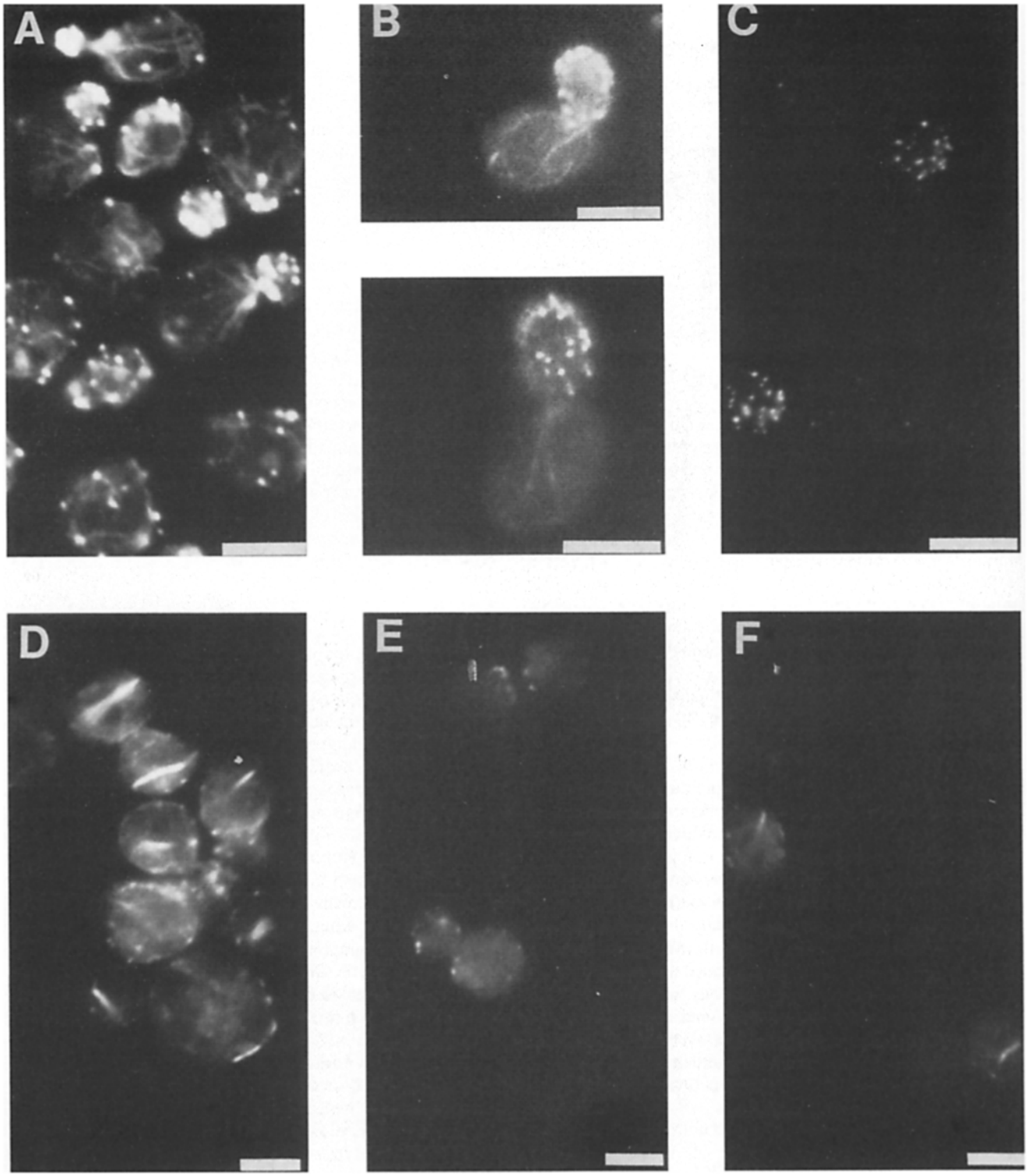

Figure 3. Localization of yeast actin-binding proteins in wild-type and *actl-2/actl-2* yeast strains. Immunofluorescence using anti-actin (*A* and *D*), anti-67- (*B* and *E*), and anti-85-kD actin-binding protein (*C* and *F*), antisera to stain the wild-type diploid DBY4866 (*A–C*) and the *actl-2/actl-2* diploid DBY1696 grown at 21°C (*D–F*). For the cells in *C*, only the bud is stained by the anti-85-kD actin-binding protein antiserum, the mother cell lies to the left of the top bud, and to the right of lower bud. Bars, 5 μm.

antibodies. The 67-kD protein is associated with both cortical actin patch structures and actin cables. The 85-kD protein is apparently associated only with the cortical actin structures associated with growing regions of the yeast cell, and not with actin cables.

The association of the 85- and the 67-kD actin-binding proteins with actin in living yeast cells was further investigated by examining the localization of these proteins in *actl-2/actl-2* mutant yeast. When yeast containing this mutation are grown at 21°C, about half of the cells contain heavy actin

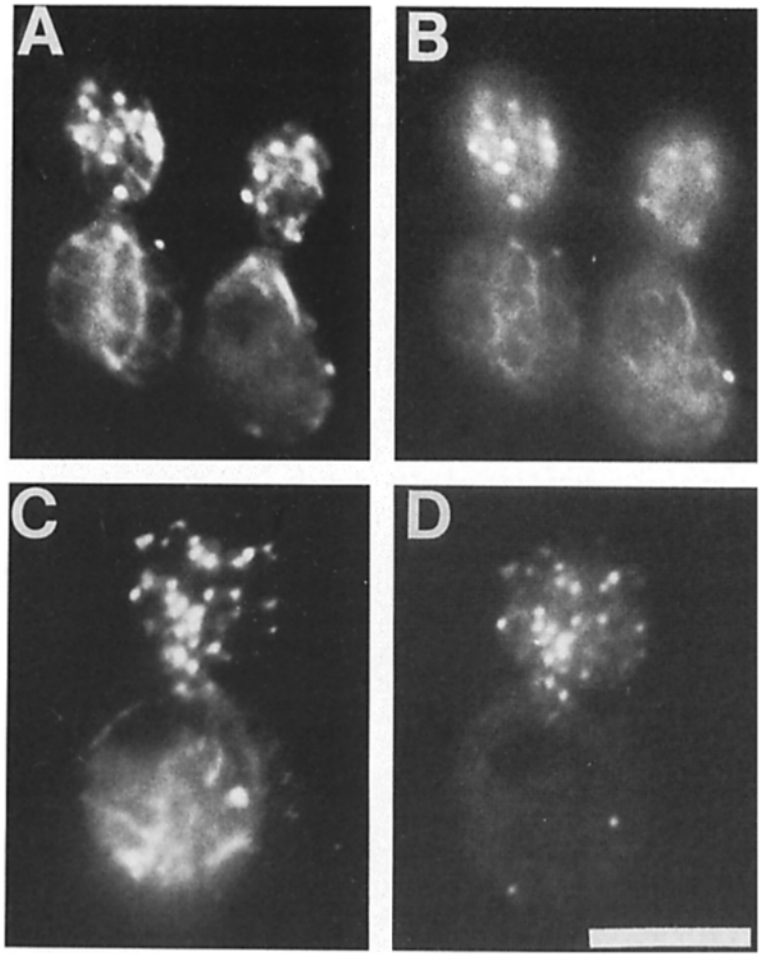

Figure 4. Colocalization of actin and actin-binding proteins by double-label immunofluorescence. (*A* and *C*) Rhodamine anti-actin immunofluorescence of the wild-type diploid DBY4866. (*B*) Fluorescein anti-67-kD actin-binding protein immunofluorescence of the same cells shown in *A*. (*D*) Fluorescein anti-85-kD actin-binding protein immunofluorescence of the same cell shown in *C*. Bar, 5 μm.

bars rather than arrays of actin cables, and cortical actin patches are less restricted to buds than is seen in wild-type cells (Novick and Botstein, 1985). Anti-actin immunofluorescence of *act1-2/act1-2* cells is shown in Fig. 3 *D* (the focal planes shown in Fig. 3, *D* and *F* optimize visualization of the actin bars at the expense of the cortical actin patches). The anti-67-kD and anti-85-kD actin-binding protein immunofluorescence images of *act1-2/act1-2* cells are shown in Fig. 3, *E* and *F*, respectively. The 67-kD protein, which associates with patches and cables in wild-type cells, associates in the mutants with cortical actin patches found in buds and in mother cells, but not with the aberrant actin bars. The 85-kD protein is associated with the cortical actin patches and with the heavy actin bars despite its apparent absence from normal actin cables in wild-type cells.

Consequences of 85-kD Actin-binding Protein Overproduction

The in vivo role of the 85-kD actin-binding protein was tested by overproduction. This first required isolating the gene encoding this protein. The gene encoding the 85-kD actin-binding protein (designated *ABP1*) was isolated by immunoscreening a λgt11 expression library (a generous gift of R. Young, Massachusetts Institute of Technology; Young and Davis, 1983) using the antiserum characterized in Fig. 2. The cloning will be described in detail later (Drubin, D. G., Z. Zhu, and D. Botstein, manuscript in preparation). The identity of the clone was verified by demonstrating that introduction of a plasmid (pRB1201) containing the cloned gene on a yeast episomal vector (pRB307) into yeast, causes an approximately fivefold elevation in levels of the 85-kD actin-binding protein, and by demonstration that expression of this protein in bacteria can be caused by transformation with pRB1201 (Drubin, D. G., Z. Zhu, and D. Botstein, manuscript in preparation).

Overproduction of the 85-kD protein is detrimental to yeast growth. The yeast strain DBY2055 was transformed with a high copy plasmid containing *ABP1* (pRB1201), or with the same plasmid lacking the *ABP1* insert (pRB307), creating the strains DBY5179 and DBY5178, respectively (see Table I). DBY5178 and DBY5179 were replica plated and grown at various temperatures under conditions selective for plasmid maintenance. DBY5178, containing only the plasmid vector, grows normally at all temperatures, but DBY5179, containing the plasmid carrying *ABP1*, grows slowly below 35°C, and does not form colonies at 37°C. Loss of pRB1201 by growth on nonselective media allows normal colony formation at 37°C, and reverses all mutant phenotypes discussed below.

Since localization of the 85-kD actin-binding protein to growing surfaces of a yeast cell (Fig. 3 *C*) suggests an involvement in cell surface growth, effect of overproduction of this protein on growth pattern and actin organization were evaluated. Anti-actin immunofluorescence of cells overproducing the 85-kD protein (strain DBY5179), and grown

at 21° or 37°C is shown in Fig. 5. At 21°C (Fig. 5 *A*) cell morphology is generally normal, although cell size is increased and a few abnormally large cells can be found in the culture. As in wild-type cells, cortical actin patches are concentrated in the growing buds. However, ~10% of the cells contain a single heavy actin bar, often emanating in the neck and terminating on the opposite side of the mother cell, in place of the network of actin cables normally seen in cells (Fig. 3 *A*).

Upon shift of DBY5179 cultures to 37°C for 30 or 180 min, cortical actin patches, normally concentrated in buds, become prominent in mother cells (Fig. 5, *B* and *C*, respectively). At the same time, mother cells, which normally show little growth, now enlarge, often at the expense of the buds; the magnification is the same in all panels of Fig. 5. These phenotypes due to overproduction of the 85-kD actin-binding protein are similar in other haploid strains transformed with pRB1201 (although temperature optima and severity of defects vary slightly); however, effects on diploid cells are less severe than on haploids (not shown). Shift of cells bearing pRB307 from 21° to 37°C has no effect on cell shape or actin organization (not shown).

Haploid and diploid yeast each have distinct budding patterns; through successive cell cycles haploids tend to bud from one region of a yeast cell surface, while diploids bud successively from one of two opposite poles of the ellipsoid yeast cell. Budding leaves behind chitin-containing scars that can be visualized using the dye calcofluor (Hayashibe and Katohda, 1973; Cabib and Bowers, 1975). Fig. 6, *A* and *B* show calcofluor-stained haploid and diploid cells, respectively. Overproduction of the 85-kD actin-binding protein causes many cells cultured at 20°C to adopt aberrant patterns. As shown in Fig. 6 *C*, and quantitated in Table II, 41% of the cells containing two or more bud scars, have scars at opposite ends. This effect appears to be quite sensitive to the temperature, being more prominent in several experiments at 20° than at 21°C. The cells in Fig. 6 *C* are distinct from the diploid cells shown in Fig. 6 *B* because the bud scars tend to be clustered at one end, as in normal haploid cells (Fig. 6 *A*), however, buds occasionally form at the wrong end of the cell. In addition, many cells containing elevated levels of the 85-kD protein stain uniformly with calcofluor at any temperature, and show no identifiable bud scars (not shown).

Discussion

Yeast Actin Cytoskeleton

We have identified three yeast actin-binding proteins by virtue of their affinity for immobilized yeast actin filaments. A 200-kD protein appears to be a yeast myosin heavy chain; it is dissociated from actin filaments by ATP, it has the correct size to be a myosin heavy chain, and a *Dictyostelium* myosin heavy chain antibody binds to it. Watts et al. (1985, 1987) previously identified a *Saccharomyces cerevisiae* myosin heavy chain. The myosin identified by Watts et al. (1985) may not be identical to the 200-kD protein identified here, because the *C. elegans* myosin antibody (monoclonal 28.2, kindly provided by Dr. H. Epstein, Baylor College of Medicine, Houston, TX) used to identify that protein as myosin-like, did not react on immunoblots with the 200-kD protein identified here by actin filament affinity chromatography, even though *C. elegans* myosin on the same blot was recognized with great sensitivity (Drubin, D. G., and D. Botstein, unpublished observation). Further experiments are needed to determine whether one or more yeast myosins exist.

A second yeast actin-binding protein of 67-kD was identified. Immunofluorescence studies show that throughout the cell cycle this protein binds along the length of actin cables and is associated with cortical actin patches in yeast. This localization suggests that the 67-kD protein is involved in

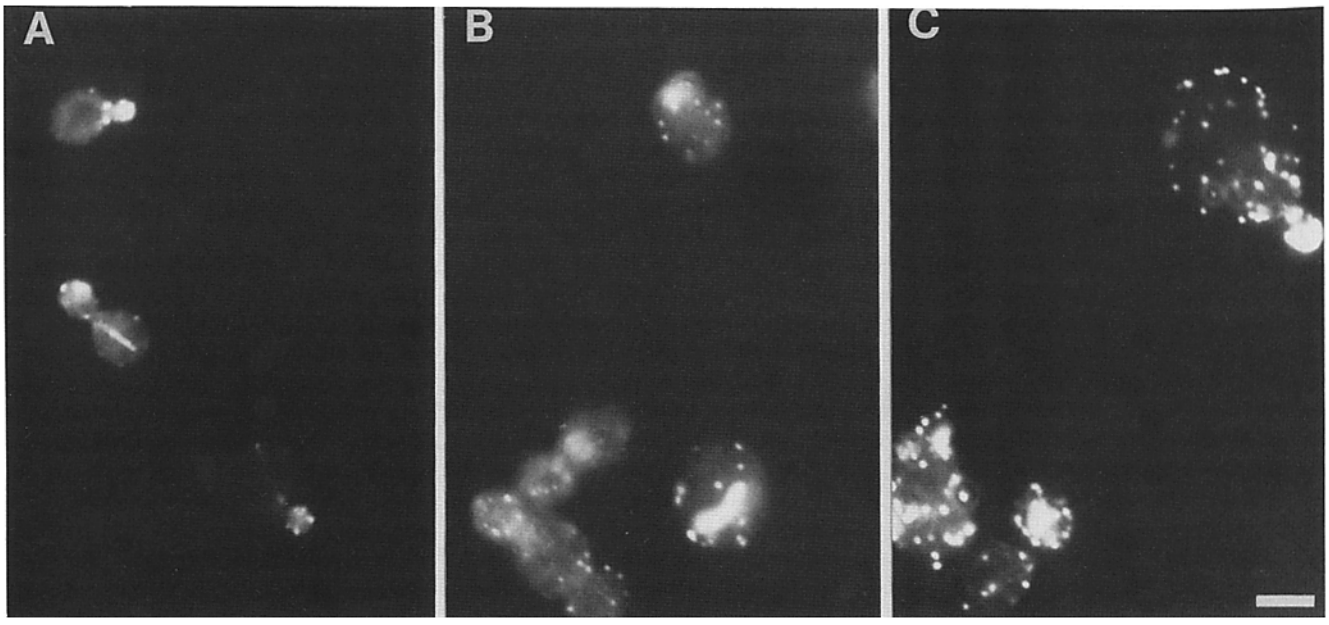

Figure 5. Effects of overproduction of the 85-kD actin-binding protein on actin organization and cell morphology. Anti-actin immunofluorescence of DBY5179 cells containing elevated levels of the 85-kD actin-binding protein. Cells were grown at 21°C (*A*), and then shifted to 37°C for 30 (*B*) or 180 min (*C*). Bar, 5 μm.

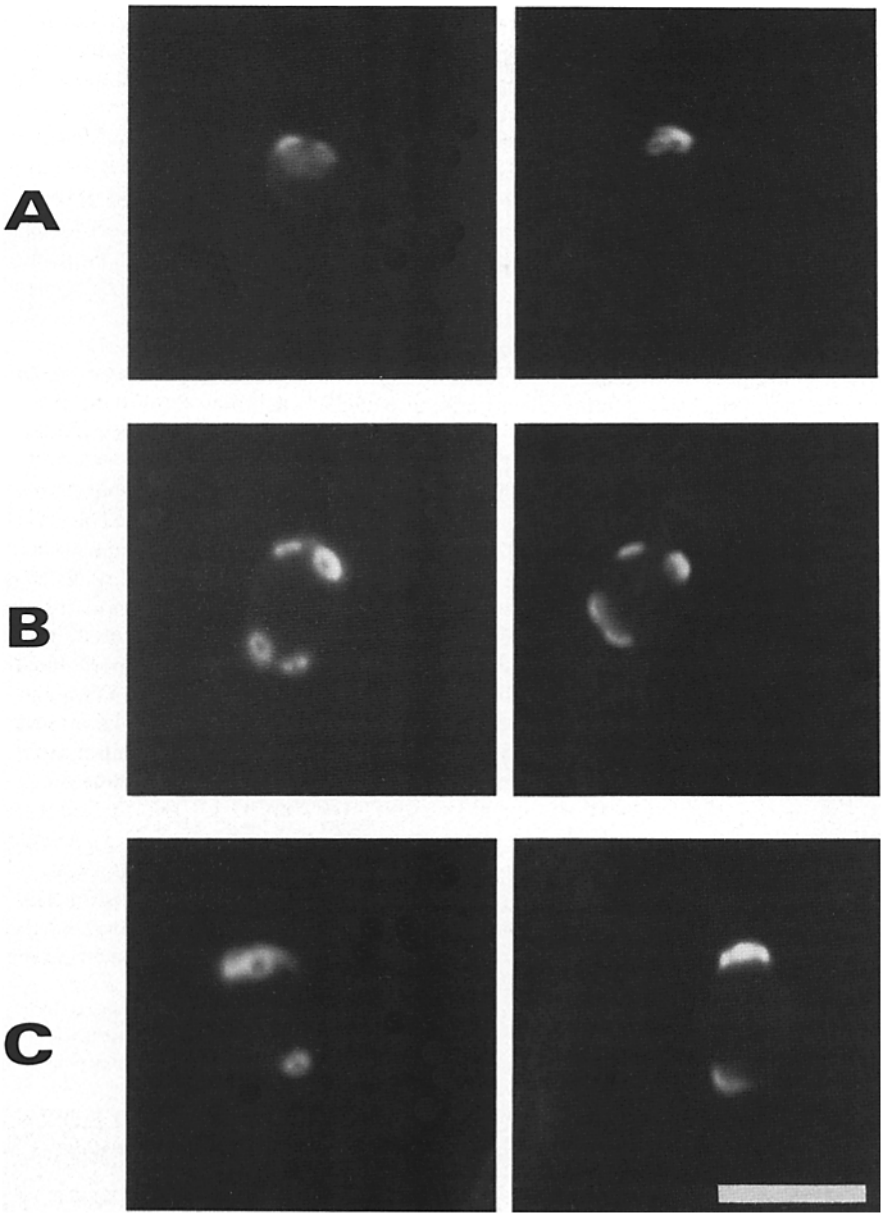

Figure 6. Effects of overproduction of the 85-kD actin-binding protein on bud scar pattern. Calcofluor staining of bud scars in the wild-type haploid strain DBY5178 (*A*), the wild-type diploid strain DBY5181 (*B*), and the haploid strain DBY5179 which contains elevated levels of the 85-kD actin-binding protein (*C*). Cells were grown at 21°C. Bar, 5 μm.

multiple actin-mediated processes, a possibility supported by recent genetic findings (Adams et al., 1988; Adams and Botstein, 1988).

In contrast to the 67-kD protein, the 85-kD protein appears to associate with only a subset of actin structures, the cortical actin patches that are concentrated at the growing surfaces of yeast cells. It is a formal possibility that the 85-kD protein associates with both actin cables and with cortical patches, but is only detected in association with patches. This possibility is not likely for the following reasons. First, the cortical patch staining by the anti–85-kD protein antiserum is brighter than the cortical patch staining by the anti–67-kD protein antiserum when viewed directly in the microscope, yet the anti–67-kD actin-binding protein antiserum very clearly stains cables, while the anti–85-kD actin-binding protein antiserum does not. Second, anti–85-kD protein antiserum stains actin bars brightly in cells containing the *act1-2* mutant allele, making it unlikely that the 85-kD protein can not be detected on normal actin cables due to fixation or antibody accessibility problems. We conclude that the 85-kD protein is associated primarily with cortical actin patches.

The identification of only three yeast actin-binding proteins by F-actin affinity chromatography may reflect a lack of protein complexity in the yeast actin cytoskeleton relative to higher cells. No additional actin-binding proteins were identified when gels containing salt-eluted fractions from actin filament columns were silver stained, despite a 50–100-fold increase in sensitivity (not shown). This apparent low complexity could greatly simplify genetic and biochemical analyses, making possible a detailed understanding of the interactions within the yeast actin cytoskeleton. When extracts from a variety of other organisms are passed over rabbit F-actin columns, using similar conditions to those used in this study, 20–40 known and potential actin-binding proteins are identified in each case (Miller, K. G., and B. M. Alberts, manuscript submitted for publication). In the present study,

actin was prepared from the same source as the extract (yeast). Use of yeast actin should optimize the binding of yeast actin-binding proteins. Indeed, *Acanthamoeba* actin-binding proteins are less efficiently retained on rabbit actin columns than are mammalian actin-binding proteins (Miller, K. G., and B. M. Alberts, manuscript submitted for publication). Also, the affinity of rabbit muscle myosin is 10-fold higher for rabbit actin filaments than for yeast actin filaments (Greer and Schekman, 1982). Thus, it appears that the amino acid residues in actin that are important for interactions with actin-binding proteins can be significantly diverged despite the high overall actin sequence conservation. Indeed, the 67-kD yeast actin-binding protein appears to bind to yeast actin filaments, but not to rabbit actin filaments, while the 200- and 85-kD yeast actin-binding proteins bind to both rabbit and yeast actin filaments (Drubin, D. G., and K. G. Miller, unpublished observations). Our search for yeast actin-binding proteins is not exhaustive; additional yeast actin-binding proteins may be identified by altering components of the column binding and elution buffers, such as Ca^{2+}.

Roles for the 85-kD Protein in Actin Organization and Yeast Morphogenesis

The association of the 85-kD actin-binding protein exclusively with actin at actively growing cell surfaces suggests a role in organizing and mediating spatially localized yeast growth. As one in vivo test of function, the 85-kD protein was overproduced in yeast. At 21°C cell morphology and cortical actin patch organization are not affected by overproduction of the 85-kD protein, although the cells are slightly larger than normal. However, the actin cables that normally form an array aligned roughly along the axis of cell growth, are replaced often by a single heavy actin bar of similar alignment. Perhaps the 85-kD protein cross-links actin cables into bars. More experiments are needed to determine the detailed structure of these bars and to elucidate the biochemical activities of the 85-kD protein.

Also at 21°C, haploid yeast cells that overproduce the 85-kD protein often bud aberrantly; normally haploid *Saccharomyces cerevisiae* cells bud successively from adjacent sites. The tendency for cells containing elevated levels of the 85-kD protein, is to have clustered budding patterns like normal haploids, but they appear to make mistakes occasionally and bud from the wrong pole. It is possible that the actin bar formation phenotype is functionally involved with the loss of the normal budding pattern. Perhaps the actin polarity is reversed in the bars, and organelle transport is directed to the wrong pole. Alternatively, increased levels of the 85-kD yeast actin-binding protein could result in the formation of improperly localized cortical actin structures. This could cause membrane insertion in an abnormal position, ultimately directing the formation of a bud in the wrong location.

When cells containing elevated levels of the 85-kD actin-binding protein are shifted from 21° to 37°C, cortical actin patches, normally restricted to buds, appear in mother cells. Coincident with this reorganization of the cortical actin cytoskeleton is a reorganization of the surface growth pattern. This result demonstrates that the 85-kD protein can affect the organization of the cortical actin cytoskeleton, and can affect the sites of cell surface growth. Determining what factors control the organization of the 85-kD protein will provide additional insights into the mechanisms responsible for organizing the yeast cell.

The association of cortical actin patches with regions of surface growth appears inviolate. When *cdc24*, *cdc42*, or *cdc43* mutants are shifted to nonpermissive growth conditions, actin patches also appear in mother cells (Adams and Pringle, 1984; Adams, 1984), and these mother cells begin to enlarge in a manner similar to that caused by overproduction of the 85-kD actin-binding protein, resulting in large unbudded cells (Pringle et al., 1984). These three *CDC* genes appear to be involved in the same processes that the cortical complexes containing actin and the 85- and 67-kD actin-binding proteins are involved in. Interestingly, some *cdc24* mutants affect budding pattern; when they are grown under permissive conditions, bud site selection becomes randomized (Sloat et al., 1981).

The yeast *Saccharomyces cerevisiae* undergoes a simple morphogenetic process to generate daughter cells. The actin cytoskeleton, which appears simple in composition by comparison to the cytoskeletons of other organisms, plays a central role in yeast morphogenesis. The overall simplicity of the system, coupled with the genetics available in yeast, hold great promise for understanding in detail the interactions responsible for the morphogenesis of the organism. By isolating the proteins that comprise the actin cytoskeleton, and the genes that encode these proteins, we are defining these interactions and determining their in vivo importance.

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