Actin and Septin Ultrastructures at the Budding Yeast Cell Cortex

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Budding yeast has been a powerful model organism for studies of the roles of actin in endocytosis and septins in cell division and in signaling. However, the depth of mechanistic understanding that can be obtained from such studies has been severely hindered by a lack of ultrastructural information about how actin and septins are organized at the cell cortex. To address this problem, we developed rapid-freeze and deep-etch techniques to image the yeast cell cortex in spheroplasted cells at high resolution. The cortical actin cytoskeleton assembles into conical or mound-like structures composed of short, cross-linked filaments. The Arp2/3 complex localizes near the apex of these structures, suggesting that actin patch assembly may be initiated from the apex. Mutants in cortical actin patch components with defined defects in endocytosis disrupted different stages of cortical actin patch assembly. Based on these results, we propose a model for actin function during endocytosis. In addition to actin structures, we found that septin-containing filaments assemble into two kinds of higher order structures at the cell cortex: rings and ordered gauzes. These images provide the first high-resolution views of septin organization in cells.

INTRODUCTION

Budding yeast has been used for over 20 yr as a model organism to study cytoskeletal function because the genes encoding many components of the cytoskeleton are conserved and because yeast cells are readily amenable to parallel genetic, biochemical, and cell biological analyses. Despite rapid advances in defining the components of the yeast cytoskeleton, its ultrastructure has been difficult to image at the resolution of the electron microscope, with the exception of microtubules (O'Toole *et al.*, 1999). This may be due to the low abundance of the cytoskeleton in yeast relative to mammalian cells, to the presence of a cell wall that complicates extraction procedures, and to high ribosome density in the yeast cytoplasm. As a result, there has been a conspicuous deficit in our understanding of the higher order organization of actin and septin polymers in vivo (Pruyne and Bretscher, 2000). Thus, although it is possible to rapidly identify and mutate cytoskeletal proteins in yeast, there is no method by which to examine the ultrastructural consequences of these mutations. This gap could be filled by developing an approach to image the yeast cytoskeleton at high resolution.

The yeast actin cytoskeleton is organized into three distinct structures: motile cortical patches that are polarized in the growing bud, actin cables that run along the mother-daughter cell axis, and a contractile cytokinetic ring at the

neck of dividing cells (reviewed in Pruyn and Bretscher, 2000). Cortical actin patches contain many of the actin-associated proteins common to all eukaryotes (Goode and Rodal, 2001) and are composed of actin filaments that undergo rapid turnover (Ayscough *et al.*, 1997). Actin is essential for yeast endocytosis (Kubler and Riezman, 1993). A number of recent studies suggest that actin participates directly in endocytosis via a dynamic, highly regulated pathway, but the precise function of actin in endocytosis is not understood (Engqvist-Goldstein and Drubin, 2003; Kaksonen *et al.*, 2003). An ultrastructural analysis of the cortical actin cytoskeleton would provide a powerful basis for understanding the function of actin in endocytosis by allowing high-resolution visual comparisons of actin and membrane structures in wild-type and mutant yeast strains. Attempts have been made to visualize yeast actin filaments by thin-section electron microscopy (Adams and Pringle, 1984; Mulholland *et al.*, 1994). However, actin filaments do not show up well in the crowded yeast cytoplasm, except for rare actin cables in *Saccharomyces cerevisiae* and pathogenic yeasts (Adams and Pringle, 1984; Kopecka *et al.*, 2001; Yamaguchi *et al.*, 2002), cytoplasmic "filasomes" distinct from cortical patches in fission yeast (Kanbe *et al.*, 1989; Takagi *et al.*, 2003), and aberrant filamentous actin structures in mutant budding yeast defective in endocytosis (Sekiya-Kawasaki *et al.*, 2003). Immunolabeled thin sections have in some cases revealed finger-like invaginations of the plasma membrane apparently entwined in an actin helix (Mulholland *et al.*, 1994). However, these structures are two-dimensional and thus provide limited information about geometry and spatial relations within cortical actin patches.

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Table 1. Yeast strains and plasmids

Strain	Description	Source
DDY902	<i>MATa his3Δ200, ade2-1, ura3-52, leu2-3112</i>	D. Drubin
BGY26	<i>MATα, his3-11,15, ura3-52, leu2-3112, ade2-1, trp1-1, ARP2::3xHA::HISMX</i>	B. Goode
BGY623	<i>MATa his3Δ200, ade2-1, ura3-52, leu2,3-112, ARP3::TEV::3xHA::HISMX</i>	B. Goode
BGY694	<i>MATα his3-11,15, ura3-52, leu2-3112, ade2-1, trp1-1, ARC18::TEV::3xHA::HISMX</i>	B. Goode
DDY957	<i>MATa, his3Δ200, ura3-52, leu2-3112, trp1-1, Δsla1::URA3</i>	D. Drubin
DDY668	<i>MATa, ura3-52, leu2-3112, Δsla2::URA3</i>	D. Drubin
HA31-9c	<i>MATa, can1-100, his3-11, ura3-52, leu2-3112, ade2-1, trp1-1, Δmyo3::HIS3, Δmyo5::TRP1</i>	L. Pon
NY1358	<i>MATa, ura3, his3, trp1, leu2, CDC3::3xHA</i>	P. Novick
YLK66	<i>MATα, leu2, ura3, his3, trp1, CDC10::GFP::kanMx6</i>	K. Tatchell
YLK189	<i>MATα, leu2, ura3, his3, trp1, Bni4::CFP::kanMx6, KCC4::YFP::HIS3</i>	K. Tatchell
Plasmids	Description	Source
Pma1::HA	CEN <i>LEU2 GAL::PMA1::HA</i>	J. Haber
Vam3::myc	2-micron <i>LEU2 pADH::myc::VAM3</i>	J. Gerst
Tlg1::myc	2-micron <i>LEU2 pADH::myc::TLG1</i>	J. Gerst
Sec22::myc	2-micron <i>LEU2 pADH::myc::SEC22</i>	J. Gerst

Another cortical cytoskeletal system in yeast is composed of septins, which are conserved GTPases that are important for cytokinesis and cell cycle regulation of polarized cell growth (reviewed in Faty *et al.*, 2002). Septins also have been postulated to form a diffusion barrier in the yeast plasma membrane to prevent free translocation of bud-specific membrane proteins into the mother cell (Barral *et al.*, 2000; Takizawa *et al.*, 2000) and to define a specialized cytokinetic plasma membrane compartment (Dobbelaere and Barral, 2004). Septins may be the structural component of a ring of 10-nm striations visible by electron microscopy in grazing sections of the yeast bud neck (Byers and Goetsch, 1976). Septins purified from *Drosophila*, mammalian, or yeast cells form short (~32-nm-long) filaments in vitro (reviewed in Faty *et al.*, 2002). These 32-nm subunits assemble end to end into >1500-nm-long paired filaments upon dialysis into physiological (75 mM) salt (Frazier *et al.*, 1998) and have a propensity to spontaneously curl longitudinally into rings the dimension of those seen in the yeast bud neck (Kinoshita *et al.*, 2002). Although, it has been proposed that septin polymerization is not required for their function in vivo (Frazier *et al.*, 1998), a recent study argues against this hypothesis (Versele *et al.*, 2004). Visualization of septin filaments at high resolution in vivo might provide important clues to the function of septin polymerization.

For many years, quick-freeze deep-etch methods have been used to visualize the cortex of cells from which the cytoplasm has been released (Heuser, 2000a). This technique might be useful for removing the ribosome-dense cytoplasm from yeast cells and for imaging structures left associated with the membrane. Here, we describe in detail the use of this method for preparing yeast cortices for deep etching and present our observations on the organization of actin and septins at the yeast cell cortex.

MATERIALS AND METHODS

Yeast Strains, Media, and Plasmid Constructions

Standard methods were used for DNA manipulations, growth and transformation of yeast (Rose *et al.*, 1989). Yeast strains, plasmids, and sources are described in Table 1.

Generating Yeast Spheroplasts

Yeast cells were grown to log phase in rich media before being spheroplasted. When selection for a plasmid was required, cells were grown to log phase in synthetic medium (and for *GAL::Pma1::HA*, induced with galactose for 6 h), and

then back-diluted for 3 h into rich medium. Five milliliters of cells (OD₆₀₀ 0.5) were pelleted and resuspended in 1 ml of spheroplasting buffer (yeast rich medium [YPD] or water containing 0.9 M sorbitol, 0.1 M potassium phosphate, pH 7.5, 28.8 mM β-mercaptoethanol, 0.05 mg/ml oxalylticase [Enzogenetics, Corvallis, OR], and 0.05 mg/ml zymolase 100T [MP Biomedicals Irvine, CA]). Cells were incubated in a rolling drum at 37°C for 1 h, and then pelleted at 5000 × g for 1 min, and washed three times with 1 ml of spheroplast wash buffer (0.9 M sorbitol and 0.1 M potassium phosphate, pH 7.5). Integrity of spheroplasts was monitored by microscopy. Cells were resuspended thoroughly in the same buffer, diluted to an OD₆₀₀ of 0.1, and processed immediately for immunofluorescence microscopy or for unroofing and electron microscopy.

Fluorescence Microscopy

The spheroplast suspension (described above) was fixed for 1 h in spheroplast wash buffer with 5% (vol/vol) formaldehyde, and then washed twice with 1 ml of spheroplast wash buffer. This suspension (15 μl) was allowed to settle for 10 min on a poly-lysine-coated glass slide. The slide was then washed 10 times with spheroplast wash buffer. Cells were extracted for 5 min with phosphate-buffered saline (PBS) containing 0.5% Triton X-100 and 0.05% SDS, and labeled with 1 U/ml rhodamine phalloidin (Molecular Probes, Eugene, OR) in PBS with 1 mg/ml bovine serum albumin (BSA) for 1 h. In Figure 1, cells were unroofed as described below, and coverslips were labeled with rhodamine-phalloidin. Cells were imaged using a Nikon E600 microscope equipped with a Roper CoolSNAP Fx (Photometrix, Tucson, AZ) charge-coupled device (CCD) camera and a Nikon numerical aperture 1.3 100× objective. Images were collected using MetaMorph software (Universal Imaging, Downingtown, PA). For Figure 7, A and B, strains expressing either an integrated *CDC10::GFP* fusion (YLK66) or *BNI4::CFP* and *KCC4::YFP* (YLK189) were created as described previously (Kozubowski *et al.*, 2003). Green fluorescent protein (GFP)-expressing cells were processed as described above with the exception that the spheroplasts were incubated for only 10 min at 37°C. Living spheroplasts were harvested by centrifugation and placed on microscope slide on a pad of 2% (wt/vol) agarose in PBS medium as described previously (Waddle *et al.*, 1996). Cells were imaged using an Olympus AX70 Provis microscope equipped with a CoolSNAP HQ CCD camera controlled by IPlab Spectrum software (Scanalytics, Fairfax, VA). For imaging GFP, a 41001 filter set (Chroma Technology, Brattleboro, VT) was used. For fluorescence imaging of cyan fluorescent protein (CFP) and yellow fluorescent protein (YFP), a JP4 filter set (Chroma Technology) was used in which the excitation and emission filters were placed in separate computer-controlled filter wheels (Ludl Electronic Products, Hawthorne, NY).

Preparation of Cortices ("Unroofing")

Custom-made circular #1 coverslips (5 mm; Erie Scientific, Portsmouth, NH) were dipped briefly into 1 mg/ml high-molecular-weight poly-lysine (Sigma Aldrich, St. Louis, MO), rinsed once in distilled water, dried on filter paper, and used within 1 h. Coverslips were placed into the wells of a flat-bottom 96-well plate and covered with the washed spheroplast suspension. The plate was centrifuged for 5 min at 3000 rpm in a Sorvall RT600B tabletop centrifuge by using 96-well plate adaptors. Coverslips were then submerged in spheroplast wash buffer in a petri dish. Coverslips looked slightly cloudy after cells had adhered to them properly. Five petri dishes were aligned on the bench, each containing the following solutions: 1) spheroplast wash buffer; 2) KH-MgE (70 mM KCl, 20 mM HEPES, pH 7.5, 5 mM MgCl₂, and 3 mM EGTA); 3) KHMgE; 4) KHMgE; and 5) KHMgE + 2% (vol/vol) electron microscopy (EM) grade glutaraldehyde (EM Scientific, Gibbstown, NJ). During transfer between dishes, care was taken not to allow air-drying of the coverslips, not

to contaminate dishes 1–4 with glutaraldehyde, and to work quickly after the first wash in KHMgE. Coverslips were washed in dishes 1 and 2 and placed in dish 3. A second coverslip was then placed slightly askew over the first and sufficient pressure was applied for 5 s with the forceps to obscure the cloudiness of the yeast on the coverslip, but not to create strong diffraction patterns between the two coverslips or to create lateral sliding forces. The coverslips were separated, washed once in dish 4, and placed cell-side up in dish 5. After all the coverslips had been processed, they were incubated in dish 5 for 10–30 min. The buffer was then aspirated from this dish, and the coverslips were washed three times with water. Coverslips that were of high quality for EM imaging had areas with faint circular impressions $\sim 1\text{--}2\text{ }\mu\text{m}$ in diameter when observed in the petri dish at $20\times$ magnification. Cell cortices had an indistinguishable appearance on the top and bottom coverslips sandwiching the cells, and both were used for imaging.

Antibody Labeling

Before antibody labeling, free aldehydes from the fixation were blocked by incubation of the coverslips for 5 min in a freshly made solution of 1 mg/ml sodium borohydride. Coverslips were then thoroughly washed three times with antibody labeling solution (ALS) (20 mM Tris, pH 8.0, and 150 mM NaCl) and transferred cell-side up to a humid chamber. Samples were blocked for 15 min in a 50- μl drop of ALS with 1 mg/ml BSA (ALS/BSA), and then incubated for 1.5 h in a 10- μl drop of ALS/BSA and the indicated dilution of antibody. Antibody dilutions were as follows: anti-hemagglutinin (HA) monoclonal 12CA5 (0.5 $\mu\text{g}/\text{ml}$), anti-myc monoclonal 9E10 (1–2 $\mu\text{g}/\text{ml}$), affinity-purified rabbit anti-yeast actin polyclonal (1:10) (Drubin *et al.*, 1988), rabbit anti-Abp1 polyclonal (1:50) (Drubin *et al.*, 1988), and anti-coronin mouse polyclonal ascites fluid (1:100) (Goode *et al.*, 1999). Coverslips were washed three times with 50 μl of ALS/BSA. Gold-labeled secondary antibodies (Ted Pella, Redding, CA) were applied for 1.5 h at a 1:20 dilution in ALS/BSA. In all experiments, secondary antibody alone was tested at least once without primary antibody to confirm specificity of the antibody labeling. To test the specificity of antibody labeling in HA-tagged cells, cells not expressing the epitope tag were labeled with primary (anti-HA) and secondary antibodies. Coverslips were washed three times with 50 μl of ALS/BSA and three times with ALS. A 50- μl drop of KHMgE with 2% (vol/vol) EM grade glutaraldehyde (EM Scientific) was applied to the coverslips for 15 min to chemically fix the antibodies to the sample. Coverslips were washed three times in the humid chamber with 50 μl of water, and then transferred to petri dishes and washed with three larger volumes of water.

Purification of *S. cerevisiae* Septins

Native septins were purified as described previously (Goode, 2002). Briefly, assembled septins were pelleted from a high-speed supernatant of wild-type yeast extracts, and then resuspended and fractionated by ion exchange (MonoQ; AP Biotech) and gel filtration (Superose 12; AP Biotech, Piscataway, NJ) chromatography.

Rapid Freeze, Deep Etch, and Electron Microscopy

Samples were prepared for electron microscopy as follows. Coverslips were rapidly frozen on a helium-cooled copper block, freeze dried at -85°C in a Cressington freeze-fracture machine (CFE-50; Ratford, Watford, United Kingdom), and rotary coated with 1.5 nm of tantalum/tungsten at a 45° angle (Figures 1 and 3–7) or with 2 nm of platinum at a 20° angle (Figures 2 and 8), both followed by coating with 3 nm of carbon without rotation (all figures). Replicas were floated off the coverslip by using 25% hydrofluoric acid, washed in water, and collected on 200-mesh copper grids coated with Formvar. Grids were photographed in a JEOL 1200-EX electron microscope at 100 kV. Digital “anaglyph” three-dimensional images from stereopairs of EM negatives were prepared as described previously (Heuser, 2000b).

RESULTS

The Structure of Yeast Cell Cortices

To visualize the cytoskeletal–membrane interface at the yeast cortex by electron microscopy, cells must be unroofed, or ripped open. Because the yeast cell wall is resistant to ripping, we used enzymatic digestion to remove it, creating spheroplasts. By fluorescence microscopy, yeast spheroplasts retained cortical filamentous actin (F-actin) patches, but they had no observable cables or rings (Figure 1A; Kopecka and Gabriel, 1995). The patches had similar dimensions to those seen in intact cells (on the order of 200 nm in diameter, near the resolution limit of light microscopy), but they were not polarized toward any side of the cell. F-actin in these patches colocalized with known components of actin patches in intact cells, including Srv2, Arp2, and Crn1 (our unpublished data). Previ-

ously, it has been shown that spheroplasts are endocytically active (Prescianotto-Baschong and Riezman, 1998), and we found that they take up the lipophilic dye FM4-64 with similar kinetics to intact cells (our unpublished data). Actin patch components are required for endocytosis in intact cells (Pruyne and Bretscher, 2000). Because spheroplasts are competent for endocytosis, the function of actin patches in endocytosis must be preserved in these specimens.

Yeast spheroplasts were prepared in parallel for immunofluorescence and for rapid freezing and freeze-drying. Spheroplasts were sandwiched between polylysine-coated coverslips, which then were pulled apart and immediately chemically fixed, leaving unroofed pieces of cortex on both coverslips. Filamentous actin structures were preserved on these fragments of cell cortex, as revealed by rhodamine-phalloidin staining (Figure 1B). Coverslips with unroofed cells were frozen on a liquid helium-cooled gold block, freeze-dried, and rotary coated with metal. In the electron microscope, fields of unroofed yeast were easily found (Figure 1C). The dimensions of a typical unroofed piece of cortex (on the order of $6\text{ }\mu\text{m}^2$) represent $\sim 10\%$ of the cortex of a single wild-type spheroplast (on the order of $60\text{ }\mu\text{m}^2$; Kopecka and Gabriel, 1995).

For clarity, all images in this work showing unlabeled samples and stereopairs are presented in negative contrast for optimal visualization of cortical structures, and all immunogold labeled images are presented in positive contrast for optimal visualization of gold particles.

We observed diverse structures on the cytoplasmic surface of the plasma membranes (Figure 2A). Crystalline particle arrays on the cytoplasmic surface of unroofed cortices were found in well-preserved specimens (Figure 2B). Mounds composed of short filaments were organized into spider-like arrays (Figure 2C). Groove-like invaginations, $0.3\text{--}0.5\text{ }\mu\text{m}$ in length (Figure 2D), folded inwards toward the cytoplasm, were visible both from inside and outside the cortex, and they were used to distinguish which side of the membrane was being viewed. These grooves previously have been observed by conventional freeze-fracture and electron microscopy of intact yeast cells, and they grow and shrink in response to osmotic pressure (Slaninova *et al.*, 2000). They therefore have been postulated to hold surplus membrane, allowing for membrane expansion and retraction (Slaninova *et al.*, 2000). Also present were large gauze-like patches (Figure 2E) composed of parallel assemblies of $0.3\text{--}0.4\text{-}\mu\text{m}$ -long filaments. Numerous vesicular structures were seen, ranging in size from 50 to 400 nm in diameter, as well as tubular structures $\sim 25\text{ nm}$ in diameter (Figure 2F). Vesicular structures of various sizes decorated with particles $\sim 15\text{--}20\text{ nm}$ in diameter were abundant and may be endoplasmic reticulum coated with ribosomes (Figure 2G). The tubule structures were continuous with the ribosome-decorated vesicles and were therefore also designated as putative endoplasmic reticulum (Figure 3C). Endoplasmic reticulum in yeast is associated with the plasma membrane (Prinz *et al.*, 2000). Structures resembling clathrin cages were not observed, although they are very abundant in similar preparations from mammalian cells (Heuser, 2000a).

To identify specific membrane-bound compartments, cortices prepared from yeast expressing epitope-tagged v- and t-SNAREs were labeled with primary and 10-nm gold-conjugated secondary antibodies. Pma1, the plasma membrane ATPase (Harris *et al.*, 1994), was distributed evenly on plasma membrane surfaces (Figure 3A). Vam3, a vacuolar t-SNARE (Wada *et al.*, 1997), was localized to abundant, large amorphous structures that were physically distinct from the cytoplasmic surface of the cortex (Figure 3B). This result suggests that our preparations partially or

Figure 1. Unroofed yeast spheroplasts contain filamentous actin. DDY902 (wild-type) yeast cells were converted to spheroplasts. (A) Intact, fixed spheroplasts were labeled with rhodamine-phalloidin. (B) Unroofed spheroplasts were fixed and labeled with rhodamine-phalloidin. (C) Low-magnification view showing optimal unroofing of yeast spheroplasts for visualization of cortical structures by electron microscopy, shown in negative contrast. Bar (C), 1 μ m.

completely release the vacuole, which then binds randomly to the coverslip surface. Sec22, a marker for the endoplasmic reticulum (Pelham, 2001), localized to membrane tubules and decorated the majority of the structures in our preparations (Figure 3C), including those shown in Figure 2, F and G, but not those shown in Figure 2, A–E. Tlg1, a marker for late Golgi and early endosomal compartments (Pelham, 2001), localized to membrane-associated structures $\sim$ 100 nm in diameter (Figure 3D, arrowheads). These

structures were smaller than those visualized by light microscopy (Figure 3D, inset). Therefore, only a subset of smaller, Tlg1-decorated structures must be preserved in our preparations.

Actin and Actin-associated Proteins Localize to Filamentous Patches Associated with the Membrane Surface

The patches shown in Figure 2C are composed of filaments, which although short, have the same diameter as metal-

Figure 2. Cortex of unroofed yeast spheroplast shown in negative contrast. (A and inset) Unroofed DDY902 (wild-type) yeast cell showing characteristic structures on the cytoplasmic surface. Colorized structures are magnified below this image: crystalline regions of plasma membrane (B), actin patches (C), invaginations (D), septin gauzes (E), and putative endoplasmic reticulum (F and G). Bars, 0.2 μ m.

coated purified actin filaments (~ 10 nm; Figure 5A). To confirm that these were actin patches, we labeled unroofed yeast cortices with anti-actin–immunogold complexes (Figure 4A). Gold complexes were densely clustered on structures ~ 200 nm in diameter, very similar to those in Figure 2C. These dimensions match well with the diameter of cortical actin patches visualized by light microscopy (also ~ 200 nm). After anti-actin–immunogold labeling, individual filaments were no longer easily resolved in these structures. Because the unroofed cortices stained with rhodamine-phalloidin (Figure 1B), which binds only to F-actin, it is likely that individual actin filaments were present but obscured by the antibody–immunogold complexes. Individual filaments were resolved in similar structures on both unlabeled cortices (Figures 2 and 5) and on cortices labeled for different actin-associated proteins (see below), suggesting that a dense labeling with anti-actin immunogold obscures the structure.

We localized three subunits of the Arp2/3 complex, Arp2::HA, Arp3::HA, and Arc18::HA (Figures 4B and 5, C–F), with anti-HA monoclonal antibodies and Abp1 (Figure 4C) and Crn1 (Figure 4D) with polyclonal antibodies. These pro-

teins localized specifically to cortical actin patches (and not to cables or the cytokinetic ring) in intact cells (Drubin *et al.*, 1988; Moreau *et al.*, 1996; Goode *et al.*, 1999) and resided on filamentous structures similar to those recognized by anti-actin antibodies on the unroofed spheroplasts. No labeling of vesicular bodies, the cytoplasmic surface of the plasma membrane, or the gauze structures seen in Figure 2E was observed for actin or actin-associated proteins. Therefore, we conclude that the patches are composed of actin filaments and correspond to cortical actin patches in intact cells.

One technique for visualizing the three-dimensional structure of an object is to create digital “anaglyphs,” by combining stereoscopic image-pairs into one image and by using optical filters to separate left and right eye views (Heuser, 2000b). Figure 5B shows digital anaglyphs revealing the three-dimensional structure of the actin patches, in which left and right eye views are best separated using red-green or red-blue eyeglasses. Patches were composed of many cross-linked 10- to 12-nm-thick filaments, and tracing of individual filaments through the structures suggests that they each extend ~ 100 nm in length. Filaments radiated from the apex of the patch and ran toward the cytoplasmic

Figure 3. Localization of subcellular markers in unroofed yeast spheroplasts. Micrographs are shown in positive contrast to allow better visualization of the gold label. Shown are unroofed DDY902 (wild-type) yeast cells carrying plasmids expressing different epitope-tagged proteins. Insets show tagged protein localization in intact, fixed spheroplasts. (A) Pma1-HA (the plasma membrane ATPase with intracellular HA tag) labeled with anti-HA/10 nm immunogold complexes. (B) Vam3-myc (a vacuolar t-SNARE). (C) Sec22-myc (an endoplasmic reticulum marker). (D) Tlg1-myc (a late Golgi/early endosome marker) labeled with anti-myc/10-nm immunogold complexes. Black arrowheads in D indicate labeled structures. Bar, 0.2 μ m.

surface, presumably around a membrane core, to the plasma membrane below. Often, a single patch had several distinct apices. The patch was sometimes asymmetric, so that the apex or apices seemed peripherally located in two-dimensional images. Both Abp1 and Crn1 localized uniformly over the entire patch (Figure 4, C and D), whereas Arp2 (Figure 5C), Arp3 (Figure 5D), and Arc18 (Figure 5, E and F) localized specifically near the apex/apices of the patch, well above the plane of the cortex. To confirm this result, we labeled cortices simultaneously for Arc18 and actin (Figure 5F). Anti-actin-immunogold complexes localized over the entire patch, obscuring the fine structure as described above, whereas Arc18 was concentrated at the apex. Given that the Arp2/3 complex binds tightly to the pointed ends of actin filaments to nucleate barbed end growth (Mullins *et al.*,

1998), these results suggest that the barbed ends of actin filaments may grow toward the base of the actin patch.

Actin Ultrastructures in Mutant Cells

We prepared cortices from yeast strains with mutations in actin-associated proteins (*Sla1*, *Sla2*, and *Myo3/5*) that cause defects in actin organization in intact cells (Holtzman *et al.*, 1993; Goodson *et al.*, 1996) and in spheroplasts (Figure 6, insets) at the light microscopic level. Unroofed cortices were labeled with anti-actin-immunogold complexes for electron microscopy. *sla1* mutant cells had large F-actin aggregates by fluorescence microscopy (Figure 6A, inset; Holtzman *et al.*, 1993; Gourlay *et al.*, 2003), and the cytoplasmic surfaces of the unroofed cells had large (~500–1000 nm) flattened arrays of actin filaments on the plasma membrane (Figure

Figure 4. Actin localization in unroofed yeast spheroplasts. Micrographs are shown in positive contrast to allow better visualization of the gold label. Arrowheads show actin structures. (A) Unroofed DDY902 (wild-type) yeast labeled with antiactin/10-nm immunogold complexes. (B) Unroofed BGY26 (ARP2::HA) yeast labeled with anti-HA/10-nm immunogold complexes. (C) Unroofed DDY902 (wild-type) yeast labeled with anti-Abp1/10-nm immunogold complexes (C) and anti-Crn1/10-nm immunogold complexes (D). Black arrowheads indicate labeled structures. Bar, 0.2 μ m.

Figure 5. Anaglyph gallery of actin patches. Micrographs are shown in negative contrast. (A) Purified human platelet actin. (B) Unroofed, unlabeled DDY902 (wild-type) yeast. (C) Unroofed BGY26 (*ARP2::HA*) yeast labeled with anti-HA/10-nm immunogold complexes. (D) Unroofed BGY623 (*ARP3::HA*) yeast labeled with anti-HA/10-nm immunogold complexes. (E) Unroofed BGY694 (*ARC18::HA*) yeast labeled with anti-HA/10-nm immunogold complexes. (F) Unroofed BGY694 (*ARC18::HA*) yeast labeled with anti-HA/10-nm and anti-actin/5-nm immunogold complexes. Bar, 0.1 μm .

6A). *sla2* mutant cells also had large F-actin aggregates by fluorescence microscopy (Figure 6B, inset; Holtzman *et al.*, 1993). However, in contrast to the *sla1* mutant, *sla2* mutant cortices had large (~ 500 – 1000 -nm) filament-coated membrane structures, which were not tightly associated with the cytoplasmic surface of the plasma membrane, but instead were raised off the surface (Figure 6B), consistent with the light microscopy observation of actin tails asso-

ciated with the cortex of *sla2* mutants (Kaksonen *et al.*, 2003). *myo3/5* mutant cells, which displayed numerous smaller actin patches by fluorescence microscopy (Figure 6C, inset), did not have obvious defects in cortical actin patch ultrastructure in the unroofed spheroplasts, although the patches looked somewhat smaller in overall size (Figure 6C). Therefore, different mutations in actin-associated proteins, which all cause defects in actin patch

Figure 6. Actin organization in unroofed mutant yeast spheroplasts. Micrographs are shown in positive contrast to better visualize the gold label. Insets show rhodamine-phalloidin labeling of intact mutant spheroplasts. (A) Unroofed DDY957 (*sla1Δ*) yeast labeled with antiactin/5-nm immunogold complexes. (B) Unroofed DDY668 (*sla2Δ*) yeast labeled with antiactin/10-nm immunogold complexes. (C) Unroofed HA31-9c (*myo3Δ*, *myo5Δ*) yeast labeled with anti-actin/10-nm immunogold complexes. Bar, 0.2 μ m.

organization and in endocytosis (Raths *et al.*, 1993; Geli and Riezman, 1996; Warren *et al.*, 2002), had distinct defects in actin patch organization at the resolution of electron microscopy.

Cortical Septin "Gauzes"

Because actin did not localize to the fibrous gauzes shown in Figure 2E, we considered based on their dimensions that they may be composed of septin filaments. First, we assessed

Figure 7. Septin localization in yeast spheroplasts. (A) Intact, live YLK66 (*CDC10::GFP*) yeast spheroplasts. (B) Intact, live YLK189 (*BNI4::CFP*, *KCC4::YFP*) yeast spheroplasts. (C) Septin ring in unroofed NY1358 (*CDC3::HA*) yeast labeled with anti-HA/10-nm immunogold complexes. (D) Septin gauze in unroofed NY1358 (*CDC3::HA*) yeast labeled with anti-HA/10-nm immunogold complexes. Micrographs are shown in positive contrast to allow better visualization of the gold label. Bars, 1 μm (A and B), 0.1 μm (C), and 0.2 μm (D).

the organization of septins in spheroplasted cells. In intact cells before bud emergence, septins organize into a ring (diameter ~ 500 – 1600 nm), which extends into an hourglass-shaped structure in the neck after bud emergence. In addition, in some mutants that disrupt septin regulation, septins form patches instead of regular rings, which lead to the hypothesis that septins organize into a patch before the formation of a ring early in the cell cycle (reviewed in Longtine and Bi, 2003). Cdc10 is one of five septin proteins expressed in vegetatively growing yeast cells. Light microscopy of spheroplasts showed that Cdc10::GFP localized primarily to patch-like structures and occasionally to partial or complete rings (Figure 7A). The patch-like structures did not resolve into rings even when examined in different focal planes. To distinguish whether patch-like structures in spheroplasts correspond to the patch that forms in intact cells, we examined localization of the septin-associated proteins Bni4 and Kcc4. These proteins localize to opposite faces of the septin ring, but colocalize in the septin patch (Kozubowski and Tatchell, personal communication). The fluorescent signals of Kcc4::YFP and Bni4::CFP partially colocalized in spheroplasts and did not seem adjacent in different focal planes (Figure 7B), suggesting that the septin structures in spheroplasts may be akin to the patch that forms in intact cells.

To determine whether the gauze structures visible by electron microscopy correspond to this septin structure, we localized anti-HA antibodies in cells expressing the HA-tagged septin Cdc3 (*CDC3::3 $\times$ HA*). This strain had no obvious growth or septin organization defects by light microscopy (our unpublished data). Immunogold anti-HA complexes localized exclusively to the fibrous gauzes and not to the actin patches or to other structures (Figure 7, C and D). Double labeling revealed no clear relationship between actin and septins by electron or light microscopy (our unpublished data). The gold particles randomly labeled the filaments in the gauze structures and also decorated occasional ring structures that seem to be formed from the same linear fibers (Figure 7C). However, gauzes were the predominant species of septin-containing structures. This result correlated well with our anti-septin immunofluorescence (our unpublished data) and GFP-septin images (Figure 7, A and B), showing that in intact spheroplasts septins are found only rarely in rings and more commonly assemble into patches.

To confirm that the septin-containing structures visible by electron microscopy were similar to those found in intact cells, we examined by electron microscopy several properties of septin structures. First, cells containing a mutation in the septin Cdc12 (*cdc12-6*) are temperature sensitive for vi-

Figure 8. (A and B) Unlabeled septin patches. Micrographs are shown in negative contrast. Double-headed arrows indicate the length of a single gauze. (C) Metal replica of purified septin filaments. (D) High-magnification of a septin gauze on the cell cortex. A single filament in the gauze has been colorized red. Bars, 0.2 μm (A and B) and 0.05 μm (C and D).

ability and do not form septin structures at the restrictive temperature (Haarer and Pringle, 1987). Consistent with this phenotype, we were unable to detect unlabeled or antibody-labeled gauzes or rings in cortices prepared from this strain (our unpublished data). Second, the fibers in the septin-containing gauzes were similar in appearance to purified metal-shadowed septin filaments (Figure 8C compared with colorized filament in Figure 8D), which pair into irregular helices, similar to negatively stained septins (Frazier *et al.*, 1998).

Figure 8 shows unlabeled septin gauzes in negative contrast, where structural details are more apparent. The main components of the gauzes were cross-linked filaments 5–8 nm in diameter. The majority of gauzes were composed of filaments with a fixed length of ~ 0.3 – $0.4 \mu\text{m}$, although they were occasionally assembled end to end into larger arrays (Figure 8B, double-headed arrows). The width of the gauzes varied from only a few filaments across to many filaments across, sometimes reaching widths of several micrometers. Occasionally, separate filaments lay across the top of the gauze. Often, a bundle of gauze-like fibers formed a ring structure that encircled the gauze or wrapped around its edge (Figure 2E). Given the similarity of the main fibers in the gauzes and rings to purified septin filaments, we conclude that gauzes consist of 300- to 400-nm-long septin filaments aligned into flat arrays with occasional septin filaments laid across or around the

array. In contrast, the septin ring consists of a gauze-like structure only three to four septin filaments thick, circumferentially aligned with cross-linking fibers running radially to create a mesh (Figure 7C).

DISCUSSION

Ultrastructure of Cortical Actin Patches in Yeast

We have elucidated the ultrastructure of yeast cortical actin patches. The filamentous patches shown in Figure 2C and Figures 4–6 are composed of actin as defined by three criteria. First, the filaments had the same diameter as metal-coated purified actin filaments ($\sim 10 \text{ nm}$; Figure 5A). Second, anti-actin-antibody/immunogold complexes were densely clustered on these structures (Figure 4A). Third, the filamentous patches were abundantly labeled by antibodies against known cortical actin patch components (Figure 4, B–D). In addition to cortical patches, yeast cells also contain actin cables, which polarize along the mother-daughter cell axis and are sometimes associated with actin patches (Karpova *et al.*, 1998, Pelham and Chang, 2001). We did not detect any actin cable-like structures in our preparations, nor are cables apparent in intact spheroplasts, which lack obvious cell polarity. Our results extend upon a previous model in which actin filaments wind around finger-like invaginations of the

plasma membrane (Mulholland *et al.*, 1994). We show that actin patches are conical or mound-like structures rising out of the plasma membrane and suggest that they are composed of short filaments arranged into an apparently cross-linked scaffold that surrounds a membrane core. At least four known actin filament cross-linking proteins, Sac6/fimbrin, Crn1/coronin, Abp140, and Scp1/calponin, localize to actin patches (Adams *et al.*, 1989; Asakura *et al.*, 1998; Goode *et al.*, 1999; Goodman *et al.*, 2003). Attempts to directly determine the orientation of actin filaments in the patches by S1 myosin fragment decoration were not successful. However, we were able to infer the orientation of filaments in the patch by examining Arp2/3 complex localization. The Arp2/3 complex nucleates barbed end assembly of actin filaments and binds tightly to actin filament pointed ends (reviewed in Welch and Mullins, 2002). Therefore, localization of Arp2/3 complex subunits near the apex of the patch (Figure 5, C–F) is consistent with the barbed ends of the actin filaments being oriented toward its base at the plasma membrane. These results are also consistent with photobleaching experiments showing that actin treadmills outward from the plasma membrane in large actin structures formed in *sla2* mutant cells (Kaksonen *et al.*, 2003).

Although actin is essential for maintaining cell polarity, cell wall integrity, and endocytosis in yeast, it is not yet clear what specific role actin patches perform in these processes. The strongest functional link for F-actin patches is to endocytosis, because many of the actin-associated proteins found at actin patches are also required for endocytosis (reviewed in Schott *et al.*, 2002; Engqvist-Goldstein and Drubin, 2003). Clathrin plays a central role in many (but not all) kinds of endocytosis (Engqvist-Goldstein and Drubin, 2003); however, we did not see clathrin-coats associated with actin structures. The role of clathrin at the yeast plasma membrane remains controversial (Baggett and Wendland, 2001), and our results do not exclude a role for clathrin in the endocytic pathway.

Recent real-time analyses of actin patch components suggest that Arp2/3 complex-stimulated actin polymerization occurs at an early step in membrane internalization (Kaksonen *et al.*, 2003). Together with these results, our observations suggest the following model for actin patch function. The short actin filaments in yeast actin patches may interface with the plasma membrane through associated proteins to form a lattice that promotes membrane invagination and vesicle formation. The Arp2/3 complex nucleates actin filaments and as the filaments grow from the point of nucleation, they become attached to the plasma membrane by actin-associated endocytic accessory proteins (such as EH-domain-containing proteins and Rvs167/amphyphysin; Raths *et al.*, 1993; Munn *et al.*, 1995; Santolini *et al.*, 1999). These proteins can bind directly or indirectly to actin filaments and either to lipids in the plasma membrane or to integral membrane proteins. The membrane then deforms into a cone or mound, either passively (due to pulling forces generated by actin assembly at these sites of attachment) or actively (due to lipid deformation activities (Takei *et al.*, 1999) of the endocytic accessory proteins attached to the actin filaments).

Mutant analysis provides additional support for this model. Deletion of *SLA1* results in large flattened actin patches (Figure 6A) and defects in endocytosis (Warren *et al.*, 2002). SH3 domains in the amino terminus of *Sla1* negatively regulate activation of the Arp2/3 complex by Las17 (yeast WASp) (Rodal *et al.*, 2003), and the large patches in *sla1* mutants may be due to a failure to attenuate actin polymerization by WASp-activated Arp2/3 complex. In addition, other domains in *Sla1* bind to integral membrane and endocytic accessory proteins (Howard *et al.*, 2002; Warren *et al.*, 2002), and the absence of such plasma membrane attachments in *sla1* cells may cause failure to de-

form the cone or mound, resulting in flattened patches. In contrast to the effects of *sla1* mutants, mutations in *SLA2* cause accumulation of expansive, raised actin structures (Figure 6B). The *sla2* mutant phenotype may therefore be due to a block in actin-dependent steps of endocytosis subsequent to the initial internalization event. In fact, real-time light microscopy of *sla2* cells reveals aberrant actin “tails” that remain attached to the cortex (Kaksonen *et al.*, 2003). Similar results were obtained in mammalian cells depleted of the *Sla2* homologue Hip1R by using RNA interference (Engqvist-Goldstein *et al.*, 2004). Our results provide a higher resolution view of these aberrant actin structures in yeast and suggest that they may consist of large, actin-coated membrane structures rather than *Listeria*-like actin comet tails, which contain no membrane component. Further analysis of actin ultrastructure in mutants of actin-associated proteins will be very informative in ordering the components of the yeast actin cytoskeleton into a functional pathway for membrane internalization.

One intriguing aspect of cortical actin patches is that they are highly motile (Doyle and Botstein, 1996; Waddle *et al.*, 1996). The importance of actin patch motility *in vivo* is not yet understood, but there is evidence that motility may be initiated by Arp2/3 complex-nucleated actin polymerization (Winter *et al.*, 1997; Kaksonen *et al.*, 2003). Our findings suggest that actin filament barbed ends generate pushing forces toward the plasma membrane (as hypothesized in Munn, 2000 and (Kaksonen *et al.*, 2003). This architecture might predict that actin patches would move into the cell interior, which is consistent with the recent finding that there is a short initial phase of motility directed into the interior of the cell, followed by a phase of long-range motility (Kaksonen *et al.*, 2003). Our preparations are likely to preserve structures involved in these initial movements away from the cell surface.

Function and Ultrastructure of Yeast Septin Networks

Here, we have shown the first electron micrographs in which septin-containing filaments have been definitively labeled in cells. These septin filaments may be analogous to the septin-dependent striations observed in grazing sections of the yeast bud neck (Byers and Goetsch, 1976) and provide strong support for septin polymerization *in vivo*, as well as a more detailed view of their higher order organization. Septins from many species form heteropolymeric filaments *in vitro* (reviewed in Faty *et al.*, 2002; Longtine and Bi, 2003), but the organization of septin structures *in vivo* has not been defined. Genetic evidence has been used to argue that polymerization is not necessary for septin function *in vivo*. In cells lacking the Cdc10 septin, the remaining septins still localize by fluorescence microscopy to discrete rings and retain most septin-dependent functions (Frazier *et al.*, 1998). In this study, septins purified from *cdc10Δ* cells did not form robust filaments *in vitro* (Frazier *et al.*, 1998). Furthermore, the 10-nm neck filaments were more difficult to detect in *cdc10Δ* cells. On the other hand, a recent study showed that septin complexes lacking Cdc10 do retain the capacity to polymerize *in vitro* (Versele *et al.*, 2004). Thus, it is possible that *in vivo* filaments do form in cells lacking Cdc10, but they are not as easy to resolve by thin-section electron microscopy. In addition, it seems unlikely that septins would have maintained the capacity to polymerize throughout evolution if this property were not important for their cellular function.

We find that in yeast spheroplasts, septins polymerize into two classes of intricate ultrastructures: rings and gauzes. Purified septins form rings, suggesting that septin polymers have an inherent propensity to curl longitudinally into rings ~500 nm in diameter (Kinoshita *et al.*, 2002). However, rings

formed from purified septins consist of relatively uniform bundles of circumferentially aligned filaments, whereas the rings in our preparations consist of a more loosely woven mesh and include filaments aligned both circumferentially and radially (Figure 7C). These results suggest that septin ring structure is more complex *in vivo* than *in vitro*. Thin-sectioning studies have revealed 10-nm striations located at wild-type yeast bud necks (Byers and Goetsch, 1976), consistent with septin filaments being aligned perpendicular to mother-daughter cell axis as a stack of rings around the bud neck. However, the following evidence suggests that the orientation of striations in the bud neck reflects regular cross-links on a "collar" of septin filaments aligned parallel to the mother-daughter cell axis. First, in mating cells, septins are found in bars aligned parallel to the projection (Longtine *et al.*, 1998), in a manner analogous to alignment along the mother-daughter cell axis in dividing cells. Second, mutations in Gin4, a cell cycle-regulated septin-associated kinase (Longtine *et al.*, 1998), and in Rts1, a cell cycle-regulated subunit of the PP2A phosphatase (Dobbelaere *et al.*, 2003) cause a shift in localization of septins in dividing cells to bars aligned along the mother-daughter axis, supporting the collar arrangement. Given this evidence for alignment of septin filaments along the mother-daughter axis in a collar, the septin gauzes in our images, by far the most prevalent septin structures, provide an intriguing parallel to septins at the bud neck. The gauzes are ~300–400 nm in length and up to several micrometers in width, although we more commonly observed smaller gauze fragments. These dimensions are consistent with a structure that could wrap around a bud neck (~3 μ m in circumference, and see "collapsed" corset in Figure 8B). Another possibility is that the septin gauzes correspond to the septin "patch" that forms early in the cell cycle or in mutants (Longtine and Bi, 2003). Bni4 and Kcc4, two septin-associated proteins, localize to opposite faces of the septin ring but colocalize in the septin patch (Kozubowski and Tatchell, personal communication). In spheroplasts, Bni4 and Kcc4 partially colocalize (Figure 7B), suggesting that in these cells septins organize into a patch-like structure. However, at the resolution of light microscopy it may not be possible to detect noncolocalization of Bni4 and Kcc4 proteins once the septin collar has collapsed.

The idea that there may be multiple types of septin structures (rings and gauzes) is consistent with the observation that exchange of septin subunits changes dramatically during the cell cycle (Caviston *et al.*, 2003; Dobbelaere *et al.*, 2003). Both rings (Figure 7C) and gauzes (Figure 8) consist of filaments organized into a mesh-like structure. However, the filaments that constitute the ring seem to be more loosely laterally associated with each other in contrast to more compact organization of gauzes. Images of filaments assembled from purified septins suggest that ~32-nm subunit exchange may occur throughout the filaments, because the ends of the ~32-nm repeats do not precisely align within a filament (Frazier *et al.*, 1998). Therefore, we predict that rings, which exhibit less lateral organization, would more easily be able to exchange subunits and thus would more readily exchange subunits early in the cell cycle, whereas gauzes, due to extensive lateral alignment of filaments, would be the more stable structures found later in the cell cycle (Dobbelaere *et al.*, 2003).

Utility of New Approaches to Image the Yeast Cortex at High Resolution

We have reported the first high-resolution images of structures assembled from actin filaments and septins at the yeast cell cortex. These images provide a long-needed advance in our understanding of the structure of the yeast cytoskeleton and bring us a step closer to a more complete understanding of

actin and septin functions *in vivo*. Ultrastructures of cytoskeletal proteins in cells have provided critical insights into the mechanisms by which cytoskeletal forces can be harnessed for diverse processes, including cell migration, flagellar beating, and mitosis. High-resolution imaging of the budding yeast cortical cytoskeleton, combined with the powerful molecular genetics unique to this organism, opens new doors for relating cytoskeletal structure to biological function.

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