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Relationship of actin organization to growth in the two forms of the dimorphic yeast *Candida tropicalis*

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Summary. *Candida tropicalis* is a dimorphic yeast capable of growing both as a budding yeast and as filamentous hyphae depending upon the source of the carbon used in the culture medium. The organization of F-actin during growth of the yeast form (Y-form) and the hyphal form (H-form) was visualized by rhodamine-conjugated phalloidin by using a conventional fluorescence microscope as well as a laser scanning confocal fluorescence microscope. In single cells without a bud or non-growing hyphae, actin dots were evenly distributed throughout the cytoplasm. Before the growth of the bud or hypha, the actin dots were concentrated at one site. During bud growth, actin dots were located solely in the bud. They filled the small bud and then filled the apical two-thirds of the cytoplasm of the middle-sized bud. During growth of the large bud, actin dots which had filled the apical half of the cytoplasm gradually moved to the tip of the bud. In the formation of the septum, actin dots were arranged in two lines at the conjunction of the bud and the mother cell. During hyphal growth, the majority of actin dots were concentrated at the hyphal apex. A line of clustered spots or a band of actin was observed only at the site where the formation of a new septum was imminent. This spatial and temporal organization of actin in both categories of cells was demonstrated to be closely related to the growth and local deposition of new cell wall material by monitoring the mode of growth with Calcofluor staining. Treatment of both forms of cells with cytochalasin A (CA) confirmed the close relationship between actin and new cell wall deposition. CA treatment revealed lightly stained unlocalized actin which was associated with abnormal cell wall deposition as well as changes in morphology. These results suggest that actin is required for proper growth and proper deposition of cell wall material and also for maintaining the morphology of both forms of cells.

Keywords: F-actin; Dimorphic yeast; *Candida tropicalis*; Cell wall deposition; Cytochalasin A; Scanning confocal fluorescence microscopy.

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Abbrevations: FM fluorescence microscopy; EM electron microscopy; rh rhodamine; CA cytochalasin A; CD cytochalasin D; PBS phosphate-buffered saline; DMSO dimethylsulfoxide; GA glutaraldehyde.

Introduction

There have been only a few studies on the organization of cytoskeletal elements in yeast and the relationship of their organization to cell cycle events. This is mainly because techniques for examining the arrangement and organization of the cytoskeleton in yeasts were not developed until quite recently. The cytochemical and immunochemical techniques regularly used to visualize cytoskeletal elements in animal and plant cells were not successful in yeast owing to the small amount of cytoskeletal elements, the small size of the cells and the presence of a fairly thick cell wall which hindered the penetration of dyes and antibodies into the cytoplasm. However, recent developments in fluorescence microscopic techniques made it possible to observe cytoskeletal elements in yeast cells, and fluorescence microscopy (FM) has several advantages over electron microscopy (EM) for elucidation of cytoskeletal organization in yeasts. The method is simple and quick and it is possible to visualize the complete image of a cytoskeleton in a cell without serial sectioning and three-dimensional reconstruction procedures which are necessary for the EM approach. Furthermore, it is easy to handle a large number of cells at various stages.

In the past few years dynamic changes in actin and tubulin during several yeast cell cycles have been revealed by FM (Kilmartin and Adams 1984, Marks and

Hyams 1985, Anderson and Soll 1986, Barton and Gull 1988, Hagan and Hyams 1988). We used this technique to obtain an overview of the actin arrangement in a dimorphic yeast *Candida tropicalis*. *C. tropicals* is a good experimental organism to show the difference in actin organization at two different phases of the dimorphism. *C. tropicals* can grow as either a yeast form (Y-form) or a hyphal form (H-form) and these two forms of growth can be easily obtained by controlling the source of carbon in the culture medium (Osumi et al. 1974). Y-form cells are formed when glucose is used as a sole carbon source and elongated H-form cells are formed when n-alkane is used as a sole carbon source. By staining the cells with rhodamine (rh)-conjugated phalloidin, actin organization was observed by conventional FM as well as laser scanning confocal FM. The serial actin staining patterns of the cells were obtained by optical sectioning of laser scanning confocal FM. Recently we reported the usefulness of laser scanning FM for the visualization of spatial organization of actin in yeast cells (Kobori et al. 1989).

The results of the present study show that organization of actin in the two forms of cells is closely associated with (1) the deposition of newly synthesized cell wall materials, (2) septum formation, and (3) the morphogenesis of both forms of cells. The involvement of actin in cell wall formation was also ascertained by treating both forms of cells with cytochalasin A (CA). CA was selected for this study because it is one of the most effective anti-fungi agents among various cytochalasins and its actin-associated functions in various fungi have been reported (Thomas 1978, Allen et al. 1980, Grove and Sweigard 1980). Also, cytochalasin D (CD) was found to be ineffective in both forms of cells of *C. tropicalis* in this study.

Materials and methods

Strain and culture conditions

A normal-alkane utilizing strain of *C. tropicals* pk 233 (ATCC 20336, Tanabe 1966) was used throughout this work. Stock cultures were maintained on agar slants of YPD medium (2% glucose, 1% Bacto-Peptone, 0.5% yeast extract). The yeast was precultured at 30 °C for 22 h in a liquid glucose medium without corn steep liquor (G-medium; Osumi et al. 1974)) to late-log phase. A semi-synchronous culture was obtained in this preculture condition in which unbudded cells comprised 80 to 90% of the population. To initiate the Y-form growth and H-form growth, cells were transferred to fresh G-medium and a hydrocarbon medium without corn steep liquor (H-medium, Osumi et al. 1974), respectively. In both cases, cells were aerobically grown by a reciprocal shaker (130 rpm/min) at 30 °C for the desired period.

Actin staining and fluorescence microscopy

Both forms of cells were fixed by adding a concentrated formaldehyde solution in phosphate buffer directly to the culture medium; final concentrations were 3.7% (wt/vol) formaldehyde and 35 mM KP buffer (35 mM potassium phosphate buffer, pH 6.8), After being fixed for 1 h at room temperature, the cells were washed three times with phosphate-buffered saline (PBS: 50 mM potassium phosphate buffer, pH 7.3, 150 mM NaCl). Cells were then permeabilized with 2% 2-mercaptoethanol containing KP buffer for 15 to 30 min by a reciprocal shaker at 100 rpm/min and then washed again in KP buffer. For visualization of F-actin, the permeabilized cells were stained with 0.3 mM rh-conjugated phalloidin (Molecular Probes, Junction City, OR, U.S.A.) in PBS for 1 h. The stained cells were mounted with KP buffer containing *P*-phenylene-diamine (1 mg/ml; Johnson and Nogueria Aranjo 1981) and 0.5 mM $MgCl_2$. For Calcofluor staining, fixed cells were placed in a mounting medium containing Calcofluor (50 mg/ml; Polyscience, Inc., Warrington, PA, U.S.A.). The drug treated cells were double-labelled with rh-conjugated phalloidin and Calcofluor by the same procedures mentioned above. Slides were viewed through an Olympus BM-2 microscope and photographed as previously described (Kobori et al. 1989). Slides with actin staining were also viewed through a scanning confocal FM (Lasersharp, MRC-500 system, Bio-Rad Microscience, Inc., Abingdon, Oxfordshire, U.K.) as previously described (Kobori et al. 1989).

Drug treatment

The effect of CA (Aldrich Chemical Co. Inc., Milwaukee, WI, U.S.A.) and CD (Sigma Chemical Co., St. Louis, MO, U.S.A.) on the two forms of cells was examined by incubating the cells in either G-medium or H-medium containing the drugs at the desired final concentration for 5.5 h. M-Medium, which has the same composition as G-medium except that glucose is replaced by galactose, was also used in order to confirm that the inhibitory effect of CA is directed at actin but not at glucose transport in Y-form cells. The cell density used at the beginning of cultivation was 1×10^6 cells/ml. CA and CD were dissolved in dimethyl sulfoxide (DMSO) and the final concentration of the solvent in the media was adjusted to 0.2% (v/v). The effect of DMSO was tested separately as a control and the concentration of the solvent used had no detectable effect on the actin configuration and morphology of the cells.

Transmission electron microscopy

The $KMnO_4$ fixation method was used in this study because the cell wall and septum are visualized at high electron density by this method whereas they are not clearly visualized when the conventional OsO_4 fixation or freeze-substitution method is used. Control cells and CA treated cells were fixed with 2% glutaraldehyde (GA) and postfixed with 1.5% $KMnO_4$ overnight at 4 °C. Specimens were dehydrated with ethanol and acetone as previously described (Osumi et al. 1974) and embedded in Quetol 653. Ultrathin sectioned samples were stained with uranyl acetate followed by lead citrate. The specimens were observed with a Hitachi H-800 electron microscope at 200 kV and a JEM-1200 EXS electron microscope at 120 kV.

Results

Actin localization during the cell cycle of yeast-form cells

Staining of F-actin with rh-conjugated phalloidin was generally satisfactory even when the procedure omitted

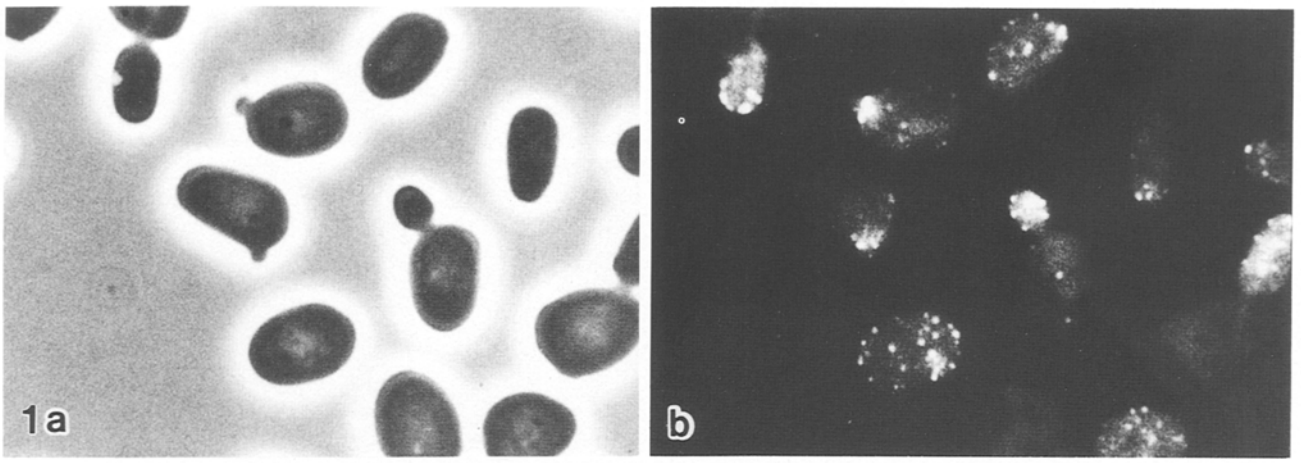


Fig. 1. Phase-contrast and fluorescence micrographs of yeast form (Y-form) growing cells taken in the same field. **a** Phase-contrast image. **b** Actin staining with rh-phalloidin, illustrating the characteristic patterns of actin organization in various stages of the cell cycle. Photographs were taken with a conventional fluorescence microscope

the removal of the cell wall, however, double staining of the non-drug treated cells was unsuccessful because the fluorescence of rh-conjugated phalloidin was weakened by the addition of Calcofluor. Figure 1 shows a typical image of single staining of actin in an asynchronous population of Y-form cells observed by conventional FM. F-actin was seen predominantly as dots measuring 0.2 to 0.5 μm in diameter. Since different distribution patterns of F-actin were observed at different phases of the cell cycle (Fig. 1), each cell was assigned to a particular point in the cell cycle using a ratio measurement of the mother bud diameter as a criterion which was found to be approximate judging from nuclear division pattern (Fig. 2). The same experiment using a semi-synchronous population indicated that the sequence of the criterion ratio was the same as the temporal sequence of the cell cycle stages. The criterion ratio was fairly constant in cells of different sizes even though the population appeared to be composed of cells of different sizes at the same stage of the cell cycle. The Y-form cells were divided into eight stages according to the mother-bud size ratio. In

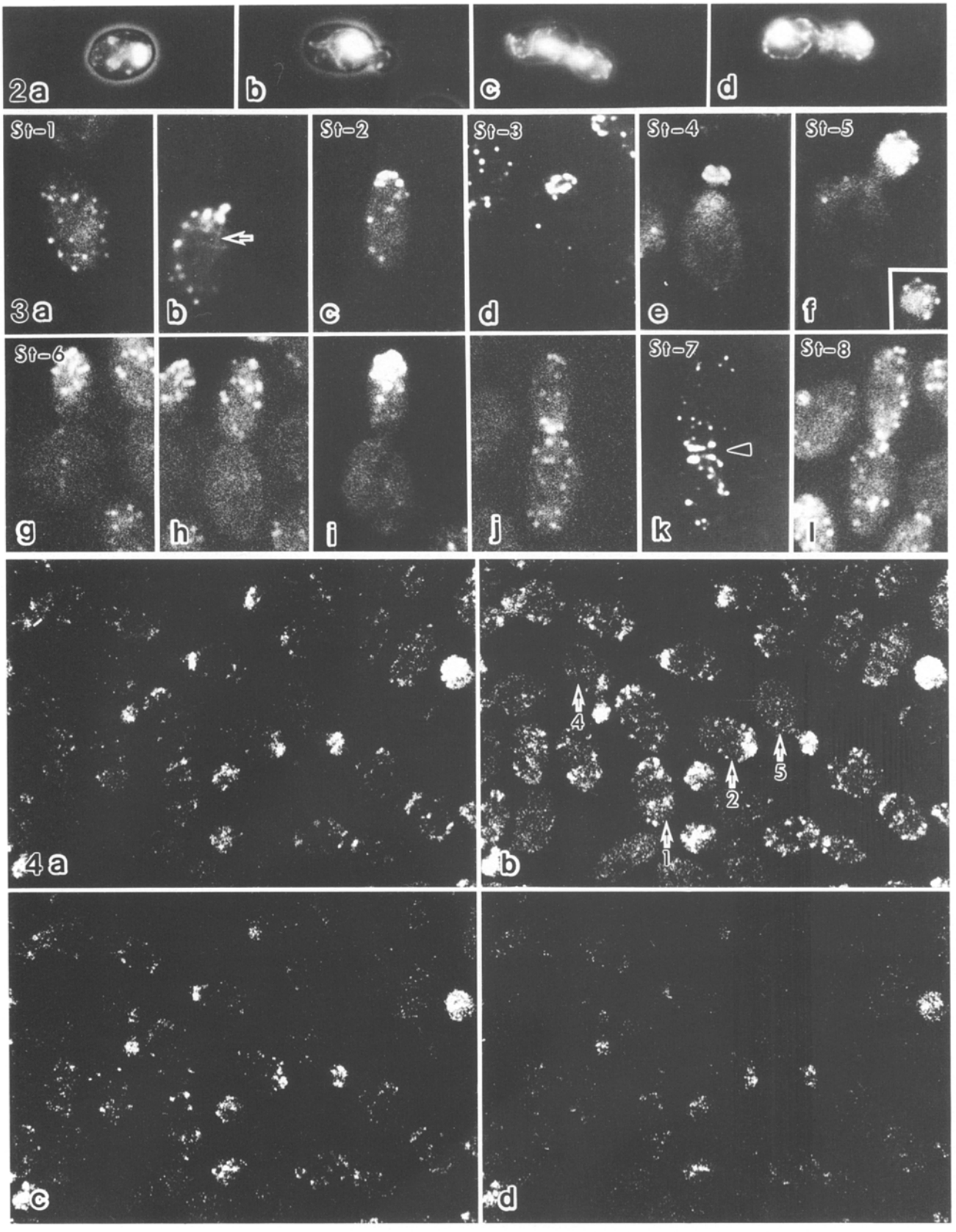
Fig. 2. Phase-4′,6-diamidino-2-phenyl-indole (DAPI) micrographs of the overall cell morphology and nuclear position during growth of *C. tropicalis* Y-form cells. The nuclear position is denoted by very bright fluorescence seen against a phase image of the cells. Mitochondrial DNA is also visualized as dots or as filaments. **a** and **b** The undivided nucleus is present in the mother cell. **c** Nuclear division has occurred. **d** The mother cell and bud both possess a daughter nuclei

Fig. 3 a–l. Actin organization during the yeast phase of the *C. tropicalis* cell cycle. Images of individual cells have been arranged according to the criteria of the mother to bud size ratio described in the text. The sequence illustrates the typical patterns of actin staining observed during the cell cycle of a yeast-form cell. The stages of the cell cycle (St-1 to St-8) are shown in the photographs. **f** The insert shows the same bud taken at low contrast, illustrating a cluster of actin dots. **b** Actin fibers (arrow) are visible only at the transition from St-1 to St-2. **k** A band or two lines of actin dots (▶) are visible at St-7

Fig. 4. Organization of actin in serial sections of an asynchronous population of the yeast cells revealed by scanning confocal fluorescence microscopy. Cells were horizontally sectioned at 0.7 μm intervals from top to bottom and four of eight serial sections are shown (**a–d**). *1, 2, 4, 5* Cell cycle stages as described in the text and Fig. 7

Fig. 5. Actin organization during the hyphal growth of *C. tropicalis*. Cells from a semi-synchronous population of unbudded Y-form cells were transferred to the hypha-forming medium (H-medium). At various time intervals, hypha-forming cells were fixed and processed for actin staining as described in Materials and methods. **a–c** Cells cultured in H-medium for 0, 2, and 3 h respectively. **d–f** Cells cultured in H-medium for 5, 8, and 10 h, respectively. **g** and **g′** Cells cultured in H-medium for 15 h. **h** and **h′** Cells cultured in H-medium for 20 h. Actin-staining pattern **g** and **h** and the corresponding phase-contrast image **g′** and **h′** observed in same field

Fig. 6. Organization of actin in serial sections of hyphal growing cells revealed by scanning confocal fluorescence microscopy. Cells were cultured in H-medium for 20 h and the fixed cells were optically sectioned at 0.7 μm intervals from top to bottom (**a–d**). **b** Actin dots are predominantly concentrated at the tips of the hyphae (arrow) and a band or line of actin dots is visible at the newly synthesized septum (▶)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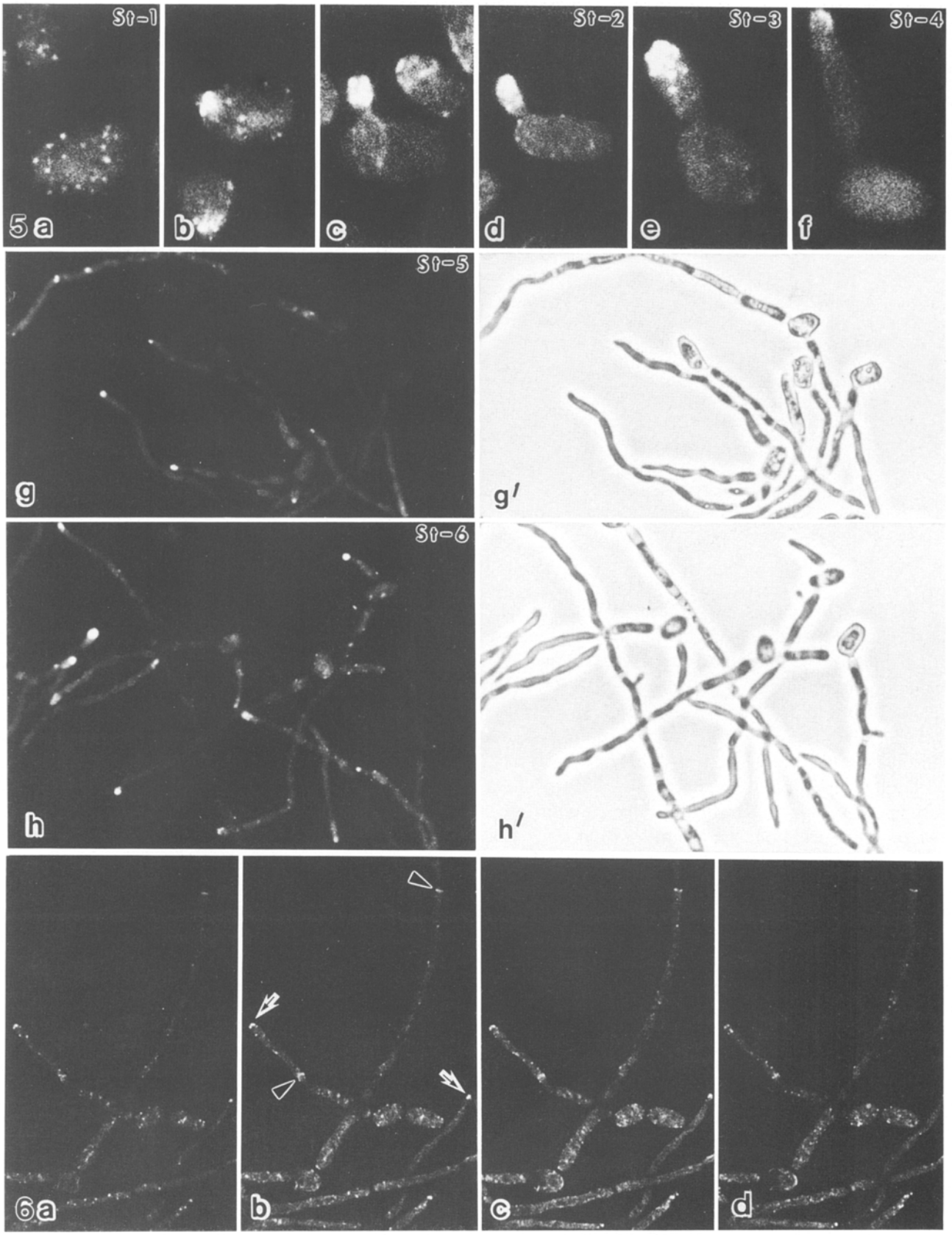
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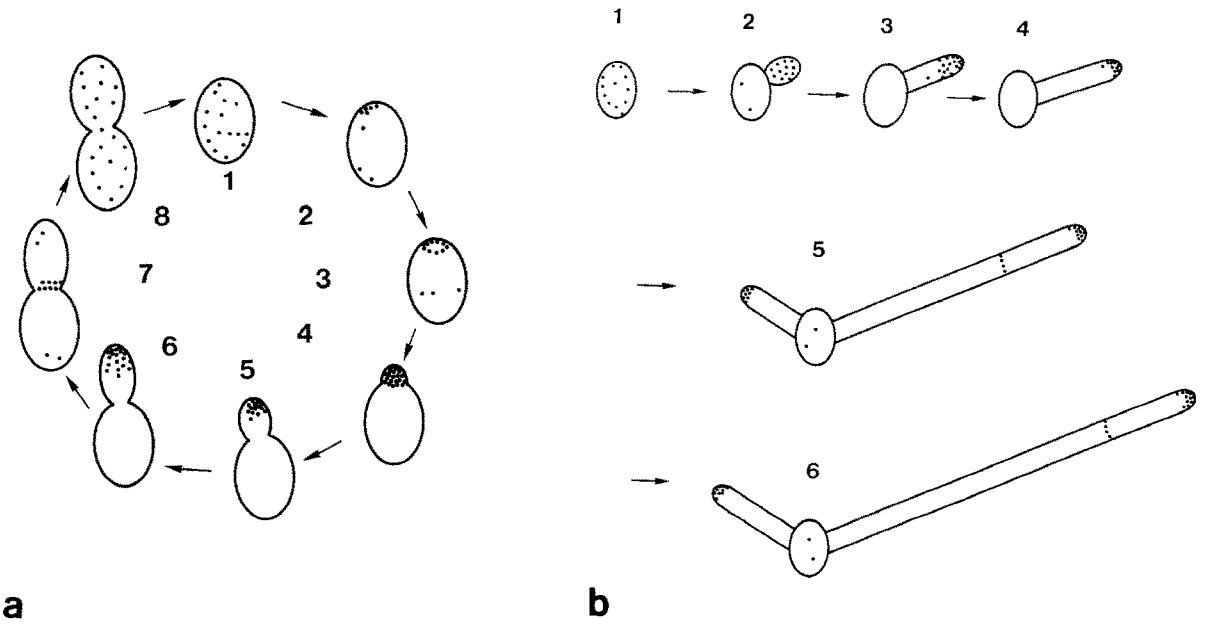


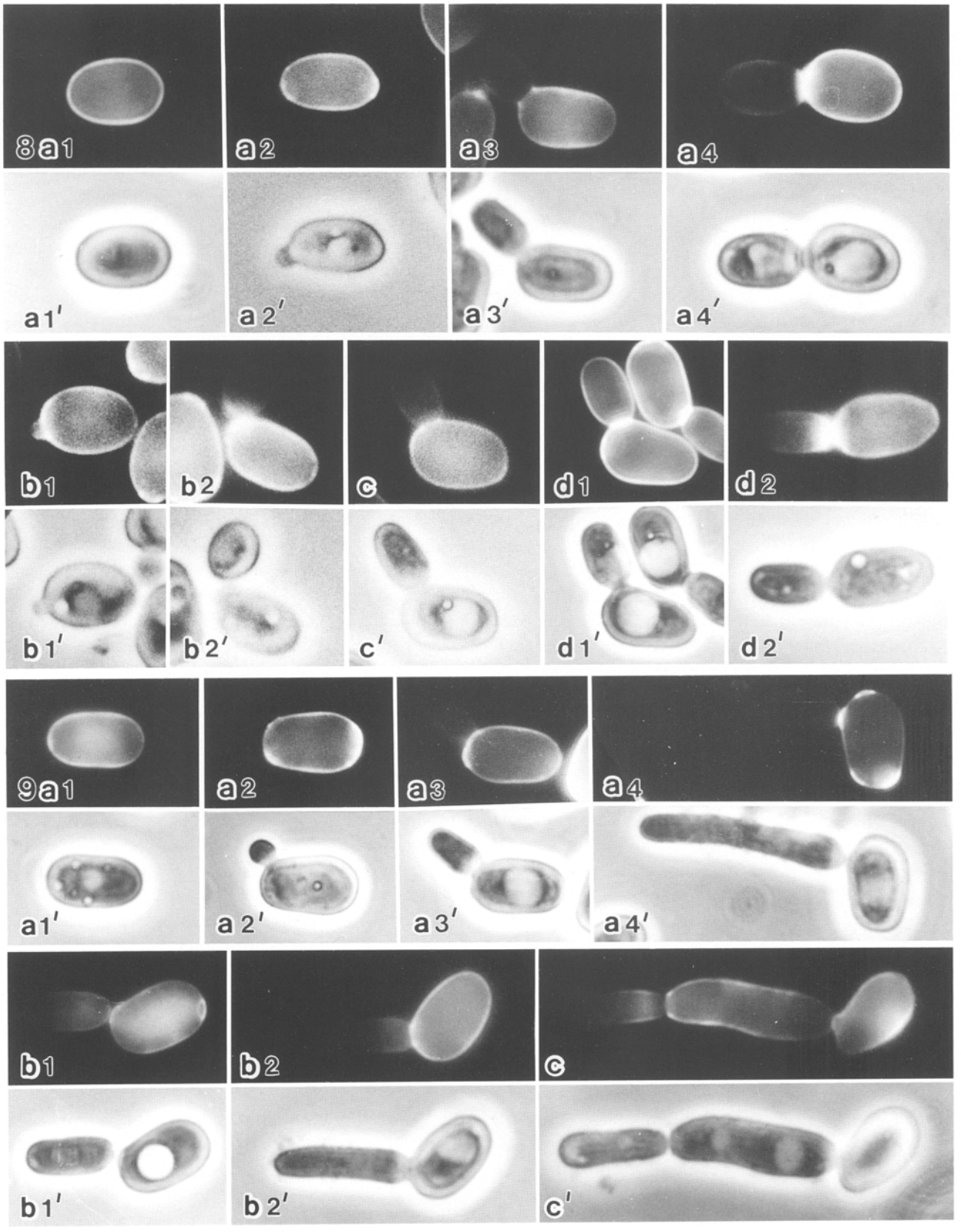
Fig. 7. Schematic diagram of the dynamic changes in actin organization during bud (**a**) and hypha (**b**) formation. Stages of the cell cycle (*1–8*) determined by a ratio measurement of the mother-bud size (see the text) (**a**). Stages of growth (*1–6*) determined by a ratio measurement of the mother-hyphae size (see the text) (**b**)

each stage the actin staining patterns of approximately 100 cells were photographed in more than 25 separate fluorescence experiments. A reconstructed sequence of actin organization during the cell cycle of Y-form cells is shown in Fig. 3. Figure 3 a shows an unbudded cell (stage 1) exhibiting uniform distribution of actin throughout the cytoplasm. Just before bud emergence, actin dots and fibers were directed towards the site of evagination (Fig. 3 b). During the cell cycle actin fibers were observed only at the transition from stage 1 to stage 2. Clustered actin dots were observed at the site on an unbudded cell where bud emergence was apparently imminent (stage 2, Fig. 3 c). Just after bud emergence (stage 3), actin dots concentrated in a ring around the neck of the bud (Fig. 3 d). When the bud attained 1/4 of the size of the mother cell (stage 4), actin dots were concentrated in the bud and filled the cytoplasm of the bud (Fig. 3 e). As the bud reached 1/2 the size of the mother cell (stage 5), the actin dots filled the apical 2/3 of the bud (Fig. 3 f). When the bud attained 2/3 the size of the mother cell (stage 6), actin dots predominated in the apical 1/2 of the bud (Fig. 3 g – i) However, in the minority of cells at stage 6, actin dots filled the cytoplasm of the bud (not shown). This actin distribution can be considered in terms of temporal transition from stage 6 to stage 7. During septum formation, actin dots were redistributed through the cytoplasm of both bud and mother cell and a concentration of fluorescence was noticed in the neck region (Fig. 3 j). Then, two lines of clustered spots or two bands were observed in the neck region (stage 7, Fig. 3 k). When the bud became the same size as the mother cell (stage 8), uniform distribution of actin dots was observed in both the bud and the mother cell (Fig. 3 l).

Laser scanning confocal FM was used to confirm the spatial organization of actin. The rh-phalloidin-stained Y-form cells were optically sectioned at 0.7 μm intervals

Fig. 8. Reconstructed sequence showing the development of buds labelled with Calcofluor. Unbudded singlets (**a 1**), small buds (**b 1**), medium-sized buds and large buds (**d 1**) which had been labelled with Calcofluor were recultured and the sequence of events was followed with ultraviolet illumination (**a 2–4**, **b 2**, **c**, **d 2**) and corresponding phase-contrast images. Details are given in the text

Fig. 9. Reconstructed sequence showing the development of hyphae labelled with Calcofluor. Unbudded singlets (**a 1**), 3 h grown hyphae and 9 h grown hyphae which had been labelled with Calcofluor were recultured and the sequence of events was observed with ultraviolet illumination (**a 2**, **3**, **b 1**, **2**, **c**) and corresponding phase-contrast images. Details are given in the text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from top to bottom. Four of eight serial sections of actin staining patterns are shown in Fig. 4. The dots were found to be prominent but filaments were scarce in all the planes of the section of each cell in all eight stages. This finding confirmed the spatial and temporal organization of actin observed with the conventional FM. A schematic diagram of actin organization in a Y-form cell is shown in Fig. 7 a.

Actin localization during the hyphal growth

The distribution of actin in filamentous hypha was examined at 0, 2, 3, 5, 8, 10, 15, and 20 h after transfer of Y-form cells to fresh H-medium. At 0 h, actin dots were scattered evenly in the cytoplasm (stage 1, Fig. 5 a). At 2 h of incubation, actin dots had moved towards the site of evagination (Fig. 5 b). At 3 h, small hyphae were formed with lengths less than 2/5 of the diameter of the mother cell. Actin dots were predominantly and evenly concentrated in the small hyphae (Fig. 5 c).

At 5 h of incubation, the small hyphae were almost half the length of the mother cell (stage 2), and clustered actin dots filled the hyphae (Fig. 5 d). At 8 h of incubation, the hypha became the same diameter as the mother cell (stage 3), and actin dots were concentrated towards the hyphal tip (Fig. 5 e). At 10 h of incubation, the hypha were 1.5 times longer than the diameter of the mother cell (stage 4), actin dots were observed solely at the hyphal tip (Fig. 5 f). Figure 5 g and g′ shows an actin-staining image of 15 h cultured cells and the corresponding phase-contrast image taken in the same field. The hyphe became 4.5 times longer than the diameter of the mother cell (stage 5), and the actin dots were predominantly concentrated at the tip of each hypha. The same actin distribution pattern was observed when the hyphae became 7 times longer than the diameter of the mother cell (stage 6) at 20 h (Fig. 5 h and h′).

Figure 6 shows the through-focus imaging of actin configuration in a 20 h cultured hypha by confocal optical sectioning. There was generally a pronounced concentration of fluorescence at the tip of the hyphae in all sections (Fig. 6 b). It is interesting to note that a line of actin dots was observed only at the site where the formation of a new septum was imminent (Fig. 6 b). As a result of viewing this line of actin as a series of horizontal images by confocal laser scanning FM, we can conceptualize three-dimensional images of the line of actin organization existing as a vertical plane. A schematic diagram of actin organization in an H-form cell is shown in Fig. 7 b.

Cell wall expansion and growth during bud and hyphal growth

The spatial and temporal organization of actin presented in Fig. 7 indicates that actin organization in the two forms of cells is basically similar. That is, during the whole process of bud and hyphal growth, actin dots are scarce in mother cells and are predominant in buds and hyphae. When a small bud or a short hypha is formed, actin dots fill the cytoplasm of both bud and hypha. With continued bud growth and hyphal elongation, actin dots are predominantly concentrated at the apical region although the region of actin localization is more restricted in the hyphae than in the bud. Similarity of actin distribution is also observed during the septum formation. This similarity of actin organization in the two forms of cells might be explained by the mode of growth and cell wall expansion of the two forms of cells, or the actin organization might be closely related to the site of new cell wall deposition. To clarify the relationship between them, the mode of growth and cell wall expansion was examined by labelling the cells with Calcoflour.

Figure 8 shows the changes in Calcofluor-staining patterns and the corresponding phase-contrast images after the cells were labelled with Calcofluor and the cells were cultured again. A sequence of events was inferred by using unbudded singlets, small buds, medium sized buds, and large buds which had been labelled. When the unbudded cells were labelled with Calcofluor (Fig. 8 a 1), the buds which subsequently formed were unlabelled (Fig. 8 a 2 – 4, and corresponding phase-contrast images of Fig. 8 a 2′ – 4′). In addition, the intensity of the fluorescence exhibited by the mother cell did not decrease detectably during bud initiation and growth. These findings suggest that growth and cell wall expansion occur predominantly in the bud and that the major portion of the cell wall material which was deposited in the growing bud is newly synthesized. In order to determine the site of cell wall expansion in the growing bud, small buds and medium buds were labelled with Calcofluor and recultured. In a small labelled bud (Fig. 8 b 1), subsequent cell wall expansion was observed at the distal tip of the growing bud (Fig. 8 b 2). Similar distal expansion of the cell wall was also observed when labelled small and medium sized buds (Fig. 8 d 1) were cultured again and grew to medium sized buds (Fig. 8 c) and large buds (Fig. 8 d 2), respectively. The timing and site of new cell wall deposition and bud growth shown in Fig. 8 coincided with the timing and site of actin accumulation during the cell cycle of a Y-form cell shown in Figs. 3

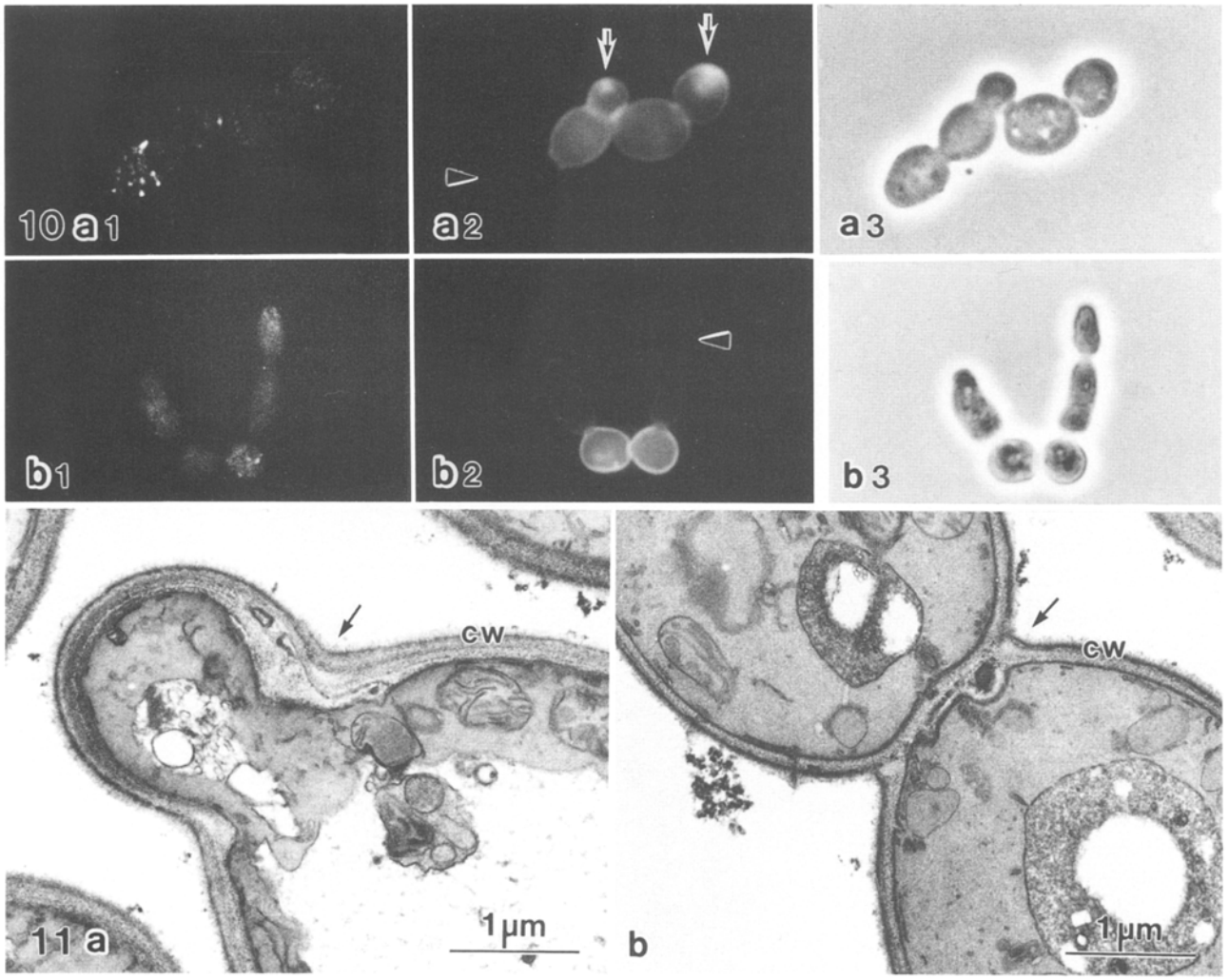

Fig. 10. Effect of CA on both forms of cells of *C. tropicalis*. **a** Treatment of Y-form cells with CA (8 μg/ml) for 5.5 h. **b** Treatment of H-form cells with CA (8 μg/ml) for 5.5 h. Images of actin staining (**1**), corresponding Calcofluor staining (**2**) and phase-contrast (**3**) taken in the same field. Arrows indicate buds showing strong fluorescence, arrowheads indicate buds showing weak fluorescence

Fig. 11. Electron micrographs of CA-treated Y-form and F-form cells. Both forms of cells were treated with CA in the same way as described in the legend for Fig. 10. Cells were fixed with GA and postfixed with $KMnO_4$. **a** A section of CA-treated Y-form cells. Irregular outgrowth of cell wall deposits (arrow) is observed. **b** A section of CA-treated F-form cells. An abnormal septum (arrow) is observed

and 7 a. This result strongly suggests that actin organization is closely related to cell wall growth in the Y-form cell.

Figure 9 shows the changes in Calcofluor-staining pattern and the corresponding phase-contrast images of H-form cells. When singlet cells were labelled with Calcofluor (Fig. 9 a 1), the hyphae which subsequently formed at 3 h (Fig. 9 a 2), 6 h (Fig. 9 a 3), and 10 h (Fig. 9 a 4) were non-fluorescent. This suggests that cell wall expansion and growth occur mainly in the hyphae. In order to determine the site of cell wall expansion in the hyphae, hyphae grown for 3 h and 9 h were labelled with Calcofluor and inoculated into fresh H-medium separately for regrowth. In hyphae stained at 3 h, subsequent development after 3 h (Fig. 9 b 1) and 6 h (Fig. 9 b 2) resulted in no fluorescence at the apical zone of the hyphae. In hyphae stained at 9 h, subsequent development after 3 h (Fig. 9 c) also resulted in no fluorescence at the apex of the hyphae. These observations indicate that cell wall expansion and deposition of new cell wall material occur at the hyphal apex during the hyphal growth. As the actin organization of H-form cells shown in Figs. 5 and 7 b coincided with the reconstructed sequence of cell wall growth shown in

Fig. 9, a close relationship between actin and hyphal growth and cell wall expansion was demonstrated.

Drug treatment

Effects of CA and CD on actin organization were examined in both forms of cells of *C. tropicalis*. CA perturbed actin organization at 1 μg/ml but CD did not perturb it even at 40 μg/ml; therefore, CA was further used to obtain evidence of the association of actin with growth. However, the disadvantage of using CA is that is has been shown to inhibit glucose transport across the cell membrane (Cooper 1987). We confirmed that the effect of CA on *C. tropicalis* was exclusively directed to actin and not to glucose transport. That is, an inhibitory effect of CA on actin was observed even when n-alkane was used as the sole carbon source for H-form cells and the same effect was observed when galactose was substituted glucose for Y-form cells. Treatment of both forms of cells with 8 μg of CA per ml for 5.5 h resulted in changes in actin organization, deposition of cell wall materials and morphology of the cells. Figure 10 a presents distribution patterns of actin in Y-form cells with the corresponding Calcofluor-staining and phase-contrast images of the same field. The concentration of actin in the buds and the septum visualized in the control cells was diminished in these CA-treated cells and faint abnormally distributed actin dots were observed (Fig. 10 a 1). The Calcofluor-staining patterns also showed abnormal deposition of cell wall materials on the buds (Fig. 10 a 2). It is interesting to note that some buds showed strong fluorescence of Calcofluor whereas others showed very weak fluorescence. As uniform intensity of Calcolfluor was observed in cells not treated with CA (Figs. 8 and 9), the different intensity of fluorescence observed in CA-treated cells might be explained by uneven distribution of Calcofluor-binding cell wall substances. It is also noteworthy that the site of strong intensity of Calcofluor not always coincided with the site of strong intensity of actin in the CA treated cell, however, cells having abnormal Calcofluor staining always showed an abnormal actin distribution pattern. A possible explanation for this is when the actin configuration is profoundly disturbed by CA, site to site correlation of localized actin and cell wall materials that existed in normal cells are lost. The morphology of the CA-treated cells was different from that of the untreated control cells. Most of the CA-treated Y-form cells showed multi-budding which was probably caused by the inability of the mother cells to separate.

In hypha-forming cells, similar changes were observed following CA treatment. The concentration of actin dots at the tip of the cells was diminished (Fig. 10 b 1) and no Calcofluor fluorescence was observed on the growing hyphae and septum (Fig. 10 b 2). Ultrathin sections of CA-treated cells allowed examination of the characteristics of the cell wall and septum in more detail. Examination of DMSO-treated controls of both forms of cells revealed a normal cell wall, septum and cell morphology (data not shown). In contrast, CA-treated cells (8 μg/ml, 5.5 h) showed irregular deposition of cell wall materials and abnormal septum formation (Fig. 11). Transmission electron micrographs revealed that the thickening of the cell wall was caused by accumulation of cell wall materials at the inner wall layer (Fig. 11 a). These results confirmed that actin is necessary for proper deposition of cell wall materials and maintaining the morphology of both forms of cells.

Discussion

Actin-staining patterns change in a characteristic way during the cell cycle of Y-form cells and during the growth of hyphae of *C. tropicalis* as diagrammed in Fig. 7. During the cell cycle of Y-form cells, the temporal and spatial organization of actin was closely associated with the timing and location of the chitin ring formation, cell wall development, and septation. During the formation of hyphae, actin dots were predominantly localized at the growing apex of hyphae and at the newly synthesized septum. A similar association of actin with the localized deposition of the cell wall has been reported for the budding yeasts *Saccharomyces uvarum* (Kilmartin and Adams 1984) and *S. cerevisiae* (Adams and Pringle 1984), the fission yeast *Schizosaccharomyces pombe* (Marks and Hymans 1985), and the dimorphic yeast *Candida albicans* (Anderson and Soll 1986). In *S. cerevisiae* evidence of a spatial association between actin and cell wall deposition was obtained from a temperature-sensitive mutant in which the mutation is in the structural genes of the actin (Novick and Botstein 1985) and also from morphogenetic mutants (Adams and Pringle 1984), In *S. pombe*, a close relationship between actin and cell wall organization was found in temperature-sensitive cell division cycle (cdc) mutants (Marks et al. 1987). We also confirmed the close relationship between actin and cell wall organization by using the reverting protoplasts of *S. pombe* as a simple model system (Kobori et al. 1989, Osumi et al. 1989). The treatment of reverting protoplasts with CD demonstrated that actin is associated

with the initiation of cell wall formation, proper depostition of cell wall materials, formation of a fibrillar network of β (1 → 3) glucan on the surfaces of the reverting protoplasts, and maintaining the normal morphology of reverting protoplasts of *S. pombe*. From these results, it is concluded that actin plays an important role in the localized deposition and growth of cell walls in yeasts.

Localization of actin during the bud growth and hyphal elongation of *C. tropicalis* was found to be basically similar. This similarity was interpreted in terms of similarity in the zones of cell wall expansion in the two forms of the dimorphic yeast. That is, the growth of the Y-form cell wall depends to a significant extent upon apical wall expansion. Also, the growth of the hyphal cell wall depends almost entirely upon an apical zone of wall expansion in a polar fashion. The apical growth of cell wall expansion in the two forms of cells coincided with apical localization of actin in the two forms of *C. tropicals* cells. This finding strengthens the idea that actin is involved in the deposition of cell wall materials and growth of the cell wall.

CA was used in this study to clarify the involvement of actin in cell wall formation. Cytochalasins and phalloidins are the best available drugs to study the role of action in cells of animals, plants and microorganisms. The specificity of phalloidin for actin and its mode of actin were well demonstrated (Cooper 1987); however, phalloidins were not used in this study. Because phalloidins have been reported not to cross the cell membrane, they are not suitable for our experiments on living cells. Among cytochalasins, CA and CD have been reported to be effective in fungi (Allen et al. 1980, Grove and Sweigard 1980), but cytochalasin B has been reported to be ineffective in fungi (Kuo and Lampen 1974). The CA perturbed the actin organization of both froms of cells of *C. tropicalis*. Its effect on *C. tropicalis* was found to be exclusively directed to actin and not to glucose transport. Therefore, the effect of CA could be interpreted as a perturbed relationship between the actin and cell wall.

The zones of cell wall expansion of *C. tropicalis* were examined by labelling the cells with Calcofluor and the apical mode of wall expansion was observed during the bud and hyphal growth. Similar apical cell wall expansion and growth has been reported for many filamentous fungi and yeasts. Recently, Staebell and Soll (1985) and Soll et al. (1985) demonstrated that apical growth accounts for more than 95% of the wall expansion during hyphal growth of *C. albicans* by monitoring the position of polylysine-coated beads firmly attached to the wall of growing cells. The apical mode of cell wall expansion was also demonstrated in bud growth of several yeasts. Tkacz and Lampen (1972) revealed the site of mannan insertion during the bud growth of two *Saccharomyces* sp. by using fluorescein-conjugated concanavalin A and found that wall expansion in *Saccharomyces* cells resembles the apical mode exhibited by filamentous fungi. Stabell and Soll (1985) showed that the apical zone accounts for roughly 70% of the surface expansion during the first two-thirds of bud growth, that the remaining third is due to general expansion and that wall expansion during the last one-third of bud growth is accomplished by general wall expansion. These results suggest that the apical mode of growth might be a general mode, applying not only to hyphal growth but also bud growth of yeasts. However, further detailed analysis is needed to elucidate the dynamics of cell wall expansion of both forms of cells of *C. tropicalis* by using various methods, such as radioautography, and monitoring the postition of cell growth by beads.

A F-actin of *C. tropicalis* was visualized mainly in the form of dots but fibers were scarce. A similar result was reported in *S. pome* (Marks and Hymans 1985). Dots of actin were predominant in all stages of the cell cycle while only a few fibers of actin were observable at 2 stages among the 8 stages of the cell cycle. However, in *Saccharomyces* spp., fibers were prominent in all stages of the cell cycle (Adams and Pringle 1984) while in *C. albicans* fibers were fairly prominent (Anderson and Soll 1986). Actin of *Saccharomyces* spp. was stained through our preparation protocol which clarified that fibers of actin were adequately preserved. We therefore concluded that a methodological difference was not the reason to explain the absence of fibers in *C. tropicalis*. Different forms of actin might depend on different species. However, we cannot exclude the possibility that fibers of actin are also present in *C. tropicalis*. Because the bundles of actin filaments are thinner than those of *Saccharomyces* spp., they cannot be detected under the resolution of fluorescence microscopy. Ultrastructure analysis is needed to clarify this.

Anderson and Soll (1986) reported on the localization of F-actin in *C. albicans*. A comparison of their study with ours reveals a similarity as well as differences in the dynamics of actin localization in the two *Candida* spp. Basically, the temporal program of organization of actin dots during the development of the buds and the hyphae were similar. However, a variety of patterns of actin localization was observed at every stage of the cell cycle during the bud formation in *C. albicans*. The

major patterns occurred in only 50% of the cell examined and one to four different minor patterns existed in each stage of the cell cycle. In contrast, patterns of actin localization converge to one major pattern at each stage of the cell cycle in *C. tropicalis*. This simple pattern of actin organization together with cell-cycle specific actin organization in *C. tropicalis* could serve as a good model in confirming and further studying the role of actin in the sequence of growth events as well as in dimorphism.

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