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The Role of Abp140p in Actin Dynamics of Budding Yeast

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In the previous studies of *Saccharomyces cerevisiae*, Abp140p (actin binding protein 140) fused to GFP has been only a protein that can label actin cables of yeast cells so far. However, the role of Abp140p in actin dynamics was remained elusive. In this study, the function of Abp140p was investigated with a deletion mutant and overexpression of GFP fused Abp140p. The deletion mutant was slightly more susceptible to Latrunculin-A (Lat-A), an actin-monomer sequestering agent, than wild type, although no significant deformation of actin structures was caused by *ABP140* deletion. Overexpression of Abp140p-GFP retarded cell growth, and produced thick and robust actin cables. Lat-A was not able to destabilize the thick actin cables, which suggests that actin dynamics was compromised in the cells with surplus of Abp140p. Therefore, Abp140p seems to stabilize actin cables together with other bundling proteins. Recently, actin cable dynamics of budding yeast was found to have a resemblance to that of filopodial tip of cultured mammalian cells. Retrograde movement of actin cables from buds to mother cells indicated local generation of the cable at bud sites. By using Abp140p-GFP, we traced the steps in the generation of a new actin cable after elimination of old cables by sodium azide. Before the appearance of a new actin cable, Abp140p-GFP concentrated in buds and disappeared, as mother cells became abundant in actin cables. Our observations provide a direct evidence of actin cable formation at buds of budding cells.

Keywords: Actin cable, Abp140p, Latrunculin-A, sodium azide, GFP, *Saccharomyces cerevisiae*, budding yeast

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Introduction

Visualization of actin structure in living cells has elucidated the mysteries of actin dynamics in various cell types. Fluorescent speckle microscopy of filamentous actin (F-actin) revealed two different actin networks at the leading edge of migrating epithelial cells (Ponti *et al*, 2004). Spatio-temporal imaging of F-actin provided new insight into a role of F-actin in spermatogenesis in *Drosophila melanogaster* (Noguchi and Miller *et al*., 2003) and relationship between F-actin and microtubule of Arabidopsis root cells (Wang *et al*., 2004). In budding yeast, an actin structure, actin patch, was visualized by fusion of green fluorescence protein (GFP) to a capping protein, Cap2p (Waddle *et al*., 1996). However, the actin cables, cross-linked bundles of actin filaments could not be seen in living yeast cells, until Yang and Pon (2002) introduced Abp140p as a target protein for GFP fusion. They suggested that establishment of bud-associated actin cables during the cell cycle was accomplished not by realignment of existing cables but by assembly of new cables within the bud or bud neck, followed by elongation. Their findings showed a relationship of actin dynamics between budding yeast and filopodium tip of mammalian cells which was observed in neuroblastoma cell lines (Mallavarapu and Michison, 1999). Dual labeling of actin cables with Abp140p-GFP and intracellular organelles with appropriate markers also revealed a role of the cables in intracellular motility of endosomes and mitochondria (Huckaba *et al*., 2004; Fehrenbacher *et al*., 2004).

Abp140p was discovered as a F-actin binding protein in budding yeast by Asakura *et al*. (1998). Abp140p does not show homology to any known actin binding protein and is expressed in cells by fusion of two ORFs (YOR239W and YOR240W) by means of a +1 translational frameshift.

Abp140p crosslinks F-actin *in vitro* and colocalizes with actin patches and cables. Cells carrying a deletion in *ABP140* showed no abnormal phenotype. In this study, to elucidate the role of Abp140 in actin dynamics of budding yeast, we observed the effect of latrunculin-A (Lat-A), an actin monomer sequestering agent, on *ABP140* deletion mutant. The phenotype of yeast cells overexpressing Abp140p was also investigated by microscopic analysis. Because of close relationship between actin cables and mitochondria (Boldogh *et al.*, 2000; Gourlay *et al.*, 2004), we examined the effects of respiratory inhibitors on actin cables by using Abp140p-GFP.

Material and methods

Yeast strains and plasmids

Abp140p-GFP was expressed 1) by tagging chromosomal *ABP140* at its C-terminus with GFP under control of the endogenous *ABP140* promoter (strain YCY006) and 2) by introducing a 2 micron multi-copy plasmid bearing *ABP140-GFP* into wild type BY4741 (MATa his3 leu2 met15 ura3) for the overexpression of Abp140p-GFP (strain YCY026). Preparation of YCY006 was described in the previous report (Yang and Pon, 2002). To prepare the plasmid for Abp140p-GFP overexpression, *ABP140* gene was amplified with the primers bearing Pac1 site and inserted to a multi-copy plasmid, pFa-His3MX6-PGAL1-GFP, at the region between GAL1 promoter and GFP gene to produce pFA-HisMX6-PGAL1-ABP140-GFP, which was introduced into BY4741 by the lithium acetate method (Ito *et al.*, 1983).

The *ABP140* deletion mutant was prepared by using a PCR-based gene deletion cassette (Longtine *et al.*, 1998). A PCR fragment containing regions homologous to the 5'end of ABP140, coding regions for the kanamycin resistance marker (KanMX6), and regions homologous to the 3'end of ABP140, was amplified from plasmid pFA6a-kanMX6 with forward primer, 5'-ATGGGTGTCGCAGATTTGATCAAG AAATTCGAAAGCATCTCGGATCCCCGGGTTAATTAA -3', and reverse primer, 5'-CTATTGAGGAACGTCAAA CACAGCTTGTACCCAGCAGCGGGAATTCGAGCTC GTTTAAAC-3'. BY4741 cells were transformed with the PCR product by using the lithium acetate method, and transformants that had integrated the PCR product were selected by growth on yeast extract/peptone/dextrose (YPD) agar medium containing 200 μg/ml geneticin. The deletion mutant (YCY016) was characterized for correct integration of the deletion cassette at the *ABP140* locus by using PCR and Western blot analysis.

Fixation and F-actin staining with phalloidin.

The method for F-actin staining is a modification of a published method (Adams and Pringle, 1984). Cells were concentrated and prefixed by incubation in 100% acetone at 0°C for 3 min. To replace the acetone with an aqueous fixative, samples were incubated in a series of solutions containing 3.7% paraformaldehyde in phosphate buffer and decreasing levels of acetone (from 75% to 10%). Samples were incubated in each solution for 2 min. At the end of incubation in the solution containing 10% acetone, samples were placed on ice for 1 min and washed three times with 3.7% paraformaldehyde previously cooled on ice. Finally, samples were incubated in 3.7% paraformaldehyde at room temperature for 45 min. The fixed cells were washed three times with PBS and stained for actin with rhodamine-phalloidin (Molecular Probes, Eugene, OR) for 45 min. Cells were then washed three times with PBS, suspended in mounting solution, mounted on microscope slides, and visualized within several hours of sample preparation.

Fluorescence imaging of Abp140p-GFP

Fluorescence images were collected using a Zeiss Axioplan II microscope equipped with a Plan-Apochromat 100′/1.4 NA objective lens, and a cooled charge-coupled device camera (Orca-100, Hamamatsu, Bridgewater, NJ). Illumination from the 100-W mercury arc lamp was controlled with a shutter (Uniblitz D122, Vincent and Associates, Rochester, NY). The camera and shutter were controlled by using IP Lab software (Scanalytics, Fairfax, VA). For analysis of colocalization of Abp140p-GFP and actin cables in fixed cells, optical z-sections were obtained at 0.3-s intervals through the entire cell with exposure times of 1.5 and 1 s per section for GFP and rhodamine channels, respectively. Optical sectioning for three-dimensional imaging was carried out by using a piezoelectric focus motor mounted on the objective lens of the microscope (Polytech PI, Auburn, MA). Out-of-focus light was removed by deconvolution of each image section by using Esee v5.1.3 (Inovision, Durham, NC). The series of deconvolved images was projected into a single plane by using National Institutes of Health Image v1.60.

Results

Colocalization of F-actin and Abp140p-GFP

F-actin structures including actin cables and patches were clearly visible by Abp140p-GFP in living yeast cells as described previously (Yang and Pon, 2002). However, the localization of Abp140p-GFP was unstable in the cells that had fixed in a conventional paraformaldehyde method (Boldogh *et al.*, 2001). In those cells, GFP signal was spread evenly in cytoplasm, which suggests the binding between Abp140p and actin filaments is relatively weak or temporary. A modified fixation method using cold acetone and papaformaldehyde could preserve the localization of Abp140p as shown in Fig. 1A. In the fixed cells, Abp140p-GFP localizes well at both cables and patches of mother cells (Fig. 1B). Different pattern of localization appeared in

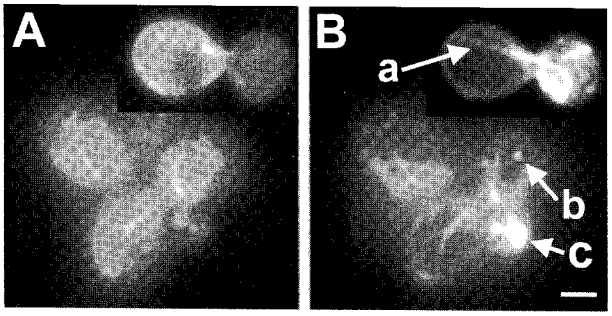

Fig. 1. Colocalization of Abp140pGFP (A) and the actin cytoskeleton (B). Cells (YCY006) were stained with rhodamine phalloidin to visualize F-actin structures. Arrow a indicates an actin cable elongating along the mother-bud axis. Arrow b and c indicate actin patches in the mother cell and the bud, respectively. (Bar, 1.5 μm).

buds with less accumulation of the fused protein at patches.

Susceptibility of *ABP140* deletion mutant to Lat-A

To investigate the function of Abp140 protein, the effect of Lat-A on the growth of the *ABP140* deletion mutant was examined by using a disc diffusion method. Growth inhibition by Lat-A appeared only at 10 mM in both BY4741 (wt) and YCY016 (abp140Ä) cells (Fig. 2). At 10 mM Lat-A, the size of clear zone of YCY016 was slightly bigger than that of BY4741, indicating that deletion of *ABP140* gene made the cells more susceptible to actin disruption. This result suggests that Abp140 protein contributes to stabilizing filamentous actin, although the activity is not essential for cell viability. The microscopic analysis of actin structures could not find any morphological difference between those two strains (data not shown).

Overexpression of GFP-fused Abp140 protein

To verify the stabilizing effect of Abp140p on filamentous actin *in vivo*, we examined the growth of cells overexpressing Abp140 protein fused with GFP. The yeast cells bearing the plasmids in which the expression of *ABP140-GFP* gene was controlled under the GAL1 promoter were grown in galactose medium, and observed under the microscope. During the growth in galactose medium, overexpression of Abp140p-GFP could be easily detected by enhanced fluorescence of cells. The yeast cells emitting intense fluorescence started to appear at the first 1 hr, and the population of cells was dominated by the strong fluorescent cells after 4 hrs, indicating that the overexpression can be achieved in most cells by incubating in galactose medium for 4 hrs. The microscopic observation revealed that Abp140p-GFP localized exclusively at actin structures and, that actin cables remained stable even by Lat-A treatment (Fig. 3). We also observed remarkably reduced growth rate of the strain overexpressing the protein (data not shown). This suggests that proper dynamics of actin structure is needed for normal growth of cells.

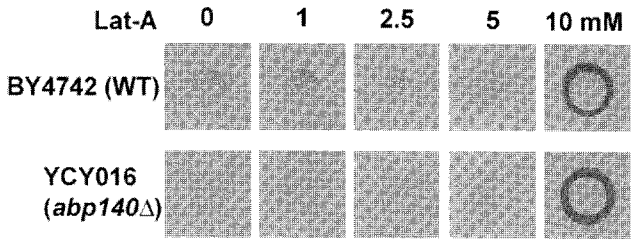

Fig. 2. Susceptibility of BY4741 (wt) and YCY016 (abp140Ä) to Lat-A. Cells were grown on YPD agar plate for 48 hrs at 30°C, on which the paper discs (7 mm) containing Lat-A of various concentrations were placed before inoculation.

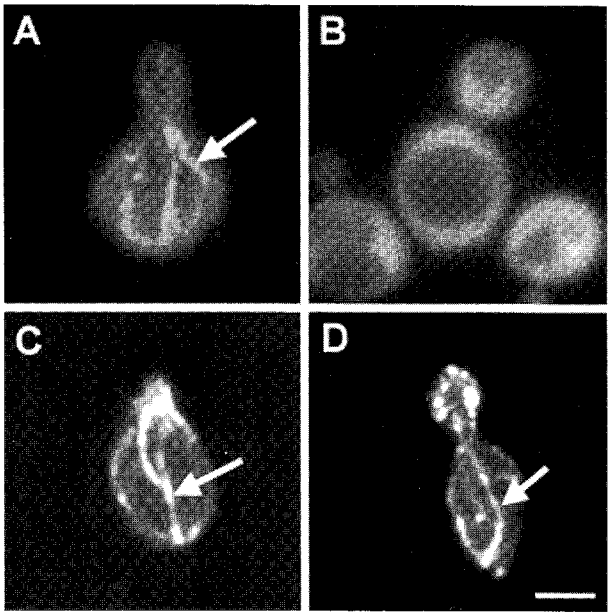

Fig. 3. Effects of Lat-A on actin structures of BY4741 (wt) (A, B) and YCY026 overexpressing Abp140p-GFP (C, D). Cells were grown in YPD medium at 30°C, washed with PBS, resuspended in galactose medium for 4 hrs, and applied to fluorescence microscopic examination of actin structures that was labeled by Abp140p-GFP (A, C). For Lat-A treatment, the cells grown in galactose medium for 4 hrs were treated with 400 μM Lat-A for 5 min. (B, D). Arrows indicate actin cables (Bar, 2 μm).

The effects of respiratory inhibitors on actin dynamics

To investigate the effects of ATP depletion on actin dynamics, we employed live cell imaging of the yeast cells expressing Abp140p-GFP. The cells were treated by 10 mM KCN, 20 uM Antimycin A, 20 uM Oligomycin or 15 mM sodium azide for 30 min in the media of non-fermented carbon source, galactose, and the morphology of actin structures was observed by fluorescence microscopy. The concentrations of the inhibitors and treatment time are enough to reduce ATP level to 50% of untreated cells. Fig. 4 shows actin structures of the ATP-depleted. KCN, Antimycin A and Oligomycin did not cause any noticeable changes in actin structures. Actin cables were well aligned along

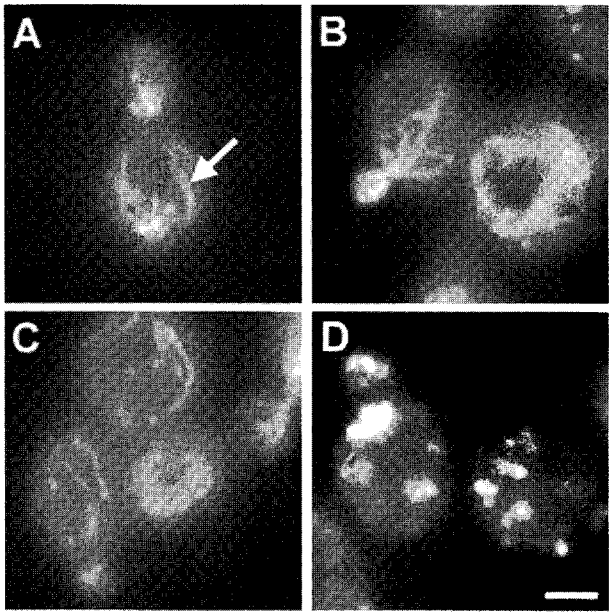

Fig. 4. Effects of respiratory inhibitors on actin structures of YCY006. Cells were exposed to 10 mM KCN (A), 20 μM Antimycin A (B), 20 μM Oligomycin (C) or 15 mM sodium azide (D) in galactose media for 30 min at 30°C and applied to microscopic analysis of actin structures that was labeled by Abp140p-GFP. The arrow indicates an actin cable (Bar, 1.5 μm).

mother-bud axis, and actin patches were mostly concentrated at buds of budding cells. There were no remarkable changes in fluorescence intensity of actin structures in the treated cells. We could find abnormal actin structures only in the azide-treated cells. Sodium azide caused removal of the actin cables and produced more numbers of patch-like structures. Furthermore, the deformation of actin morphology occurred even at 3 min after exposure to azide.

Actin cable regeneration after removal of sodium azide

In previous studies, actin cables were reported to generate from presumptive bud sites and buds of yeast cells during late G1 and G2 phases (Yang and Pon, 2002). To obtain a direct evidence of the locally restricted generation of actin cables in living cells, we traced recovery of actin structures in azide-treated cells after elimination of sodium azide from the medium. Fig. 5 shows the time-lapse images of a budding cell producing new actin cables. We found several steps involved in reappearance of actin cables. First, patch-like structures disappeared rapidly. This phenomenon could not be seen by live cell imaging that needed at least 2 minutes for the preparation for microscopic observation. Second, fluorescence concentrated in buds. The state of increasing fluorescence could not either be observed in our imaging process, but there were already highly fluorescent buds when the cells were picked for microscopic observation. Third, new actin cables appeared from the fluorescent buds. As fluorescent actin cables became abundant in mother cells, fluorescence of buds diminished gradually. This reciprocal change of fluorescence between buds and mother cells indicates that new cables are produced from the bud sites. Furthermore, new actin cables elongated from buds to mother cells were found in the budding cells. Taken all altogether, those steps in cables formation point out buds as the unique sites of actin cable regeneration.

Discussion

The previous *in vitro* study of Abp140p revealed that the protein was able to cross-link actin filaments in to a bundle (Asakura *et al.*, 1998). Because of the localization at actin cables and patches in cells, it is assumed that Abp140p contributes to formation of those two actin structures. The fact that there is no characteristic phenotype in the null mutant suggests the existence of functionally overlapped proteins (i.e., fimbrin). The increased susceptibility of the deletion mutant to Lat-A (Fig. 2) might be an evident of partial protection of actin bundles. The protective effect was also verified by the test of Lat-A sensitivity in cells overexpressing Abp140p. The thick actin cables induced by overexpression of Abp140p-GFP were not disrupted by Lat-A (Fig. 3), which means that depolymerization of actin filaments was blocked by surplus Abp140p. This result is an evident for the active participation of Abp140p in actin dynamics of yeast cells.

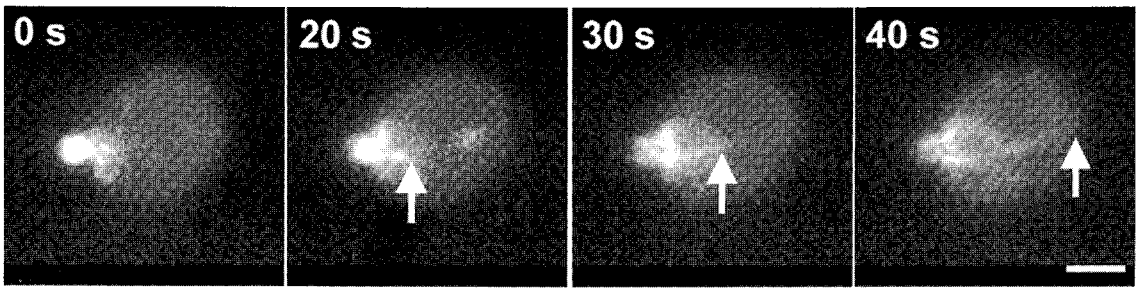

Fig. 5. Time-lapse images of regeneration of actin cables in a budding cell (YCY006). Cells were treated with 15 mM sodium azide for 3 min in galactose medium, washed with PBS, resuspended in fresh medium, and placed under the fluorescence microscope. The total time from removal of azide (resuspension in fresh medium) to acquisition of the first image was 2 min. Arrows indicate the tip of a newly generated actin cable from bud (Bar, 1.5 μm).

The role of ATP in conversion between F-actin and G-actin has been well studied by biochemical approaches and actin visualization of ATP-depleted cells (Pollard, 1986; Shelden *et al.*, 2002). Profilin facilitates ADP-actin to ATP-actin which is added to barbed end of actin filaments, and the loss of Pi resulting in ADP-actin in actin filaments increases binding affinity of the filaments to cofilin, an actin depolymerizing factor (Paavilainen *et al.*, 2004). Therefore, ATP and ADP theoretically seem to play a role in polymerization and depolymerization of actin, respectively. However, that was not the case in various types of mammalian cells. Antimycin A-treated cells accumulated filamentous actin at the sites of focal adhesion and cell-cell interaction with simultaneous decrease G-actin, although the underlying mechanism is not well understood (Atkinson *et al.*, 2004). In yeast cells, as shown Fig. 4, respiratory inhibitors (i.e., Antimycin A, potassium cyanide and Oligomycin) did not affect actin morphology, whereas sodium azide disrupted actin cables in several minutes. Although azide caused deformation of actin structures, it is not likely that ATP-depletion affected actin cables for two reasons. First, the other inhibitors did alter actin structures. Second, azide affected actin cables in less than 3 min that is too short to reduce intracellular ATP level significantly. Thus, azide seems to exert its effect in a more direct way rather than ATP depletion, of which mechanism is not understood.

A previous study about actin cable dynamics in living cells have proposed that the cables are generated in buds and translocated to mother cells (Yang and Pon, 2002). The localization of formin proteins at buds was also consistent with their observation (Evangelista *et al.*, 2002). Nevertheless, there is no direct evidence indicating buds as only sites for cable formation in budding cells. In this study, we attempted to visualize newly formed actin cables followed by disruption of actin structures. To disrupt actin filaments of yeast cells, Lat-A has been exclusively used because of its highly specific binding affinity to actin monomers. Generally, actin structures disappear in several minutes by Lat-A treatment. However, the high affinity to actin monomers retarded the recovery to a normal state of actin dynamics after removal of Lat-A, which makes it difficult to use the reagent in the study of actin reformation. Here, we employed azide for disruption and regeneration of actin cables, since azide induced rapid response of actin. Strikingly, regeneration of actin cables was as fast as disruption, reinforcing our hypothesis that actin deformation by azide is not through ATP depletion. We also found local synthesis and vetorial movement of actin cables from buds to mother cells, although this observation still lacks molecular explanation.

Dynamics of yeast actin cables that we observed in this study have a resemblance to actin in filopodia of mammalian cells. Actin bundles displayed a retrograde movement from the apex of filopodia to the center of cells (Mallavarapu and Mitchison, 1999). Extensive further study of yeast actin cables in a molecular level may help to dissect the process involved in actin dynamics in filopodia.

Acknowledgements

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