Initial Polarized Bud Growth by Endocytic Recycling in the Absence of Actin Cable–dependent Vesicle Transport in Yeast

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The assembly of filamentous actin is essential for polarized bud growth in budding yeast. Actin cables, which are assembled by the formins Bni1p and Bnr1p, are thought to be the only actin structures that are essential for budding. However, we found that formin or tropomyosin mutants, which lack actin cables, are still able to form a small bud. Additional mutations in components for cortical actin patches, which are assembled by the Arp2/3 complex to play a pivotal role in endocytic vesicle formation, inhibited this budding. Genes involved in endocytic recycling were also required for small-bud formation in actin cable-less mutants. These results suggest that budding yeast possesses a mechanism that promotes polarized growth by local recycling of endocytic vesicles. Interestingly, the type V myosin Myo2p, which was thought to use only actin cables to track, also contributed to budding in the absence of actin cables. These results suggest that some actin network may serve as the track for Myo2p-driven vesicle transport in the absence of actin cables or that Myo2p can function independent of actin filaments. Our results also show that polarity regulators including Cdc42p were still polarized in mutants defective in both actin cables and cortical actin patches, suggesting that the actin cytoskeleton does not play a major role in cortical assembly of polarity regulators in budding yeast.

INTRODUCTION

Cell polarization is crucial for many cellular processes in both single-celled and multicellular organisms, including localized membrane growth, directional cell migration, and differentiation. The direction of cell polarization is determined by specific spatial cues provided by the environment (such as chemoattractant gradients) or cell history (such as bud scars in yeast; Drubin and Nelson, 1996). The initial signals for the establishment of polarity are first detected at the plasma membrane and then transmitted via signaling pathways to various cellular targets. A major cellular target for these signaling events is the actin cytoskeleton, which undergoes rearrangements to bring about polarization (Hall and Nobes, 2000).

The budding yeast S. cerevisiae is an excellent model system for studies of dynamics of the actin cytoskeleton because yeast has a relatively simple actin cytoskeleton and offers powerful experimental tools. Throughout the yeast cell cycle, precisely choreographed changes in the organization of the actin cytoskeleton underlie spatial control of cell surface growth and thereby determine cell morphology. Extension of the cell surface is preceded by the polarized organization of two actin filament–containing structures: actin cables and cortical actin patches (Pruyne and Bretscher, 2000b). The small GTPase Cdc42p is a key member of the upstream signaling network for spatial organization of these actin structures. Cdc42p is active in its GTP-bound form, but is inactive in its GDP-bound form. Cdc42p-GTP interacts with various effector molecules to assemble actin filaments, to promote docking and fusion of secretory vesicles and to coordinate signaling events leading to bud formation (Pruyne and Bretscher, 2000a; Brennwald and Rossi, 2007; Park and Bi, 2007). In the initial stage of budding, Cdc24p and Bem1p play pivotal roles in the activation of Cdc42p as well as its recruitment to the bud site. Cdc24p is a GDP/GTP exchange factor that activates Cdc42p, and Bem1p acts as a scaffold at the bud site by interacting with Cdc24p and Cdc42p-GTP (Pruyne and Bretscher, 2000a; Irazoqui et al., 2004; Park and Bi, 2007).

Actin cables consist of parallel bundles of actin filaments stabilized by tropomyosins (Tpm1p and Tpm2p) and serve as tracks for type V myosin Myo2p-driven transport of secretory vesicles, vacuoles, Golgi membranes, proteins, and RNAs (Bretscher, 2003). The nucleation and assembly of actin cables require the action of the formins Bni1p and Bnr1p. Formins are a family of highly conserved eukaryotic proteins that are implicated in a wide range of actin-based processes. They contain two conserved juxtaposed formin homology (FH) domains, FH1 and FH2. The proline-rich FH1 domain binds to the actin monomer–binding protein profilin (Pfy1p), whereas the FH2 domain is sufficient for actin filament nucleation in vitro (Evangelista et al., 2003). The FH2 domain also binds to the filament’s barbed end, modulating its elongation and protecting it from capping.
proteins (Zigmond, 2004). Bni1p is part of a 12S complex termed the polarisome, which also includes Spa2p, Pia2p, and Bud6p (Sheu et al., 1998). Polarisome components are required for apical growth; in their absence, cells fail to confine the growth site to a small region during initial bud emergence and bud growth. Formation and reorganization of cortical actin patches are regulated by cortical patch-like protein structures, including the Arp2/3 complex and several of its activators, as well as endocytic adaptors and scaffolds (Pruyne and Bretschner, 2000b). The Arp2/3 complex nucleates new actin filaments from the side of existing filaments to form a branched actin filament network (Goley and Welch, 2006). Activators of Arp2/3 in yeast includes the Wiskott-Aldrich syndrome protein (WASP) homologue Las17p, type I myosins Myo3p and Myo5p, and an Eps15 homology (EH) protein Pan1p (Weaver et al., 2003). Cortical actin patches and their associated protein function in the internalization process of endocytosis (Engvist-Goldstein and Drubin, 2003); actin patches assemble at the plasma membrane as the endocytic vesicle forms, and many proteins are recruited to the actin patch, including endocytic adaptors Sla1p, Sla2p, End3p, and Pan1p (Kaksonen et al., 2003). However, roles of Arp2/3-mediated actin assembly in cell polarity remain obscure in budding yeast.

In this study, we show that yeast cells form a small bud in the absence of actin cables, indicating that they can polarize without actin cable–dependent vesicle transport. Additional mutations in genes for actin patch components inhibited this polarized growth. Our results suggest that the Arp2/3 system contributes to polarized growth by promoting endocytic membrane recycling.

MATERIALS AND METHODS

Strains and Plasmids

Yeast strains used in this study are listed in Table 1. Yeast strains carrying complete gene deletions (ura3-52, trp1-901, and his3-11,050) were constructed by introducing PCR-amplified alleles disrupted with KanMX4. The entire open reading frame of BEM1 was deleted from the linearized plasmid p406S2G (pRS406-SPA2-GFP; Arkowitz and Lowe, 2004). The entire open reading frame of NDD1 was deleted from the linearized plasmid p406S2G (pRS406-NDD1-GFP). Strains carrying pRS416-GFP-SNC1 was constructed by introducing PCR-amplified alleles disrupted with KanMX4 in BY4741 (Winzeler et al., 1999). All strains constructed by PCR-based procedures as described (Longtine et al., 1998; Goldstein and McCusker, 1999).

RESULTS

Loss of Actin Cables in Temperature-sensitive bni1 bnr1 Mutants Causes Characteristic Growth Arrest with a Small Bud

We previously constructed strains that harbored temperature-sensitive (ts) bni1 alleles in the bnr1Δ background by random mutagenesis (Kadota et al., 2004). All of these mutant strains showed a similar small-budded phenotype at the restrictive temperature (see below; our unpublished results). We selected one such mutation, bni1-116 and analyzed it further. The temperature-sensitive growth phenotype of the bni1-116 bnr1Δ mutant is shown in Figure 1A. Bni1-116p contained the amino acid substitutions (V1475A, K1498E, and D1511N) within the FH2 domain (Kadota et al., 2004). After shift to 35°C, actin cables disappeared from the bni1-116 bnr1Δ mutant cells within 5 min (Figure 1B). GFP-tagged Myo2p also disappeared from polarized growth sites, such as the bud tip and cytokinesis site in bni1-116 bnr1Δ mutant cells 5 min after temperature shift (data not shown). These results indicate that Bni1-116p is incapable of nucleating actin and assembling actin cables at the restrictive temperature.

Interestingly, most bni1-116 bnr1Δ cells were arrested with a small bud at the restrictive temperature (Figure 1C). After a 160-min incubation of an asynchronous culture at 37°C, the population of small-budded cells increased to 84% (Figure 1C). These results show that the bni1-116 bnr1Δ mutant is able to complete cytokinesis once past the small-bud stage, but polarized growth is impeded soon after small-bud formation. This indicates that actin cables are not required for cytokinesis or polarized bud growth once the cells have proceeded past the small-bud stage. We examined the time course of small-bud formation at 37°C after release from G1 arrest. Wild-type and bni1-116 bnr1Δ cells were treated with α-factor for 2 h at 25°C, and the G1-arrested cells were released at 37°C. After 40 min, 16% of the wild-type cells formed a small bud (0.23 ± 0.24 μm in length, n = 125), whereas the bni1-116 bnr1Δ cells did not (Figure 1D). After 60 min, 30% of the bni1-116 bnr1Δ cells formed a small bud;
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these buds were 0.35 ± 0.12 μm in length (n = 116), whereas the buds of the wild-type cells were 2.4 ± 0.57 μm in length (n = 121). After 160 min, 96% of the bni1-116 bnr1Δ cells were arrested with a small bud, as were those in asynchronous culture shifted to 37°C for 160 min. These results indicate that the small-budded phenotype is common to all three bni1-ts alleles in at least two genetic backgrounds.

Analysis of genetic differences between two genetic backgrounds might give us a clue about the mechanism of small-bud formation in the bni1-116 bnr1Δ mutant. Therefore, we included the tpm1-2 tpm2Δ (ABY944) strain that has a large un budded phenotype at 37°C (Pruyne et al., 1998) in this study. We first examined whether the tpm1-2 tpm2Δ mutant in our YEF73 genetic background would exhibit the small-budded phenotype. We backcrossed the ABY944 tpm1-2 tpm2Δ (ABY) strain to our wild-type haploid (YKT39) three times to obtain a tpm1-2 tpm2Δ (YEF) mutant. The morphology of the parental tpm1-2 tpm2Δ (ABY) mutant and the tpm1-2 tpm2Δ (YE F) mutant was examined after α-factor-arrest-and-release at 37°C (Figure 2B). After 80 min, 19% of the tpm1-2 tpm2Δ (YE F) cells formed a small bud, and after 160 min, 64% were arrested with a small or medium bud, although 31% exhibited unbudded morphology. Compared with the bni1-116 bnr1Δ mutant, the tpm1-2 tpm2Δ (YE F) mutant was arrested with a larger bud with a wider bud neck; the tpm2-2 allele may be somewhat leaky, or formin-polymerized actin filaments may retain some function without tropomyosins. In contrast, most (83%) of the parental ABY944 tpm1-2 tpm2Δ mutant cells exhibited unbudded morphology as described previously (Pruyne et al., 1998), and only a small percentage (17%) formed a small bud after a 160-min incubation. When tpm1-2 tpm2Δ haploid progeny from a diploid between tpm1-2 tpm2Δ (ABY) and our wild type (YKT39) were examined microscopically at 37°C, small-budded and unbudded phenotypes were observed with a
similar frequency (7:8) at a random spore basis. These results suggest that a single genetic trait is responsible for the small-budded versus unbudded phenotype.

We next examined whether this morphological difference between the two genetic backgrounds would also be observed for the bni1-116 bnr1Δ mutants. The tpm1-2 tpm2Δ (ABY) mutant was crossed with the bni1-116 bnr1Δ (YEF) mutant. The resulting diploid was tetrad-dissected, and 30 bni1-116 bnr1Δ progeny were morphologically examined. When cells that were exponentially growing at 25°C were shifted to 37°C for 3 h, 16 clones exhibited the small-budded phenotype (>85% were arrested with a small bud), whereas 14 clones exhibited the unbudded phenotype (>85% were arrested without a bud). The morphologies of representative clones are shown in Figure 2C. When these morphologically different clones were crossed, the resulting diploids showed the small-budded phenotype (Figure 2C, bottom panel). Taken together, these results imply that the ABY genetic background carries a single recessive mutation that is responsible for the defects in budding in the absence of actin cables. This gene seems to be involved in endocytosis (see below).

We wanted to exclude the possibility that the small-bud formation was due to leakiness of the bni1 and tpm1 ts alleles. The bni1-116 bnr1Δ mutations were combined with the tpm1-2 tpm2Δ or pfy1-116 mutations to construct a quadruple or triple mutant. Pfy1p stimulates formin-induced actin assembly in vitro, which requires its interactions with

Figure 1. The bni1-116 bnr1Δ mutant shows growth arrest with a small bud. (A) Temperature-sensitive growth in the bni1-116 bnr1Δ mutant. Strains were streaked onto YPDA plates, followed by incubation at 25°C for 3 d or at 35°C for 2 d. Strains were wild type (WT, YKT38), bni1Δ (YKT382), bnr1Δ (YKT390), and bni1-116 bnr1Δ (YKT503). (B) Filamentous actin structures in the bni1-116 bnr1Δ mutant. Strains were grown in YPDA medium at 18°C and then shifted to 35°C for 5 min. Cells were fixed and stained for filamentous actin with TRITC-phalloidin. Strains were wild type (YKT7) and bni1-116/bni1-116 bnr1Δ/bnr1Δ (YKT458). (C) Growth arrest with a small bud in bni1-116 bnr1Δ cells. Exponentially growing wild-type (YKT38) and bni1-116/bnr1Δ (YKT503) cells were shifted to 37°C and fixed with 3.7% formaldehyde at the indicated time point. The graph shows the percentage of cells with the bud size that was categorized as described in Materials and Methods. The right panel displays images of cells after a 160-min incubation. (D) Time course of small-bud formation after release from G1 arrest. Wild-type (YKT38) and bni1-116 bnr1Δ (YKT503) cells were arrested with α-factor as described in Materials and Methods and released into fresh medium at 37°C, followed by fixation with 3.7% formaldehyde at the indicated time point. The graph shows the percentage of cells with the bud size as in C. Right, images of cells after incubation for the indicated time periods. Bars, 5 μm.
the FH1 domain and actin monomers (Sagot et al., 2002b; Kovar et al., 2003; Frings et al., 2003). Exponentially growing cells were treated with α-factor at 25°C, and the G1-arrested cells were released at 37°C for 160 min. As shown in Figure 3, 89% of the tpm1-2 tpm2Δ bni1-116 bnr1Δ cells formed a small bud. Similarly, 86% of the pfy1-116 bni1-116 bnr1Δ cells formed a small bud. The pfy1-116 single mutant was not exclusively arrested with a small bud (47% small budded), because this mutant grew slowly at 37°C (data not shown). These results suggest that yeast cells possess a system to promote polarized growth independent of actin cables.

**Polarized Localization of Cdc42p and Polarity Regulators Is Normal in bni1-116 bnr1Δ Mutant Cells**

It was reported that the tpm1-2 tpm2Δ mutant exhibited defects in polarized localization of Cdc42p (Pruyne et al., 2004; Irazoqui et al., 2005; Zajac et al., 2005); all of these studies used the tpm1-2 tpm2Δ mutant in the ABY genetic background. However, our results suggest that Cdc42p might be localized normally to the bud tip in an actin cable-independent manner. This would also be consistent with the fact that Cdc42p can be polarized in the presence of an actin inhibitor latrunculin-A (Ayscough et al., 1997). To examine this further, exponentially growing bni1-116 bnr1Δ cells were transferred to 37°C for 160 min and stained with affinity-purified polyclonal antibodies against Cdc42p (Kozminski et al., 2000). As expected, Cdc42p was localized at the bud tip in 87% of small-budded bni1-116 bnr1Δ cells (Figure 4A); under our staining conditions, Cdc42p was detected in 93% of small-budded wild-type cells. We examined the initial polarization of Cdc42p in cells released from G1-arrest (Figure 4B). Forty minutes after release, 28% of wild-type cells polarized Cdc42p at the presumptive bud site, but only 2% of bni1-116 bnr1Δ cells showed Cdc42p polarization (data not shown). Sixty and 160 min after release, 30 and 76% of bni1-116 bnr1Δ cells polarized Cdc42p, respectively. This time course for Cdc42p polarization, which is delayed compared with the wild type, is consistently similar to that for budding in bni1-116 bnr1Δ cells (Figure 4D). To examine whether the delay in Cdc42p polarization in the bni1-116 bnr1Δ cells was caused by the delayed cell cycle progression, we monitored GFP-fused Spc42p, a component of the spindle pole body (SPB; Adams and Kilmartin, 1999). When G1-arrested cells were released at 37°C for 60 min, the
...and the resulting triple or quadruple mutants were examined. (A) Cdc42p polarization in an asynchronous culture of bni1-116 bnr1Δ cells. Exponentially growing wild-type (YKT38) and bni1-116 bnr1Δ (YKT503) cells were shifted to 37°C, and incubated for 160 min, followed by fluorescence immunostaining using the anti-Cdc42p antibody. In wild type, small-budded cells were selected and shown. (B) Time course of polarization of GFP-tagged polarity regulators in the bni1-116 bnr1Δ mutant. Strains examined were Spc42p-GFP–expressing wild type (YKT1584), and Exo70p-GFP–expressing wild type (YKT1570) and bni1-116 bnr1Δ (YKT1574). Bars, 5 μm.

**Figure 4.** Cdc42p and polarity regulators are polarized to the bud emergence site and the bud tip in the bni1-116 bnr1Δ mutant. (A) Cdc42p polarization in an asynchronous culture of bni1-116 bnr1Δ cells. Exponentially growing wild-type (YKT38) and bni1-116 bnr1Δ (YKT503) cells were shifted to 37°C, and incubated for 160 min, followed by fluorescence immunostaining using the anti-Cdc42p antibody. In wild type, small-budded cells were selected and shown. (B) Initial polarization of Cdc42p in an actin cable–deficient mutant. α-factor–arrested cells were released into fresh medium at 37°C, except for the tpm1-2 tpm2Δ (ABY) mutant (YKT1684), followed by fixation with 3.7% formaldehyde at the indicated time point. YKT1684 was released at 35°C because Spc42p was not duplicated at 37°C (our unpublished results). Cdc42p was visualized as described above. Cell cycle progression was examined by monitoring duplication of Spc42p-GFP. The graph shows the percentage of cells with Spc42p-GFP duplication (Spc42p) and polarized Cdc42p at the bud emergence site or the bud tip (Cdc42p). Bottom, images of representative cells with polarized or nonpolarized Cdc42p 60 min after release. Arrowheads, Cdc42p localized to cortical sites other than the budding site in the bni1-116 bnr1Δ mutant. Strains examined were Spc42p-GFP–expressing wild type (YKT1514), bni1-116 bnr1Δ (YKT1550), tpm1-2 tpm2Δ (YEF)(YKT1552), and tpm1-2 tpm2Δ (ABY) (YKT1684). (C) Polarized localization of polarity regulators in the bni1-116 bnr1Δ mutant. Strains were cultured and fixed, and polarization of each GFP-tagged polarity regulator was scored as described in B. Bottom, images of representative cells that polarized a GFP-fused protein after a 60-min incubation. The strains examined were Bem1p-GFP–expressing wild type (YKT1438) and bni1-116 bnr1Δ (YKT1562), Spa2p2p–expressing wild type (YKT570) and bni1-116 bnr1Δ (YKT1570), and Exo70p-GFP–expressing wild type (YKT1570) and bni1-116 bnr1Δ (YKT1574). Bars, 5 μm.

Spc42p-GFP dot was duplicated in 27 and 12% of wild-type and bni1-116 bnr1Δ cells, respectively (Figure 4B), indicating that loss of actin cable assembly causes a delay in cell cycle progression. In our strain background, the tpm1-2 tpm2Δ mutant polarized Cdc42p and duplicated Spc42p-GFP in a manner similar to that in the bni1-116 bnr1Δ mutant; 60 and 160 min after release, 42 and 82% of tpm1-2 tpm2Δ cells, respectively, polarized Cdc42p. In contrast, in the ABY genetic background, Cdc42p was poorly polarized even after 160 min as reported previously (Pruyne et al., 2004; Irazoqui et al., 2005; Zajac et al., 2005). We noticed that the bni1-116 bnr1Δ mutant, but not the tpm1-2 tpm2Δ or bnr1Δ mutant (data not shown), accumulated Cdc42p to some cortical regions in addition to the bud tip (Figure 4B, arrowheads).

To confirm that bud formation in the bni1-116 bnr1Δ mutant occurs through a normal polarization process, we monitored the localization of other polarity regulators, Bem1p, Spa2p, and Exo70p. Exo70p is a component of the exocyst complex that is essential for exocytosis (TerBush et al., 1996). Most components of the exocyst associate with the exocytic vesicles and are delivered to the bud tip in an actin cable–dependent manner, whereas a portion of Exo70p, like Sec3p, is transported to the bud tip in the absence of actin cables (Boyd et al., 2004). When G1-arrested bni1-116 bnr1Δ cells were released at 37°C, these polarity regulators were polarized in time courses similar to that of Cdc42p (Figure 4C), whereas Bem1p in the tpm1-2 tpm2Δ mutant with the ABY genetic background was poorly polarized (data not shown). Taken together, these results strongly suggest that the processes of initial cell polarization can occur independently of transport along actin cables.

**Small-Bud Formation in the bni1-116 bnr1Δ Mutant Requires Arp2/3-dependent Actin Polymerization**

F-actin assembly seems to be essential for bud formation, because treatment with latrunculin-A prevents bud formation (Ayscough et al., 1997; our unpublished results). Arp2p is a subunit of the Arp2/3 complex, which is the major known contributor to actin nucleation in vivo in yeast (Winter et al., 1999). Myo3p/5p, type I myosins, are one of activators for the Arp2/3 complex (Evangelista et al., 2000; Lechler et al., 2000). We next examined the involvement of Arp2/3-mediated actin assembly in small-bud formation in the bni1-116 bnr1Δ mutant. The temperature-sensitive arp2-2 or myo3Δ myo5-1 mutations were combined with the bni1-116 bnr1Δ mutation, and the resulting triple or quadruple mutants were examined for small-bud formation after release from G1 arrest with α-factor. We noticed that the arp2-2 single mutant could not bud at all when released at 37°C, and this was due to the inhibition of cell cycle progression, as assessed by Spc42p-GFP duplication (data not shown). When released at 36°C, however, 45% of the arp2-2 mutant cells duplicated Spc42p-GFP after a 160-min incubation, albeit with a lower efficiency than that observed in the bni1-116 bnr1Δ mutant cells (88%). Under these conditions (36°C, 160 min), 37% of the arp2-2 cells formed a small or medium bud, whereas 97% of the bni1-116 bnr1Δ cells formed only a small bud (data not shown). In contrast, only 4% of the arp2-2 bni1-116 bnr1Δ cells formed a small bud (Figure 5A), even though Spc42p-GFP duplication was seen in 59% of these cells. For myo3Δ myo5-1, α-factor–arrested cells were released at 37°C, because this mutant initiated budding at 37°C. The cells that started budding progressed into the medium-budded stage; 17 and 25% of the cells formed a small and medium buds, respectively, after a 160-min incubation. Under these conditions (37°C, 160 min), Spc42p-GFP duplication was observed in 46% of the cells. However, only a minor fraction (6%) of...
the myo3Δ myo5-1 bni1-116 bnr1Δ mutant cells formed a small bud, although 54% duplicated Spc42p-GFP. These results suggest that Arp2/3p-dependent actin polymerization is required for the bud formation in the absence of actin cables.

In addition to the Arp2/3 complex and Myo3/5p, numerous proteins are involved in endocytosis as adaptors, scaffolds, and regulators of actin assembly (Pruyne and Bretscher, 2000b). We next examined whether these proteins are also involved in small-bud formation in the bni1-116 bnr1Δ mutant. Sla2p, a homologue of mammalian HIP1 (Huntingtin-interacting protein 1), regulates rearrangement of actin patch assembly (Engqvist-Goldstein and Drubin, 2003). End3p and Sla1p are members of the Pan1p complex, which links cargo proteins to clathrin-coated pits and sites of actin assembly in ubiquitin- and NPFXD-dependent endocytosis (Howard et al., 2002; Miliaras et al., 2004; Kaksonen et al., 2006). We wanted to use an allele of SLA2 that is defective in endocytosis but is not defective for growth, to eliminate possible secondary effects caused by a more severe allele; the sla2Δ mutant exhibited a severe temperature-sensitive growth defect and did not duplicate Spc42p-GFP even at 35°C after α-factor arrest-and-release (our unpublished results). The sla2-82 mutant, which produces a mutant Sla2p protein lacking the C-terminal half (amino acids 491–968), shows a defect in endocytosis, but grows normally at 37°C (Yoshiuchi et al., 2002; Miliaras et al., 2004; Kaksonen et al., 2006). The sla2Δ mutant in the BY4743 background as well as in the YEF473 background. Data shown are from cells in the BY4743 background.

**Figure 5.** Small-bud formation in the bni1-116 bnr1Δ mutant requires Arp2/3-dependent actin assembly. (A) The arp2-2 and myo3Δ myo5-1 mutations inhibit small-bud formation in the bni1-116 bnr1Δ mutant. α-factor-arrested cells were released into fresh medium at 36°C (arp2-2) or 37°C (myo3Δ myo5-1), followed by fixation at the indicated time point. The graph shows the percentage of cells with Spc42p-GFP duplication (Spc42p) and the bud size as categorized in Materials and Methods. Bottom, cells after an 80-min (arp2-2 and myo3Δ myo5-1) or a 160-min (arp2-2 bni1-116 bnr1Δ and myo3Δ myo5-1 bni1-116 bnr1Δ) incubation after release. Strains examined were Spc42p-GFP–expressing arp2-2 (YKT1553), arp2-2 bni1-116 bnr1Δ (YKT1554), myo3Δ myo5-1 (YKT1555), and myo3Δ myo5-1 bni1-116 bnr1Δ (YKT1556). (B) The sla2-82 mutation inhibits small-bud formation in the bni1-116 bnr1Δ mutant. Spc42p-GFP–expressing sla2-82 (YKT1557) and sla2-82 bni1-116 bnr1Δ (YKT1558) cells were arrested with α-factor, released into fresh medium at 37°C, fixed at the indicated time point, and analyzed as in A. Bottom, images of cells after an 80-min (sla2-82) or a 160-min (sla2-82 bni1-116 bnr1Δ) incubation after release. (C) The NPFXD-mediated endocytosis adaptors End3p and Sla1p are not required for small-bud formation in the bni1-116 bnr1Δ mutant. α-factor–arrested end3Δ bni1-116 bnr1Δ (YKT1600) and sla1Δ bni1-116 bnr1Δ (YKT1601) cells were released into fresh medium at 37°C, followed by a 160-min incubation. Numbers indicate the percentage of small-budded cells. It was reported that the sla1Δ mutant exhibited temperature-sensitive growth (Holtzman et al., 1993). The sla1Δ mutant in the BY4743 background did not grow at 37°C, but the sla1Δ mutant in the YEF473 background grew at 37°C (data not shown). We confirmed efficient small-bud formation in the sla1Δ bni1-116 mutant.
defects in GFP-Snc1p endocytosis. GFP-Snc1p localization was examined in the budding-deficient tpm1-2 tpm2Δ (ABY) mutant. Wild-type (YKT38), bni1-116 bnr1Δ (YKT305), tpm1-2 tpm2Δ (YEF) (YKT476), and tpm1-2 tpm2Δ (ABY) (YKT286) strains were transformed with pRS416-GFP-SNC1, and exponentially growing transformed cells were shifted to 37°C, followed by a 1-h incubation. (B) The budding defect in bni1-116 bnr1Δ mutants is associated with defects in GFP-Snc1p endocytosis. GFP-Snc1p localization was examined in bni1-116 bnr1Δ progeny shown in Figure 2C as described in A. Bars, 5 μm.

Figure 6. The tpm1-2 tpm2Δ mutant in the ABY944 background is defective in endocytosis of GFP-Snc1p. (A) Endocytosis of GFP-Snc1p is inhibited in the budding-deficient tpm1-2 tpm2Δ (ABY) mutant. Wild-type (YKT38), bni1-116 bnr1Δ (YKT305), tpm1-2 tpm2Δ (YEF) (YKT476), and tpm1-2 tpm2Δ (ABY) (YKT286) strains were transformed with pRS416-GFP-SNC1, and exponentially growing transformed cells were shifted to 37°C, followed by a 1-h incubation. (B) The budding defect in bni1-116 bnr1Δ mutants is associated with defects in GFP-Snc1p endocytosis. GFP-Snc1p localization was examined in bni1-116 bnr1Δ progeny shown in Figure 2C as described in A. Bars, 5 μm.

Culture; more than 80% of the arp2-2, myo3Δ myo5-1, and sla2-82 cells with bni1-116 bnr1Δ were arrested without a bud after a 3-h incubation at nonpermissive temperatures (our unpublished results). Taken together, these results suggest that the assembly of cortical actin patches can promote polarized bud growth by endocytosis in a manner independent of NPFXD-mediated endocytosis.

These results prompted us to examine endocytosis in the tpm1-2 tpm2Δ (ABY) mutant. Snc1p, an exocytic v-SNARE, cycles from the plasma membrane through early endosomes to the TGN along the endocytic recycling pathway (Lewis et al., 2000). GFP-fused Snc1p is normally localized to polarized sites, such as a bud or a cytokinesis site, where exocytosis is actively occurring (Figure 6A, wild type). When shifted to 37°C for 1 h, GFP-Snc1p accumulated intracellularly in the bni1-116 bnr1Δ (YEF) mutant as well as in the tpm1-2 tpm2Δ (YEF) mutant (Figure 6A). Interestingly, GFP-Snc1p was seen as one or two large dots rather than uniform cytoplasmic staining. These structures may be endosomal compartments, because GFP-Snc1p was localized to similar large structures when coexpressed with the RFP-tagged Gcs1p (Robinson et al., 2006), which is an ADP ribosylation factor (Arf)-GTPase–activating protein involved in the endocytic recycling pathway (Robinson et al., 2006; Sakane et al., 2006). In these compartments, endocytic recycling seems to be normal, because GFP-Snc1p was also localized to the plasma membrane (Robinson et al., 2006); we confirmed that small-bud formation also occurred in GFP-Snc1p–expressing bni1-116 bnr1Δ and tpm1-2 tpm2Δ mutants (our unpublished results). This dot localization of Snc1p may be caused by the overexpression of GFP-tagged Snc1p, because neither clustered nor large membrane structures, but rather secretory vesicles were observed in EM-sectioning of bni1-116 bnr1Δ cells (data not shown). Nonetheless, there may be a mechanism that affects the morphology or localization of GFP-Snc1p–containing endosomal membranes when exocytotic vesicle transport is inhibited, because a large dot of GFP-Snc1p was not seen in wild-type cells (Figure 6A).

Surprisingly, in the tpm1-2 tpm2Δ (ABY) mutant, GFP-Snc1p was uniformly localized to the plasma membrane and to some punctate structures beneath the plasma membrane that were reminiscent of early endosomal structures. These results suggest that the tpm1-2 tpm2Δ (ABY) mutant is somewhat deficient in endocytosis. Linkage of the unbudded phenotype to defects in endocytosis was confirmed in the bni1-116 bnr1Δ progeny described in Figure 2C from the diploid of bni1-116 bnr1Δ (YEF) and tpm1-2 tpm2Δ (ABY); see Figure 6B. We further observed the GFP-Snc1p localization in an additional four small-budded and four unbudded bni1-116 bnr1Δ progeny and obtained the same results (our unpublished results). These results are consistent with the notion that endocytosis is involved in small-bud formation in the absence of actin cables. Interestingly, defective endocytosis was not observed for the α-factor transporter Ste6p-GFP, which is normally endocytosed and transported to the vacuole (Kelm et al., 2004); Ste6p-GFP was localized to the vacuole in the tpm1-2 tpm2Δ (ABY) mutant as well as in the wild type at 37°C (data not shown). One interesting possibility is that the mutation in the ABY genetic background might affect endocytosis specific to the endocytosis-recycling pathway, which is required for small-bud formation in the absence of actin cables (see below).

One interesting possibility for the budding defects in the arp2-2 bni1-116 bnr1Δ mutant cells would be that Cdc42p and other polarity regulators were not polarized in these cells. We examined the localization of polarity regulators in end (arp2-2, myo3Δ myo5-1, or sla2-82) bni1-116 bnr1Δ mutants released from G1 arrest at the nonpermissive temperature. After 60- and 80-min incubations, Cdc42p was localized at the presumptive bud site or at the bud tip in arp2-2, myo3Δ myo5-1, and sla2-82 mutants, and the Cdc42p polarization to the presumptive bud site was also observed in end mutants carrying bni1-116 bnr1Δ mutations (Figure 7). To confirm that these Cdc42p polarizations occurred through normal budding processes, we also monitored polarization of Bem1p-GFP, Spa2p-GFP, and Exo70p-GFP. Similar to Cdc42p, these polarity regulators were localized to the presumptive bud site. These results indicate that the initial cell polarization occurs normally in a mutant defective in both Arp2/3- and formin-mediated actin assemblies.

Small-Bud Formation in the bni1-116 bnr1Δ Mutant Requires the Endocytic Recycling Pathway

Endocytosis is important for recycling secreted proteins and lipids to reuse them and to redirect growth throughout the cell cycle (Pruyne and Bretscher, 2000b); endocytic recycling
is one plausible mechanism that might underlie small-bud formation in the bni1-116 bnr1Δ mutant. We therefore examined the involvement of proteins that regulate the endocytic recycling pathway in small-bud formation in bni1-116 bnr1Δ cells (Figure 8A). Tlg2p, an endosomal/trans-Golgi network (TGN) t-SNARE, is required for efficient fusion of early endosome-derived vesicles to the TGN (Lewis et al., 2000). Vps54p is a component of the Golgi-associated retrograde protein (GARP) complex and is responsible for tethering vesicles derived from endosomes to the TGN (Siniossoglou and Pelham, 2001; Conibear et al., 2003). Both Tlg2p and Vps54p are involved in the endocytic recycling pathway. In contrast, Pep8p (Vps26p), a component of the retromer, functions in the late endosome-to-TGN retrieval pathway, but not in the early endosome-to-TGN retrieval pathway (Lewis et al., 2000; Reddy and Seaman, 2001). Tlg2Δ and pep8Δ mutants grew normally at 37°C, whereas the vps54Δ mutant showed a weak growth defect at 37°C in our strain background (data not shown). When released for 60 min at 37°C from G1 arrest, these mutants budded normally and progressed through the cell cycle in a normal manner except that the vps54Δ mutant exhibited a slight delay. In contrast, neither tlg2Δ bni1-116 bnr1Δ nor vps54Δ bni1-116 bnr1Δ cells formed a bud even 160 min after release, whereas pep8Δ bni1-116 bnr1Δ cells formed a small bud in a manner similar to that of bni1-116 bnr1Δ (Figure 8A). The time course for Sec4p-GFP duplication in each triple mutant was similar to that in the bni1-116 bnr1Δ mutant except for a slight delay in the vps54Δ bni1-116 bnr1Δ mutant. These results indicate that the early endosome-to-TGN, but not the late endosome-to-TGN, retrograde transport pathway is required for small-bud formation in the absence of actin cable assembly.

We next examined the localization of polarity-regulating factors in the tlg2Δ bni1-116 bnr1Δ mutant. G1-arrested cells were released at 37°C, and localization of Cdc42p, Bem1p-GFP, Spa2p-GFP, and Exo70p-GFP was examined. As shown in Figure 8B, these polarity regulators were localized at the presumptive bud site with efficiency comparable to that in the bni1-116 bnr1Δ mutant (see Figure 4). Thus, although actin cables and endocytic recycling have redundant functions for polarized bud growth, they do not seem to play important roles for the initial assembly of polarity regulators.

Myc2p Is Required for Small-Bud Formation in the bni1-116 bnr1Δ Mutant

The endocytic recycling pathway for small-bud formation might use an unknown specialized pathway distinct from the conventional post-Golgi secretory pathway. We examined the effect of a late secretory pathway mutation, sec4-2 (a mutation in a Rab family GTPase associated with post-Golgi secretory vesicles, Salminen and Novick, 1987), on bud formation. The sec4-2 mutant in the YEF473 genetic background was constructed by repeated backcrosses. G1-arrested cells were released at 33°C, because sec4-2 cells did not duplicate the Spc42p-GFP dot at 35°C (data not shown). When released, most sec4-2 cells (99%) did not form a bud even after 160 min (Figure 9A), indicating that Sec4p is required for budding irrespective of actin cables. Taken together with the results that small-bud formation requires Tlg2p, the endosomal/TGN t-SNARE (Figure 8A), these results suggest that, in the bni1-116 bnr1Δ mutant, endocytic vesicles for small-bud formation pass through the TGN, followed by formation of Sec4p-associated vesicles for delivery to the

Figure 7. Cdc42p and polarity regulators are polarized in the bni1-116 bnr1Δ mutant that is defective for Arp2/3-mediated actin polymerization. α-factor-arrested cells were released into fresh medium at 37°C, followed by fixation at the indicated time point. The graph shows the percentage of cells with polarization of Cdc42p or polarity regulators. Cdc42p and polarity regulators were visualized by immunofluorescence staining and GFP-tagging, respectively. Right, images of representative cells that polarized Cdc42p or GFP-fused polarity regulators after a 60-min incubation after release. Strains used were tlg2Δ (YKT476), tlg2Δ bni1-116 bnr1Δ (YKT1546), myo3Δ myo5-1 (YKT91), myo3Δ myo5-1 bni1-116 bnr1Δ (YKT1547), sla2-82 (YKT850), and sla2-82 bni1-116 bnr1Δ (YKT1548), and GFP-tagged polarity regulator (Bem1p, Spa2p, and Exo70p)-expressing versions of these strains. Bar, 2 μm.
bud. We also examined the localization of Bem1p-GFP in the sec4-2 mutant. Bem1p-GFP was polarized to the presumptive bud site in 45% of cells 60 min after release, suggesting that the late secretory pathway is not essential for the initial polarization of Bem1p. However, 160 min after release, Bem1p-GFP disappeared from the polarized sites in most cells (92%), suggesting that continuous vesicle flow is required for the maintenance of polarized Bem1p. Sustained polarization of Bem1p-GFP 160 min after release in bni1-116 bnr1Δ cells (Figure 4C) seems to be supported by polarized transport of vesicles containing a membrane-bound polarity regulator (e.g., Cdc42p).

Even though bud formation in the bni1-116 bnr1Δ mutant was inefficient, it may still be dependent on directional transport of vesicles. We examined the role of Myo2p for bud formation. The myo2-66 allele encodes a protein with a single amino acid substitution (E511K) in an actin-binding motor domain (Lillie and Brown, 1994), whereas myo2-12 and myo2-20 alleles encode proteins with amino acid substitutions in a cargo-binding tail domain (Schott et al., 1999). These myo2 mutants in the YEF473 genetic background were constructed by repeated backcrosses. G1-arrested myo2-66 cells were released at 32°C, because the myo2-66 cells did not duplicate the Spc42p-GFP dot at 33°C (data not shown), whereas the myo2-12 and myo2-20 cells were released at 36.5 and 37°C, respectively. When released, most myo2-66 (94%), myo2-12 (90%), and myo2-20 (88%) cells did not form a bud even after 160 min (Figure 9B), indicating that Myo2p is also required for budding irrespective of actin cables. These results suggest that, in the bni1-116 bnr1Δ mutant, Myo2p transports vesicles along actin filaments that are formin-independent or that it has functions that are not dependent on actin filaments. Bem1p-GFP was also polarized to the presumptive bud site in myo2 mutant cells (Figure 9B), suggesting that Myo2p-mediated transport is not essential for polarized localization of Bem1p. However, polarized local-

Figure 8. Endocytic recycling is required for small-bud formation in the bni1-116 bnr1Δ mutant. (A) A mutation in a gene that is involved in the endocytic recycling pathway inhibits small-bud formation in the bni1-116 bnr1Δ mutant. α-factor-arrested cells were released into fresh medium at 37°C, followed by fixation at the indicated time point. The graph shows the percentage of cells with Spc42p-GFP duplication (Sp42p) and the bud size categorized as in Materials and Methods. Bottom, images of cells after a 160-min incubation after release. Strains examined were Spc42p-GFP–expressing tlg2Δ (YKT1539), tlg2Δ bni1-116 bnr1Δ (YKT1560), vps54Δ (YKT1602), vps54Δ bni1-116 bnr1Δ (YKT1603), pep8Δ (YKT1605), and pep8Δ bni1-116 bnr1Δ (YKT1606). Bar, 5 μm. (B) Cdc42p and polarity regulators are polarized to the presumptive bud site in the tlg2Δ bni1-116 bnr1Δ mutant. α-factor-arrested cells were released into fresh medium at 37°C, followed by fixation at the indicated time point. The graph shows the percentage of cells showing polarization of polarity regulators. Bottom, images of representative polarized cells in the tlg2Δ bni1-116 bnr1Δ mutant after a 60-min incubation after release. Cdc42p was visualized by fluorescence immunostaining and other polarity regulators were visualized by GFP-tagging. tlg2Δ and tlg2Δ bni1-116 bnr1Δ mutants used were YKT955 and YKT1549 for Cdc42p, YKT1572 and YKT1573 for Bem1p-GFP, YKT1592 and YKT1593 for Spa2p-GFP, and YKT1582 and YKT1583 for Exo70p-GFP, respectively. Bar, 2 μm.
Myo2p are required for budding but not for Bem1p-GFP polarization. α-factor–arrested cells were released into fresh medium at 33°C, followed by fixation at the indicated time point. Left, an image of cells after a 160-min incubation after release, and the number indicates the percentage of cells having small-budded cells. The graph shows the percentage of cells with Spc42p-GFP duplication (Spc42p) and polarized Bem1p-GFP (Bem1p). Right, representative cells with polarized Bem1p-GFP after a 60-min incubation. Strains examined were sec4-2 mutants expressing Spc42p-GFP (YKT1608) or Bem1p-GFP (YKT1611). Bars, 5 and 2 μm for left and right panels, respectively. (B) Requirement of Myo2p for budding but not for Bem1p-GFP polarization. α-factor–arrested cells were released into fresh medium at 32°C (myo2-66), 36.5°C (myo2-12), or 37°C (myo2-20), followed by fixation at the indicated time point. The results are presented as described in A. Strains examined were myo2-66, myo2-12, and myo2-20 mutants expressing Spc42p-GFP (YKT1610, YKT1679, and YKT1677, respectively).

Figure 9. The late secretory pathway and the type V myosin Myo2p are required for bud formation. (A) Requirement of Sec4p for budding but not for Bem1p-GFP polarization. α-factor–arrested cells were released into fresh medium at 33°C, followed by fixation at the indicated time point. Left, an image of cells after a 160-min incubation after release, and the number indicates the percentage of small-budded cells. The graph shows the percentage of cells with Spc42p-GFP duplication (Spc42p) and polarized Bem1p-GFP (Bem1p). Right, representative cells with polarized Bem1p-GFP after a 60-min incubation. Strains examined were sec4-2 mutants expressing Spc42p-GFP (YKT1608) or Bem1p-GFP (YKT1611). Bars, 5 and 2 μm for left and right panels, respectively. (B) Requirement of Myo2p for budding but not for Bem1p-GFP polarization. α-factor–arrested cells were released into fresh medium at 32°C (myo2-66), 36.5°C (myo2-12), or 37°C (myo2-20), followed by fixation at the indicated time point. The results are presented as described in A. Strains examined were myo2-66, myo2-12, and myo2-20 mutants expressing Spc42p-GFP (YKT1610, YKT1679, and YKT1677, respectively).

Myo2p are required for budding but not for Bem1p-GFP polarization. Myo2p-GFP signals could be seen at the presumptive bud site or the bud tip in 18% of bni1-116 bnr1Δ cells. However, Myo2p-GFP did not polarize in bni1-116 bnr1Δ mutants carrying mutations in the endocytosis-recycling pathway, including myo3a, myo5-1, sla2-82, and tlg2Δ (Figure 9C), and this was the case even 160 min after release (our unpublished results). These results may suggest that the endocytosis-recycling pathway is required for localization or maintenance of a factor that is involved in polarized localization of Myo2p. One intriguing possibility is that this factor is involved in the assembly of actin structures for Myo2p-driven vesicle transport.

DISCUSSION

Initial Polarized Bud Growth in the Absence of Actin Cables

In this study, we have shown herein that the budding yeast can form a small bud even in the absence of actin cables, which are assembled by formins and are stabilized by tropomyosins. Our results parallels the observation made in the fission yeast Schizosaccharomyces pombe; cells lacking the formin for3p still grow quite well without actin cables (Feierbach and Chang, 2001). Therefore, it seems that both budding and fission yeast possess a conserved mechanism to promote polarized growth in an actin cable-independent manner and that actin cables function as a facilitator rather than an essential factor for polarized growth. This actin cable-independent bud formation may represent a fundamental mechanism for initial polarization in eukaryotic cells.

Our finding of small-bud formation in the bni1-116 bnr1Δ mutant was unexpected, because the previously reported bni1-11 bnr1Δ and tpm1-2 tpm2Δ mutants exhibited defects in budding (Pruyne et al., 1998; Evangelista et al., 2002). We have shown that these discrepancies were possibly due to a single recessive mutation in the ABY genetic background used in the above studies. This mutation, which rendered our bni1-116 bnr1Δ mutant defective for small-bud formation, partially inhibited endocytosis of GFP-Snc1p, consis-

or Bem1p-GFP (YKT1612, YKT1680, and YKT1681, respectively). (C) Polarized localization of Myo2p-GFP in the bni1-116 bnr1Δ mutant requires the endocytosis-recycling pathway and a regulator of the Arp2/3p-dependent actin polymerization. α-factor–arrested strains were released into fresh medium at 37°C, followed by fixation after a 60-min incubation after release. Myo2p-GFP–expressing strains were bni1-116 bnr1Δ (YKT1791), myo3A myo5-1 bni1-116 bnr1Δ (YKT1614), sla2-82 bni1-116 bnr1Δ (YKT1615), and tlg2Δ bni1-116 bnr1Δ (YKT1617). Numbers indicate the percentage of cells having polarized Myo2p-GFP. Bar, 2 μm.
tent with the notion that the endocytosis-recycling pathway is required for small-bud formation in the absence of actin cables.

The Endocytosis-recycling Pathway as an Engine to Promote Polarized Membrane Growth

We have shown that the Arp2/3 complex and its activators, whose major role seems to be to promote endocytosis, are required for small-bud formation in the absence of actin cables. Small-bud formation also required factors (Tlg2p and Vps54p) that are involved in early endosome-to-TGN vesicle transport. These results strongly suggest that the budding yeast possesses a second mechanism for polarized growth: budding by endocytosis and recycling of vesicles. Endocytic recycling plays a pivotal role in cell morphogenesis in various cell types (Lecuit and Pilot, 2003; Georgiaou et al., 2008; Kleine-Vehn and Friml, 2008; Lee et al., 2008; Taberi-Talesh et al., 2008; Higuchi et al., 2009). Our results suggest that a mechanism for cell polarization by endocytic recycling is conserved throughout evolution. Thus, yeast cells would provide a useful model system for dissection of the molecular mechanisms underlying the endocytosis-recycling pathway for cell polarization.

Specificity of the endocytic recycling pathway for small-bud formation was suggested by our observations that small-bud formation required neither Slap1p nor End3p, components of the Slap1p/Pan1p/End3p endocytic complex (Tang et al., 1997; Tang et al., 2000), which are involved in ubiquitin- and NPFXY-dependent endocytosis (Howard et al., 2002; Miliaras et al., 2004). A mutation in the ABY genetic background inhibited endocytosis of GFP-Snc1p, but not that of Ste6p-GFP, which is known to be endocytosed in a ubiquitin-dependent manner (Kölling and Losko, 1997; Kelm et al., 2004). Identification of this gene would be an interesting next step to analyze the molecular mechanisms of endocytic recycling-promoted polarized growth.

Endocytic recycling and actin cable-dependent vesicle transport has been implicated in polarization of various membrane proteins such as the v-SNARE Snc1p (Valdez-Taubas and Pelham, 2003), the chitin synthase III Chs3p (Chuang and Schekman, 1996; Holthuis et al., 1998; Valdivia et al., 2002), the cell wall stress sensor Wsc1p (Piao et al., 2007), the a-factor receptor Ste3p (Chen and Davis, 2000), and the phospholipid translocase Dnf1p (Saito et al., 2004). We have first demonstrated that the endocytic recycling pathway is also involved in membrane remodeling (polarized growth) in budding yeast. Two plausible functions could be envisioned. One is to maintain a membrane protein required for polarized growth at the polarized site. These membrane proteins may include those involved in exocytosis or actin reorganization. Interestingly, a mutation in the ABY genetic background partially inhibited endocytosis of the v-SNARE Snc1p, but not that of Ste6p. Efficient recycling of Snc1p may be required for small-bud formation when actin cable-dependent vesicle transport is compromised. The other function may be to supply new membranes to the bud. In this case, endocytosis from the mother plasma membrane, but not from the bud membrane, would be required for efficient polarized growth. However, we have shown that the post-Golgi Sec4p-dependent exocytotic pathway is required for small-bud formation. These results suggest that newly synthesized membranes can also contribute to small-bud formation by merging at the TGN with endocytic membranes. Nonetheless, we should not rule out the possibility that a specific TGN compartment is generated from endocytic membranes and that this TGN compartment is used for small-bud formation in the absence of actin cable-dependent vesicle transport.

Roles of endocytic recycling for small-bud formation and polarized localization of membrane proteins suggest that early endosomes could be localized in a polarized manner. Yeast early endosomes are observed as scattered dotty structures, but we cannot discriminate between early endosomes for vacuolar sorting and those for endocytic recycling (Lewis et al., 2000). It was previously reported that the early endosome-to-TGN transport was specifically blocked in cdc50 mutants (Wiederkehr et al., 2000; Furuta et al., 2007). These mutants accumulated enlarged early endosomal membranes, probably due to defects in vesicle budding from early endosomes. Interestingly, these enlarged endosomal membranes were localized in the bud or near the bud neck (Wiederkehr et al., 2000; Furuta et al., 2007), suggesting a mechanism that localizes endosomal membranes in a polarized manner. It is an interesting question whether this polarized localization of endosomal membranes is independent of actin cables.

Roles of the Actin Cytoskeleton in the Polarized Membrane Growth in the Absence of Actin Cables

One important question is how exocytotic vesicles are transported in a polarized manner in formin and tropomyosin mutants. The myo2 mutants exhibiting a budding defect and Myo2p-GFP was localized to the bud tip in the bni1-116 bnr1A mutant, suggesting that Myo2p transports vesicles toward the bud tip along some type of actin structure in the absence of actin cables. On the other hand, it was shown that a small fraction of Myo2p could be localized to the bud site possibly by passive diffusion in the presence of an actin inhibitor latrunculin-A (Ayscough et al., 1997), and we confirmed their results for Myo2p-GFP in our strain background (data not shown). Therefore, further work is needed to know whether Myo2p is transported along actin structures in the absence of actin cables. However, efficient polarized growth in the for3 mutant does not seem to be accounted for by passive diffusion. Interestingly, a Sla2p homologue is required for polarized cell growth in Schizosaccharomyces pombe (Castagnetti et al., 2005; Ge et al., 2005). In addition, involvement of a WASP homologue and type I myosins in polarized growth has also been shown in the pathogenic fungus Candida albicans; mutants in these genes exhibit defects in hyphal growth (Oberholzer et al., 2002; Walther and Wendland, 2004). It is an intriguing possibility that these potential Arp2/3 regulators including those in budding yeast might regulate actin reorganization for polarized vesicle transport.

Recently, it was reported that budding yeast cells formed a small bud in the absence of actin assembly (Sahin et al., 2008). In this report, quiescent cells from a 7-d-old culture were released into fresh medium containing 200 μM actin inhibitor latrunculin-A. In our α-factor arrest-and-release assay, 100 μM latrunculin-A completely inhibited budding as also reported for cells in stationary phase (data not shown; Ayscough et al., 1997; Bi et al., 1998). Seven-day-old G0 cells might be physiologically very different from cells in the growth cycle, as suggested by the authors, but it would be difficult to imagine that a cell achieves efficient budding with only a cortical polarity scaffold and passive diffusion of secretory vesicles. It seems that there should be an alternative route to actin guidance in the budding from 7-d-old cells.
Roles of the Actin Cytoskeleton for Initial Cell Polarization

Assembly of polarity regulators in the absence of the actin cytoskeleton was first reported by Ayscough et al. (1997). They showed that Cdc42p and Bem1p were localized to the incipient bud site with wild-type kinetics when G0-arrested cells were released in the presence of latrunculin-A. Our current results are consistent with their results. Cdc42p and polarity regulators were polarized normally in the absence of components for two major actin structures in yeast, actin cables and cortical actin patches (e.g., in the arp2-2 bni1-116 bnr1 Δ mutant). Therefore, it seems that the scaffolding activities of upstream regulators including Cdc24p and Bem1p are enough for the initial assembly of Cdc42p. A mutant deficient only in actin cables (e.g., the tpm1-2 tpm2 Δ mutant) also polarized Cdc42p and even formed a small bud. In contrast, the tpm1-2 tpm2 Δ mutant in the ABY genetic background, Cdc42p was initially polarized but rapidly dispersed (Irazoqui et al., 2005); we confirmed that the tpm1-2 tpm2 Δ (ABY) mutant failed to polarize Cdc42p after α-factor arrest-and-release (Figure 4B). Irazoqui et al. (2005) proposed that this dispersal was caused by endocytosis of Cdc42p and demonstrated that the Cdc42p dispersal was suppressed by a blockade of endocytosis. How endocytosis of Cdc42p is involved in Cdc42p polarization is an interesting question. Endocytosis can antagonize Cdc42p polarization by promoting dispersal of Cdc42p from a polarized site, but endocytosis and subsequent recycling of Cdc42p to the polarized site can promote Cdc42p polarization as hypothesized by Marco et al. (2007). Therefore, the endocytosis-recycling pathway could be a third pathway for Cdc42p polarization in addition to the scaffold-mediated assembly pathway and the actin cable–dependent vesicle transport pathway (Wedlich-Soldner et al., 2004). We showed that the tpm1-2 tpm2 Δ (ABY) mutant was partially defective in endocytosis of GFP-Snc1p. If the endocytosis-recycling pathway plays some role in the maintenance of Cdc42p at the polarized site in the actin cableless mutant, partial defects in endocytosis would enhance dispersal of Cdc42p by slowing down the redelivery of Cdc42p to the plasma membrane. In contrast, strong inhibition of endocytosis would leave Cdc42p localized at the polarized site as demonstrated by Irazoqui et al. (2005) and this study.

In conclusion, we think that the role of the actin cytoskeleton for the initial polarization of Cdc42p remains obscure. However, in a mutant in which the scaffold-mediated assembly pathway is compromised (e.g., the bni1 mutant), treatment with latrunculin-A inhibits polarization of Cdc42p (Wedlich-Soldner et al., 2004; our unpublished results), indicating that actin-dependent processes play a compensating role for the initial polarization of Cdc42p. Whether this compensation is brought about by actin cable (formins) and/or cortical actin patch (Arp2/3)-dependent processes remains a fascinating question.

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