The polarity-establishment component Bem1p interacts with the exocyst complex through the Sec15p subunit

Y. Ellen France[1], Charles Boyd[2], Jeff Coleman[2] and Peter J. Novick[2,*]

[1]Department of Molecular, Cellular and Developmental Biology, Yale University, New Haven, Connecticut 06520, USA
[2]Department of Cell Biology, Yale University School of Medicine, New Haven, Connecticut 06520, USA
*Author for correspondence (e-mail: peter.novick@yale.edu)

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Summary

Spatial regulation of the secretory machinery is essential for the formation of a new bud in *Saccharomyces cerevisiae*. Yet, the mechanisms underlying cross-talk between the secretory and the cell-polarity-establishment machineries have not been fully elucidated. Here, we report that Sec15p, a subunit of the exocyst complex, might provide one line of communication. Not only is Sec15p an effector of the rab protein Sec4p, the master regulator of post-Golgi trafficking, but it also interacts with components of the polarity-establishment machinery. We have demonstrated a direct physical interaction between Sec15p and Bem1p, a protein involved in the Cdc42p-mediated polarity-establishment pathway, confirming a prior two-hybrid study. When this interaction is compromised, as in the case of cells lacking the N-terminal 138 residues of Bem1p, including the first Src-homology 3 (SH3) domain, the localization of green fluorescent protein (GFP)-tagged Sec15 is affected, especially in the early stage of bud growth. In addition, Sec15-1p, which is defective in Bem1p binding, mislocalizes along with Sec8p, another exocyst subunit. Overall, our evidence suggests that the interaction of Sec15p with Bem1p is important for Sec15p localization at the early stage of bud growth and, through this interaction, Sec15p might play a crucial role in integrating the signals between Sec4p and the components of the early-polarity-establishment machinery. This, in turn, helps to coordinate the secretory pathway and polarized bud growth.

Key words: Membrane traffic, Sec15p, Bem1p, Exocyst, Cell polarity

Introduction

The spatial and temporal regulation of the secretory apparatus is essential for the establishment of a new bud in *Saccharomyces cerevisiae*. At the core of post-Golgi vesicle trafficking is Sec4p, one of the founding members of the small rab GTPase family. As master regulators, rab proteins are thought to interact with multiple effectors to coordinate downstream events and to impart specificity to membrane trafficking at the levels of vesicle budding, transport, tethering and fusion to the target membrane. One effector of Sec4p is the exocyst – an octameric complex composed of Sec3p, Sec5p, Sec6p, Sec8p, Sec10p, Sec15p, Exo70p and Exo84p – that has been implicated in tethering secretory vesicles to the plasma membrane. Sec15p has been shown to interact genetically as well as physically with activated Sec4p, making it a documented effector of Sec4p (Guo et al., 1999b; Salminen and Novick, 1989). All components of the exocyst complex localize to sites of active exocytosis (Guo et al., 1999a; Guo et al., 1999b; TerBush et al., 1996). However, six of the eight subunits are brought to exocytic sites on secretory vesicles, whereas the remaining two, Sec3p and Exo70p, can be recruited to these sites on the plasma membrane independently of ongoing membrane traffic (Boyd et al., 2004; Finger et al., 1998). Analysis of the interactions between the subunits within the exocyst has revealed that these proteins form a highly ordered assembly, which may be crucial for its function as a tethering factor (Guo et al., 1999). The different subunits are thought to have distinct functions in the regulation of vesicle attachment to the target membrane, presumably through their specific interactions with additional factors. Supporting this hypothesis, many interactors of the exocyst complex have emerged over the recent years, both in yeast and mammalian cells, indicating the dynamic and versatile nature of this complex in various cellular contexts (Riefler et al., 2003; Rogers et al., 2004; Sans et al., 2003; Shin et al., 2000; Sugihara et al., 2002; Toikkanen et al., 2003; Vega and Hsu, 2003; Vik-Mo et al., 2003; Wang et al., 2004; Zhang et al., 2001).

The establishment of cell polarity is a complex process in which cortical landmarks lead to the local activation of Rho-family GTPases that then direct the polarization of the actin cytoskeleton as well as the spatial regulation of the secretory apparatus. Cdc42p, a member of the Rho GTPase family, is a key regulator of cell polarity establishment. The recruitment of Cdc42p to the cell surface by Bem1p and its local activation by the exchange factor Cdc24p in response to positional cues and cell-cycle signals are crucial events in establishing cell polarity (Butty et al., 2002; Casamayor and Snyder, 2002). Evidence of functional links between polarity-establishment components and the secretory apparatus has been emerging in

recent years. In yeast, Sec3p has been shown to interact with both Rho1p and Cdc42p, whereas Exo70p interacts with Rho3p (Guo et al., 2001; Robinson et al., 1999; Zhang et al., 2001). In addition, once polarity has been initially established, the secretory pathway functions to reinforce and stabilize the original polarity cues by bringing additional molecules of Cdc42p to sites of polarization (Wedlich-Soldner et al., 2004).

A recent large-scale two-hybrid interaction study (Drees et al., 2001) has revealed novel interactions between the polarity-establishment machinery and the secretory apparatus, supporting the hypothesis that these two processes are indeed tightly linked. We have pursued the reported identification of three core components of polarity establishment, namely Bem1p, Rsr1p and Cdc24p, as putative Sec15p-interacting proteins. Here, we identify Bem1p – a protein that is crucial in the recruitment and maintenance of many of the key components involved in establishment of early polarity – as a bona fide, direct binding partner of Sec15p. Bem1p is required for maintaining the localization of Cdc42p, a key regulator of polarity establishment; Cdc24, the exchange factor for Cdc42p; and several other proteins involved in generating polarity (Butty et al., 2002; Gulli et al., 2000; Irazoqui et al., 2003; Peterson et al., 1994; Wedlich-Soldner et al., 2004). In this study, we demonstrate that Sec15p interacts with Bem1p both genetically and physically. We propose that the Sec15p-Bem1p interaction provides an additional link between the polarity-establishment machinery and the secretory apparatus.

Results

Prompted by the yeast two-hybrid results, we first explored genetic interactions between *SEC15* and *BEM1*. The *bem1-3* mutant is considered to be a null allele with a nonsense mutation resulting in premature termination after only 70 amino acids. Although *BEM1* is not essential, *bem1-3* mutant cells grow slowly at 25°C or 30°C and are severely impaired above 34°C (Chenevert et al., 1992). We found that a high copy number *SEC15* plasmid suppressed the temperature sensitivity of a *bem1-3* mutant at both 34°C and 37°C (Fig. 1). When similar amounts of yeast were plated at restrictive temperatures, single *bem1-3* colonies carrying the *SEC15* plasmid were larger and were also more numerous than those lacking the plasmid at both permissive and restrictive temperatures. We also introduced a high copy *SEC4* plasmid to determine whether we might observe a similar effect because Sec4p is an upstream activator of Sec15p. Indeed, we also observed suppression of *bem1-3* by the *SEC4* plasmid. We observed no suppression of the temperature sensitivity of *sec15-1* by a high copy number *BEM1* plasmid (data not shown). Upon PCR-based genomic sequencing of the *sec15-1* allele, we determined that a point mutation of a single base pair (G to T) at position 2506 resulted in premature termination of the protein and loss of the last 76 amino acids at the C-terminus, which is consistent with the observation that the mutant protein is shifted to a higher mobility by SDS-PAGE analysis (Salminen and Novick, 1989).

To evaluate the physical interaction between Sec15p and Bem1p, we performed glutathione *S*-transferase (GST) pull-down assays starting with yeast lysates (Fig. 2). Owing to the low abundance of Sec15p in the cell, Sec15p-GST was overproduced under the control of the strong *GAL1* promoter in wild-type yeast and purified using glutathione-Sepharose affinity chromatography. To identify the region involved in Bem1p interaction, we generated several versions of Sec15p truncated from the N-terminal region and also took advantage of the mutant protein encoded by *sec15-1*, which is truncated at the C-terminus (Fig. 2A). The sizes of all the purified Sec15p constructs, as determined by SDS-PAGE and either Coomassie Brilliant Blue staining or western blot analysis, were close to those predicted by their sequence. Bem1p was found to co-precipitate with full-length Sec15p-GST from yeast lysates, whereas Sec15-1p, lacking the last 76 amino acids, failed to precipitate Bem1p, suggesting that the residues 834-910 of Sec15p are important for the interaction with Bem1p (Fig. 2B). By contrast, the C-terminal truncation of 76 residues did not severely reduce the interaction of Sec15-1p with Sec10p, the nearest neighbor of Sec15p within the exocyst complex. On the basis of western blot analysis, we determined that the pull-down efficiency of Sec15-GST was approximately 10% of the total Sec15-GST expressed in yeast, and that 1% of Bem1p was co-precipitated with Sec15-GST. Next, we performed the pull-down protocol using a limited C-terminal region (residues 740-910) and were still able to observe Bem1p binding (Fig. 2C). However, the interaction of Sec15p with Sec10p was abrogated when as little as the first 82 residues were removed, despite the fact that there was a similar amount of Sec10p present in the lysates, suggesting that this interaction requires the region encompassing residues 1-82.

On the basis of the GST pull-down experiment results, we conclude that the extreme N-terminal region of Sec15p is required for the interaction with Sec10p that links Sec15p to the rest of the exocyst complex, whereas the region encompassing the C-terminal 170 residues is necessary and sufficient for interaction with Bem1p (Fig. 2D). This observation is consistent with previous results from our laboratory indicating that Sec15-1p could be co-immunoprecipitated with the rest of the exocyst using an epitope tag on Sec8p (TerBush et al., 1996). Since different regions of Sec15p are required for the Bem1p and Sec10p interactions, these results suggest that Bem1p might be able to interact with the assembled exocyst complex through Sec15p. To test this hypothesis, we conducted binding experiments in which we mixed bacterial, recombinant Bem1p, tagged with GST and immobilized to glutathione-Sepharose beads, with exocyst complex, purified from yeast lysates using a modification of published TAP purification

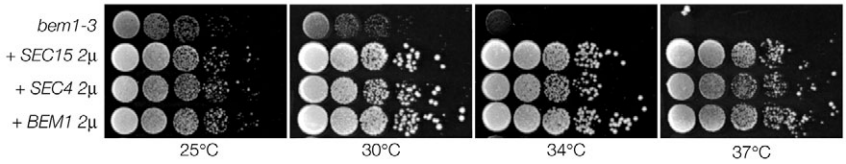

Fig. 1. The *bem1-3* growth defect is suppressed by both *SEC15* and *SEC4* multi-copy plasmids. *S. cerevisiae bem1-3* cells (NY1444) carrying 2 μ *SEC15*, 2 μ *SEC4* or 2 μ *BEM1* plasmids (pNB192, pNB142 and pNB1227, respectively) were spotted onto YPD plates along with *bem1-3* cells carrying no plasmid. The plates were incubated at 25°C, 30°C, 34°C and 37°C for 3 days and scanned.

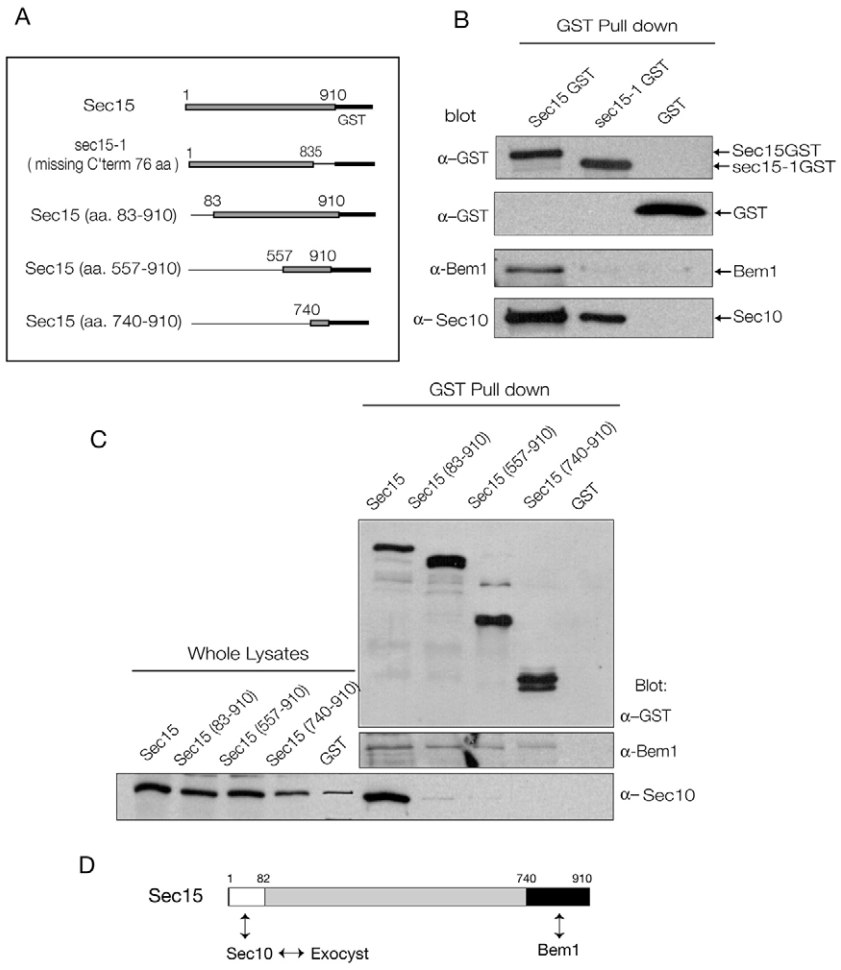

Fig. 2. The C-terminus of Sec15p physically interacts with Bem1p, whereas the N-terminus of Sec15p is required for the interaction with Sec10p. Cell growth, yeast lysate preparations, GST pull-down and western blotting procedures were as described in the Materials and Methods. (A) Schematic diagram of Sec15p constructs employed in this study. (B) Sec15-Bem1 complexes. Glutathione-Sepharose pull-down products from lysates of cells expressing GST-fusion products of Sec15 (Sec15 GST; NY2559) and Sec15-1 (sec15-1 GST; NY2560), and GST (NY2561), were analyzed by standard SDS-PAGE and western blotting with the indicated antibodies. (C) Glutathione-Sepharose pull-down products from lysates of cells expressing GST-fusion products of the Sec15p constructs described in A were analyzed by standard SDS-PAGE and western blotting with the indicated antibodies. The purified Sec15-GST constructs were resolved on an SDS-PAGE gel and the gel was analyzed by western blotting using anti-GST antibodies. Lysates were loaded to indicate that there were similar amounts of Sec10p present in all lysates although only the full-length Sec15p containing the extreme N-terminal region can pull-down Sec10p. (D) Diagram of the protein-protein interactions of Sec15.

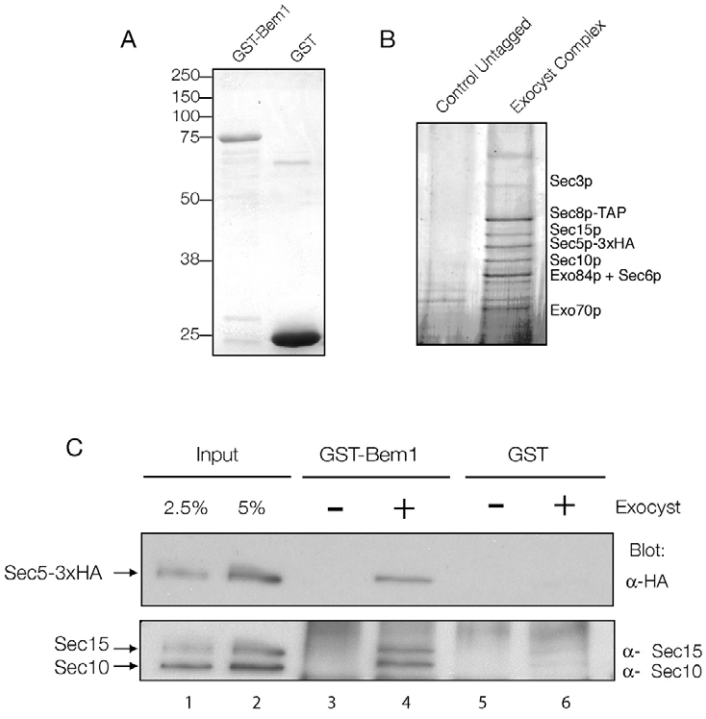

Fig. 3. Bem1p can interact with the exocyst complex through Sec15p. (A) Purified recombinant GST-Bem1p (GST-Bem1) and GST were resolved by SDS-PAGE and the gel was stained with Coomassie Brilliant Blue. Proteins of the predicted molecular masses were observed. (B) Exocyst complex purified from yeast strain NY2521 was resolved by SDS-PAGE and the gel was silver stained to visualize the subunits. (C) GST-Bem1p (GST-Bem1) or GST immobilized on glutathione-Sepharose beads was incubated with the purified exocyst complex as described in the Materials and Methods. Lanes 1 and 2 represent 2.5% and 5%, respectively, of the exocyst complex used in the binding reactions. The beads were washed four times with the binding buffer and the bound proteins were eluted with SDS-PAGE sample buffer. The eluates were then resolved by SDS-PAGE, and western blot analysis was performed using anti-HA (to detect Sec5-3xHA), anti-Sec15 and Sec10 antibodies.

protocols (Puig et al., 2001; Rigaut et al., 1999) (see Materials and Methods) (Fig. 3). The eight polypeptide bands comprising the exocyst complex were confirmed by silver staining, as well as by western blot analysis using available antibodies raised against some of the subunits (Fig. 3B). Indeed, we confirmed by immunoblotting using antibodies raised against several different subunits of the exocyst that GST-Bem1p could pull-down the assembled exocyst complex (Fig. 3C). Sec5p, a subunit that does not directly interact with Sec15p, was detected in our pull-down analysis, indicating that the exocyst complex that has been added to binding mixtures remained intact throughout the protocol. In addition, all three subunits that we analyzed were precipitated with similar efficiency, supporting our conclusion that Bem1p can interact with the fully assembled exocyst complex through its interaction with Sec15p.

Both Sec15p and Bem1p are known to be present in large complexes and to have multiple interacting partners (Bender et al., 1996; Bose et al., 2001; Guo et al., 1999b; Lyons et al., 1996; Peterson et al., 1994; TerBush et al., 1996; TerBush and Novick, 1995). To determine if the Bem1p-Sec15p interaction we observed by the GST pull-down assay is direct or is mediated by intermediary factors, we performed in vitro binding assays with recombinant Bem1p and the C-terminal fragment of Sec15p, which are both expressed in bacteria (Fig. 4A). It was found that 6-histidine (6xHis)-tagged, full-length Bem1p bound efficiently to the GST-Sec15p C-terminal fragment immobilized on glutathione-Sepharose beads, but not to the GST control (Fig. 4B). Thus, the Bem1p-Sec15p interaction occurs in the absence of other yeast proteins, indicating that the interaction is direct.

In an attempt to define further the domain of Bem1p that is involved in interacting with Sec15p, we generated recombinant bacterial Bem1 proteins that lacked either the first 128 residues [which includes the first Src-homology 3 (SH3) domain] or residues 1-220 (including both the first and the second SH3 domain). The second SH3 domain has been implicated in binding several other proteins (Fig. 4A) (Bender et al., 1996; Bose et al., 2001; Leeuw et al., 1995; Peterson et al., 1994). Whereas the full-length Bem1p efficiently bound to the C-terminus of Sec15p, the N-terminally truncated Bem1 proteins did not exhibit detectable binding, suggesting that the N-terminal region of Bem1p is important for interaction with Sec15p (Fig. 4C). Next, in order to determine whether the fragment missing in the N-terminally deleted Bem1p constructs is not only necessary, but also sufficient, for binding, a small fragment consisting of only the first 138 residues was cloned and expressed for the binding experiment (Fig. 4D). Indeed, the Sec15 C-terminus immobilized to the glutathione beads pulled down the Bem1p N-terminal fragment, whereas GST immobilized to beads alone did not. This result indicates that the N-terminal 138 residues, which include the first SH3 domain, are necessary and sufficient to mediate Sec15p interaction.

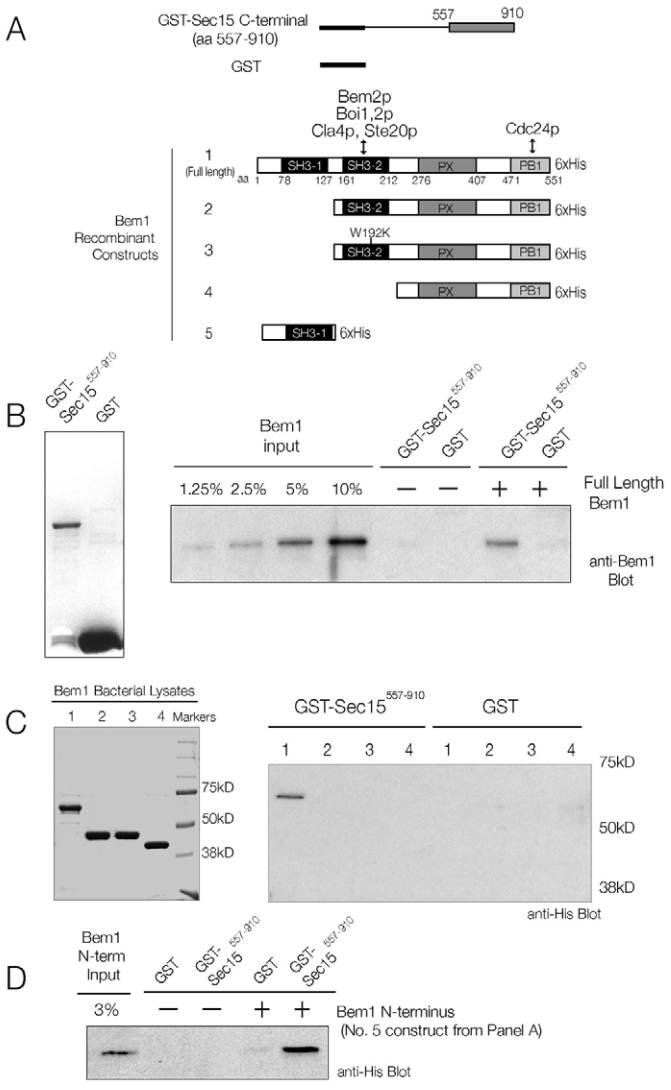

Fig. 4. The C-terminus of Sec15p directly interacts with Bem1p, and the N-terminus of Bem1p is necessary and sufficient for Sec15p interaction. (A) Schematic diagram of recombinant proteins used in this study. Several proteins that have been reported to interact with specific domains of Bem1p are indicated. SH3, Src-homology 3; PX, Phox homology domain; PB1, Phox and Bem1 domain; 6xHis, 6-histidine tag. (B) The Sec15p C-terminal region can directly interact with Bem1p. The C-terminus of Sec15 (residues 557-910) was fused to GST (GST-Sec15$^{557\text{-}910}$), purified and immobilized on the resin. Bem1p was fused to a 6xHis tag and purified from baterial lysates. These proteins were used in an in vitro binding assay as described in the Materials and Methods. The panel on the left shows purified recombinant GST-Sec15$^{557\text{-}910}$ and GST constructs resolved by SDS-PAGE and stained with Coomassie Brilliant Blue. Proteins of appropriate molecular masses were observed. The C-terminal fragment of Sec15 immobilized to glutathione-Sepharose beads was incubated with bacterially purified Bem1p. The beads were then washed and the bound proteins were resolved by SDS-PAGE and detected by western blot analysis using anti-Bem1p serum. (C) The interaction with Sec15p requires the N-terminal region of Bem1p. The panel on the left shows N-terminally truncated alleles of Bem1p expressed in bacteria. For binding studies, crude bacterial lysates containing these truncated constructs were incubated with GST-Sec15$^{557\text{-}910}$ immobilized on glutathione beads. The beads were washed and the bound products were resolved by SDS-PAGE and detected by western blot analysis using anti-His monoclonal antibody. (D) The N-terminal 138 residues are necessary and sufficient for mediating the Sec15p interaction. The crude bacterial lysates containing the minimal 138 amino acids at the N-terminus of Bem1 were incubated with GST-Sec15$^{557\text{-}910}$ on glutathione beads and the binding assay was performed in similar fashion to panel C.

The Bem1p-Sec15p interaction involves the N-terminal region of Bem1p, containing an SH3 domain, and the C-terminal region of Sec15p, containing a proline-rich region between residues 773 and 800. Since the proline-rich region is a potential binding site for the first SH3 domain of Bem1p, we mutated the proline residues to alanine residues within the region of Sec15p. However, yeast cells expressing this allele as the sole copy of *SEC15* did not display any obvious defects in secretion or polarity, nor was there any effect on Bem1p binding in vitro (data not shown). It is therefore likely that the interaction between Sec15p and Bem1p involves regions other than the putative proline-rich patches at the C-terminus of Sec15p and might also require sequences outside the first SH3 domain of Bem1p.

The localization of many key components of the polarity-establishment apparatus, such as Cdc42p and Bem1p, is known to be at least partially independent of polarized actin cables, whereas the localization of proteins that rely exclusively on vesicular traffic for their transport is entirely actin dependent (Ayscough et al., 1997). We examined the localization of green fluorescent protein (GFP)-tagged Sec15 (Sec15-GFP) to determine whether the localization mode of Sec15p differs from that of other subunits of the exocyst such as Sec8p, which was previously reported to be actin dependent. We observed that Sec15p was able to maintain a polarized localization under conditions in which actin was disrupted, either by a mutation in the actin structural gene *ACT1* (*act1-3*) or by treatment with the actin-disrupting agent latrunculin A (Lat-A) (Fig. 5A,B). In both cases, the disrupted state of polarized actin was confirmed by staining with TRITC-conjugated phalloidin.

To determine if Sec15-GFP could establish new sites of polarized localization independent of actin function, we observed cells as they were recovering from G0 arrest in the presence or absence of Lat-A. We were thereby able to determine whether polarized actin cables were required for the initial targeting of Sec15p to an incipient budding site. Although the cells were unable to grow new buds owing to the presence of Lat-A, in many cells Sec15-GFP was found to localize to a distinct patch by 2 hours (Fig. 5C). The positive control cells, treated with DMSO alone, fully recovered from G0 arrest and began to grow a new bud by 2 hours. We also performed the same experiment on cells expressing Sec8-GFP (Fig. 5D). Consistent with a previous report (Ayscough et al., 1997), Sec8-GFP clearly showed sensitivity to actin perturbation in polarity establishment. By quantification of these observations (Fig. 5E), we conclude that a pool of Sec15-GFP could establish and maintain a polarized localization independent of actin cables. This clearly distinguishes the mode of Sec15p localization from that of Sec8p.

To evaluate the physiological significance of the interaction of Sec15p with Bem1p, we generated a strain where *BEM1* was replaced by a plasmid expressing a mutant *bem1*$^{SH3\text{-}1\Delta}$ whose N-terminal 138 residues including the first SH3 domain were deleted. The plasmid containing *bem1*$^{SH3\text{-}1\Delta}$ was able to compliment the growth defects of *bem1Δ* at all temperatures we tested and its growth rate was comparable with that of the wild-type strain. However, upon close examination by measuring and analyzing the axial ratio, we observed that *bem1*$^{SH3\text{-}1\Delta}$ has a mild polarity defect – a significant percentage of cells were round when compared with the wild-type strain (Fig. 6A). Because the N-terminal 138 residues of Bem1p are necessary and sufficient to interact with Sec15p, we determined whether Sec15-GFP could still polarize in an actin-independent manner when the interaction with Bem1p was specifically disrupted. The *bem1*$^{SH3\text{-}1\Delta}$ cells were first grown to early log phase at 25°C, then treated either with 200 μM Lat-A or the control solvent DMSO, and the localization of Sec15-GFP was examined (Fig. 6B). Interestingly, the *bem1*$^{SH3\text{-}1\Delta}$ cells showed a partial defect in the maintenance of polarized Sec15-GFP localization even with the mock treatment, and the loss of localization was most apparent in unbudded cells and small budded cells, whereas the localization at the bud neck was the least affected (Fig. 6C). Next, we examined the localization of Sec15-GFP in G0-released *bem1*$^{SH3\text{-}1\Delta}$ cells with Lat-A treatment to determine whether the loss of the N-terminal 138 residues of Bem1p also disrupts the initial establishment of Sec15-GFP localization during bud emergence (Fig. 6D). After isolating a G0 population from the stationary culture, we released the cells in the presence or absence of Lat-A at 25°C for 2 hours. Consistent with our observation of an asynchronous population, *bem1*$^{SH3\text{-}1\Delta}$ cells already exhibited a significant defect in establishing Sec15-GFP localization at the presumptive bud site with mock treatment, which was exacerbated with Lat-A treatment (Fig. 6D,E).

In a recent study, we showed that Sec15p normally arrives at exocytic sites by vesicular transport, which is an actin-dependent process (Boyd et al., 2004). To understand the mechanism behind the actin-independent and Bem1-dependent Sec15p localization observed here, we conducted fluorescence recovery after photobleaching (FRAP) experiments to determine the dynamics of Sec15-GFP at bud sites in the presence or absence of actin cables (Fig. 7). We added Lat-A dissolved in DMSO to an exponentially growing culture of a strain expressing Sec15-GFP as the sole copy of *SEC15*. Cultures were incubated with 200 μM Lat-A at room temperature for 30 minutes, then diluted with an equal volume of melted low-melting-point (LMP) agarose at 37°C before being mounted on a slide for photobleaching and observation. The final Lat-A concentration during photobleaching was thus 100 μM. Cells treated with DMSO alone exhibited a photobleaching recovery tau of 22±5 seconds (n=15 cells). When treated with Lat-A, the same strain exhibited a recovery tau more than four times longer: 90±16 seconds (n=13 cells) (Fig. 7A,B). This indicates that Sec15-GFP actively localizes in the absence of actin cables; however, the mechanism used in this situation is much slower than that used when the actin cable network is intact. The recovery in the presence of Lat-A is approximately twice as efficient as when Lat-A is absent (32% compared with 15%) (Fig. 7C). This probably reflects the smaller fraction of Sec15-GFP in the bud tips prior to bleaching of Lat-A-treated cells.

We further tested the role of the Sec15p-Bem1p interaction by examining the subcellular localization of a GFP-tagged *sec15-1* allele defective in Bem1p binding. In wild-type cells, all exocyst subunits localize to exocytic sites: at the emerging bud site and the tips of small buds early in the cell cycle; and at the neck separating mother/daughter cells late in the cycle. Wild-type Sec15-GFP localization was examined as a positive control, and as expected it was found to localize to sites of active exocytosis at both 25°C and 37°C (Fig. 8A, top two panels). However, the localization of Sec15-1–GFP was

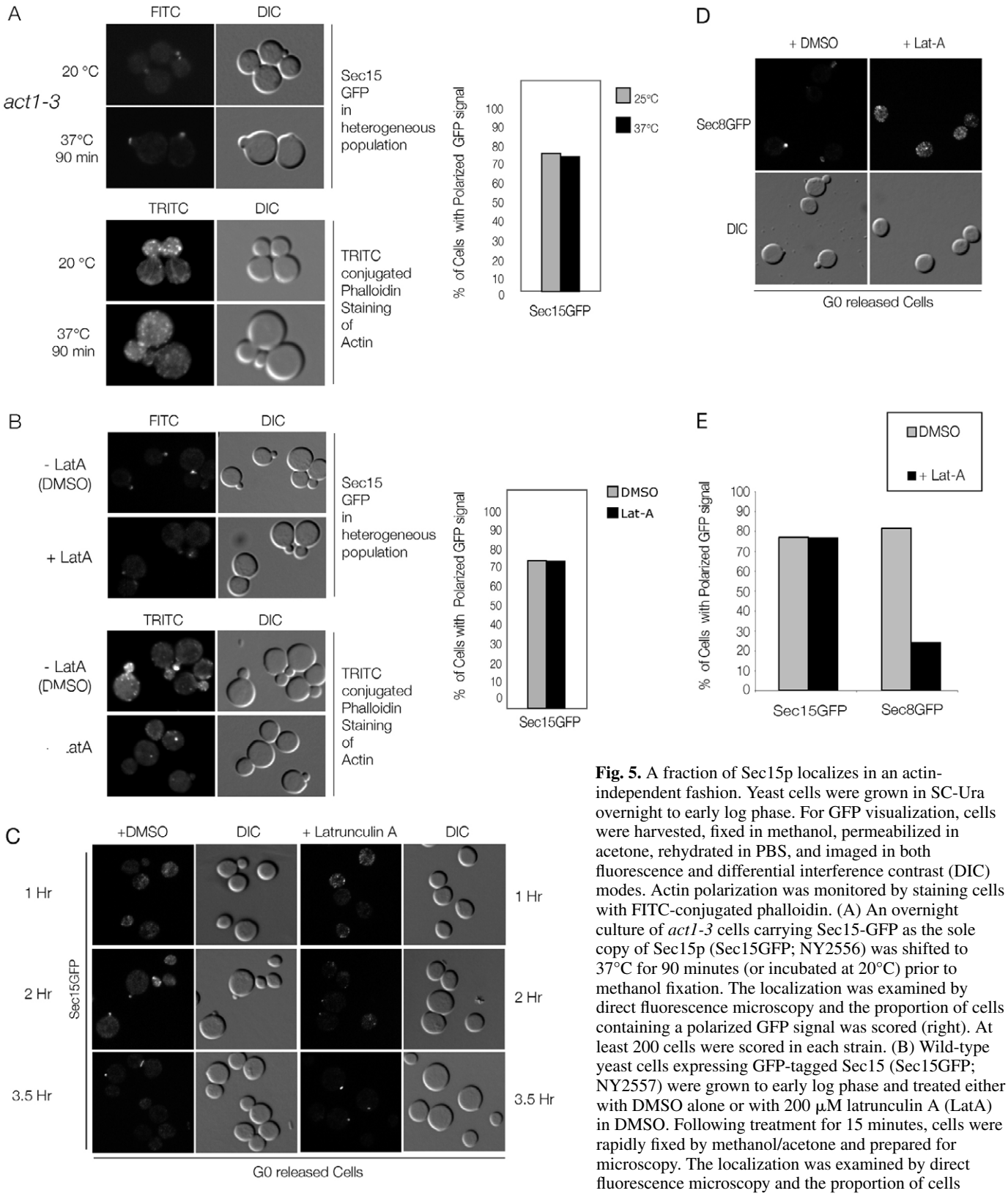

Fig. 5. A fraction of Sec15p localizes in an actin-independent fashion. Yeast cells were grown in SC-Ura overnight to early log phase. For GFP visualization, cells were harvested, fixed in methanol, permeabilized in acetone, rehydrated in PBS, and imaged in both fluorescence and differential interference contrast (DIC) modes. Actin polarization was monitored by staining cells with FITC-conjugated phalloidin. (A) An overnight culture of *act1-3* cells carrying Sec15-GFP as the sole copy of Sec15p (Sec15GFP; NY2556) was shifted to 37°C for 90 minutes (or incubated at 20°C) prior to methanol fixation. The localization was examined by direct fluorescence microscopy and the proportion of cells containing a polarized GFP signal was scored (right). At least 200 cells were scored in each strain. (B) Wild-type yeast cells expressing GFP-tagged Sec15 (Sec15GFP; NY2557) were grown to early log phase and treated either with DMSO alone or with 200 μM latrunculin A (LatA) in DMSO. Following treatment for 15 minutes, cells were rapidly fixed by methanol/acetone and prepared for microscopy. The localization was examined by direct fluorescence microscopy and the proportion of cells containing a polarized GFP signal was scored (right). At least 200 cells were scored in each strain. (C) The localization of Sec15-GFP (Sec15GFP) in wild-type cells (NY2557) exiting G0 at 25°C in the absence or presence of latrunculin A was examined at time points of 1, 2 and 3.5 hours. (D) The localization of Sec8-GFP (Sec8GFP; NY2558) in wild-type cells exiting G0 was examined and the cells were observed after 3.5 hours of incubation in the absence or presence of latrunculin A (Lat-A). (E) Quantification of the GFP fluorescence to assess the percentage of cells incubated in the absence or presence of Lat-A that exhibited polarized Sec15-GFP (Sec15GFP) or Sec8-GFP (Sec8GFP) proteins. At least 200 cells were scored in each strain.

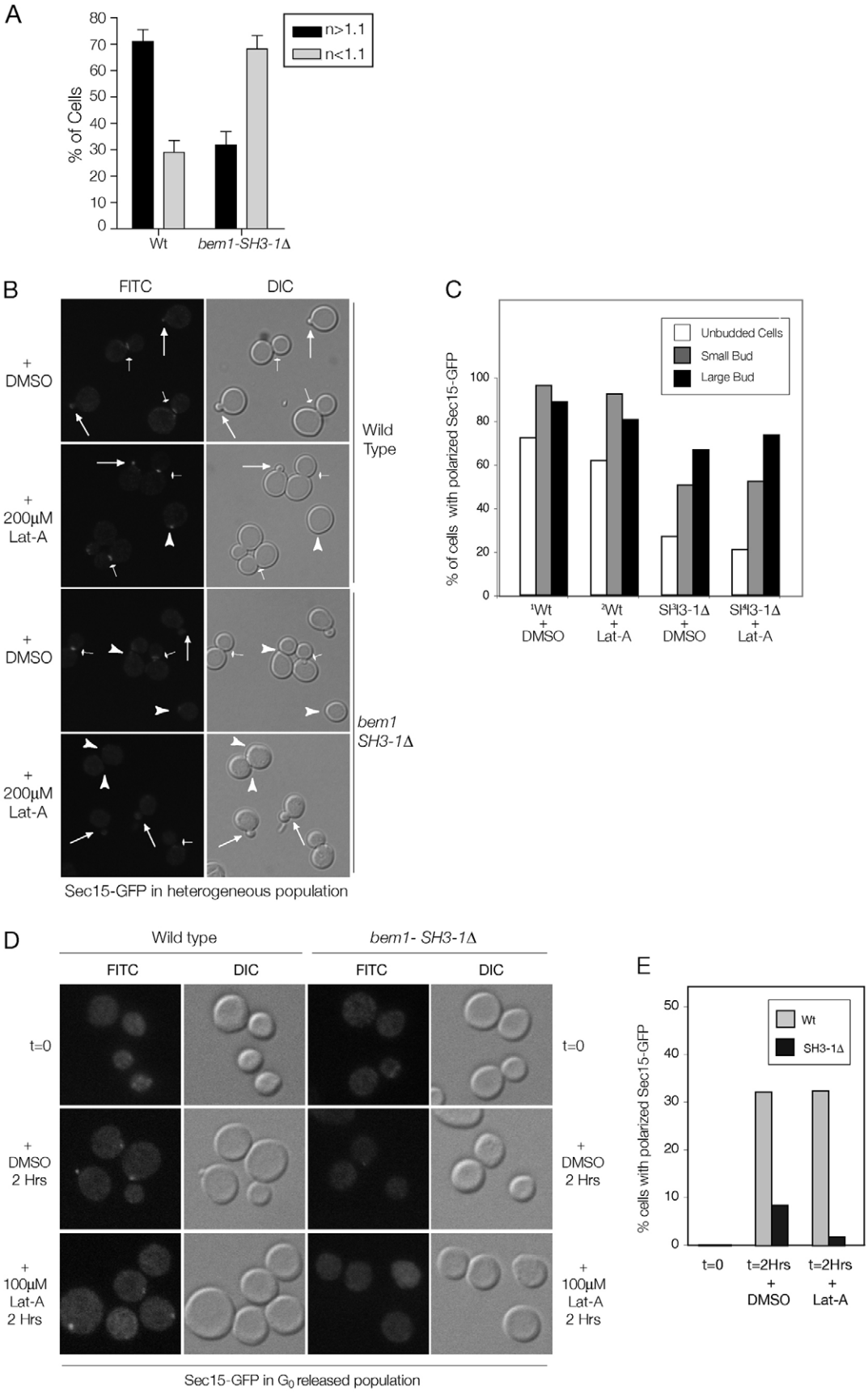

Fig. 6. See next page for legend.

Fig. 6. *bem1*$^{SH3\text{-}1\Delta}$ mutant cells have a mild polarity defect and the polarized localization of Sec15-GFP is disrupted in *bem1*$^{SH3\text{-}1\Delta}$ mutant cells. (A) The percentage of yeast mother cells with oval-shaped wild-type versus round morphology was determined as described in the Materials and Methods for wild-type (Wt) and *bem1*$^{SH3\text{-}1\Delta}$ mutant (*bem1-SH3-1Δ*) cells. For each dataset, the yeast strains were grown in parallel and DIC images were taken to measure ratios (n) of length to width. The average and s.d. were calculated from two independent datasets, and at least 200 cells were measured per strain and dataset. (B) *bem1*$^{SH3\text{-}1\Delta}$ mutant (*bem1-SH3-1Δ*; NY2568) and wild-type (NY2557) strains containing Sec15-GFP were grown overnight at 25°C to early log phase and treated either with DMSO alone or with 200 μM latrunculin A (Lat-A). After 15 minutes of treatment, cells were rapidly fixed by methanol/acetone and mounted for microscopy. The localization was examined by direct fluorescence microscopy. Examples of cells with a small bud are marked with large arrows and those with a large bud are marked with smaller arrows. Unbudded cells are marked with arrowheads. (C) Wild-type (Wt) and *bem1*$^{SH3\text{-}1\Delta}$ (SH3-1Δ) cells were categorized based upon their pattern of Sec15-GFP polarized localization – the presumptive bud, bud tip or the bud neck. The graph depicts the percentage of the population within each category. At least 200-300 cells were scored in each strain. (D) The localization of Sec15-GFP in synchronized wild-type and *bem1*$^{SH3\text{-}1\Delta}$ (*bem1-SH3-1Δ*) cells released from G0 at 25°C in the absence or presence of Lat-A was examined at time points of 0 and 2 hours. (E) Quantification of GFP fluorescence, used to assess the percentage of cells that exhibited polarized Sec15-GFP in the presence or absence of Lat-A treatment. At least 200 cells were scored in each strain.

significantly altered at both temperatures. Sec15-1–GFP was very faint at the permissive temperature and, if visible, the localization appeared to be cytoplasmic (Fig. 8A). The normal localization to small bud tips or bud necks was rarely observed. At the restrictive temperature of 37°C, the Sec15-1–GFP signal formed a disorganized array of cytoplasmic puncta distributed over both mother and daughter cells. Overall, the microscopic analysis indicates that the last 76 residues, which are missing in Sec15-1p and are involved in Bem1p interaction, are also important for the proper localization of Sec15p to exocytic sites. Since a dramatic effect is seen even at the permissive temperature, it is unlikely to be an indirect effect of a disruption in membrane traffic or cell growth. Next, we examined whether the mislocalization of Sec15-1p affects the localization of other exocyst subunits, such as Sec8p. The wild-type Sec8-GFP localization is comparable with that of Sec15-GFP and other exocyst subunits, and the signal is rather bright (Fig. 8C, top two panels). However, in a *sec15-1* background, the localization of Sec8-GFP was altered. At permissive temperature, the Sec8-GFP signal was very faint, reminiscent of the localization of Sec15-1–GFP, whereas at restrictive temperature Sec8-GFP was found to be in a punctate pattern (Fig. 8C, bottom two panels). Therefore, we conclude that the C-terminus of Sec15p is important both for the localization of Sec15p and that of other exocyst subunits.

Discussion

As a membrane-tethering complex, the exocyst must associate with both the target membrane and incoming secretory vesicles. Several lines of evidence indicate that most exocyst subunits, including Sec15p, are associated with secretory vesicles as they approach sites of exocytosis. The exceptions – Sec3p and, to some extent, Exo70p – associate with the plasma membrane at sites of exocytosis independent of actin-dependent vesicle delivery, and are therefore considered to constitute spatial landmarks. Exocyst complex assembly occurs as vesicles are delivered to sites marked by Sec3p and Exo70p, thereby tethering the incoming vesicle to the plasma membrane in preparation for exocytic fusion. The localization of this spatial landmark is cell-cycle dependent, as sites of secretion and cell-surface expansion shift through the cycle. This dynamic localization implies a tight functional link to the polarity-establishment machinery; yet, the relationship between secretion and polarity establishment has not been thoroughly defined. Sec3p is known to interact with Rho1p (Guo et al., 2001), Cdc42p (Zhang et al., 2001) and Bud4p (Osman et al., 2002), and each of these components is important in exocyst localization. Additionally, Exo70p interacts with Rho3p (Adamo et al., 1999; Robinson et al., 1999). Here, we explore an interaction between Sec15p and Bem1p that provides an additional line of communication between the vesicle-tethering and polarity-establishment machineries.

If two of the subunits of the exocyst interact with regulators of cell polarity, why would there be a need for an additional subunit, Sec15p, to interact with a component of the polarity-establishment machinery? Although Sec3 is thought to serve as a spatial landmark at the target membrane, *SEC3* is not an essential gene (Finger et al., 1998; Wiederkehr et al., 2003). Furthermore, upon overexpression of either Sec4p or Sec1p, neither *SEC3* nor *EXO70* are essential (Wiederkehr et al., 2004). In a *sec3Δ* or *exo70Δ* background, Sec8p still localizes in a polarized manner, albeit the localization is not as tightly focused as in the case of the wild-type cells. Thus, there is reason to believe that Sec3p or Exo70p might not be the only exocyst subunits that serve as spatial landmarks or that function under the direct regulation of the polarity-establishment machinery. The additional link provided by the interaction of Sec15p with Bem1p might increase the robustness of the system.

In addition to interacting with Bem1p, Sec15p also binds to the rab GTPase Sec4p, as well as to Sec10p within the exocyst complex. On the basis of these observations, Sec15p can be considered to be a crucial element that implements the targeting specificity promoted by Sec4p signaling. Sec15p provides a physical link between activated Sec4p on the vesicle surface and the remaining subunits of the exocyst, and in addition provides a link to Bem1p at the cell cortex. In this way, Sec15p is poised to facilitate cross-talk between the secretory pathway and the polarity-establishment machinery. One prediction of this model is that the localization of the vesicle-associated exocyst subunits would be altered by severing the link between Sec15p and Bem1p. Indeed, our study demonstrates that the Sec15-1p truncation of the extreme C-terminal region compromises the interaction with Bem1p and results in mislocalization of both the mutant protein and another exocyst subunit, Sec8p. Consistent with this observation, the level of exocyst assembly is reduced significantly in a *sec15-1* strain (TerBush and Novick, 1995). Although we cannot exclude the possibility that the reduced level of assembly and the mislocalization reflect an additional defect of the *sec15-1* allele, we have demonstrated that loss of the Sec15p-interacting domain of Bem1p largely blocks the actin-independent localization of Sec15-GFP, further supporting an in vivo role for the observed Bem1p-Sec15p interaction.

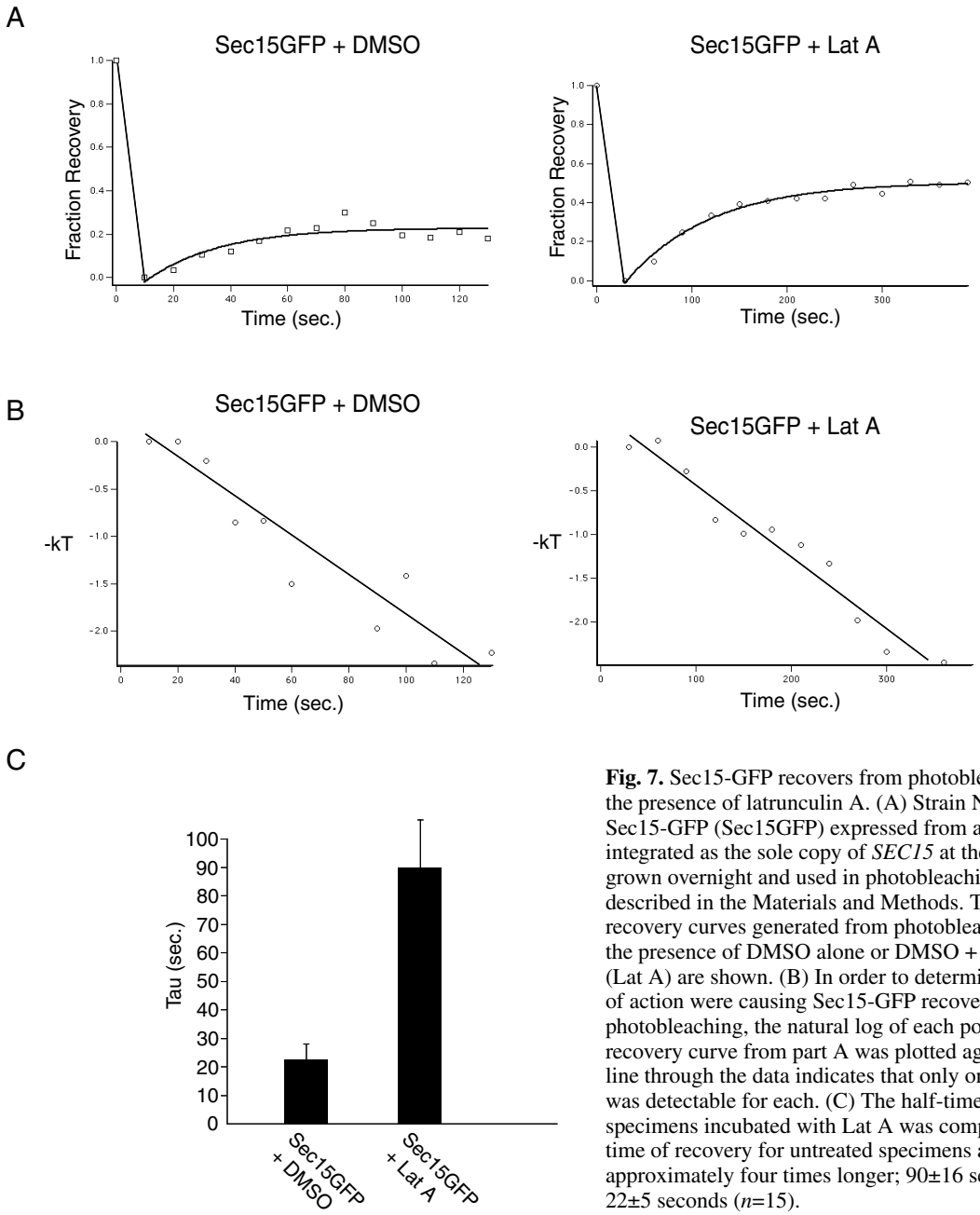

Fig. 7. Sec15-GFP recovers from photobleaching more slowly in the presence of latrunculin A. (A) Strain NY2445 containing Sec15-GFP (Sec15GFP) expressed from a fusion construct integrated as the sole copy of *SEC15* at the *SEC15* locus was grown overnight and used in photobleaching experiments as described in the Materials and Methods. Two representative recovery curves generated from photobleaching Sec15-GFP in the presence of DMSO alone or DMSO + 200 μM latrunculin A (Lat A) are shown. (B) In order to determine how many modes of action were causing Sec15-GFP recovery after photobleaching, the natural log of each point from the example recovery curve from part A was plotted against time. The straight line through the data indicates that only one mode of recovery was detectable for each. (C) The half-time of recovery for specimens incubated with Lat A was compared with the half-time of recovery for untreated specimens and found to be approximately four times longer; 90±16 seconds (n=13) versus 22±5 seconds (n=15).

Bem1p is required to maintain the Cdc42p module at the incipient budding site and is therefore essential for the proper function of the Cdc42p signaling cascade that leads to the initiation and stabilization of cell polarity. As a protein that links several key polarity factors, Bem1p is an attractive candidate to participate in a localized interaction with a component of the secretory machinery. Although the Bem1p-Sec15p interaction involves the N-terminal region of Bem1p (containing an SH3 domain) and the C-terminal region of Sec15p (containing a proline-rich region), mutation of the proline residues to alanine residues did not yield any obvious defects in secretion or polarity, nor was there any effect on Bem1p binding in vitro, suggesting that the interaction involves other structural elements.

FRAP experiments have demonstrated that, under normal conditions, the dynamics of Sec15-GFP is similar to that of the secretory marker Sec4p-GFP, and that the recovery in the presence of Lat-A is strongly reduced in efficiency within the time frame of about one minute (Boyd et al., 2004). These experiments have helped to establish that the normal mode by which Sec15p arrives at exocytic sites is by actin-dependent vesicle traffic. However, we report here that a portion of Sec15p still localizes to exocytic sites under conditions that disrupt polarized actin cables and hence block vesicle trafficking. How do we reconcile these two observations? We performed additional FRAP experiments to detect a possible slow, actin-independent recovery mode by taking images at much longer intervals in the presence of Lat-A and observed that, indeed, Sec15-GFP recovers to some degree, albeit with much slower kinetics than normal. Therefore, we conclude that

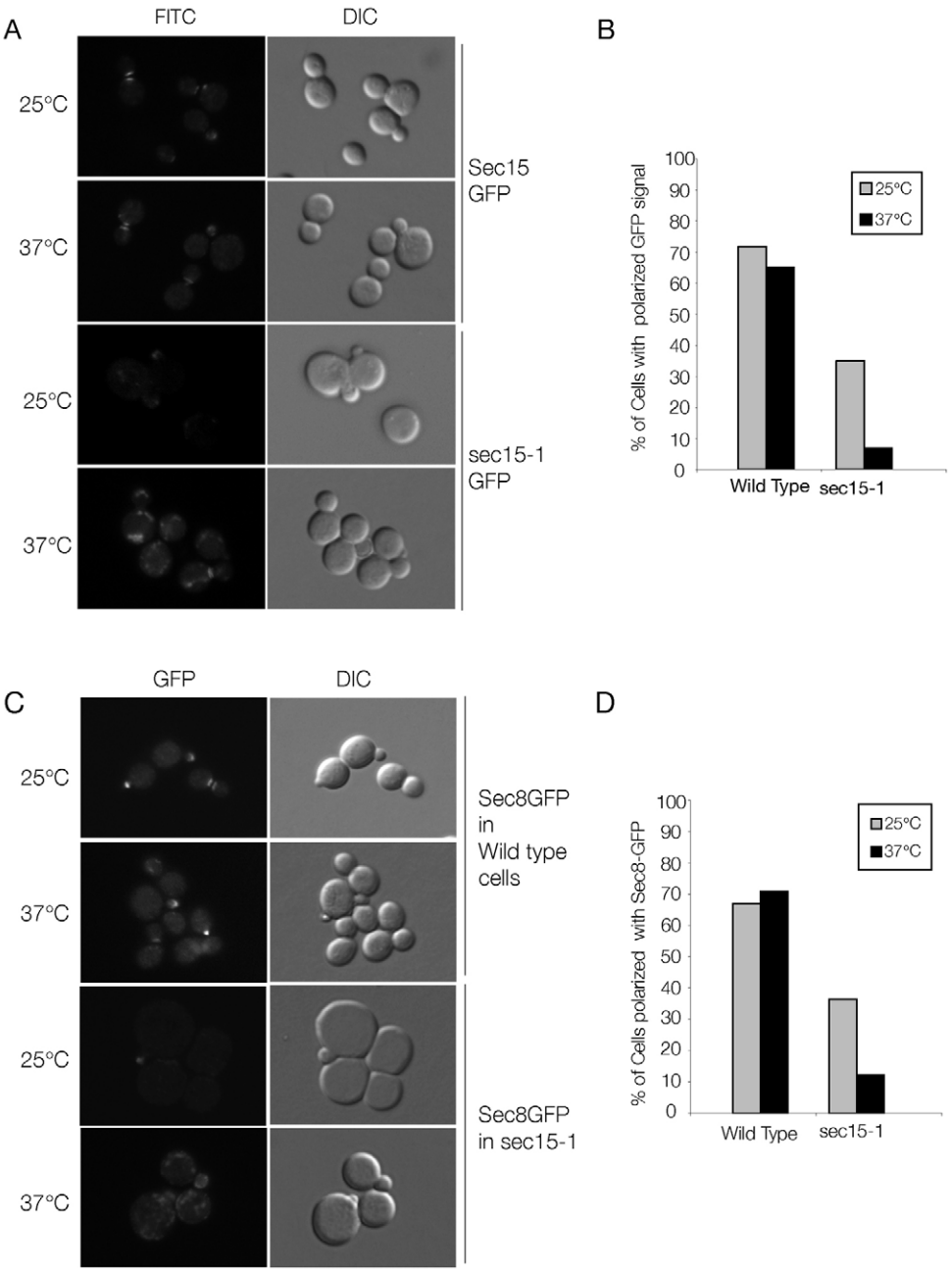

Fig. 8. Sec15-1–GFP mislocalizes as does Sec8-GFP in a *sec15-1* background. (A) GFP-tagged *sec15-1* (sec15-1 GFP; NY2565) or *SEC15* (Sec15 GFP; NY2557) cells were shifted to restrictive temperature (37°C) or grown at permissive temperature (25°C) for 1.5 hours and processed for GFP fluorescence detection. Cells were visualized by direct fluorescence microscopy. (B) The proportion of cells containing a polarized GFP signal from the experiment shown in panel A was scored. At least 200 cells were scored in each strain. (C) Cells expressing GFP-tagged Sec8 (Sec8GFP), either in a wild-type (NY2558) or *sec15-1* (sec15-1; NY2566) background were shifted to restrictive (37°C) or grown at permissive (25°C) temperature for 1.5 hours and processed for the direct GFP fluorescence detection. The localization was visualized by direct fluorescence microscopy. (D) The proportion of cells containing a polarized GFP signal from the experiment shown in panel C was scored. At least 200 cells were scored in each strain.

the normal physiological mechanism for Sec15p localization depends on vesicular traffic, although Sec15p can display slow, actin-independent localization to the incipient budding site. Since the actin-independent localization is largely blocked by removal of the Sec15p-interacting domain of Bem1p, we conclude that this pathway relies on the interaction of Sec15p with Bem1p. Our current model is that Sec15p rides vesicles along with most other subunits of the exocyst to the exocytic site marked by Sec3p and Exo70p. Upon arrival, the interaction with Bem1p provides an additional link to the target site, which helps to stabilize the cortical association of the exocyst complex. In the case of the Sec15-1p protein or the N-terminal truncation of Bem1p, where the interaction between Bem1p and Sec15p is compromised, the vesicles carrying Sec15p and other subunits are delivered to the target site, but the association with the cortex is not as stable as in wild type, and hence a defect in exocyst localization is observed. In the presence of Lat-A, Sec15p might be recruited directly from the cytosol to the bud tip by binding Bem1p.

In a very recent publication, Zajac et al. also reported actin-independent localization of Sec15-GFP (Zajac et al., 2005). However, they observed that the initial localization of Sec15-GFP following recovery from G0 arrest was blocked by the addition of latrunculin B (Lat-B), whereas we observed localization in the presence of Lat-A using similar conditions. We have confirmed that the difference reflects the use of Lat-A versus Lat-B (data not shown). This difference appears to be related to a phenomenon reported by Irazoqui et al. (Irazoqui et al., 2005). They reported that complete disassembly of actin by Lat-A allows polarization of Cdc42p following recovery from

Table 1. Yeast strains used in this work

NY64	*Mat a ura3-52 sec15-1*
NY180	*Mat α ura3-52 leu2-3, 112*
NY603	*Mat α ura3-52 leu2-3, 112 pep4::URA3 GAL+*
NY1444	*Mat α bem1-3 ura3 leu2 ade2 ades3 lys2* (y461 from A. Bender)
NY2445	*Mat a ura2-52, leu2-3, 112 his3Δ200 SEC15GFP [SEC15GFP URA3]*
NY2553	*Mat α bem1-3 ura3 leu2 ade2 ade3 lys2+[2 μ SEC15: URA3;* pNB192*]*
NY2554	*Mat α bem1-3 ura3 leu2 ade2 ade3 lys2+[2 μ SEC4: URA3;* pNB142*]*
NY2555	*Mat α bem1-3 ura3 leu2 ade2 ade3 lys2+[2 μ BEM1: URA3*; pNB1227*]*
NY2556	*Mat a ura3-52 act 1-3 SEC15:: [SEC15GFP URA3*; pNB1228*]*
NY2557	*Mat α ura3-52 leu2-3, 112 SEC15:: [SEC15GFP URA3*; pNB1228*]*
NY2558	*Mat α ura3-52 leu2-3, 112 SEC8:: [SEC8GFP URA3*; pNB1235*]*
NY2559	*Mat α ura3-52 leu2-3, 112:: [GALp-SEC15-GST LEU2*; pNB1229*] pep4::URA3 GAL+*
NY2560	*Mat α ura3-52 leu2-3, 112::[GALp-sec15-1-GST LEU2*; pNB1230*] pep4::URA3 GAL+*
NY2561	*Mat α ura3-52 leu2-3, 112::[GALp-GST LEU2*; pNB1154*] pep4::URA3 GAL+*
NY2562	*Mat α ura3-52 leu2-3, 112::[GALp-sec15 (aa.83-910)-GST LEU2*; pNB1231*] pep4::URA3 GAL+*
NY2563	*Mat α ura3-52 leu2-3, 112::[GALp-sec15 (aa.557-910)-GST LEU2*; pNB1232*] pep4::URA3 GAL+*
NY2564	*Mat α ura3-52 leu2-3, 112::[GALp-sec15 (aa.740-910)-GST LEU2*; pNB1233*] pep4::URA3 GAL+*
NY2521	*Mat α ura3-52 leu2-3, 112 SEC8::[SEC8TAP LEU2] SEC5::[SEC5 3xHA HIS3MX6] GAL+*
NY2565	*Mat α ura3-52 leu2-3, 112 SEC15::[sec15-1GFP URA3*; pNB1234*]*
NY2566	*Mat a ura3-52 sec15-1 SEC8::[SEC8GFP URA3*; pNB1235*]*
NY2567	*Mat α ura3-52 leu2-3, 112 bem1Δ::kanMX*
NY2568	*Mat α ura3-52 leu2-3, 112 bem1Δ::kanMX SEC15:: [SEC15GFP LEU2*; pNB1241*]*
NY2569	*Mat α ura3-52 leu2-3, 112 bem1Δ::kanMX SEC15:: [SEC15GFP LEU2; pNB1241] +[CEN URA3 bem1$^{SH3\text{-}1\Delta}$; pNB1242]*

G0 arrest, whereas Lat-B, which disrupts actin cables but not actin patches at endocytic sites, prevents polarization of Cdc42p. They have proposed that an initial, actin-independent localization of Cdc42p is reinforced by actin-cable-mediated exocytosis and is dispersed by actin-patch-mediated endocytosis. Lat-A blocks both pathways, leaving the initial patch of Cdc42p intact, whereas Lat-B blocks only exocytosis, allowing endocytic dispersal of the initial patch. We propose that Bem1p localization is regulated in a similar fashion to Cdc42p, and the localization of Sec15p observed in our experiments reflects direct binding of Sec15p from the cytosol to Bem1p.

Overall, these findings contribute to an understanding of how two different cellular machineries work in a cooperative manner to achieve the correct positional targeting of secretory cargo in an efficient manner. Recently, a *sec15* mutant was identified in a screen for genes required for synaptic specificity in *Drosophila* (Mehta et al., 2005). The targeting defect evident in the neurons of this mutant suggests that the role of Sec15 in specifying certain areas of the cell cortex for membrane expansion may be conserved through evolution. Identification of novel exocyst interactors in different organisms and in different tissues of multicellular organisms may have a significant impact on our current understanding of the spatial regulation of exocytosis mediated by this tethering complex.

Materials and Methods

Yeast strains, media and reagents

The genotypes of the *Saccharomyces cerevisiae* strains used in this study are listed in Table 1. Cells were grown in yeast extract peptone (YP) medium containing 1% bactoyeast extract and 2% bactopeptone (Difco laboratories) with 2% glucose (YPD). For the overproduction of Sec15-GST proteins, cells were first grown in YP-2% glycerol overnight, and then switched to YP-2% galactose to induce the expression of Sec15-GST proteins. The cell density was determined by measuring the absorbance of cell suspensions at 600 nm (model Ultrospec 3000 Pro; Amersham Pharmacia Biotech). Transformations were performed by the lithium acetate method (Gietz et al., 1992)

Strain construction and molecular biology techniques

General molecular biological methods were as described previously (Sambrook et al., 1989). Bacterial *Escherichia coli* strains XL1-Blue (Stratagene) and DH5α were used for cloning purposes, and recombinant proteins were produced in the BL21 and BL21(DE3) strains.

To tag the genomic copy of *SEC15* with GFP at the C-terminus, the last 1.8 kb of the *SEC15* coding sequence was amplified by PCR and cloned into either pRS305 or pRS306, yeast integrating vectors containing *LEU2* and *URA3*, respectively. PCR-amplified GFP was ligated just behind the *SEC15* C-terminus. The resulting plasmid pNB1228 was cut at a unique *Sex*AI site within the *SEC15* coding sequence and used for yeast transformation. To generate a *sec15-1* allele tagged with GFP, the 3′ primer was designed in such a way to exclude the last 228 bp of the *SEC15* coding sequence and subcloned into the same vector described above, with GFP subcloned into the same position, generating pNB1234. The constructs were verified by sequencing.

A plasmid that expresses Sec15p with a GST tag under the *GAL1* promoter (pNB1229) was constructed as follows; a PCR reaction was performed to amplify the GST sequence from the commercial pGEX vector and placed into pNB529 (pRS305 vector with *GAL1* promoter and ADH terminator sequence). A DNA fragment encoding *SEC15* was PCR amplified using pNB192 (2 μ *SEC15* plasmid) as a template. The product (*SEC15*; 2.7 kb) was digested with *Bam*HI and *Xho*I, and ligated into the vector containing the GST tag, resulting in pNB1229. pNB1229 was linearized with *Afl*II and integrated into the *LEU2* locus of the protease-deficient *pep4*::*URA3* yeast strain NY603 (leaving endogenous *SEC15* intact), resulting in NY2559. All the truncation constructs of the *SEC15* coding region were amplified using pNB192 as a template and subcloned into the same vector between *Bam*HI-*Xho*I sites, and have a GST tag at the C-terminus. For the overexpression of GST-tagged Sec15 proteins behind the *GAL1* promoter, the cells were first grown in YP-2% glycerol overnight, and the protein expression was induced by adding 2% galactose into the culture and allowing it to grow for another 5-6 hours.

To create NY2568 (*bem1*$^{SH3\text{-}1\Delta}$), the entire open reading frame of *BEM1* was first replaced with the KanMX6 module as described previously (Longtine et al., 1998), generating NY2567 (*bem1*Δ). NY2567 was transformed with a low copy plasmid containing *BEM1* missing the first SH3 domain (pNB1242), which was generated by PCR amplification and subcloning of the *BEM1* coding region between the base pairs 415-1656. For Sec15-GFP visualization in *bem1*$^{SH3\text{-}1\Delta}$, the genomic copy of Sec15 was tagged with GFP at the C-terminus through homologous recombination, generating NY2569.

Yeast GST pull-down assays

For typical pull-down experiments, 75-100 OD_{600} units of yeast overexpressing GST, Sec15p-GST (NY2559) or GST-tagged Sec15 truncation constructs under the *GAL1* promoter were resuspended in lysis buffer containing phosphate-buffered saline (PBS), Triton X-100 (0.5%, v/v), 5 mM DTT and protease inhibitors. Cells were disrupted in a bead beater using 0.5 mm zirconia/silica beads (beads and instrument from Biospec Products). Lysates were then cleared by centrifugation at 10,000 ***g*** for 10 minutes at 4°C. Triton X-100 was adjusted to 1% (v/v) and supernatants were incubated with 400 μl of 50% (v/v) slurry of glutathione-Sepharose 4B (Amersham Pharmacia Biotech) beads for 2 hours at 4°C with nutation. After incubation, the beads were spun at 500 ***g*** and washed four times with 1.5 ml ice-cold PBS buffer, and bound products were analyzed by standard SDS-PAGE and western blotting analysis.

In vitro binding assay

In order to produce recombinant proteins, the C-terminal coding region of Sec15p (amino acids 557-910) was amplified and fused to GST (GST-Sec15$^{557\text{-}910}$) using

the pGEX vector system (Amersham Biosciences), whereas Bem1p was fused to His6 by subcloning the complete coding sequence into pET21-a (Novagen) by PCR amplification. The fusion proteins were purified from *Escherichia coli* according to the manufacturer's protocol and used for in vitro binding assays. For a typical binding experiment, the GST-Sec15$^{557\text{-}910}$ fusion protein immobilized to glutathione-Sepharose beads (5 μl of beads; estimated amount of GST-Sec15$^{557\text{-}910}$ on beads was 1.25 μg) was incubated with 5 μM Bem1p in binding buffer (1× PBS buffer containing 1 mg/ml ovalbumin, 10 mM β-mercaptoethanol, and 0.1% IGPAL-30) for 1 hour at room temperature. The total volume of incubation mixture was 200 μl. After the resin was washed four times with the binding buffer excluding ovalbumin, bound products were resolved by SDS-PAGE.

To analyze binding of various Bem1p deletion constructs, GST-Sec15$^{557\text{-}910}$ immobilized on the beads was incubated with crude bacterial lysates expressing Bem1p constructs for 1 hour at room temperature. The beads were washed and bound products were analyzed. For the detection of Bem1p in the western blot analysis, either anti-Bem1p serum (dilution 1:3000) or anti-His monoclonal antibodies (Cell Signaling) (dilution and direction according to the manufacturer) were used.

To test the interaction between Bem1p and the exocyst complex, exocyst proteins were isolated by tagging Sec8p with a Tandem Affinity Purification (TAP) tag at the C-terminus through homologous recombination. Fusion proteins were isolated using the method described in Puig et al. (Puig et al., 2001) with some modifications. Instead of using a French press, cells (grown to OD_{600} 1.5) were lysed in a buffer of 20 mM Pipes (pH 6.8), 150 mM NaCl, 1 mM EDTA, 0.2 mM phenylmethylsulfonylfluoride (PMSF), 10 μM antipain, 20 μM aprotinin, 20 μM chymostatin, 20 μM leupeptin, 20 μM pepstatin A and 10 mM β-mercaptoethanol using a Bead Beater (Biospec Products). The 30 ml chamber was half-filled with 0.5 mm glass beads (Biospec) and run 4× 1 minute. NP-40 (IGPAL CA-630; Sigma) was added (0.5%, v/v) and the lysates were incubated at 4°C for 15 minutes and then centrifuged at 30,000 ***g*** for 15 minutes. The method for protein isolation in Puig et al. (Puig et al., 2001) was followed except 20 mM Pipes (pH 6.8) was substituted for Tris-HCl in all buffers, the TEV incubation was allowed to proceed overnight at 4°C, and the concentration of EGTA in the elution buffer was increased to 10 mM. Bacterially purified GST-Bem1p was immobilized to the glutathione-Sepharose beads and incubated with the purified exocyst complex for 4 hours at 4°C. The beads were washed four times, and analyzed by SDS-PAGE and western blot.

Morphological analysis

For each dataset, the yeast strains were grown in parallel and differential interference contrast (DIC) images were taken to measure ratios of length to width. The length and width of yeast cells were measured using the NIH Image 1.62 program as described in Wiederhehr et al. (Wiederhehr et al., 2005). The average and s.d. were derived from two independent datasets, and cells with an axial ratio greater than 1.1. were considered round. At least 200 cells were measured per strain and dataset (total >400 cells per strain).

Epifluorescence microscopy for localization analysis

For Sec15-GFP and Sec8-GFP localization studies, 4-8 OD_{600} units of yeast cells were grown overnight and diluted in fresh synthetic complete (SC) medium the next morning, then further incubated at 25°C or 37°C. Cells were first resuspended in ice-cold 10 mM Tris-HCl pH 7.5, 10 mM sodium azide/fluoride wash buffer after 90 minutes of incubation at 25°C or 37°C, and centrifuged at low speed for 5 minutes at 4°C. The pellet was immediately fixed in cold methanol and incubated at –20°C for 10 minutes. After a centrifugation step, the cells were briefly resuspended in cold acetone. The fixed cells were washed three times in PBS before the analysis. The cell suspension was mixed with an equal volume of 1.6% NuSieve GTG low-melting-point (LMP) agarose (FMC BioProducts) and mounted on a glass slide.

Experiments using Lat-A treatment were performed as described in Ayscough et al. (Ayscough et al., 1997). All cells were examined with a Zeiss Axioplan2 upright fluorescence microscope using a 63× Plan Neofluor apochromatic oil-immersion objective with N.A. 1.3. Images were captured with a Hammamatsu ORCA ER-cooled CCD camera and analyzed; if appropriate, images were enhanced with Open lab software from Improvision and Adobe Photoshop software.

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