The GAP activity of Msb3p and Msb4p for the Rab GTPase Sec4p is required for efficient exocytosis and actin organization

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Polarized growth in Saccharomyces cerevisiae is thought to occur by the transport of post-Golgi vesicles along actin cables to the daughter cell, and the subsequent fusion of the vesicles with the plasma membrane. Previously, we have shown that Msb3p and Msb4p genetically interact with Cdc42p and display a GTPase-activating protein (GAP) activity toward a number of Rab GTPases in vitro. We show here that Msb3p and Msb4p regulate exocytosis by functioning as GAPs for Sec4p in vivo. Cells lacking the GAP activity of Msb3p and Msb4p displayed secretory defects, including the accumulation of vesicles of 80–100 nm in diameter. Interestingly, the GAP activity of Msb3p and Msb4p was also required for efficient polarization of the actin patches and for the suppression of the actin-organization defects in cdc42 mutants. Using a strain defective in polarized secretion and actin-patch organization, we showed that a change in actin-patch organization could be a consequence of the fusion of mistargeted vesicles with the plasma membrane.

Introduction

Polarization of cell growth is critical for generating distinct cellular domains and is ultimately responsible for the diversity of cell types, tissues, and organs. Thus, cell polarity is essential for development and differentiation in many organisms (Drubin and Nelson, 1996). In the budding yeast Saccharomyces cerevisiae, polarized cell growth is thought to occur in a hierarchical manner. At the beginning of the cell cycle, the small GTPase Cdc42p and its regulators such as the guanine nucleotide–exchange factor (GEF) Cdc24p are clustered at a specific region of the cell cortex, marking the site for polarity establishment (Johnson, 1999). Cdc42p effectors, including the p21-activated kinases, Ste20p and Cla4p; the formin, Bni1p; and the structurally related proteins, Gic1p and Gic2p; are then recruited to the cortical site to polymerize and/or organize the actin cytoskeleton, including actin cables and actin patches, at the presumptive bud site (Pruyne and Bretscher, 2000b). Actin patches are thought to mediate endocytosis (Munn, 2000), whereas actin cables are thought to function as tracks along which post-Golgi vesicles are transported from the mother cell to the daughter cell (Pruyne and Bretscher, 2000a).

Secretion in eukaryotes occurs in multiple, sequential steps, each of which is controlled by a distinct Rab GTPase. In S. cerevisiae, the Rab GTPase Sec4p plays a central role in polarized secretion from Golgi to the plasma membrane and is thought to act by tethering secretory vesicles to the plasma membrane via its effector, the exocyst, a multisubunit protein complex (Guo et al., 1999). Like other Ras-family members, Sec4p cycles between an inactive GDP- and active GTP-bound state. This cycling is regulated by its GEF, Sec2p (Walch-Solimena et al., 1997), and presumably by its GAP(s), whose identity has not been determined.

Msb3p and Msb4p are a pair of structurally related proteins that localize to the sites of polarized growth (Bi et al., 2000). Overexpression of Msb3p or Msb4p suppresses cdc24-Ts and cdc42-Ts mutants, although the mechanism underlying this suppression is not clear. Both Msb3p and Msb4p have a Rab GAP domain and indeed display a GAP activity toward a number of Rab GTPases in vitro (Albert and Gallwitz, 1999, 2000). However, the in vivo Rab targets of Msb3p and Msb4p are not known. In this report, we present multiple lines of evidence to indicate that Msb3p and Msb4p regulate exocytosis by functioning as GAPs for Sec4p in vivo. In contrast to the general view that polarized actin cytoskeleton guides secretion to a specific cellular domain, we also
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present evidence to indicate that a primary defect in polarized secretion can cause defects in polarized actin organization. Thus, polarized actin organization and polarized secretion appear to reinforce each other.

Results

Deletion of MSB3 and MSB4 causes a secretory defect

Because Msb3p and Msb4p display a GAP activity toward a number of Rab GTPases in vitro, a substrate promiscuity that is common among most known Rab GAPs, we decided to define the in vivo Rab target(s) of Msb3p and Msb4p by the following approaches. First, we examined possible protein-trafficking defects in cells lacking both Msb3p and Msb4p. We found that msb3Δ msb4Δ cells accumulated a large number of vesicles at 24°C, whereas wild-type and single-mutant cells had none or very few vesicles (Fig. 1 A and Fig. 4 A). The vesicles were 90.00 ± 11.77 nm (n = 204) in diameter, falling within the range of 80–100 nm post-Golgi secretory vesicles (Novick and Schekman, 1979). In most cells, the vesicles appeared to be distributed randomly in the mother and the daughter (Fig. 1 A, bottom left). In a few cells, vesicles were concentrated in the daughter (Fig. 1 A, top right). These data suggest that Msb3p and Msb4p must share a function in secretion.

The second approach used to assess a possible secretory defect in msb3Δ msb4Δ cells was to monitor the secretion of invertase, a sucrose-metabolizing enzyme, and of Bgl2p, an endo-β-1,3-glucanase required for cell wall organization and biogenesis (Mrsa et al., 1993). When invertase secretion was

Figure 1. Deletion of MSB3 and MSB4 causes a secretory defect. (A) Wild-type (YEF473) and msb3Δ msb4Δ (YEF1631) cells were grown in YPD media at 24°C and processed for electron microscopy. Bars, 0.5 μm. (B) Invertase secretion. Wild-type (YEF473A) and msb3Δ msb4Δ (YEF1289) cells were induced for secretion of invertase at 24°C. The percentage of external (Ext, secreted) pool versus total invertase (Ext + Int) was measured at indicated times after induction. (C) Bgl2p secretion. Wild-type (YEF473A), msb3Δ msb4Δ (YEF1289) and sec6–4 (BY37) cells were grown at 24°C, or shifted to 37°C for 1 h. The amounts of internal and external pools of Bgl2p were analyzed by Western blotting with anti-Bgl2p antibody. Samples of external pool were loaded only half the amount as those of internal pool.
The ratio of secreted invertase versus total invertase (external plus internal) was mildly, but consistently lower in \(\text{msb3}\Delta \text{msb4}\Delta\) cells than in wild-type cells, with the difference peaking around 45 min after induction (Fig. 1 B). In contrast, Bgl2p was accumulated efficiently to the periplasmic region during the course of induction (Fig. 1 C). These data suggest that most vesicles accumulated in \(\text{msb3}\Delta \text{msb4}\Delta\) cells carry Bgl2p and a small fraction of vesicles carry invertase.

### Genetic evidence for the involvement of Msb3p and Msb4p in exocytosis and for Msb3p and Msb4p functioning as GAPs for Sec4p in vivo

If Msb3p and Msb4p play a role in exocytosis, deletion or overexpression of \(\text{MSB3}\) and \(\text{MSB4}\) may display genetic interactions with some of the late secretory mutants. Indeed, deletion of \(\text{MSB3}\) and \(\text{MSB4}\) produced synthetic inhibitory effects on cell growth with \(\text{sec3}\Delta\text{–2}\) and \(\text{sec9}\Delta\text{–4}\) mutants at 30°C, but not with \(\text{sec1}\Delta\text{–1}, \text{sec2}\Delta\text{–41}, \text{sec6}\Delta\text{–4}\), and \(\text{sec6}\Delta\text{–4}\) mutants (Fig. 2 A). In addition, overexpression of \(\text{MSB3}\) or \(\text{MSB4}\) inhibited the growth of \(\text{sec2}\Delta\text{–41}\) cells at 30°C, but not of any other late secret mutants, including \(\text{sec1}\Delta\text{–1}, \text{sec3}\Delta\text{–2}, \text{sec4}\Delta\text{–8}, \text{sec5}\Delta\text{–24}, \text{sec6}\Delta\text{–4}, \text{sec8}\Delta\text{–9}, \text{sec9}\Delta\text{–4}, \text{sec10}\Delta\text{–2},\) and \(\text{sec15}\Delta\text{–1}\) (Fig. 2 B). In contrast, overexpression of Gyp1p, a GAP for Ypt1p that is involved in ER to Golgi transport but also exhibits a GAP activity toward Sec4p in vitro (Du et al., 1998; Du and Novick, 2001; De Antoni et al., 2002), did not inhibit the growth of \(\text{sec2}\Delta\text{–41}\) cells at 30°C (Fig. 2 B). These results suggest that Msb3p and Msb4p are involved in exocytosis and can antagonize the function of Sec2p.

Because Msb3p and Msb4p display a GAP activity toward Sec4p in vitro and appear to colocalize with Sec4p at the sites of polarized growth during the cell cycle, it seemed likely that Msb3p and Msb4p might participate in the regulation of exocytosis by functioning as GAPs for Sec4p in vivo. To test this hypothesis, we took advantage of the observation that a \(\text{sec4}\Delta\text{–Q79L sec15}\Delta\text{–1}\) double mutant is inviable at 25°C (Walworth et al., 1992). The Q79L mutation shifts Sec4p toward its GTP-bound form by decreasing the intrinsic GTPase activity, but this Sec4p mutant is still responsive to GAP action (Walworth et al., 1992; Du et al., 1998). Sec15p, an effector of Sec4p, is thought to mediate the role of Sec4p in the assembly of the exocyst (Guo et al., 1999). We reasoned that if Msb3p and Msb4p are physiological GAPs for Sec4p, their overexpression might suppress the synthetic lethality between \(\text{sec4}\Delta\text{–Q79L sec15}\Delta\text{–1}\) by decreasing the level of GTP-bound Sec4p.

To examine this possibility, we constructed a \(\text{sec4}\Delta\text{–Q79L sec15}\Delta\text{–1}\) double mutant harboring an \(\text{URA3}\)-marked plasmid carrying wild-type \(\text{SEC4}\). A LEU2-marked multicopy plasmid carrying either \(\text{MSB3}\) or \(\text{MSB4}\) was transformed into the tester strain and assayed for its ability to replace the \(\text{SEC4}\)-containing plasmid by examining cell growth on plates containing 5FOA, a chemical that selects for cells that have lost the \(\text{URA3}\)-containing plasmid (Fig. 2 C). Multicopy \(\text{MSB3}\), but not \(\text{GYP1}\) or \(\text{MSB4}\), was able to suppress the \(\text{sec4}\Delta\text{–Q79L sec15}\Delta\text{–1}\) mutant, supporting the hypothesis that Msb3p functions as a GAP for Sec4p in vivo.

### Msb3p and Msb4p function as GAPs for Sec4p by an arginine finger–like mechanism

GAPs for Ras, Rho, and Rab GTPases all contain an invariant arginine residue (the “finger arginine”) in the catalytic domain that is critical for their GAP activities (Ahmadian et al., 1997; Albert et al., 1999). Because Msb3p and Msb4p...
contain an arginine residue (R282 in Msb3p and R200 in Msb4p) at the corresponding position, we decided to examine whether Msb3p and Msb4p function on Sec4p by a similar mechanism. In addition, we hoped that Msb3p and Msb4p mutants deficient in the GAP activity toward Sec4p might offer an opportunity to distinguish the role of Msb3p and Msb4p in secretion from their role in actin organization. For these reasons, we substituted the arginine residue in Msb3p and Msb4p for either phenylalanine or lysine and determined the properties of the mutant proteins.

To facilitate protein purification, Msb3p, Msb4p, and their derivatives were all tagged with six histidines at their COOH termini. The tagged Msb3p and the arginine mutants were expressed from a galactose-inducible promoter in yeast cells overexpressing His3-tagged Msb3p wild-type (WT) or arginine mutants were analyzed by Western blotting with anti-His, antibody. (B and C) Time course of hydrolysis of Sec4p-bound GTP stimulated by the wild-type or the arginine mutants of Msb3p (B) or by the wild-type or the arginine mutants of Msb4p (C). (D) The arginine mutants of Msb3p and Msb4p express normally. Yeast strain JGY18 (msb3Δ msb4Δ cdc42–201, pRS316-CDC42) carrying plasmid YEp181–3HA-MSB3, YEp181–3HA-MSB3-R282F, YEp181–3HA-MSB3-R282K, YEp181–3HA-MSB4, YEp181–3HA-MSB4-R200F, or YEp181–3HA-MSB4-R200K was analyzed for the expression of wild-type and the arginine mutants of Msb3p and Msb4p. The mitochondrial outer membrane protein Isp42p was used as a loading control. (E) The arginine mutants of Msb3p localize normally. YEp181–3HA-MSB3, YEp181–3HA-MSB3-R282F, YEp181–3HA-MSB3-R282K was transformed into YEF1619 (msb3Δ msb3Δ). Transformants were grown at 24°C and processed for immunofluorescence with anti-HA antibody. (F) The GAP activity of Msb3p and Msb4p is required for their in vivo function. YEp181 alone, or carrying 3HA-tagged MSB3, MSB4, or their respective arginine mutants, was transformed into strain JGY18 (msb3Δ msb4Δ cdc42–201, pRS316-CDC42). Transformants were replica plated onto a SC-Leu+5FOA plate and incubated at 24°C for 4 d. (G) The GAP activity of Msb3p and Msb4p is required for their inhibitory effects on a sec4–41 mutant. YEp181 alone, or carrying 3HA-MSB3, 3HA-MSB4, 3HA-MSB4-R200K was transformed into JGY28B (sec4–41). Transformants were streaked onto SC-Leu plates and incubated for 3 d at 24°C. (H) The GAP activity of Msb3p is required for the suppression of the sec15–1 s4-Q79L mutant. YEp181 alone, or carrying 3HA-MSB3 or 3HA-msb3-R282K, was transformed into strain JGY86A (sec15–1 s4-Q79L, pRS316-SEC4). Transformants were replica plated onto a SC-Leu+5FOA plate and incubated at 24°C for 3 d.

The GAP activity of Msb3p and Msb4p is essential for their in vivo function

Despite the drastic reduction in their GAP activity toward Sec4p, the arginine mutants of Msb3p and Msb4p were expressed at normal levels (Fig. 3 D) and localized to the sites of polarized growth like the wild-type proteins (Fig. 3 E, and unpublished data). These data suggest that a significant loss of GAP activity. These data suggest that Msb3p and Msb4p function as GAPs for Sec4p by an arginine finger-like mechanism.
viable with a loss-of-polarity phenotype (Bi et al., 2000). The second assay is based on our new observation that msb3Δ msb4Δ is synthetically lethal with cdc42–201, a newly isolated temperature-sensitive cdc42 allele (Zhang et al., 2001). These two tester strains were kept alive by introducing an URA3-marked plasmid that carries either wild-type GIC1 for the first assay or CDC42 for the second assay. HA-tagged Msb3p, Msb4p, and their arginine mutants expressed from a LEU2-marked, high-copy plasmid in the tester strains were assayed for their ability to replace the URA3-marked plasmids on SC-Leu+5FOA plates. Plasmids carrying the arginine mutants of MSB3 or MSB4 failed to replace the URA3-marked plasmids in both assays, in direct contrast to the plasmids carrying wild-type MSB3 or MSB4 (Fig. 3 F and unpublished data). These data indicate that the GAP activity of Msb3p and Msb4p is essential for their in vivo function(s).

Overexpression of Msb3p-R282K and Msb4p-R200K mutants also failed to inhibit the growth of sec2–41 cells at

$$\text{Figure 4. Loss of the GAP activity of Msb3p and Msb4p causes vesicle accumulation and a defect in actin organization.}$$

(A) MSB3 msb4Δ (JGY184), msb3-R282K msb4Δ (JGY190), msb3Δ MSB4 (JGY51), and msb3Δ msb4-R200K (JGY127) cells were grown in YPD media at 24°C and processed for electron microscopy. Please note that the appearance of vesicles varies from batch to batch due to possible variations in sample preparations. Bars, 0.5 μm. (B) Wild-type (YEF473), msb3Δ msb4Δ (YEF1631), MSB3 msb4Δ (JGY184), msb3-R282K msb4Δ (JGY190), msb3Δ MSB4 (JGY71), and msb3Δ msb4-R200K (JGY130) diploid cells were grown at 24°C and stained for F-actin.

Table II. The GAP activity of Msb3p is required for the suppression of the actin-organization defects in cdc42-Ts mutants

| Plasmids                  | YEF115 (cdc42–I) host strain | YEF2258 (cdc42–201) host strain |
|---------------------------|-----------------------------|---------------------------------|
|                           | (24°C) 0 min                | 38.5°C 5.3 h                    | (24°C) 0 min | 35.5°C 6 h |
|                           | Budded cells | Actin polarized | Budded cells | Actin polarized | Budded cells | Actin polarized | Budded cells | Actin polarized |
| YEplac181                 | 3                        | 8                             | 6             | 20              | 5             | 3              | 8             | 6              |
| YEp181-3HA-MSB3           | 4                        | 9                             | 42            | 75              | 6             | 4              | 48            | 38             |
| YEp181-3HA-MSB3-R282K     | 5                        | 10                            | 13            | 39              | 7             | 3              | 7             | 6              |
| YEp181-3HA-MSB1           | 4                        | 6                             | 29            | 60              | 7             | 3              | 20            | 19             |

The enriched, unbudded population of cells harboring indicated plasmids were grown in fresh media at indicated temperatures for the indicated lengths of time before assaying for the suppression of the budding and the actin-organization defects (see Materials and methods for detail). More than 600 cells were scored to determine the percentage of budded cells by DIC microscopy, and at least 200 cells were scored for actin organization through F-actin staining. Shown here is one set of representative results from three independent experiments performed in each cdc42-Ts host strain. Representative cells are shown in Fig. 5B.
32°C (Fig. 3 G), suggesting that the GAP activity of Msb3p and Msb4p is required for antagonizing the function of Sec2p. The arginine mutant of Msb3p also failed to suppress the synthetic lethality between sec4-79L and sec15–1 (Fig. 3 H), further supporting the notion that it is the GAP activity of Msb3p toward Sec4p, not merely the presence of Msb3p, that is responsible for the suppression.

The GAP activity of Msb3p and Msb4p is required for efficient exocytosis and polarized actin organization

To determine whether vesicle accumulation in msb3Δ msb4Δ cells was due to the absence of the proteins or the loss of their GAP activity, we examined two pairs of haploid strains, JGY184A (MSB3 msb4Δ) and JGY190A (msb3-R282K msb4Δ), and JGY51 (msb3Δ MSB4) and JGY127A (msb3Δ msb4-R200K). In most MSB3 msb4Δ or msb3Δ MSB4 cells, either no or just a few vesicles (usually <10 vesicles per cell section) were detected (Fig. 4 A, left). In contrast, most msb3-R282K msb4Δ or msb3Δ msb4-R200K cells accumulated a large number of vesicles similar to those observed in msb3Δ msb4Δ cells (Fig. 4 A, right). These data indicate that the loss of the GAP activity of Msb3p and Msb4p is responsible for vesicle accumulation.

Cells of msb3Δ msb4Δ strain are rounder in shape, heterogeneous in size, and have a partially disrupted actin cytoskeleton (Bi et al., 2000). Actin patches in these cells tended to delocalize into the mother side at early stages of the cell cycle when the patches should be predominantly concentrated in the buds (Fig. 4 B, compare columns 1 and 2). Actin cables were clearly present and largely well organized in msb3Δ msb4Δ cells. However, some cables appeared to be shorter, and sometimes misoriented in the mutant strain (Fig. 4 B, compare columns 1 and 2).

To determine whether the GAP activity of Msb3p and Msb4p is required for actin-patch organization, MSB3, msb3-R282K, MSB4, or msb4-R200K was integrated into the msb3Δ msb4Δ mutant at the msb3Δ or msb4Δ locus, respectively. The integrants with MSB3 or MSB4 showed normal cell morphology and actin-patch organization (Fig. 4 B, columns 3 and 5). Interestingly, the integrants with msb3-R282K or msb4-R200K displayed similar defects in cell morphology and actin-patch organization as the msb3Δ msb4Δ mutant did (Fig. 4 B, columns 4 and 6), suggesting that the GAP activity of Msb3p and Msb4p is required for actin-patch organization.

Multicopy MSB3 is known to suppress the growth defect of cdc42–1 cells at the nonpermissive temperature (Bi et al., 2000) (Fig. 5 A, top). We found that multicopy MSB3 also suppressed the growth defect of another cdc42–1 allele, cdc42–201 (Fig. 5 A, bottom). In addition, the budding and the actin-organization defects in both cdc42–1 Ts mutants were largely suppressed by multicopy MSB3 (Fig. 5 B) (Table II). Interestingly, multicopy msb3-R282K failed to suppress both the budding and the actin-organization defects of the two cdc42–1 Ts mutants (Fig. 5, A and B) (Table II), which were not defective in secretion per se as indicated by EM studies (Fig. 5 C). These results suggest that the GAP activity of Msb3p is required for the suppression of the actin-organization defects in cdc42 mutants. Together with the results described in the previous section, these data raise an intriguing question.
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possibility that polarized secretion may be normally involved in modulating polarized actin organization.

A defect in actin-patch organization can be a consequence of a primary defect in polarized secretion

The fact that the GAP activity of Msb3p is required to rescue both the secretory and the actin-patch-organization defects in msb3Δ msb4Δ cells raises the possibility that the actin-patch disorganization in msb3Δ msb4Δ cells might be a consequence of the fusion of mistargeted vesicles with the plasma membrane of the mother cells. To examine this possibility, we took advantage of mutants in TPM1 and TPM2, which encode two isoforms of tropomyosin that are required specifically for the formation of actin cables but not actin patches (Pruyne et al., 1998). When tropomyosins are conditionally inactivated, all actin cables are lost within one minute. As a result, secretory vesicles are no longer transported to the daughter cell, but instead fuse with the plasma membrane of the mother cell. After inactivation of tropomyosins for 30–60 min, actin patches become randomly distributed in both the mother and the daughter cells (Fig. 6, A and B) (Pruyne et al., 1998).

To test our hypothesis, we examined the distribution of actin patches in sec6–4 tpm1–2 tpm2Δ cells. At the restrictive temperature, secretory vesicles in this mutant are no longer delivered to the bud due to the loss of actin cables. In addition, vesicles in this mutant fail to fuse with the plasma membrane due to the inactivation of Sec6p, a component of the exocyst that is essential for vesicle tethering. We observed that, upon shifting to 36°C for 60 min, 51% of the triple mutant cells still displayed a polarized organization of actin patches in comparison to 88% of the tpm2Δ cells, 85% of the sec6–4 cells, and 0% of the tpm1–2 tpm2Δ cells (Fig. 6, A and B). These results suggest that the actin-patch disorganization in tpm1–2 tpm2Δ cells, and, by extrapolation, in msb3Δ msb4Δ cells, depends on the fusion of secretory vesicles in the mother cells with the plasma membrane.

One possible explanation for the polarized organization of actin patches in sec6–4 tpm1–2 tpm2Δ cells is that the lifespan of the “old patches” (existed before the temperature shift) in the buds of the small-budded cells is significantly increased. We measured the lifespan of actin patches in four different strains at two different temperatures, 20°C and 36°C, using Abp1p-GFP as a marker for the patches (Fig. 6, C and D). At 20°C, actin patches in all four strains displayed a similar lifespan, ~16 s. At 36°C, the lifespan of actin patches in both tropomyosin mutants (tpm2Δ and tpm1–2 tpm2Δ) were ~9 s. In contrast, the lifespan of actin patches in both mutants carrying sec6–4 (sec6 tpm1–2 tpm2Δ, and sec6–4) were ~21 s. We also observed that the lifespan of actin patches in the mother and the daughter compartments of the same cell were virtually identical for all four strains at both temperatures. These data suggest that blocking exocytosis at 36°C increases the lifespan of actin patches, but this increase alone is not sufficient to explain the polarized actin-patch organization in the sec6 tpm1–2 tpm2Δ mutant.

Discussion

Msb3p and Msb4p are involved in exocytosis by functioning as GAPs for Sec4p in vivo

The challenge for studying Rab GAPs is twofold. First, most Rab GAPs in yeast are not essential for cell viability. Cells
lacking a single known Rab GAP in yeast, Gyp1p, Mdr1p/Gyp2p, Gyp3p, Gyp6p, Gyp7p, or Gyp8p, produce no obvious defects in protein trafficking (Strom et al., 1993; Vollmer and Gallwitz, 1995; Du et al., 1998; Albert and Gallwitz, 1999; Vollmer et al., 1999; Du and Novick, 2001; De Antoni et al., 2002). Second, all known Rab GAPs show substrate promiscuity in vitro assays, and with the exception of Ypt1p-GAPs (Du and Novick, 2001; De Antoni et al., 2002), no evidence for their in vivo function has been obtained.

We took multiple approaches to assess whether Msb3p and Msb4p are Sec4p-specific GAPs in vivo. First, deletion of MSB3 and MSB4 together, more specifically, inactivation of the GAP activity of Msb3p and Msb4p caused a defect in exocytosis. Second, msb3Δ msb4Δ cells displayed synthetic interactions with late secretory mutants. Third, multicopy MSB3 suppressed the synthetic lethality of a sec4-Q79L sec15–1 mutant, presumably, by reducing the level of GTP-bound Sec4p. Fourth, multicopy MSB3 or MSB4 inhibited the growth of sec2–41 cells, but not of any other late sec mutants. Finally, among the 11 Rab GTPases in S. cerevisiae, only Sec4p shares a localization profile and mutant phenotype (post-Golgi vesicle accumulation and exocytosis defect) with Msb3p and Msb4p. We further demonstrate here that Msb3p and Msb4p most likely act on Sec4p by an arginine finger–like mechanism. Together, these results provide compelling arguments for the involvement of Msb3p and Msb4p in exocytosis by functioning as GAPs for Sec4p in vivo.

Deletion of MSB3 and MSB4, presumably leading to a higher level of Sec4p-GTP in the cell, caused significant accumulation of 100-nm vesicles and of the endoglucanase Bgl2p in the cell, but produced little effect on invertase secretion. A similar effect on invertase secretion is also observed in a strain carrying sec4-Q79L, in which Sec4p is predominantly in the GTP-bound form (Walworth et al., 1992). These data suggest that a defect in GTP hydrolysis by Sec4p affects secretion of different cargoes differentially, which is consistent with a previous report that there are at least two distinct populations of post-Golgi vesicles accumulated in late sec mutants: a minor population carries invertase, whereas the major population carries Bgl2p (Harsay and Bretscher, 1995). Similar differential effect on secretion is also observed in a cdc42-Ts mutant (Adam et al., 2001). Because Msb3p, Msb4p, and Cdc42p are all involved in polarized growth and because Msb3p and Msb4p interact with Cdc42p genetically (Bi et al., 2000) and biochemically (unpublished data), it is possible that one role of Msb3p, Msb4p, and Cdc42p in polarized growth is to regulate secretion of Bgl2p, a cell wall–remodeling enzyme that is needed during cell-surface expansion.

The GAP activity of Msb3p and Msb4p and its implication in spatial regulation of exocytosis

Our EM studies indicate that in most msb3 msb4 mutant cells, vesicles are distributed randomly within the cell, and in a few cells, vesicles are preferentially localized to the daughter cell. This pattern of vesicle accumulation would be consistent with a defect in vesicle transport and/or tethering. Vesicle transport from Golgi membrane to bud tip requires the function of vesicle-associated Sec4p-GTP, whose formation is catalyzed by the vesicle-associated GEF Sec2p, presumably using the cytosolic pool of Sec4p-GDP as the substrate. Vesicle tethering requires the function of Sec4p-GTP and its effector, the exocyst (Guo et al., 1999). In the absence of Msb3p and Msb4p, Sec4p would be primarily in the GTP-bound form and less Sec4p-GDP would be recycled back to the cytosol. This raises the question as to how an alteration in the ratio of GTP-bound versus GDP-bound Sec4p results in vesicle accumulation. We imagine two major scenarios. First, the decreased recycling of Sec4p-GDP to the cytosol in msb3 msb4 mutant cells could be responsible for the vesicle accumulation. In this case, less Sec4p-GDP from the cytosol is recruited to the Golgi site to be converted to Sec4p-GTP on Golgi membranes and/or vesicles. Consequently, the efficiency of vesicle transport is compromised. The second scenario is that the increased amount of Sec4p-GTP in msb3 msb4 mutant cells could be responsible for the vesicle accumulation. In this case, the increased level of Sec4p-GTP might hold the exocyst in place for longer periods of time so that reduced recycling of exocyst components would cause a defect in additional rounds of vesicle tethering. It is also possible that the disassembly of the vesicle-tethering complex is a prerequisite for the formation of the trans-SNARE complexes between Golgi membrane–derived vesicles and the plasma membrane, which leads to vesicle fusion. Because the assembly of the exocyst depends on Sec4p-GTP (Guo et al., 1999), its disassembly may depend on the hydrolysis of Sec4p-bound GTP. Thus, a deficiency in GTP hydrolysis may cause a secretory defect by indirectly preventing efficient formation of the SNARE complexes.

To distinguish between the two scenarios, we reasoned that, if the Sec4p-GDP recycling is the key, multicopy wild-type SEC4 should suppress the morphological defect of the msb3Δ msb4Δ sec4-Q79L mutant, because more Sec4p-GDP would be generated in the cell due to the intrinsic GTPase activity of the wild-type Sec4p. In contrast, if the absolute amount of Sec4p-GTP is critical, multicopy SEC4 should exacerbate the morphological defect of the msb3Δ msb4Δ sec4-Q79L mutant, because more Sec4p-GTP would be produced in the cell due to the increased concentration of Sec4p protein. We found that multicopy SEC4 suppressed the morphological defect of the triple mutant reasonably well (unpublished data), thus favoring the first scenario. However, this result cannot rule out the second scenario with certainty.

The cellular locations of the GEF and the GAPs for Sec4p provide direct clues on how the activity of Sec4p, and hence the exocytosis, is spatially regulated (Fig. 7 A). Sec2p, the GEF for Sec4p, colocalizes with Sec4p on the secretory vesicles. This colocalization is thought to ensure Sec4p in its GTP-bound form and less Sec4p-GDP would be recycled back to the cytosol. This raises the question as to how an alteration in the ratio of GTP-bound versus GDP-bound Sec4p results in vesicle accumulation. We imagine two major scenarios. First, the decreased recycling of Sec4p-GDP to the cytosol in msb3 msb4 mutant cells could be responsible for the vesicle accumulation. In this case, less Sec4p-GDP from the cytosol is recruited to the Golgi site to be converted to Sec4p-GTP on Golgi membranes and/or vesicles. Consequently, the efficiency of vesicle transport is compromised. The second scenario is that the increased amount of Sec4p-GTP in msb3 msb4 mutant cells could be responsible for the vesicle accumulation. In this case, the increased level of Sec4p-GTP might hold the exocyst in place for longer periods of time so that reduced recycling of exocyst components would cause a defect in additional rounds of vesicle tethering. It is also possible that the disassembly of the vesicle-tethering complex is a prerequisite for the formation of the trans-SNARE complexes between Golgi membrane–derived vesicles and the plasma membrane, which leads to vesicle fusion. Because the assembly of the exocyst depends on Sec4p-GTP (Guo et al., 1999), its disassembly may depend on the hydrolysis of Sec4p-bound GTP. Thus, a deficiency in GTP hydrolysis may cause a secretory defect by indirectly preventing efficient formation of the SNARE complexes.

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A defect in polarized secretion can lead to a defect in polarized actin organization

Our studies led to two interesting findings. First, the GAP activity of Msb3p and Msb4p is required for efficient exocytosis and actin-patch organization. Second, the GAP activity of Msb3p is required for the suppression of the actin-organization defects in cdc42–1 and cdc42–201 cells. These observations raise an intriguing question: could a defect in polarized secretion cause a defect in actin organization? In 

| A | B|
|---|---|
| **A. Spatial regulation of exocytosis** | **B. Coupling exocytosis to actin patch organization** |
| ![Diagram A](image1.png) | ![Diagram B](image2.png) |

Figure 7. Models on the spatial regulation of exocytosis and on the coupling of exocytosis to actin-patch organization. (A) Role of Msb3p and Msb4p in spatial regulation of exocytosis. Secretory vesicles carrying Sec4p-GTP, Sec2p (GEF for Sec4p), and actin-patch clustering and/or assembly factor X are delivered to the bud tip by Myo2p (a type V myosin) along actin cables. Msb3p and Msb4p at the bud tip stimulate the hydrolysis of Sec4p-bound GTP, leading to the recycling of Sec4p for next round of exocytosis. The cargo molecule X, which could include the polarity proteins Cdc42p, Rho1p, and the actin-binding protein Aip3p/Bud6p, is delivered to the bud tip to reinforce the polarized organization of actin patches, which, in turn, may be involved in the recycling of the exocytic machinery such as the exocyst for next round of secretion. (B) Fusion of an exocytic vesicle with the plasma membrane leads to the formation and/or trapping of an actin patch at the fusion site. In msb3Δ msb4Δ cells, vesicles that are not transported out of the mother compartment can fuse with the plasma membrane (not depicted in the diagram). Vesicles that are not tethered at the bud tip (green arrows) can diffuse into the mother side and fuse with the plasma membrane, depositing their cargos, including factor X, at the fusion sites for the generation and/or trapping of actin patches. In a tropomyosin mutant (black arrow), the vesicles are not transported to the bud and thus fuse with the plasma membrane of the mother compartment, leading to the reorganization of actin patches as described for msb3Δ msb4Δ cell.

brane, causing depolarized growth. Meanwhile, actin patches are gradually reorganized from the small buds to the entire cell cortex (Pruyne et al., 1998). However, when the actin cables and the vesicle-tethering/fusion process are simultaneously inactivated in the sec6–4 tpm1–2 tpm2Δ cells, actin patches remain in the small buds. This result suggests that the reorganization of actin patches in tpm1–2 tpm2Δ cells depends on ongoing exocytosis and, likely, endocytosis, both of which are defective in sec6–4 and all other late sec mutants (Riezman, 1985).

Actin-patch polarization in the small buds of the sec6–4 tpm1–2 tpm2Δ cells at 36°C can be explained by one of the three possibilities: patch motility is blocked; the lifespan of the patches is increased dramatically; and the putative patch-clustering factor and/or patch-assembly factor remains in the small bud in the absence of continuous exocytosis and endocytosis. Our data support the third possibility.

Patch motility is unlikely to be the answer. First, patches are highly labile structures with a life span of ~10 s (Smith et al., 2001; Carlsson et al., 2002). Second, most patches display random motion and a few display directed motion. Third, patches have an average speed of 0.49 ± 0.30 μm/s. Together, most patches would have disassembled before they can cross the bud neck. Thus, it has been concluded that the organization of actin patches is due to the assembly of the patches at the sites of polarized growth (Smith et al., 2001).

The lifespan of actin patches in different mutants cannot explain the observed phenotype either, because the lifespan of the patches in sec6–4 tpm1–2 tpm2Δ cells increases only two-fold over that in tpm1–2 tpm2Δ cells at 36°C.

The patch polarization in sec6–4 tpm1–2 tpm2Δ cells and patch organization in general can be explained by assuming that secretory vesicles carry factors that are required for actin-patch clustering and/or assembly; and that local concentration of these factors depends on the balance of exocytosis and endocytosis (Fig. 7 B). During bud growth of wild-type cells, actin-patch polarization occurs over endocytosis; these factors accumulate dynamically at the sites of polarized growth, leading to polarized actin-patch organization. In the tropomyosin mutants, these putative factors are deposited over the entire cell surface through ongoing depolarized exocytosis, and the high concentration of these factors at the “old buds” is eliminated through active endocytosis, which is not blocked in these mutants (Pruyne et al., 1998); thus, leading to the reorganization of actin patches. In the sec6–4 tpm1–2 tpm2Δ and sec6–4 mutants, exocytosis and endocytosis are blocked together; the high concentration of the putative factors in the old buds remains; thus, actin patches are still polarized, and are still going through their dynamic assembly and disassembly cycle. These putative factors could include polarity proteins such as Cdc42p and Rho1p, and actin-binding proteins such as Aip3p/Bud6p, because their accumulation at the active growth sites depends on intact secretory pathway (McCaffrey et al., 1991; Jin and Amberg, 2000; Wedlich-Soldner et al., 2003).

Our finding on the cause-effect relationship between vesicle fusion and the organization of actin patches has profound biological implications. Exocytosis and endocytosis are intimately coupled in many biological systems including S. cerevisiae (Riezman, 1985), Drosophila (Roos and Kelly, 1999), and...
mammals (Sudhof, 2000), but the mechanisms are unclear. In S. cerevisiae, many components of the actin patches are required for the internalization step of endocytosis (Munn, 2000); thus, our finding could provide a concrete means to spatially link exocytosis to endocytosis (Fig. 7, A and B): fusion of the exocytic vesicles with the plasma membranes leads to the clustering of actin patches at the fusion site, which mediates endocytosis to recycle the exocytic machinery. This finding also explains why the actin patches and the ends of the actin cables, which mediate exocytosis, are always in close proximity with each other (Karpova et al., 1998).

Materials and methods

Strains and media
Yeast strains used in this study are listed in Table III. Standard culture media and genetic techniques were used (Guthrie and Fink, 1991). 1 mg/ml 5-fluoroorotic acid (5FOA) (Angus Buffers and Biochemicals) was added to media to select for the loss of URA3-containing plasmids.

Construction of plasmids and DNA mutagenesis
Plasmids used in this study include YEp181 (2 μ, LEU2), pRS316 (CEN, URA3), pFA6a-kanMX6 (Longtine et al., 1998), pYES2-MSB3 (Albert and Pringle, 1996), and pRS423-SEC4 (Bi and Pringle, 1996). pFA6a-kanMX6 (Longtine et al., 1998), YEp181–3HA-MSB3 and YEp181–3HA-MSB4 (Bi et al., 2000), and pRS316-SEC4 (2 μ, HIS3). Plasmids YEp181-SEC4 and pRS316-SEC4 were constructed by inserting a 1.5-kb BamHI-EcoRI fragment carrying SEC4 from pRS423-SEC4 into YEp181 and pRS316 at the corresponding sites, respectively.

Table III. Yeast strains used in this study

| Name     | Genotype                        | Source                        |
|----------|---------------------------------|-------------------------------|
| YEF473   | a/α his3/3 his3 leu2/2 lys2/2 trp1/trp1 ura3/ura3 | (Bi and Pringle, 1996)         |
| YEF473A  | a his3 leu2/2 trp1 ura3         | Segregant from YEF473         |
| YEF115   | a his4 leu2/2 trp1 ura3 gal2/cdc42-1 | J. Pringle                    |
| YEF1264  | As YEF473 except MSB3/MSB3Δ::HIS3 MSB4/MSB4Δ::TRP1 | (Bi et al., 2000)             |
| YEF1289  | a his3 leu2/2 trp1 ura3/3 his3 msb4Δ::TRP1 | (Bi et al., 2000)             |
| YEF1291  | a his3 leu2/2 trp1 ura3 msb3Δ::HIS3 msb4Δ::TRP1 | (Bi et al., 2000)             |
| YEF1563  | As YEF473 except MSB3/MSB3Δ::HIS3 MSB4/MSB4Δ::GIC1/gic1-Δ1::LEU2/GIC2/gic2-Δ2::TRP1 | YEF1269 × CCY1042-12B |
| YEF1619  | As YEF473 except MSB3/MSB3Δ::HIS3 MSB4/MSB4Δ::TRP1 | YEF1239 × YEF1304             |
| YEF1631  | As YEF473 except MSB3/MSB3Δ::HIS3 MSB4/MSB4Δ::TRP1 | (Bi et al., 2000)             |
| YEF2258  | a his3 leu2/2 trp1 ura3 cdc42-201 | (Zhang et al., 2001)          |
| ABY971   | As ABY973 except tpn1-2::LEU2 | (Pruyne et al., 1998)         |
| ABY973   | a/α tpn1-2::HIS3/tpn1-2::HIS3 his3Δ200/his3Δ200 leu2-3,112/leu2-3,112 lys2-801/lys2-801 trp1-1/trp1-1 ura3/ura3-52/ura3-52 | (Prueyne et al., 1998)         |
| ABY999   | As ABY973 except tpn1-2::LEU2 | P. Brenwald                   |
| BY37     | a ura3-52 sec6-4                 | P. Brenwald                   |
| BY45     | a ura3-52 sec6-15-1             |                               |
| JGY10    | a his3 leu2/2 trp1 ura3 msb3Δ::HIS3 msb4Δ::HIS3 gic1-Δ1::kanMX6 gic2-Δ2::TRP1 (pCC904-GIC1) | This study                     |
| JGY18    | a his3 leu2/2 trp1 ura3 msb3Δ::HIS3 msb4Δ::TRP1 cdc42-201 (pRS316-CDC42) | Segregant from JGY13          |
| JGY22B   | a his3 leu2/2 trp1 ura3 sec6-2   | This study                     |
| JGY30A   | a his3 leu2/2 trp1 ura3 sec6-4   | This study                     |
| JGY31B   | a his3 leu2/2 trp1 ura3 sec9-4   | This study                     |
| JGY32B   | a his3 leu2/2 trp1 ura3 sec3-2   | This study                     |
| JGY37B   | a his3 leu2/2 trp1 ura3 sec3-2   | This study                     |
| JGY39A   | a his3 leu2/2 trp1 ura3 sec6-4   | This study                     |
| JGY40A   | a his3 leu2/2 trp1 ura3 sec9-4   | This study                     |
| JGY48A   | a his3 leu2/2 trp1 ura3 sec6-4   | This study                     |
| JGY51    | a msb3Δ::HIS3 msb4Δ::3HA-MSB4 his3 leu2/2 trp1 ura3 | This study                     |
| JGY71    | As YEF473 except msb3Δ::HIS3/MSB4::3HA-MSB4::msb4Δ::3HA-MSB4 | This study                     |
| JGY73    | a his3 leu2/2 trp1 ura3 sec4-3Q79L | This study                     |
| JGY82B   | a his3 leu2/2 trp1 ura3 sec15-1 | This study                     |
| JGY86A   | a his3 leu2/2 trp1 ura3 sec15-1 sec4-3Q79L (pRS316-SEC4) | This study                     |
| JGY127A  | a msb3Δ::HIS3 msb4Δ::3HA-MSB4::R200K his3 leu2/2 trp1 ura3 | This study                     |
| JGY130   | As YEF473 except msb3Δ::HIS3/MSB4::msb4Δ::3HA-MSB4::R200K/msb4Δ::3HA-MSB4::R200K | This study                     |
| JGY184A  | a msb3Δ::3HA-MSB3::msb4Δ::TRP1 his3 leu2/2 trp1 ura3 | This study                     |
| JGY184   | As YEF473 except msb3Δ::3HA-MSB3/MSB4::TRP1 his3 leu2/2 trp1 ura3 | This study                     |
| JGY190A  | a msb3Δ::3HA-MSB3-R282K/3HA-MSB3-R282K his3 leu2/2 trp1 ura3 | This study                     |
| JGY190   | As YEF473 except msb3Δ::3HA-MSB3-R282K/3HA-MSB3-R282K his3 leu2/2 trp1 ura3 | This study                     |
| JGY381   | a/α his3/3 his3 leu2/2 leu2 ura3/3 ura3 TRP1/trp1 sec6-4/sec6-4 | This study                     |
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Ts mutants, cells grown exponentially at 24°C were shifted to restrictive temperatures for 1 h, and quickly put under an objective lens heated to 36°C for 30 min, which was followed by standard dehydration. Cell pellets were embedded in Spurr’s resin. Thin sections were cut and processed for electron microscopy. Cells were viewed with a JEOL 1010 electron microscope and photographed at 80 kV. The size of vesicles was measured at 100,000× magnification.

Invertase secretion assay
Yeast cells were grown to exponential phase in YPD media containing 5% dextrose at 24°C. Approximately 1.5-A600 units of cells were collected, washed twice with YPD media containing 0.1% dextrose, and resuspended in 1.5 ml of the same media. Secretion of invertase was induced for various time at 24°C. Cells from each time point were washed with and resuspended in 1 ml of ice-cold 10 mM NaN4 solution. A600 of this cell suspension was measured. 20 μl of cell suspension was directly applied to assay medium on ice for 10 min at 37°C. A600 was used to assay internal pool of invertase. The external and internal pools of invertase activity were assayed by following the protocol described by Adamo et al. (1999).

Bgl2p secretion assay
Yeast cells were grown to midlog phase at 24°C. Half of the culture was kept at 24°C and the other half was shifted to 37°C for 1 h. At the end of shift, NaN4 and NaF were added to ~20 mM each to all cultures. 25-A600 units of cells from each culture were washed in 20 ml NaN4/20 mM NaF twice and resuspended in 830 μl of spermidine solution (100 mM Tris-HCl, pH 7.5, 1.6 M sorbitol, 12 mM NaN4, 0.1% β-mercaptoethanol, 200 μg/ml zymolyase 100-T). Spheroplasts were gently pelleted at 2,000 rpm for 5 min. The top 830 μl of the supernatant (external Bgl2p pool) was transferred to a new tube and 170 μl of 6X sample buffer (0.35 M Tris-HCl, pH 6.8, 30% glycerol, 10% SDS, 93 mg/ml DTT, 0.1 mg/ml bromophenol blue) was added to it, and the samples were boiled immediately for 10 min. The pellet (internal Bgl2p pool) was resuspended in 6X sample buffer and boiled for 10 min. 50 μl of samples were separated by a 12.5% SDS-polyacrylamide gel, probed with a rabbit α-Bgl2p antibody, and detected by ECL.

Determination of the organization and life span of actin patches
To determine the lifespan of actin patches, plasmid pBR2139 carrying an NH2-terminal tagged GFP-TUB1 was linearized by Sgrl and integrated at the arac locus in yeast strains ABY971, ABY973, ABY999, and JCY381, respectively. The resulting strains were inoculated in 25 ml of SC-Ura media and incubated at 36°C until ~50% cells from the positive samples became budded. Cells were then fixed with 4% formaldehyde at the restrictive temperatures for 15 min. For 4°C controls, the remaining enriched cells were fixed in 4% formaldehyde at 24°C for 1 h. Fixed cells were stained for F-actin and DNA.

Enrichment of un budded cells
Cdcl42-Ts mutant cells harboring YEpplac181-based plasmids were grown on SC-Leu plates at 24°C for 4 d. Cells were then scraped off plates and resuspended in 25 ml of 1 M sorbitol + 50% SC-Leu. Unbudded cells were enriched by repeated centrifugation at 800 rpm for 1 min until more than 90% of cells was unbudded. 5–10-A600 units of the enriched cells were transferred into 40 ml SC-Leu media and incubated at restrictive temperatures until ~50% cells from the positive samples became budded. Cells were then fixed with 4% formaldehyde at the restrictive temperatures for 15 min.

Electron microscopy
Yeast cells were grown in YPD media to early log phase at 24°C. For cdcl42-Ts mutants, cells grown exponentially at 24°C were shifted to restrictive temperatures for 1 h, and were prefixed with 1.6% of glutaraldehyde in culture for 10 min. 10–20-A600 units of fixed cells were used in 1 ml fixative (2% glutaraldehyde in PBS buffer, pH 7.4) at 24°C for 30 min, which was followed by additional 30 min with fresh fixative. Cells were then spheroplasted and fixed with 1% glutaraldehyde in PBS buffer, pH 7.4 at 4°C overnight. Spheroplasts were washed in 0.1 M cacodylate buffer and postfixed twice with ice-cold solution containing 0.5% OsO4 and 0.8% potassium ferricyanide on ice for 10 min each time. Spheroplasts were then washed with distilled water and incubated in 2% uranyl acetate at 24°C for 30 min in dark, which was followed by standard dehydration. Cell pellets were embedded in Spurr’s resin. Thin sections were cut and processed for electron microscopy. Cells were viewed with a JEOL 1010 electron microscope and photographed at 80 kV. The size of vesicles was measured at 100,000× magnification.

Production of Msb3p and Msb4p proteins and measurement of their GAP activity
Production of His6-tagged Msb3p and its arginine mutants from yeast, and His6-tagged Msb4p and its arginine mutants from E. coli, and measurement of the GAP activity were all performed as described previously (Albert and Gallwitz, 1999; Albert and Gallwitz, 2000).

Immunoblotting and immunofluorescence microscopy
For immunoblotting, the mouse monoclonal anti-HA antibody HA.11 (Berkley Antibody Company), or the rabbit anti-Isp42p polyclonal antibodies were used. Proteins were detected with the ECL Western blotting detection reagents.

For localization of HA-tagged Msb3p or Msb4p, yeast cells grown exponentially in SC-Leu media at 24°C were fixed for formaldehyde and processed for immunofluorescence microscopy as described by Pringle et al. (1991). Mouse anti-HA antibody HA.11 and the secondary Cy3-conjugated donkey anti-mouse IgG antibody were used. For visualizing the actin cytoskeleton, yeast cells were fixed for 4% formaldehyde for 1 h at 24°C and stained with rhodamine-phalloidin (Molecular Probes). DNA was stained with 1 μg/ml bisBenzimidze (Sigma-Alrich). Differential interference contrast (DIC) and fluorescence microscopy were performed using a Nikon Microscope ECLIPSE E800 (Nikon Corporation) with a 60x plan apo objective. The images were acquired using Image-Pro Plus software (Media Cybernetics).

Msb3p and Msb4p in exocytosis and actin organization
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