Regulation of Cell Polarity by Interactions of Msb3 and Msb4 with Cdc42 and Polarisome Components

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In Saccharomyces cerevisiae, polarized growth depends on interactions between the actin cytoskeleton and the secretory machinery. Here we show that the Rab GTPase-activating proteins (GAPs) Msb3 and Msb4 interact directly with Spa2, a scaffold protein of the “polarisome” that also interacts with the formin Bni1. Spa2 is required for the polarized localization of Msb3 and Msb4 at the bud tip. We also show that Msb3 and Msb4 bind specifically to Cdc42-GDP and Rho1-GDP in vitro and that Msb3 and Rho GDP dissociation inhibitor act independently but oppositely on Cdc42. Finally, we show that Msb3 and Msb4 are involved in Bni1-nucleated actin assembly in vivo. These results suggest that Msb3 and Msb4 regulate polarized growth by multiple mechanisms, directly regulating exocytosis through their GAP activity toward Sec4 and potentially coordinating the functions of Cdc42, Rho1, and Bni1 in the polarisome through their binding to these GTPases. A functional equivalent of the polarisome probably exists in other fungi and mammals.

Cell polarity is essential for the development and differentiation of most unicellular and multicellular organisms. Core mechanisms underlying cell polarity appear conserved from yeasts to humans at both the conceptual and the molecular level (46). In the budding yeast Saccharomyces cerevisiae, polarized cell growth is achieved through a multistage process. The cell first selects a cortical site for cell polarization, then marks that site with polarity establishment proteins, and finally polarizes the actin cytoskeleton, including the actin cables and patches, at the marked site. The actin cables then direct secretion to the chosen site to form a bud (53, 54).

Cdc42, a conserved Rho GTPase, affects polarized actin organization and secretion in both S. cerevisiae and mammalian cells (2, 3, 32, 36, 45, 49, 50, 72). In S. cerevisiae, Cdc42 plays an essential role in polarity establishment (31, 54). Conditional inactivation of Cdc42 with temperature-sensitive mutations in CDC42 or CDC24, which encodes the guanine nucleotide exchange factor (GEF) for Cdc42, results in complete loss of polarized actin organization and secretion (3, 64, 75). The role of Cdc42 in polarized secretion is mediated by its role in actin organization as well as by its direct interaction with the secretory machinery (2, 72).

Genetic studies suggest that Cdc42 controls polarized growth by at least two parallel pathways, an S. cerevisiae-specific (Gic1/Gic2) pathway and an evolutionarily conserved (Bni1) pathway (9). Gic1 and Gic2, a pair of structurally related Cdc42 effectors unique to S. cerevisiae, are implicated in polarized growth, because deletion of both genes together causes temperature-sensitive growth, with cells arrested as large, round, and unbudded cells at the restrictive temperature (12, 14). Bni1, another effector of Cdc42 and other small GTPases (15, 18, 35), is a member of the formin family that is involved in actin cable formation in S. cerevisiae (19, 60). The binding of the Rho GTPases to Bni1 is thought to relieve an autoinhibitory loop formed between the N-terminal and C-terminal portions of Bni1 such that the C-terminal fragment including the FH2 domain is able to nucleate actin cable formation (15, 55, 61). Deletion of BNI1 does not cause cell lethality but affects polarized growth by eliminating the formation of bud tip-oriented actin cables (18, 35, 56, 60). However, deletion of BNI1 and GIC1 GIC2 together causes cell lethality at all temperatures (9).

Polarisome is a protein complex that affects polarized growth in S. cerevisiae, but the underlying mechanisms are not clear. Polarisome consists of Spa2, Bni1, Bud6, Pea2, and perhaps additional proteins (63). Spa2 interacts with all the known components through distinct domains and is thus considered a scaffold protein of the polarisome (63, 67). The formin Bni1 binds directly to the C-terminal, SHD-V region of Spa2 (21, 59) (see Fig. 1A). Bud6 is an actin monomer-binding protein that promotes Bni1-stimulated actin assembly in vitro (6, 44). The biochemical function of Pea2 is not known (66). All components of the polarisome localize to the sites of polarized growth. Deletion of each component causes a defect in bipolar bud site selection and a moderate defect in polarized growth at all temperatures, resulting in rounder cells, and cells with any of these components deleted show synthetically lethal interaction with gic1Δ gic2Δ cells (30, 39, 62, 63, 66, 70). These data suggest that the polarisome and Gic1/Gic2 may act in parallel to control polarized growth.

Msb3 and Msb4 are members of the TBC/PTM/GYP protein family, which is characterized by a Rab GTPase-activating protein (GAP) domain (4, 5, 9, 42, 47, 71). Indeed, Msb3 and Msb4 display GAP activity toward the Rab GTPase Sec4 in vitro and in vivo, and this GAP activity is required for efficient exocytosis (4, 5, 22). Increased dosages of Msb3 and Msb4 suppress cdc42 and calc42 mutations (9); thus, Msb3 and Msb4 are well positioned to coordinate Rho and Rab functions. The
behavior of Msb3 and Msb4 in the cell cycle is very similar to that of the polarisome components (9). Both Msb3 and Msb4 localize to the sites of polarized growth; deletion of both genes together causes a defect in bipolar bud site selection and a moderate defect in polarized growth; and msb3Δ msb4Δ cells are synthetically lethal with gic1Δ (to be described elsewhere) (forward primer, 5'-CGAAAATCGTGACAGGTC

TABLE 1. Yeast strains used in this study

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**MATERIALS AND METHODS**

**Strains and growth conditions.** Yeast strains used in this study are listed in Table 1. Standard culture media and genetic methods were used (25). *Escherichia coli* strains DH12S (Life Technologies, Gaithersburg, MD) and BL21 (Invitrogen Life Technologies, Carlsbad, CA) were used as hosts for plasmid manipulation and recombinant protein expression, respectively.

**Plasmid and strain constructions.** All the two-hybrid plasmids carrying different fragments of *SPA2* fused to the LexA DNA binding domain (BD) were kindly provided by Michael Snyder (Yale University) (63). The activation domain (AD)-containing plasmids used in the experiment for which results are shown in Fig. 1B were pG4-5-MSB3, pG4-5-MSB3-N220, pG4-5-MSB4, and pG4-5-MSB3-N144, which were constructed by PCR amplifying and cloning full-length *MSB3*, the 5' end fragment of *MSB3* (encoding the first 220 amino acids [aa]), full-length *MSB4*, or the 5' end fragment of *MSB4* (encoding the first 144 aa), respectively, into pG4-5 (2 μm TRP1 pGAL1) (20). All the maltose-binding protein (MBP) gene fusions, including MBP-MSB3, MBP-MSB3-R282K, MBP-MSB3-N (aa 1 to 220), MBP-MSB4, MBP-MSB4-N (aa 1 to 1444), MBP-PXL1, and MBP-GHC2, were constructed by PCR amplifying and cloning the full-length genes indicated or fragments of those genes into the pMAL-c2 vector (New England Biolabs, Beverly, MA). The glutathione S-transferase (GST)-SPA2 fusion (encoding the first 150 aa of Spa2) was constructed by cloning a 5' end fragment of *SPA2* into the pGEX-4T-1 vector (Amersham Biosciences Corp., Piscataway, NJ). Plasmids pEGKT-MSB3 and pEGKT-MSB4 were constructed by cloning PCR-amplified full-length MSB3 and MSB4 under the control of the pGAL1 promoter into the pEGKT vector (2 μm UR43) (43). Plasmid Yep181-GFP-MSB3 (2 μm LEU2) was constructed by replacing the 3'4 fragment in Yep181-3HA-MSB3 (9) with an in-frame green fluorescent protein (GFP) fragment. Plasmid YepGFP* pBD8 (2 μm LEU2) carries BUD8 fused to an enhanced version of GFP (27). Plasmids expressing recombinant GST-Cdc42, GST-Rho1, GST-Rho3, and GST-Rho4 in E. coli were kindly supplied by Wei Guo (University of Pennsylvania). Plasmids pYES2-MSB3 and pYES2-MSB3::R282K (22) carry pGAL1-controlled MSB3 and MBP [GST], respectively, cloned into pYES2 (2 μm UR43) (Invitrogen). Plasmid Yep181-3HA-MSB3-R282K (2 μm LEU2) carries hemagglutinin (HA)-tagged mb3ΔR282K (22). Plasmid pRS316-GAL1-CDC24 (CEN UR4) carries a pGAL1-controlled CDC24. Plasmids Yep352-GAL1-3pBD-BNI1 (2 μm UR43) and Yep352-GAL1-GST-BNI1 (2 μm UR43) carry pGAL1-controlled GFP-tagged or GST-tagged BNI1, respectively. Different alleles of BNI1 (W1363A and W1374A; K1601D; W1363A, W1374A, and K1601D (see Fig. 4D) were introduced into Yep352-GAL1-GFP-BNI1 using standard PCR-based site-directed mutagenesis. The RD1 gene was PCR amplified from yeast genomic DNA (primers CGCGCGATCCATGGCCGAAGAATGACGAGCTTA and CGCGCCGCGACCTACATTGTGCATTTCAAGAAGA) with Klenow fill-in and cloned into pGEX-2TP (kindly provided by Wei Guo’s laboratory at the University of Pennsylvania) to generate plasmid pGAL1-TRP1. The correct fitness of all the plasmid-based constructions was confirmed by DNA sequencing. All oligonucleotide primers were purchased from Integrated DNA Technologies (Corvalle, IA). Sequences of these primers and details of the plasmid constructions are available upon request.

Complete deletions or tagged versions of SPA2 or tagged versions of BUD6 or PE2 were constructed by using a PCR-based method (38) in either the Yep473 background (11), generating strains YEF4264 (a spa2Δ::His3), YEF2482 (a spa2Δ::TRP1), YEF3758 (a SPA2::MYC-HIS3), YEF3760 (a SPA2::MYC-HIS3 PE2A::HA-TRP1), and YEF3761 (a SPA2::MYC-HIS3 PE2A::HA-TRP1), or the Yep319 background (a cdc24-11), generating strain Yep3759 (a cdc4-11) (11), and Yep3761 (a cdc4-11 ribi::KANMX) was constructed by PCR amplifying the ribi::KANMX locus from a strain in the Research Genetics collection into Yep319.

Strain JGY1290 (spa2Δ::150) was constructed as follows. First, a pair of primers was used to PCR amplify the fusion of Spa2 to a Cen4-kanMX6-URA3 (Masa1243 (a myo1α::UR3-KanMX) (to be described elsewhere) (forward primer, 5'-CGAAAAATCGTGACAGGTC 

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the template. The PCR product was digested with BamHI and SalI enzymes (the restriction sites were included in the PCR primers) and cloned into pBlueScript KS(+) (Stratagene, La Jolla, CA) at the corresponding sites, resulting in pBS-SPA2. Third, a pair of primers corresponding to the regions upstream of the 2nd and downstream of the 150th codon of SPA2 (forward, 5′-5′/Sphi/AACAT CACAAATCTCTCCTGACTAACA-3′; reverse, 5′-5′/Sphi/CATGTATAC TCTTCTTTCCTGTTATCTG-3′) was used to amplify pBS-SPA2. The PCR product was treated with T4 DNA polymerase and blunt end ligated to generate plasmid pBS-SPA2-Δ150, carrying the spa2-Δ150 allele, in which the coding sequence for amino acids 2 to 150 of SPA2 is deleted. Fourth, pBS-SPA2-Δ150 was digested with BamHI and SalI, and the digestion mixture was transfected into YEp4048 yeast. A “kan” colonies were checked by PCR and sequencing to confirm the correctness of the allele replacement in strain JOY1290.

**GST pulldown assay.** Strain YEF5760 (a SPA2-MYC-HIS3 BUD6-HA-TRP1) or YEF3761 (a SPA2-MYC-HIS3 PICH1-LA-HA-TRP1) carrying the pEGKT, pEGKT-MSB3, or pEGKT-MSB4 vector was grown to exponential phase in synthetic complete medium without uracil (SC-Ura medium) containing 2% rafinose at 30°C. Galactose was added to each culture to a final concentration of 3% to induce expression of the GST fusions for 4 h. Cells were then harvested by centrifugation and washed with sorbitol buffer (100 mM NaCl, 10 mM MgCl2, 5 mM DTT, and 0.1% Triton X-100) at 4°C for 1 h. The beads were washed five times with the sorbitol buffer containing 0.2% NP-40, and proteins bound to the beads were eluted with sodium dodecyl sulfate (SDS) sample buffer. Samples were then analyzed by 7.5% SDS-polyacrylamide gel electrophoresis (PAGE), followed by a standard immunoblotting procedure using enhanced chemiluminescence reagents. Primary antibodies were mouse monoclonal antibodies against GST, MYC, and HA (Covance Research Products, Richmond, CA) and a rabbit anti-Bni1 polyclonal antibody kindly provided by Mark Longtine (Oklahoma State University). Secondary antibodies used were horseradish peroxidase-conjugated rabbit anti-mouse immunoglobulin G (IgG) and horseradish peroxidase-conjugated goat anti-mouse IgG (Jackson ImmunoResearch Laboratories, West Grove, PA).

**Protein interaction assays.** (i) Two-hybrid interactions. Strain Y1026 (a leu2-3,112) carrying different BD-SPA2 fusions (63) were mated on yeast extract-peptone-dextrose (YPD) plates with strain Y860 (a leu2-3,112) carrying different AD-MSb3 fusions, which are under the control of a galactose-inducible system. After mating, the mating reactions were replicated onto SC-His-Trp plates to select for diploids, which were subsequently replicated onto SC-His-Trp-Ade plates containing 1% rafinose plus 2% galactose and incubated at 30°C for more than 3 days. Positive interactions were confirmed by growth on the plates and negative interactions were confirmed by a standard filter assay for β-galactosidase activity (data not shown). (ii) In vitro protein-binding assays. All recombinant proteins used in this study were purified from the protease-deficient E. coli strain BL21. For GST fusion proteins, glutathione-Sepharose 4B (Amersham Biosciences Corp.) was used for purification. For MBP fusions proteins, amylose resin (New England Biolabs) was used for purification.

(iii) Binding of Msb3 and Msb4 to Spa2. Five to 10 µg of purified MBP-Msb3, MBP-Msb4, MBP-Msb-N (aa 1 to 220), MBP-Msb-N (aa 1 to 144), and MBP-Pdi1 was added to amylose beads and incubated individually with 20 µg of purified GST-SPA2 (aa 1 to 150) in binding buffer (20 mM Tris-HCl, pH 7.4, 100 mM NaCl, 5 mM EDTA, 5 mM dithiorthiourea [DTT], and 0.1% Triton X-100) at 4°C for 1 h. The beads were washed five times with the binding buffer, and the bound proteins were eluted from amylose beads with SDS sample buffer. Samples were analyzed by SDS-PAGE, and proteins were detected by Coomassie blue staining and immunoblotting with an anti-GST antibody.

(iv) Binding of Msb3 and Msb4 to Rho GTPases. GST fusion proteins of Cdc42, Rho1, and Rho4 were first loaded with GDP or GTP-S. To do this, 15 µg of a GST fusion protein was first incubated in the preloading buffer (20 mM Tris-HCl, pH 7.4, 100 mM NaCl, 5 mM EDTA, 5 mM DTT, and 10 mM GDP or GTP-S) at 30°C for 30 min, followed by the addition of MgCl2 to a final concentration of 25 mM, and the reaction mixture was incubated at 24°C for 15 min. After nucleotide loading, GST fusion proteins were added to the amylose beads containing 5 to 10 µg of MBP fusion proteins, including MBP-Msb3, MBP-Msb-N, MBP-Msb-R282K, MBP-Msb4, and MBP-Gic2. After incubation at 4°C for 2 h, samples were washed five times with washing buffer (20 mM Tris-HCl, pH 7.4, 100 mM NaCl, 10 mM MgCl2, 5 mM DTT, and 0.1% Triton X-100), and the bound proteins were eluted from the beads with SDS sample buffer. Samples were analyzed by 12% SDS-PAGE, and proteins were detected by Coomassie blue staining and immunoblotting with an anti-GST antibody.

### RESULTS

**Msb3 and Msb4 bind directly to the N-terminal region of Spa2.** Comprehensive two-hybrid analysis indicates that full-length Msb3 and Msb4 interact with full-length Spa2 (16). Because Msb3, Msb4, and Spa2 may function in the same pathway (see the introduction), we decided to characterize these interactions further.

First, we used full-length Msb3 and Msb4 in two-hybrid assays against a host of Spa2 fragments (63) to map their binding sites on Spa2. Msb3 and Msb4 showed identical interaction profiles with the Spa2 fragments; their strongest interactions were with the first 150 amino acids of Spa2 (Fig. 1B, top panel; also data not shown), which is also called SHD-I (Spa homology domain I) (59). SHD-I contains two tandem repeats of a 31-amino-acid motif named SDR (Spa2 direct repeat) or SHD (Fig. 1A) that exists in yeast and mammalian proteins (33, 59, 73). The function of the SDR motif is not known, although the SDR-containing region of the mammalian Arf GAP GIT1 has been shown to interact directly with the Rac GEF, βPix, and also with Piccolo, a core component of the neuronal cytoskeletal matrix assembled at the active zones (33, 73).

Next, we found that the N-terminal portions of Msb3 (aa 1 to 220) and Msb4 (aa 1 to 144) lacking the Rab GAP domain (Fig. 1A) interacted with the Spa2 fragments in a pattern similar to that of their full-length proteins (Fig. 1B, lower panel; also data not shown). The N-terminal portions of Msb3 (aa 1 to 424) and Msb4 (aa 1 to 335) carrying most of the Rab GAP domain interacted only with the SHD-I region of Spa2, not with full-length Spa2 or with other fragments of Spa2 (data not shown). The C-terminal portions of Msb3 (aa 446 to 633) and Msb4 (aa 357 to 492) did not interact with any of the Spa2 fragments at all (data not shown). Together, these results indicate that the N-terminal portions of Msb3 and Msb4 are able to interact with the SHD-I region of Spa2.
FIG. 1. Msb3 and Msb4 bind directly to the N-terminal region of Spa2. (A) Molecular motifs of Msb3, Msb4, and Spa2. (B) The N-terminal portion of Msb3 interacts with the SHD-I region of Spa2 by two-hybrid assay. Different DNA binding domain-Spa2 fragment fusions (BD:SPA2) were tested against the activation domain-Msb3 fusion AD:MSB3 (full length) and AD:MSB3-N220 (aa 1 to 220). The numbers to the left and right of the image refer to the amino acid residues of different Spa2 fragments. It is not clear why BD:SPA2(1-530) failed to interact with AD:MSB3. (C) The N-terminal, non-GAP region of Msb3 or Msb4 binds to the SHD-I region of Spa2 in vitro. Recombinant MBP, MBP-Msb3, MBP-Msb3-N (aa 1 to 220), MBP-Msb4, MBP-Msb4-N (aa 1 to 144), and MBP-Pxl1 (another control) were purified from E. coli, bound to amyllose beads, and incubated with equal amounts of GST-Spa2 (aa 1 to 150). MBP fusions and their associated partners were purified from amyllose beads, separated by SDS-PAGE, and then either stained with Coomassie blue (top and bottom panels) or immunoblotted with an anti-GST antibody to detect the bound GST-Spa2 (middle panel). (D) Strain YEF3760 (a SPA2::MYC BUD6::HA) or YEF3761 (a SPA2::MYC PE2::HA) carrying the pEGKT vector (GST), pEGKT-Msb3 (GST-Msb3), or pEGKT-Msb4 (GST-Msb4) was grown to the exponential phase in liquid SC-Ura medium containing 2% raffinose. Galactose was added to each culture to a final concentration of 3% to induce the expression of the GST fusions for 4 h at 30°C. GST, GST-Msb3, and GST-Msb4 were expressed well in the cell lysates prepared from each strain (left panel). Equal amounts of glutathione-agarose beads were incubated with equal amounts of cell lysates from each strain to pull down the GST fusions. The amounts of Spa2-MYC, Bni1-HA, and Pea2-HA in the pulldown precipitates were determined by immunoblotting with antibodies against MYC, Bni1, and HA, respectively (right panel).
To determine whether Msb3 and Msb4 bind to Spa2 directly, full-length Msb3 and Msb4 and their N-terminal portions were expressed as MBP fusion proteins, and the SHD-I region of Spa2 was expressed as a GST fusion protein. These recombinant proteins were purified from E. coli and were examined for their interactions in vitro. A given amount of GST-Spa2(1-150) (Fig. 1C, bottom panel) was incubated individually with approximately the same amounts of MBP-Msb3, MBP-Msb3-N (aa 1 to 220), MBP-Msb4, and MBP-Msb4-N (aa 1 to 144) or with larger amounts of MBP and MBP-Pxl1 (a paxillin-like protein in yeast) as controls. The MBP fusions were pulled down with amylose beads and washed, and the bound proteins were eluted with SDS sample buffer and subjected to SDS-PAGE analysis. Coomassie blue staining revealed that full-length Msb3 and Msb4 and their N-terminal portions bound to GST-Spa2(1-150) at a ratio of approximately 1:1 (Fig. 1C, top panel). Neither MBP alone nor MBP-Pxl1 bound to Spa2. The identity of GST-Spa2 in the pulldown proteins was confirmed by Western-blot analysis with an anti-GST antibody (Fig. 1C, middle panel). These results demonstrate that the N-terminal regions of Msb3 and Msb4 bind directly to the SHD-I region of Spa2.

Spa2 is required for the clustering of Msb3 and Msb4 at the bud tip. To determine the functional consequences of disrupting the Msb3-Spa2 interaction, we compared the localization patterns of GFP-tagged Msb3 in wild-type and spa2Δ cells as well as in other polarisome mutants. In small-budded wild-type cells, Msb3 localized to the bud tip as a “cap” (Fig. 2A, cell 1, and 2B), whereas in large-budded cells, Msb3 localized to the entire cortex of the bud (Fig. 2A, cell 2, and 2B). These localization results are similar to those obtained by an indirect immunofluorescence method with cells carrying HA-tagged Spa2 and Msb3 (9), except that the GFP-tagged Msb3 showed a clear plasma membrane association (Fig. 2A), which may reflect a higher sensitivity of the latter approach.

In contrast to its localization pattern in wild-type cells, Msb3-GFP completely failed to cluster at the bud tip in spa2Δ cells (Fig. 2A, cells 3 and 4, and 2B), even though Msb3-GFP was expressed at a similar levels in wild-type and spa2Δ cells (Fig. 2C). A similar phenotype was also observed with Msb4-GFP (data not shown). Because the two-hybrid and in vitro protein-binding assays both indicate that Msb3 and Msb4 bind to the region comprising the first 150 aa of Spa2, we asked whether this binding is responsible for the clustering of Msb3 at the bud tip. We found that Msb3 also failed to cluster in spa2Δ cells at any stage of budding (Fig. 2A, cells 5 and 6, and 2B). Significantly, Msb3 still localized preferentially to the bud and was still membrane associated, suggesting that the mechanisms for the targeting and membrane association of Msb3 are still intact even in the absence of Msb3 clustering at the bud tip. In other polarisome mutants, including bni1Δ, bud6Δ, and pea2Δ cells, Msb3 delocalized slightly from the bud tip (data not shown), i.e., Msb3 occupied a relatively larger surface zone near the bud tip in these mutants than in wild-type cells, but was still polarized in the bud cortex compared to its localization in spa2Δ cells. This subtle defect was difficult to quantitate. Likewise, the polarisome components, including...
Spa2, Bni1, Bud6, and Pea2 (all GFP-tagged proteins), localized to a relatively larger surface area near the bud tip of an *msb3Δ*/*H9004* or *msb4Δ*/*H9004* cell (data not shown). These results suggest that Spa2 is required for the clustering of Msb3 at the bud tip but is not required for its membrane association or its targeting to the bud cortex.

Deletion of individual components of the polarisome results in distinct defects in the trafficking of the bipolar-budding marker Bud8. Mutations in polarisome components, including Spa2, Bni1, and now Msb3 and Msb4, cause defects in bipolar budding (9, 65, 70). To determine the molecular basis for this common defect, we examined the localization of the distal-pole marker Bud8 in polarisome mutants. In wild-type cells, Bud8-GFP localized efficiently to the tip of a small bud as a “cap” (27) (Fig. 3A and B). In *spa2Δ* cells, the localization pattern of Bud8-GFP was largely normal (27) (Fig. 3A), but for reasons that are not clear, the percentage of cells with this relatively normal localization was significantly reduced in comparison to that for wild-type cells (Fig. 3B). In *bni1Δ* cells, Bud8-GFP was not localized to the bud tip (27, 48) (Fig. 3A and B), even though Bud8-GFP was expressed at similar levels in *bni1Δ* and wild-type cells (Fig. 3C). The localization of Bud8-GFP in *msb3Δ msb4Δ* cells was more complex (Fig. 3A and B); Bud8-GFP was delivered to the daughter compartment in most cells, but it often failed to localize to the bud tip as a cap; instead, Bud8-GFP was present as a cluster of particles near the bud tip or in the entire bud cortex. These particles probably represent post-Golgi secretory vesicles, which have been visualized recently in *msb3Δ msb4Δ* cells (22). Together, these results indicate that deletion of individual components of the polarisome differentially affects the trafficking and/or targeting of the bipolar-budding marker Bud8 to the bud tip. These results also suggest that different components of the polarisome play unique roles in regulating polarized protein delivery.

**Msb3 and Msb4 promote Bni1-nucleated actin filament assembly in vivo.** During the course of our experiments on Bni1, one of the two formins in *S. cerevisiae*, we observed that overexpression of GFP-tagged full-length Bni1 in wild-type cells formed actin “rings” and “aggregates” (Fig. 4A and B), which appeared morphologically different from the actin “loops” or “rings” associated with overexpression of the C-terminal half of Bni1 containing the FH1-FH2 domains (55). After induction of GFP-Bni1 expression from a galactose-inducible promoter for 1 to 2 h, some cells formed a Bni1 ring that was invariably associated with F-actin (Fig. 4A), providing direct evidence that Bni1 can colocalize with an actin structure in vivo. After induction of GFP-Bni1 for 4 to 6 h, most of the wild-type cells formed one or multiple Bni1 aggregates (Fig. 4B). In addition, most wild-type cells contained at least one Bni1 aggregate that colocalized with F-actin (Fig. 4B and C). Such association between the Bni1 aggregates and F-actin was slightly affected by the deletion of Spa2 or Bud6, two components of the polarisome, but was severely affected by the deletion of Msb3 and Msb4 together (Fig. 4B and C). These

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**FIG. 3.** Deletion of individual polarisome components causes distinct defects in the trafficking of the bipolar-budding marker Bud8 to the bud tip. (A and B) Localization of Bud8-GFP in wild-type and polarisome mutant cells. (A) Wild-type diploid cells (WT; YEF473) or other diploid cells homozygous for either *spa2Δ* (YEF2468), *msb3Δ msb4Δ* (YEF1631), or *bni1Δ* (YHH799) carrying plasmid YEpGFP*-BUD8* were grown exponentially in SC-Leu medium at 24°C and then observed by fluorescence microscopy. (B) At least 100 cells (with a bud smaller than one-third of the mother size) were scored for each strain. (C) Expression of Bud8-GFP in wild-type and polarisome mutant cells was determined by immunoblotting with an anti-GFP antibody. It is not clear why the expression of Bud8-GFP was increased in the *spa2Δ* strain. Porin (Por1) was used as the loading control.
FIG. 4. Msb3 and Msb4 may promote Bni1-stimulated actin assembly in vivo. (A) Overexpression of GFP-Bni1 causes cortical Bni1 ring formation that is always associated with F-actin. Wild-type (WT) cells (YEF473) carrying plasmid YEp352-GAL1-GFP-BNI1 were grown exponentially in SC-Ura medium containing 2% raffinose at 30°C. Galactose was added to the culture to a final concentration of 3% to induce the expression of GFP-Bni1 for 1 h. Cells were fixed with 70% ice-cold ethanol and stained with rhodamine-conjugated phalloidin (10) to visualize F-actin. (B) Prolonged overexpression of GFP-Bni1 causes the formation of Bni1 aggregates, which often associate with F-actin. WT (YEF473), spa2Δ (YJZ358), and mlsb3Δ mlsb4Δ (YEF1631) cells carrying YEp352-GAL1-GFP-BNI1 were processed as for panel A to visualize the colocalization of GFP-Bni1 and F-actin, except that the induction time for GFP-Bni1 was 4 h. (C) The percentages of cells from the strains listed in the legend to panel B that had at least one GFP-Bni1 aggregate colocalized with F-actin were counted after induction of GFP-Bni1 for 4 or 6 h. At least 200 cells were scored for each strain at each time point. (D) Nucleation-deficient alleles of BNI1 reduce the association between GFP-Bni1 aggregates and F-actin. Experiments were performed as for panel C except that only the WT (YEF473) strain carrying different alleles of BNI1 on plasmid YEp352-GAL1-GFP-BNI1 was used. (E) Cofilin is not in the GST-Bni1-induced F-actin aggregates. Cells of the WT (YEF473) strain carrying plasmid YEp352-GAL1-GST-BNI1 were induced as for panel A at 30°C for 6 h before being processed for immunofluorescence with anti-cofilin (Cof1) antibodies and for actin staining with Alexa-phalloidin. The arrow indicates an out-of-focus F-actin spot. (F) Msb3-GFP colocalizes with GST-Bni1-induced F-actin aggregates. Cells of the WT (YEF473) strain carrying YEp181-GFP-MSB3 and YEp352-GAL1-GST-BNI1 were induced as for panel A at 30°C for 6 h, fixed with formaldehyde, and then visualized for GFP-Msb3 and F-actin.
results suggest that Msb3 and Msb4 are required for efficient association between GFP-Bni1 aggregates and F-actin. Furthermore, we found that induction of GFP-BNI1 expression by galactose for 6 h at 30°C in msb3Δ msb4Δ cells (YEplac181) carrying YEpplac181 alone (vector), YEp181-3HA-MSB3 (MSB3), or YEp181-3HA-MSB3-R282K (msb3R282K) resulted in the colocalization of GFP-Bni1 aggregates with F-actin in 20%, 44%, and 42% of the cells, respectively. This result suggests that the role of Msb3 in the Bni1–F-actin association appears to be independent of its GAP activity toward Sec4.

To distinguish whether the F-actin associated with the GFP-Bni1 aggregates is due to the nucleation activity of Bni1 or to the trapping of existing actin filaments by the GFP-Bni1 aggregates, we introduced various mutations in the FH2 domain of Bni1 that are known to eliminate the nucleation activity of Bni1 in vitro (44, 69) into our GFP-Bni1-overexpressing construct. Overexpression of either the GFP-Bni1W1363A W1374A or the GFP-Bni1K1601D mutant decreased the association between the GFP-Bni1 aggregates and F-actin significantly; the latter mutant had a more dramatic effect (Fig. 4D). The triple mutant (W1363A W1374A K1601D) showed an effect similar to that of the K1601D single mutant in this assay (Fig. 4D). These results suggest that the F-actin associated with the GFP-Bni1 aggregates is largely due to Bni1 nucleation activity.

We also found that overexpression of GST-Bni1 induced the formation of F-actin aggregates that colocalized with the GST-Bni1 aggregates by indirect immunofluorescence (data not shown). The F-actin aggregates induced by GST-Bni1 do not contain cofilin, a protein specifically associated with actin shown). The F-actin aggregates induced by GST-Bni1 (data not shown) or the GFP-Bni1K1601D mutant decreased the association between the GFP-Bni1 aggregates and F-actin significantly; the latter mutant had a more dramatic effect (Fig. 4D). The triple mutant (W1363A W1374A K1601D) showed an effect similar to that of the K1601D single mutant in this assay (Fig. 4D). These results suggest that the F-actin associated with the GFP-Bni1 aggregates is largely due to Bni1 nucleation activity.

How do Msb3 and Msb4 facilitate Bni1-nucleated actin filament assembly in vivo? One possibility is that Msb3 and Msb4 bind directly to Bni1 to regulate its nucleation activity. We could not confirm a previously reported two-hybrid interaction between Msb3 and Bni1 (16). In addition, we failed to detect any significant interaction between purified MBP-Msb3, MBP-Msb4, and GST-tagged Bni1 fragments that cover different regions of Bni1 (data not shown). Furthermore, in an in vitro actin polymerization assay with 75 nM Bni1-FH1-FH2-C fragment and 2 μM G-actin (50% pyrenyl-actin), even as much as 8 to 10 μM MBP-Msb3 and MBP-Msb4 failed to stimulate Bni1-nucleated actin polymerization (data not shown). Thus, it is unlikely that Msb3 and Msb4 regulate actin filament assembly by binding directly to Bni1.

Msb3 and Msb4 bind specifically to the GDP-bound form of Cdc42 and Rho1. Previously, we have shown that MSB3 and MSB4 stimulate Cdc42 function genetically (9, 22). Thus, it is possible that Msb3 and Msb4 regulate Bni1-nucleated actin filament assembly through Cdc42, which is thought to activate Bni1 nucleation activity by directly binding to the N-terminal region of Bni1 (15, 18). To examine this possibility, we first asked whether Msb3 and Msb4 could bind directly to Cdc42. GST-Cdc42 was purified from E. coli and was either stripped off the nucleotide with EDTA or loaded with GDP or GTP-S. Msb3, Msb4, and the positive control Gic2 were purified from E. coli as MBP fusions. As expected, Gic2, a known effector of Cdc42 (12, 14), bound to Cdc42-GTP specifically (Fig. 5A). In contrast, Msb3 and Msb4 bound to Cdc42-GDP specifically (Fig. 5A). We also found that Msb3 bound specifically to Rho1-GDP, not Rho3 or Rho4. Experiments were performed as for panel A. (C) Msb3R282K still binds preferentially to Cdc42-GDP. The experiment was performed as for panel A.
the Cdc42 interaction and Rab GAP activities of Msb3 are separable.

Msb3 is not a GEF for Cdc42 and does not promote polarized growth through inhibition of Rho GDI function. What is the function of the binding of Msb3 to Cdc42-GDP? Only the GEF and the Rho GDI are known to bind to the GDP-bound form of Cdc42 or Rho1 (34, 75). Thus, one possibility is that Msb3 functions as a GEF for Cdc42 or stimulates the GEF activity of Cdc24 toward Cdc42. To determine whether Msb3 is a GEF for Cdc42, we overexpressed wild-type Msb3 or Msb3 lacking the Rab GAP activity in wild-type cells and measured the Cdc42-GTP level in cell lysates using a GST-PBD pulldown assay (13). As expected, overexpression of Cdc42 or hyperactivation of Cdc42 by a mutation (13) caused a significant increase in the Cdc42-GTP level (Fig. 6). In contrast, overexpression of Msb3 or its GAP-deficient form did not change the gross level of Cdc42-GTP (Fig. 6). The failure to detect a possible small increase in Cdc42-GTP in the presence of Msb3 overexpression could be due to the high rate of GTP hydrolysis by Cdc42 that is enhanced by its GAPs. Thus, we performed the same GST-PBD pulldown assay with a strain lacking all three Cdc42 GAPs (Bem3, Rga1, and Rga2) to enhance our chance for such detection. The same profile of Cdc42 activation by different proteins was obtained for the triple GAP mutant as for the wild-type strain (data not shown). These data suggest that Msb3 does not function as a GEF for Cdc42, a finding consistent with the fact that Msb3 lacks a putative GEF domain. In addition, co-overexpression of Cdc24 and Msb3 or its GAP-deficient form did not increase the level of Cdc42-GTP over that caused by overexpression of Cdc24 alone (data not shown), suggesting that Msb3 does not enhance the GEF activity of Cdc42.

Another possibility is that Msb3 helps to localize and/or maintain a pool of Cdc42-GDP at the bud tip, protecting this pool of Cdc42 from being extracted by Rho GDI into the cytosol. This pool of Cdc42-GDP would be poised for activation at the correct cellular location when Cdc42 GEF, which localizes at the same site, is activated by a cell cycle signal. This hypothesis is consistent with the observations that Msb3 and a fraction of Rd11, the sole Rho GDI in *S. cerevisiae*, localize to the sites of polarized growth in the cell cycle (9, 58). This hypothesis also predicts that Msb3 would genetically antagonize Rho GDI function and biochemically protect Cdc42 from being extracted from its membrane pool by Rho GDI. Deletion of *RD11* does not produce any obvious phenotype, but overexpression of Rd11 was reported to cause cell lethality (41). Surprisingly, we found that overexpression of Rd11 did not cause cell lethality in our wild-type strain but merely made the cells rounder. This result suggests that, in wild-type cells, the GTP exchange reaction of Cdc42 is sufficiently efficient to counteract the effect of GDI overexpression. If so, compromising the exchange reaction of Cdc42 by mutations of its GEF might allow visualization of a GDI overexpression effect. Indeed, overexpression of Rd11 in a cdc24-Ts (cdc24-11) mutant caused cell lethality even at the permissive temperature, with cells arrested as large, round, and unbudded cells (Fig. 7A and 7B, left). This now provides a genetic assay by which we can monitor the relationship between Msb3 and Rd11.

Simultaneous overexpression of Msb3 and Rd11 in cdc24-11 cells still caused cell lethality (data not shown). However, overexpression of Msb3 or its GAP-deficient derivative, Msb3R282K, resulted in a significant increase in the population of cells with an elongated bud, indicative of polarized growth and Cdc42 activation (22% cells with an elongated bud for the strain overexpressing Msb3 and Rd11, 19% for the strain overexpressing Msb3R282K and Rd11, and 2% for the strain overexpressing Rd11 alone; n = 800 for each strain) (Fig. 7B, right). This result suggests that Msb3 antagonizes the function of Rd11 in Cdc42 activation in a manner that is largely independent of its Rab GAP activity.

To determine whether Msb3 antagonizes Rd11 function by acting independently but oppositely on Cdc42-GDP or by simply inhibiting Rd11 function, we conducted the following experiments. First, deletion of *MSB3* or its GAP-deficient derivative, Msb3R282K, showed weak to no suppression of cdc24-11 lethality at 32°C (Fig. 7C) but not at 35°C (data not shown), suggesting that Rd11 is not simply an inhibitor of Cdc42 function and that Msb3 cannot promote polarized growth simply by inhibiting Rd11 function. Second, multicityc *MSB3* suppressed cdc24-11 at 32°C (Fig. 7C) but not at 35°C (data not shown), and multicityc *msb3* lacking its GAP activity (msb3R282K) showed weak to no suppression of cdc24-11 lethality, but obvious suppression of the cdc24-11 morphological defect, at 32°C (Fig. 7C and data not shown). Similarly, multicityc *MSB3* and *msb3R282K* showed strong and weak suppression of cdc24-11 rd11Δ lethality at 32°C, respectively (Fig. 7C), suggesting that Msb3 can promote polarized growth independently of its GAP activity and of Rd11 function. These results, together with our previous reports (9, 22), also suggest that both the GAP activity and a GAP-independent activity of Msb3 (the latter possibly being its interaction with Cdc42-GDP) are required for the maximal suppression of cdc24 and cdc42 mutants. Finally, Rd11 is known to extract both Cdc42 and Rho1 from their membrane pools (17, 41). Under conditions where GST-Rd11 purified from *E. coli* extracted Cdc42 from the membrane pools into the cytosol (Fig. 7D, supernatant after centrifugation at 100,000 × g [100K Supt.]), we failed to detect any protection from Cdc42 extraction by GST-Rd11 in either wild-type (four independent experiments) or rd11Δ (two independent experiments) strains overexpressing either Msb3 or Msb3R282K (Fig. 7D). Together, these results suggest that Msb3 and Rd11 act independently but oppositely on Cdc42. These results also imply that the binding of Msb3 to Cdc42-GDP might simply be involved in capturing...
and/or maintaining a pool of Cdc42-GDP at the sites of polarized growth, a hypothesis that remains to be tested.

DISCUSSION

Polarisome modulates polarized growth near the bud tip by multiple mechanisms and thus determines the local cell shape. Cell polarity in *S. cerevisiae* can be dissected into global polarity (the mother-daughter polarity) and local polarity (the polarity within the daughter cell). Global polarity is responsible for making a bud and determines the overall cell morphology, including the size ratio of the mother compartment to the daughter compartment. Global polarity is largely determined by Cdc42-mediated polarization of the actin cytoskeleton (3, 31, 54), but the underlying mechanisms are still not fully elucidated.
Local polarity, i.e., the asymmetry in the daughter cell, which reflects the growth mode of the daughter cell over time, determines the shape of the bud. It has been generally accepted that the bud undergoes apical growth early in the cell cycle (late G1 to early G2), making the bud longer, and then switches to isotropic growth in G2 to make the entire bud larger until late anaphase or telophase, when cell growth is redirected to the bud neck to promote cytokinesis (37, 54). If this were accurate, a spindle-shaped bud would be produced. However, the distal hemisphere of the bud resembles a dome rather than a cone, suggesting that cell growth is not directed toward a fixed point at the bud tip but rather that there might be a dynamic structure that modulates polarized secretion near the bud tip to control the local shape. Polarisome might be the dynamic structure, since live-cell imaging reveals that the polarisome component Bni1 fused with GFP moves as a “spot” and/or a “cap” around the cortex of a tiny or a small bud. The location and duration of the Bni1 “spot” correlate perfectly with the location and duration of polarized growth toward the bud tip (51).

Previously, we have shown that Msb3, Msb4, and the polarisome component Bni1 function in the same pathway downstream of Cdc42 (9) (Fig. 8A). Other genetic studies position Spa2 in the same pathway as Bni1 (9, 30). Thus, it appears that the polarisome and the Gic1/Gic2 effectors define at least two pathways by which Cdc42 regulates actin organization and/or polarized cell growth. This hypothesis also implies that Cdc42 could be partitioned into multiple complexes to regulate cell polarity. While the mechanisms underlying the role of Gic1 and Gic2 in actin organization or cell polarity remain unknown, our current study suggests concrete mechanisms by which the polarisome regulates cell polarity.

In summary, we have demonstrated that Msb3 and Msb4 bind specifically to the N-terminal region of Spa2 (Sph1 [7, 59], a Spa2-related protein in *S. cerevisiae*, has not been examined for interactions with Msb3 and Msb4 in our laboratory) as well as to the GDP-bound form of Cdc42 and Rho1. These new results have led to a simple model of the function of the polarisome (Fig. 8B). In essence, the polarisome is a dynamic, multifaceted protein complex that determines local cell shape, in this case the shape of the bud. Bni1 regulates polarized growth by stimulating actin cable formation near the bud tip. Msb3 and Msb4 regulate polarized growth via two routes. One route is the creation of a local pool of Cdc42-GDP near the bud tip; this pool of Cdc42 can be locally converted to Cdc42-GTP by the polarisome (Fig. 8B). In yeast and mammalian cells, (A) Cdc42 controls polarized actin organization by at least two parallel pathways, the yeast-specific Gic1/Gic2 pathway and the evolutionarily conserved “polarisome” pathway. This model is supported by mutant phenotypes, protein localization, protein-protein interactions, and genetic interactions. (B) The Spa2-based polarisome regulates polarized growth in *S. cerevisiae* by at least two coordinated yet distinct mechanisms. First, Msb3 and Msb4 bind to the SH-D-I region of Spa2 to regulate the recycling of Sec4 and thus exocytosis. Second, Msb3 and Msb4 bind to Cdc42-GDP and Rho1-GDP (not depicted here) to facilitate the local activation of these GTPases. The activated GTPases then activate Bni1, which is bound to the SH-D-V of Spa2, to direct the formation of actin cables. The actin cables then guide secretory vesicles toward the polarisome at the bud tip. (C) The GIT1-based protein complex at the focal adhesion regulates Arf6 cycling and thus membrane trafficking through its N-terminal Arf GAP domain. It also regulates Rac and Cdc42 activity through its SH-D domain, which is bound to the Rac GEF, pPIX. pPIX also binds to PAK, effector of both Rac and Cdc42. It is not known how GIT1 is connected to Rho and the formins to regulate stress fiber formation.

**FIG. 8.** Model for the role of polarisome in polarized growth in yeast and comparison with its possible “functional equivalent,” the focal adhesion, in mammalian cells. (A) Cdc42 controls polarized actin organization by at least two parallel pathways, the yeast-specific Gic1/Gic2 pathway and the evolutionarily conserved “polarisome” pathway. This model is supported by mutant phenotypes, protein localization, protein-protein interactions, and genetic interactions. (B) The Spa2-based polarisome regulates polarized growth in *S. cerevisiae* by at least two coordinated yet distinct mechanisms. First, Msb3 and Msb4 bind to the SH-D-I region of Spa2 to regulate the recycling of Sec4 and thus exocytosis. Second, Msb3 and Msb4 bind to Cdc42-GDP and Rho1-GDP (not depicted here) to facilitate the local activation of these GTPases. The activated GTPases then activate Bni1, which is bound to the SH-D-V of Spa2, to direct the formation of actin cables. The actin cables then guide secretory vesicles toward the polarisome at the bud tip. (C) The GIT1-based protein complex at the focal adhesion regulates Arf6 cycling and thus membrane trafficking through its N-terminal Arf GAP domain. It also regulates Rac and Cdc42 activity through its SH-D domain, which is bound to the Rac GEF, pPIX. pPIX also binds to PAK, effector of both Rac and Cdc42. It is not known how GIT1 is connected to Rho and the formins to regulate stress fiber formation.
GTP by its GEF upon instruction by a cell cycle signal. This locally activated Cdc42 will in turn activate Bni1 to stimulate actin cable formation. The second route is direct regulation of exocytosis by facilitating the recycling of Sec4 near the bud tip (22). The role of Spa2 is to bring Bni1 and Msb3 and Msb4 into close proximity by interacting with both sets of proteins via distinct domains: the N-terminal SHD-I for Msb3 and Msb4 and the C-terminal SHD-V for Bni1 (21).

This model integrates three basic observations of this study: Msb3-Spa2 interaction, the binding of Msb3 to Cdc42-GDP, and the stimulatory effect of Msb3 on Bni1-nucleated actin assembly. One key feature of this model remains to be tested: what is the function of Msb3-Cdc42 interaction? From our genetic analyses, Msb3 positively regulates Cdc42 function (9, 22) (this study), yet data presented here indicate that Msb3 is not a GEF or an enhancer of the GEF for Cdc42 and that Msb3 and Rdi1 act independently, but oppositely, on Cdc42. Our working hypothesis is that Msb3 captures and/or maintains a pool of Cdc42-GDP at the bud tip, preparing for local activation of Cdc42, and that Msb3 and Rdi1 dynamically control the level of Cdc42-GDP at the bud tip. Recent evidence suggests that Cdc42 at the sites of polarized growth could be reinforced through delivery of Cdc42 by a secretory pathway, providing a positive feedback mechanism for polarity establishment and maintenance (56, 68). These data also suggest a need for capturing Cdc42, perhaps in its GDP-bound form, from the secretory pathway. Msb3 could fulfill this hypothetical role. Rho1 is in the GDP-bound form prior to its arrival at the plasma membrane (1). Even at the plasma membrane, Rho1 occupies a much larger zone of the bud tip than the active portion of Rho1 does (1), suggesting that there are factors that can maintain Rho1-GDP near the bud tip prior to its GEF action. Again, Msb3 could be part of these putative factors.

The polarisome: conservation and divergence through evolution. Genomic studies suggest that only 15 to 20% of the known genomes represent essential genes (24). The rest of the genomes encode nonessential genes that make the essential processes run with optimal efficiency and accuracy in response to internal and external cues. The genes encoding the components of the polarisome belong to the latter class. The function of the polarisome is not to determine the global polarity but to modulate polarized growth locally. Is the regulation of local polarity by polarisome a universal feature among eukaryotes?

The core components of the polarisome, Spa2, Bni1, Bud6 (6, 44), and Msb3, appear to be conserved in other fungi including the fission yeast Schizosaccharomyces pombe and the dimorphic fungus Candida albicans (http://www.yeastgenome.org/). Functional analysis of the polarisome components in other fungi is still in its infancy. For3, the Bni1 homolog in S. pombe, localizes to the sites of polarized cell growth, modulates local cell shape, and is required for actin cable formation (20). CaSpa2, the Spa2 homolog in C. albicans, also localizes to the sites of polarized growth during its budding or hypha-growing cycle (74). Deletion of CaSPA2 causes thickening of the hyphae but does not block overall hyphal initiation and growth, suggesting that CaSpa2 does not determine the global polarity but modulates local polarized growth. Remarkably, only the SHD-I (Msb3 binding) and SHD-V (Bni1 binding) regions of CaSpa2 are highly homologous to the corresponding regions of S. cerevisiae Spa2, whereas other portions of the molecules are poorly conserved. These results suggest that not only does the polarisome containing the core components Spa2, Bni1, and Msb3 likely exist in other fungi, but also the same kind of protein-protein interactions in the polarisome are conserved.

The “functional equivalent” of the polarisome in filamentous fungi could be the complex structure called Spitzenkorper (SPK). SPK is an electron-dense structure located near the hyphal tip. It consists of a filamentous core likely made of F-actin and peripherally localized membrane-bound vesicles with a diameter of 70 to 120 nm (28, 29). Computer-enhanced video microscopy and computer modeling of hypha tip growth indicate that SPK is a dynamic structure that functions as a vesicle supply center, which modulates polarized growth to determine the local shape, the tip of a hypha (8, 23, 57). No molecular components except actin have been associated with SPK in fungi. It would be extremely interesting to determine whether SPK uses the same types of molecules as the polarisome does to carry out its functions or whether it employs different sets of molecules to perform a similar function.

In mammalian cells, some protein complex in the focal adhesion may function similarly to the polarisome (Fig. 8C). The Arf GAP protein GIT1 is a scaffold-like protein that localizes to the focal adhesion site by interacting with paxillin through its C terminus. The SHD region of GIT1 interacts with βPIX, a Rac GEF, which is known to interact with PAK, an effector of Rac and Cdc42. GIT1 also has an Arf GAP domain that acts on Arf6 to regulate membrane trafficking between the plasma membrane and the recycling endosome (40, 73). Thus, it appears that the GIT1 protein complex regulates membrane trafficking through a GAP domain, is linked to Cdc42 and Rac through an SHD domain, and must somehow associate with the actin stress fibers, whose formation is regulated by Rho and formins. The current thought is that the GIT1 protein complex is involved in promoting cell migration by stimulating focal adhesion disassembly (40, 73). A similar protein complex involving GIT1 and its binding partners also exists in the cytoskeletal matrix assembled at the active zones of neurons that are involved in neurotransmitter release (33). It becomes increasingly clear that there are three types of molecules involved in fundamental processes: the evolutionarily conserved molecules (such as Cdc42 and formins), the molecules with conserved domains only (GIT1, Spa2, and Msb3), and the species-specific molecules. This type of molecular diversity allows a fundamental process to be coordinated with or regulated by a species-specific mechanism. Further investigation will paint a clearer picture of the conservation and divergence among polarisome, Spitzenkorper, and the GIT1 protein complex.

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