Identification of Novel, Evolutionarily Conserved Cdc42p-interacting Proteins and of Redundant Pathways Linking Cdc24p and Cdc42p to Actin Polarization in Yeast

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In the yeast Saccharomyces cerevisiae, Cdc24p functions at least in part as a guanine-nucleotide-exchange factor for the Rho-family GTPase Cdc42p. A genetic screen designed to identify possible additional targets of Cdc24p instead identified two previously known genes, MSB1 and CLA4, and one novel gene, designated MSB3, all of which appear to function in the Cdc24p–Cdc42p pathway. Nonetheless, genetic evidence suggests that Cdc24p may have a function that is distinct from its Cdc42p guanine-nucleotide-exchange factor activity; in particular, overexpression of CDC42 in combination with MSB1 or a truncated CLA4 in cells depleted for Cdc24p allowed polarization of the actin cytoskeleton and polarized cell growth, but not successful cell proliferation. MSB3 has a close homologue (designated MSB4) and two more distant homologues (MDR1 and YPL249C) in S. cerevisiae and also has homologues in Schizosaccharomyces pombe, Drosophila (pollux), and humans (the oncogene tre17). Deletion of either MSB3 or MSB4 alone did not produce any obvious phenotype, and the msb3 msb4 double mutant was viable. However, the double mutant grew slowly and had a partial disorganization of the actin cytoskeleton, but not of the septins, in a fraction of cells that were larger and rounder than normal. Like Cdc42p, both Msb3p and Msb4p localized to the presumptive bud site, the bud tip, and the mother-bud neck, and this localization was Cdc42p dependent. Taken together, the data suggest that Msb3p and Msb4p may function redundantly downstream of Cdc42p, specifically in a pathway leading to actin organization. From previous work, the BNI1, GIC1, and GIC2 gene products also appear to be involved in linking Cdc42p to the actin cytoskeleton. Synthetic lethality and multicopy suppression analyses among these genes, MSB, and MSB4, suggest that the linkage is accomplished by two parallel pathways, one involving Msb3p, Msb4p, and Bni1p, and the other involving Gic1p and Gic2p. The former pathway appears to be more important in diploids and at low temperatures, whereas the latter pathway appears to be more important in haploids and at high temperatures.

INTRODUCTION

A central feature of morphogenesis in many types of cells is cell polarization, which involves the asymmetric organization of the cytoskeleton, secretory system, and plasma membrane components along an appropriate axis (Drubin and Nelson, 1996). A protein of central importance in cell polarization is Cdc42p, a member of the Rho/Rac family of Ras-related small GTPases (Valencia et al., 1991). Cdc42p was first identified in yeast by analysis of a temperature-sensitive mutant that was defective in cell polarization and bud emergence and thus formed large, round, isotropically growing cells at restrictive temperature (Pringle and Hartwell, 1981; Adams and Pringle, 1984; Pringle et al., 1986; Adams et al., 1990; Johnson and Pringle, 1990). It was subsequently found to be remarkably highly conserved (≥76% identity in amino acid sequence) in other types of eukaryotic cells, including humans (Johnson, 1999). In both yeast and animal cells,
Table 1. *Saccharomyces cerevisiae* strains used in this study

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Cdc42p appears to participate in a variety of signaling pathways, including those leading to the actin cytoskeleton and (at least in yeast) of the septins of the neck filaments (Adams and Pringle, 1984; Pringle et al., 1986, 1995; Amatruda and Cooper, 1992; Cvrčková et al., 1995; Li et al., 1995; Van Aelst and D'Souza-Schorey, 1997; Hall, 1998; Aspenström, 1999; Johnson, 1999). Thus, it is important to define both the proteins that regulate Cdc42p activity and the proteins that serve as effectors for the various Cdc42p-mediated signaling pathways.

Intensive efforts during the past few years have resulted in considerable progress along these lines. For example, it seems clear that Cdc42p-family proteins are activated by members of the Dbll family of guanine-nucleotide-exchange factor (GEF) proteins (Cerione and Zheng, 1996; Whitehead et al., 1997), represented in yeast by Cdc24p (Hartwell et al., 1974; Sloot et al., 1981; Adams and Pringle, 1984; Zheng et al., 1994b), and that their return to the GDP-bound state involves the action of specific GTPase-activating proteins (GAPs) (Lamarche and Hall, 1994), which in yeast include Bem3p (Zheng et al., 1994b) and Rga1p/Dhm1p (Stevenson et al., 1995; Chen et al., 1996). The activity of yeast Cdc42p may also be regulated both by Rdi1p, a GDP-dissociation inhibitor (Koch et al., 1997), and by the still-mysterious Zds1p and Zds2p proteins (Bi and Pringle, 1996).

In addition, many candidate Cdc42p effectors have been identified in yeast and other organisms. These include the p21-activated (PAK) family of protein kinases (Sells and Chernoff, 1997), represented in yeast by Ste20p, Cla4p, and Skm1p (Cvrčková et al., 1995; Simon et al., 1995; Zhao et al., 1995; Peter et al., 1996; Benton et al., 1997; Leberer et al., 1997; Martin et al., 1997; Eby et al., 1998); the Wiscott–Aldrich syndrome protein (WASP) family (Bi and Zigmond, 1999), represented in yeast by Lsm17p/Bee1p (Li, 1997; Naqvi et al., 1998); the formin homology (FH) domain proteins (Frazier and Field, 1997; Wasserman, 1998), represented in yeast by Bni1p and Bnr1p (Jansen et al., 1996; Zahner et al., 1996; Evangelista et al., 1997; Imamura et al., 1997; Fujiiwara et al., 1998; Umikawa et al., 1998); the IQGAPs (Machesky, 1998), represented in yeast by Igq1p/Cyk1p (Epp and Chant, 1997; Lippincott and Li, 1998; Osman and Cerione, 1998; Shannon and Li, 1999); and phosphoinositide 3-kinase (Zheng et al., 1994a). Many of these proteins share a conserved Cdc42p/Rac-interactive-binding (CRIB) domain (Burbelo et al., 1995), and recent studies in yeast have identified additional CRIB domain proteins, Gi1p and Gi2p, as functionally redundant Cdc42p effectors that are involved in the polarization of the actin cytoskeleton (Brown et al., 1997; Chen et al., 1997a).

Despite this progress, it seems likely that additional Cdc42p-interacting proteins remain to be identified, and the details of the Cdc42p effector pathways remain poorly understood. Indeed, in some cases, such as the question of whether the PAK kinases are involved in actin polarization, the available data appear contradictory (Cvrčková et al., 1995; Leberer et al., 1997; Peter et al., 1996; Benton et al., 1997; Eby et al., 1998; Hall, 1998; Johnson, 1999). In other cases, such as that of the yeast Msb1p protein (Bender and Pringle, 1989, 1991; Mack et al., 1996), the available data...
identified, MSB3, in conjunction with Cdc42p. The one novel gene identified all encode proteins that appear to function as other hypothetical targets of Cdc24p. However, the other types of proteins. In the study reported here, we include additional Rho-type GTPases (Hart et al., 1995; Tanaka and Takai, 1998) or other targets other than Cdc42p. Such targets might include additional Rho-type GTPases (Hart et al., 1994; Horii et al., 1994; Cid et al., 1995; Tanaka and Takai, 1998) or other types of proteins. In the study reported here, we undertook genetic screens that were designed to identify such other hypothetical targets of Cdc24p. However, the genes identified all encode proteins that appear to function in conjunction with Cdc42p. The one novel gene identified, MSB3, along with its homologue MSB4, defines a pathway that appears to function redundantly with that involving Gic1p and Gic2p in the control of actin polarization. Although our studies failed to identify specific additional targets of Cdc24p, they did provide additional indirect evidence suggesting that such targets may exist.

### MATERIALS AND METHODS

#### Strains, Growth Conditions, and Genetic and Recombinant DNA Methods

Yeast strains used in this study are listed in Table 1; the construction of strains containing deletions and/or tagged genes is described below. Cells were grown on YM-P or YPD rich liquid medium, solid YPD medium, or selective media (Lillie and Pringle, 1980; Guthrie and Fink, 1991), as indicated; 2% glucose was used as carbon source except where noted. Where noted, 1 M sorbitol was added to solid media to enhance the suppression of Ts° phenotypes (Bender and Pringle, 1989), or 1 mM 5-fluoroorotic acid (Toronto Research Chemicals, North York, Ontario, Canada) was added to select for the loss of URA3-containing plasmids (Sikorski and Boeke, 1991). Escherichia coli strain DH12S (Life Technologies, Gaithersburg, MD) was used as a plasmid host. E. coli strain MC1066a (leuB600 trpC9830 pytF74::Tn5 Kanr ara hsdR hsdM* srl:Tn10 recA13) was used to select plasmids carrying the yeast LEU2 gene (Sand-baken and Culbertson, 1988). E. coli was grown under standard conditions (Sambrook et al., 1989).

Standard methods of yeast genetics and recombinant DNA manipulation (Sambrook et al., 1989; Guthrie and Fink, 1991) were used except where noted. Enzymes were purchased from New England Biolabs (Beverly, MA), and oligonucleotide primers were from Integrated DNA Technologies (Coralville, IA). For physical mapping, 32P-labeled DNA fragments were used to probe a filter carrying the completed genome (Chen et al., 1997b; Stillman et al., 1992). Restriction endonucleases were used to construct plasmids in yeast. Strains containing deletions and/or tagged genes are described below. Detailed information about these methods is available in a number of reviews (Sambrook et al., 1989; Guthrie and Fink, 1991).

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* Derived from plasmids described by Čvrčková et al. (1995).
Plasmids

Plasmids used in this study are listed in Table 2 or described where appropriate below. Plasmid YEps352-CD24 was constructed by subcloning an ~1.7-kb BamHI–HindIII fragment that carries CDC42 from YEps351-CD24 (Ziman et al., 1991) into the corresponding sites of YEps352. The genomic DNA library contains yeast Sac3A fragments inserted at the BamHI site of YEps13 (DeMarini et al., 1997). Plasmids YEps13-MSB1, YEps13-CLA4*, and YEps13-MSBX were isolated from this library in the genetic selection described below. Plasmid pEWP1 was constructed by subcloning an ~2.9-kb CLA4*-carrying HindIII–SalI fragment (both sites from the vector) from YEps13-CLA4* into the corresponding sites of pBluescript KS(+). Plasmid YEps352-42CLA4* was constructed by subcloning an ~2.5-kb EcoRI fragment (one site from the vector, the other from the insert, 1024 bp upstream of the CLA4 start codon) from pEWP1 into the EcoRI site of YEps352-CD24. Plasmid YEplac-MSBX was constructed by subcloning an ~3.4-kb SacI–BamHI fragment (both sites from the insert) from YEps13-MSBX into the corresponding sites of YEplac181. Plasmid YEplac-MSBD3 was constructed by deleting a 1238-bp BglII fragment integral to MSB3 from YEplac-MSB3; this deletion results in an in-frame stop codon immediately downstream of the BglII site and thus eliminates the entire COOH terminus of Msb3p starting from codon 115. Plasmid YEplac-ORF1/2 was constructed by subcloning an ~4.1-kb HindIII fragment (one site from the vector, the other from the insert) from YEps13-MSBX into the HindIII site of YEplac181. Plasmid YEplac-MSB4 was constructed by cloning an ~3.9-kb PCR fragment into the EcoRI and XbaI sites of YEplac181, using corresponding sites that had been incorporated into primers MSB4-forward and MSB4-reverse (Table 3). The PCR reaction was carried out using the Expand long template PCR system (Boehringer Mannheim, Indianapolis, IN) and genomic DNA from strain YEF473 as template.

To construct plasmids encoding Msb3p tagged with a triple-hemagglutinin (3HA) epitope, the ~3.4-kb SacI–BamHI fragment from plasmid YEps-MSB3 (see above) was cloned into the corresponding sites of pALTER-1 (Promega, Madison, WI). Using the protocol recommended by Promega and primer MSB3-NotI (Table 3), a NotI site was then introduced immediately downstream of the MSB3 start codon. The ~3.4-kb SacI–BamHI fragment from the resulting plasmid was cloned into the corresponding sites of Yeplac211, and a NotI fragment encoding the 3HA epitope (Tyers et al., 1991) was cloned into the NotI site of the resulting plasmid, yielding plasmid Yeplac-3HA-MSB3, which encodes an in-frame fusion of 3HA and MSB3. The SacI–SalI fragment carrying 3HA-MSB3 from plasmid Yeplac-3HA-MSB3 was then cloned into the corresponding sites of Yeplac181, yielding plasmid Yeplac-3HA-MSB3. Plasmids encoding a 3HA-tagged Msb4p were constructed similarly, using the ~3.9-kb EcoRI–XbaI fragment from Yeplac-MSB4 (see above) and primer MSB4-NotI (Table 3); this yielded plasmids Yeplac-3HA-MSB4 and Yeplac-3HA-MSB4. A plasmid encoding a fusion of green fluorescent protein (GFP) to Cdc24p was constructed similarly, using the ~1.7-kb BamHI–HindIII fragment carrying CDC42 from Yeplac351-CD24 (Ziman et al., 1991), primer CDC24-NotI (Table 3), and a cassette encoding GFP (De Virgilio et al., 1996); this yielded plasmid Yeplac-GFP-CDC42.

To construct plasmid pCC1107, the ~3.4-kb SacI–BamHI fragment containing MSB3 from Yeplac-MSB3 was cloned into SacI–BamHI-digested pSM217. To construct plasmid pCC1108, the ~3.9-kb EcoRI–HindIII fragment containing MSB4 from Yeplac-MSB4 was cloned into EcoRI–HindIII-digested pSM217. To construct plasmid pCC904, the ~1.7-kb Ncol fragment containing GIC1 (Chen et al., 1997a) was blunt ended using T4 DNA polymerase and cloned into Smal-digested pSM217. To construct plasmid pCC967, the ~3.1-kb EcoRI–BglII fragment containing GIC2 (Chen et al., 1997a) was cloned into EcoRI–BamHI-digested pSM217.

Construction of Strains Containing Deletions and Tagged Genes

Complete deletions of the MSB3, MSB4, MDR1, YPL249C, CDC42, BEM3, RGA1, and CDC24 coding regions were constructed using the PCR method described by Baudin et al. (1993). A pair of hybrid primers (Table 3) was used in each PCR reaction. The 5’-ends of the primers corresponded to sequences immediately upstream or downstream of the coding region to be deleted, whereas the 3’-ends of the primers corresponded to vector sequences flanking either HIS3 or TRP1 in plasmid pRS310 or pRS304 (Sikorski and Hieter, 1989), respectively. The amplified fragments were transformed into strain YEF473, selecting stable His+ or Trp+ transformants. The success of the deletion constructions was confirmed by PCR using a forward primer (Table 3) that corresponded to sequences upstream of the particular coding region and a reverse primer that was the same as the one used in making the deletion. In addition, a complete deletion of CDC24 was constructed by transforming strain YEF1223 with a construct carrying the TRP1 marker (generated by PCR as described above), yielding strain YEF1265. The success of this construction was confirmed by Southern blot analysis.

Strains expressing 3HA-tagged Msb3p, 3HA-tagged Msb4p, and GFP-tagged Cdc42p were constructed as follows. Plasmid Yplac-3HA-MSB3 (see above) was linearized within URA3 with Apal and transformed into strains YEF1239 and YEF1242, selecting for stable Ura+ transformants, which were then mated to generate strain YEF1474. Similarly, plasmid Yplac-3HA-MSB4 was linearized within URA3 with Ncol and transformed into strains YEF1247 and YEF1249, and the resulting Ura+ transformants were mated to yield strain YEF1475. To construct strain YEF1517, one copy of CDC42 was deleted in strain YEF473, using the PCR method as described above. The resulting strain was transformed with plasmid Yplac-GFP-CDC42 (see above) that had been linearized within URA3 using EcoRV, and a segregant containing both the cdc42 deletion and GFP-CDC42 was selected.

Morphological Observations, Antibodies, and Protein Methods

Differential interference contrast (DIC) and fluorescence microscopy were performed using a Zeiss (Thornwood, NY) Axioskop or a Nikon (Garden City, NY) Microphot SA microscope with a 60x Plan-apo objective. Immunofluorescence and staining of chitin with Calcofluor were performed as described previously (Pringle, 1991; Ford and Pringle, 1991) after fixing cells by adding formaldehyde directly to the culture medium (final concentration, 3.7%); bisBenzamidine (Sigma, St. Louis, MO) was included in the immunofluorescence mounting medium to visualize DNA. Except where noted, actin rings and patches were visualized by staining cells with 20 U/ml rhodamine-conjugated phalloidin (Molecular Probes, Eugene, OR) for 5 or 30 min (Adams and Pringle, 1991).

The primary antibodies used included goat anti-actin antibodies (Karpova et al., 1993); the rat monoclonal anti-tubulin antibody YOL1/34 (Kilmartin et al., 1982; Kilmartin and Adams, 1984; Accurate Chemical and Scientific, Westbury, NY); rabbit polyclonal antibodies to Cdc11p (Ford and Pringle, 1991), Cdc24p (Pringle et al., 1995, Park et al., 1997), and Isp42p (Kassenbrock et al., 1993); and the mouse monoclonal anti-HA-epitope antibody HA.11 (Berkeley Antibody Company, Richmond, CA). The secondary antibodies used included rhodamine-conjugated donkey anti-goat-immunoglobulin G (IgG), fluorescein isothiocyanate (FITC)-conjugated goat anti-rabbit-IgG, FITC-conjugated rat anti-mouse-IgG, rhodamine-conjugated goat anti-mouse-IgG, and FITC-conjugated goat anti-rat-IgG (all from Jackson ImmunoResearch Laboratories, West Grove, PA).

For immunoblot analysis, protein extracts were prepared by vortexing cells repeatedly with acid-washed glass beads (425–600 μm) in buffer (50 mM Tris–HCl, pH 7.5, 400 mM NaCl, 5 mM EDTA, 1% NP-40) containing a mixture of protease inhibitors (20 μg/ml phe- nylmethanesulfonyl fluoride, 1 μg/ml leupeptin, 1 μg/ml pepstatin

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A, 1 μg/ml aprotinin [all from Sigma]). The glass beads were allowed to settle, and the extracts were transferred to new tubes, diluted 1:1 with 5× SDS-lysis buffer (Laemmli, 1970), boiled for 10 min, and separated electrophoretically on a 10% SDS-polyacrylamide gel. Proteins were then transferred electrophoretically to nitrocellulose paper (Schleicher & Schuell, Keene, NH). Strips of nitrocellulose that carried different sizes of proteins were probed either with anti-Cdc24p or (as a loading control) with anti-Isp42p antibodies, using alkaline phosphatase–conjugated goat anti-rabbit-IgG (Jackson ImmunoResearch Laboratories) as secondary antibody.

RESULTS

Identification of a cdc24 ts Mutation that Is Not Suppressed by Multicopy CDC42

It is currently unclear whether Cdc24p has targets in addition to Cdc42p (see INTRODUCTION). We reasoned that if such additional targets exist, it might be possible to identify a cdc24 ts mutation that is not suppressed by multicopy CDC42 alone but is suppressed by overexpression of the hypothetical target or by simultaneous overexpression of Cdc42p and the hypothetical target. Four previously isolated but incompletely characterized cdc24 ts mutants (carrying the alleles cdc24-10, cdc24-11, cdc24-12, and cdc24-13) were examined. Strains YEF316, YEF319, YEF323, and YEF327, along with the control strain YEF313 (cdc24-4), were transformed with plasmid YEp352-CDC42 and tested for growth at 37°C in the presence of 1 M sorbitol. Under these conditions, all of the cdc24 ts mutations but cdc24-11 were suppressed, suggesting that the primary or exclusive defect caused by the mutations other than cdc24-11 is in the GEF activity for Cdc42p. In contrast, although cdc24-11 might only cause a more severe defect of the same type, it might also cause a defect in a distinct function (or functions) of Cdc24p and thus provide an opportunity to identify such a function genetically.

Isolation of Multicopy Suppressors of cdc24-11

In an initial attempt to identify such a distinct function by isolating multicopy suppressors of cdc24-11, strain YEF319

Table 3. Oligonucleotide primers used for gene deletion, cloning, and tagging

<table>
<thead>
<tr>
<th>Name of primer</th>
<th>Sequence of primer</th>
</tr>
</thead>
<tbody>
<tr>
<td>MSB3-forward</td>
<td>GCCCTATCAAGACCTGTCTGCTAATCCAACCCGAGAGATCGATTGTACTGAGAGTGCACC</td>
</tr>
<tr>
<td>MSB3-reverse</td>
<td>CTTGAATTATTTAGTATTTGCTGTATACTAGTTTTATTTACTGTGCGGTATTTCACACCG</td>
</tr>
<tr>
<td>MSB4-forward</td>
<td>CACTTTTACAATCACAGAAGGCAAAACCCGGCATGACAGGGATTGTACTGAGAGTGCACC</td>
</tr>
<tr>
<td>MSB4-reverse</td>
<td>TTATTTTACACAGTTGCGCTTTTTATCACGGTTGCGCTTACTGTGCGGTATTTCACACCG</td>
</tr>
<tr>
<td>MDR1-forward</td>
<td>AAAAGCACTGTACCTACAATTTAAGACCGAACC</td>
</tr>
<tr>
<td>MDR1-reverse</td>
<td>TTAGTTAGTATGTTAATACTGTGCGGTATTTCACACCG</td>
</tr>
<tr>
<td>YPL249C-forward</td>
<td>AGCTCCTACACCAGTGTAAAGTAGAACGTTAATAGAGCAGATTGTACTGAGAGTGCACC</td>
</tr>
<tr>
<td>YPL249C-reverse</td>
<td>GCAGAAGAAGTACCATTGC</td>
</tr>
<tr>
<td>CDC24-forward</td>
<td>GCCCTATCAAGACCTGTCTGCTAATCCAACCCGAGAGATCGATTGTACTGAGAGTGCACC</td>
</tr>
<tr>
<td>CDC24-reverse</td>
<td>CTTGAATTATTTAGTATTTGCTGTATACTAGTTTTATTTACTGTGCGGTATTTCACACCG</td>
</tr>
<tr>
<td>BEM3-forward</td>
<td>TATATTCTAGAATTTACACTCAACTAATACAGACGAGATTGTACTGAGAGTGCACC</td>
</tr>
<tr>
<td>BEM3-reverse</td>
<td>GTACAAGAAGAAAGGATAG</td>
</tr>
<tr>
<td>RGA1-forward</td>
<td>AGTTATATAAAGGGGTCTTAAACATTTCCCAAGGGCAGGCTTTAGAGG</td>
</tr>
<tr>
<td>RGA1-reverse</td>
<td>AGCAGGCAGTATGCTAATTAACAGCGAAGGATAGG</td>
</tr>
<tr>
<td>CDC24-forward</td>
<td>GTGAAAGAGGATAGTACCCAGGACCGGATAGG</td>
</tr>
<tr>
<td>CDC24-reverse</td>
<td>GTCAAGAAGAAAGGATAG</td>
</tr>
</tbody>
</table>

The underlined sequences correspond to those immediately upstream or downstream of the coding region to be deleted. All sequences are written from 5' to 3'.

a The EcoRI (MSB4-forward-2) and XbaI (MSB4-reverse-2) sites are underlined.

b The NotI sites are underlined.

A, 1 μg/ml aprotinin [all from Sigma]). The glass beads were allowed to settle, and the extracts were transferred to new tubes, diluted 1:1 with 5× SDS-lysis buffer (Laemmli, 1970), boiled for 10 min, and separated electrophoretically on a 10% SDS-polyacrylamide gel. Proteins were then transferred electrophoretically to nitrocellulose paper (Schleicher & Schuell, Keene, NH). Strips of nitrocellulose that carried different sizes of proteins were probed either with anti-Cdc42p or (as a loading control) with anti-Isp42p antibodies, using alkaline phosphatase–conjugated goat anti-rabbit-IgG (Jackson ImmunoResearch Laboratories) as secondary antibody.

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Isolation of Multicopy Suppressors of cdc24-11

In an initial attempt to identify such a distinct function by isolating multicopy suppressors of cdc24-11, strain YEF319
(leu2 ura3 cdc24-11) was transformed with a YEp13-based genomic DNA library. Transformants were plated on SC-Leu medium containing 1 M sorbitol and incubated at 37°C. Among ~45,000 transformants, 22 were found to have plasmid-dependent growth at 37°C. Twenty of these transformants also grew on SC-Leu plates without sorbitol at 37°C. Probing Southern blots of these 22 plasmids with a labeled CDC24 fragment showed that they all carried CDC24, suggesting that no single gene on a high-copy plasmid, other than CDC24 itself, can suppress the temperature-sensitive lethality of cdc24-11. The two plasmids that allowed growth only in the presence of sorbitol at 37°C presumably contained copies of CDC24 that encoded proteins of lower activity or were expressed at a lower level.

If cdc24-11 causes defects both in a CDC42-mediated function and in a second function of Cdc24p, it might be possible to suppress cdc24-11 by simultaneous overexpression of CDC42 and the gene mediating the second function. Thus, strain YEF319 harboring plasmid YEp352-CDC42 was transformed with the YEp13-based genomic DNA library, plated on SC-Leu-Ura medium, incubated overnight at 23°C (to allow transformants to recover), and shifted to 37°C for several days. One transformation plate was kept at 23°C to estimate the total number of transformants obtained. Transformants forming colonies at 37°C presumably contained multicopy plasmids that could suppress cdc24-11 in the presence of the CDC24-CDC42 plasmids. Plasmids were recovered from these transformants into E. coli strain MC1066a (to select for the YEp13-based plasmids) and retransformed into strain YEF319 containing YEp352-CDC42 to confirm the suppression phenotype. Among ~92,000 transformants, 50 yielded plasmids that reproducibly allowed growth of the test strain on SC-Leu-Ura medium at 37°C.

Transformants containing these 50 plasmids were streaked on SC-Leu medium containing 5-fluoroorotic acid at 23°C to select for cells that had lost plasmid YEp352-CDC42. The resulting clones were streaked on SC-Leu medium with and without 1 M sorbitol at 37°C to test for suppression of cdc24-11. This divided the 50 suppressor plasmids into two classes. The first class contained 36 plasmids that allowed the cdc24-11 mutant to grow at 37°C even in the absence of multicopy CDC42. With 18 of these plasmids, growth at 37°C was also sorbitol independent. Based on the results of the first multicopy suppressor screen with the cdc24-11 allele (see above), these 18 plasmids were presumed to carry CDC24 itself. The other 18 plasmids in this class were likely to carry CDC24 itself, and this was confirmed by Southern blot analyses. The second class contained 14 plasmids that did not suppress cdc24-11 in the presence of YEp352-CDC42, even in the presence of 1 M sorbitol. These plasmids potentially identified genes that encode additional targets of Cdc24p.

The 14 plasmids were analyzed by restriction mapping and by probing Southern blots with labeled fragments of CDC24, CDC42, and MSB1. None was found to carry CDC24 or CDC42. Twelve plasmids appeared to have overlapping inserts as judged by restriction mapping, and all 12 hybridized to the MSB1 probe. In addition, the insert from one of these plasmids was shown to hybridize to X' clones (see MATERIALS AND METHODS) from the MSB1 region; this plasmid was designated YEp13-MSB1. Sequencing and hybridization to the X' clones showed that one of the remaining two plasmids contained a truncated allele of CLA4; this plasmid was designated YEp13-CLA4*. Sequencing the ends of the insert DNA in the final plasmid and comparing these sequences to the genome database showed that the insert was derived from a region on the left arm of chromosome XIV not previously known to carry genes related in function to CDC24 and CDC42. This plasmid was designated YEp13-MSBX.

**Suppression of cdc24 and cdc42 by MSB1 and CLA4**

To explore further the interactions among Msb1p, Cla4p, Cdc24p, and Cdc42p, we examined in detail the patterns of multicopy suppression. As indicated above, neither high-copy CDC42 by itself (Figure 1A, sector 4) nor high-copy MSB1 by itself (Figure 1A, sector 6) could suppress cdc24-11 at 37°C, but the presence of both genes in high copy number resulted in good suppression (Figure 1A, sector 2). Similarly, high-copy CLA4* by itself did not suppress cdc24-11 (Figure 1A, sector 5), although it suppressed well in combination with high-copy CDC42 (Figure 1A, sector 1). One possible explanation of these suppression patterns is that cdc24-11 may be defective in two pathways, one involving CDC42 and the other involving MSB1 and/or CLA4*. Consistent with this hypothesis is the observation that high-copy MSB1 and CLA4* together did not suppress cdc24-11 in the absence of high-copy CDC42 (Figure 1A, sector 3). However, it is also possible that cdc24-11 is defective only in the Cdc42p-mediated pathway, but that high-copy MSB1 or CLA4* can somehow enhance the function of this pathway in the presence of high-copy CDC42. This latter hypothesis is supported by the observations that high-copy MSB1 or CLA4* alone suppressed cdc42-1 well in the presence of 1 M sorbitol (Figure 1B, sectors 4 and 5) but did not suppress cdc42-12 (Figure 1B, sectors 1 and 2) or several other cdc42 alleles (our unpublished results) under the same conditions. Other evidence also supports the hypothesis that Msb1p and Cla4p are involved in the same pathways(s) as Cdc42p (see DISCUSSION).

Sequence analysis revealed that the truncated CLA4* allele encodes a protein of 369 amino acids that lacks the entire kinase domain from the COOH-terminal half of the protein but has the PH and PAK domains in the NH2-terminal region (Figure 1C). Interestingly, high-copy full-length CLA4 (in plasmid YEp352-CLA4) did not suppress cdc42-7 by itself and did not suppress cdc24-11 in conjunction with high-copy CDC24 (our unpublished results) (see DISCUSSION).

**Suppression of cdc24 and cdc42 by MSB3**

Plasmid YEp13-MSBX has an ~6.5-kb insert that contains three previously uncharacterized open reading frames (Figure 2A). Subcloning and deletion analysis showed that ORF YNL293W was responsible for the suppression of cdc24-11 in conjunction with high-copy CDC42 (Figure 2B). In the absence of high-copy CDC42, neither YEp13-MSBX nor YEpLac-MSB3 (containing YNL293W) could suppress any of the cdc24 alleles tested (cdc24-4, cdc24-10, cdc24-11, cdc24-12, and cdc24-13) at 37°C even in the presence of 1 M sorbitol (Figure 2C, sectors 1–4; our unpublished results). In contrast, YEp13-MSBX or YEpLac-MSB3 alone could suppress cdc24-2 efficiently at 37°C even in the absence of sorbitol (Figure 2C, sectors 5 and 6; our unpublished results). These
observations suggest that Ynl293Wp, like Msb1p and Cla4p, is involved in the Cdc24p–Cdc42p pathway. By analogy with other genes identified as multicopy suppressors of budding defects, we designated YNL293W as MSB3.

**Homology of Msb3p to Proteins from Yeast and Other Organisms**

Database searches using the deduced amino acid sequence of Msb3p (633 amino acids) revealed that this protein belongs to a widely distributed family of proteins that share a homologous region of ~110 amino acids (Figure 3A). In addition to Msb3p, *Saccharomyces cerevisiae* contains three other members of this family, one of which, the ORF YOL112W gene product, is particularly closely related to Msb3p (51% sequence identity over its full 492 amino acids) and is thus designated Msb4p (Figure 3A). The other two *S. cerevisiae* family members, Mdr1p (950 amino acids; Serpe and Kosman, unpublished *Saccharomyces* Genome Database entry, 1996) and the product of ORF YPL249C (894 amino acids), are less closely related to Msb3p (Figure 3A). In addition, two members of this family have been identified in *Schizosaccharomyces pombe*, one in *Drosophila melanogaster*,

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**Figure 1.** Suppression of *cdc24*<sup>ts</sup> and *cdc42*<sup>ts</sup> mutations by multicopy MSB1 or multicopy truncated CLA4. The transformants described below were streaked onto SC-Leu-Ura plates with 1 M sorbitol (A) or SC-Leu plates with 1 M sorbitol (B) and incubated at the indicated temperatures. (A) *cdc24-11* strain YEF319 was transformed with (1) YEpl352-CDC42 and YEpl13-CLA4, (2) YEpl352-CDC42 and YEpl13-MSB1, (3) YEpl13-CLA4 and pPB191 (high-copy MSB1), (4) YEpl352-CDC42 and YEpl13, (5) YEpl24 and YEpl13-CLA4, or (6) YEpl24 and YEpl13-MSB1. (B) *cdc24-12* strain YEF323 (1–3) and *cdc42-1* strain DJTD2–16D (4–6) were transformed with (5 and 6) YEpl13, (2 and 5) YEpl13-CLA4, or (1 and 4) YEpl13-MSB1. (C) Structures of full-length Cla4p and of the truncated Cla4p encoded by plasmid YEpl13-CLA4.

**Figure 2.** Suppression of *cdc24*<sup>ts</sup> and *cdc42*<sup>ts</sup> mutations by multicopy MSB3. (A) Physical maps of the inserts in plasmid YEpl13-MSBX and in the subclones tested for suppression. All *Hind*III (H), *Sac*I (S), *Bgl*II (G), and *Bam*HI (B) sites are shown. (B and C) The transformants described below were streaked onto SC-Leu-Ura plates with 1 M sorbitol (B) or SC-Leu plates with 1 M sorbitol (C) and incubated at the indicated temperatures. (B) *cdc24-11* strain YEF319 harboring plasmid YEpl352-CDC42 was transformed with (1) YEpl13, (2) YEpl-MSB3D, (3) YEpl-MSB3, or (4) YEpl-ORF1/2. (C) *cdc24-13* strain YEF327 (1 and 2), *cdc24-12* strain YEF323 (3 and 4), and *cdc42-1* strain DJTD2–16D (5 and 6) were transformed with (2, 4, and 6) YEpl13 or (1, 3, and 5) YEpl13-MSBX.
Figure 3. Alignment of Msb3p-homologous sequences from different organisms using the Genetics Computer Group (Madison, WI) Pile-Up program plus some manual adjustments. (A) The complete sequences of Msb3p, Msb4p, and SpMsb3p are shown except for the COOH terminus of Msb3p and the NH₂ and COOH termini of SpMsb3p; for the other proteins, only the regions of clear homology to Msb3p are shown (213 amino acids for Mdr1p; 105–122 amino acids for Pollux, Ypl249Cp, and tre17). Black boxes indicate amino acids identical to those in Msb3p; gray boxes indicate amino acids similar to those in Msb3p (D/E; Q/N; R/K; S/T; I/L/V). Overlining indicates the possible transmembrane domains (see text). The proteins depicted (with accession numbers) include the four proteins from S. cerevisiae (Msb3p [U23084], Msb4p [S51885], Mdr1p [Z72885], and Ypl249Cp [Z67751]); one from S. pombe (SpMsb3p [the product of a previously uncharacterized ORF on cosmid SPCC4G3 from chromosome III]); one from D. melanogaster (Pollux [U50542]); and one from H. sapiens (tre17 [U33005]). Other known proteins showing this region of homology include one from S. pombe (Z56276); seven from C. elegans (Z78539, U67954, Q09445, U49940, U42831, U41540, and U29244); one from M. musculus (Tbc1 [U33005]); and one from H. sapiens (D13644). (B) The sequences of Pollux, Ypl249Cp, and tre17 are shown for the region immediately downstream of the portions of these proteins shown in A. Black boxes indicate amino acids identical in two or more of the proteins; gray boxes indicate amino acids similar (as in A) in two or more of the proteins.
seven in Caenorhabditis elegans, one in Mus musculus, and two in Homo sapiens (Figure 3). One of the S. pombe proteins (here designated SpMsb3p; 635 amino acids) is highly homologous to Msb3p and Msb4p (35% sequence identity over the full-length proteins; 47–50% sequence identity over the central region of 310 amino acids), suggesting that the function(s) of Msb3p and Msb4p may be evolutionarily conserved. Thirteen amino acids are absolutely conserved among the seven sequences aligned in Figure 3A. Interestingly, Pollux, Ypl249Cp, and tre17 also share a region of homology with each other (although not with Msb3p, Msb4p, SpMsb3p, and Mdr1p) in a region immediately downstream from that in which all seven proteins are homologous (Figure 3B). Little is known about the function of this family of proteins (see DISCUSSION).

The program TM-pred (Hofmann and Stoffel, 1993) predicts two potential membrane-spanning domains in Msb3p (residues 320–337 and 424–440; indicated by overlining in Figure 3A), two in Msb4p (residues 238–255 and 335–351, corresponding to Msb3p 320–327 and 424–440), four in Mdr1p (residues 122–141, 326–343, 400–416, and 427–444, the middle two of which correspond to Msb3p 320–327 and 424–440), and two in Ypl249Cp (residues 561–576 and 595–612). The program of Lupas (1996) identifies one region in Msb3p (residues 236–270, p = 0.35) with some potential to form coiled coils and two regions in Ypl249Cp (residues 729–810, p = 0.99; residues 822–876, p = 0.99) with strong potential to form coiled coils. Potential coiled coils were not identified in Msb4p or Mdr1p. The functions of these regions remain to be determined.

**Functional Analysis of MSB3, MSB4, MDR1, and YPL249C**

The similarity in sequence between Msb4p and Msb3p suggested that Msb4p might also interact with Cdc24p and/or Cdc42p. As one test of this hypothesis, we amplified MSB4 by PCR and cloned it into a high-copy vector, generating plasmid YEplac-MSB4, which was then assayed for its ability to suppress cdc24 and cdc42 mutations. High-copy MSB4 suppressed cdc24-11 effectively in conjunction with high-copy CDC42 at 35.5°C (Figure 4A, sector 2); however, unlike MSB3, high-copy MSB4 fails to suppress cdc24-11 (our unpublished results).

To explore further the functions of Msb3p and its homologues, we examined the phenotypic consequences of precise deletions of MSB3, MSB4, MDR1, and YPL249C, alone and in combination. None of the four single mutants displayed any obvious growth defect or morphological abnormalities at temperatures ranging from 23 to 37°C (Figure 5, A, sectors 2 and 3, C, and D; our unpublished results). Strains deleted for all four genes or any combination of two or three genes (see Table 1 for a list of strains constructed and examined) were viable over the same temperature range, indicating that these four genes are not essential for viability under laboratory conditions. However, the msb3 msb4 double mutants (as well as the quadruple mutant and triple mutants containing both the msb3 and msb4 deletions) grew significantly more slowly than did the wild-type strain at 23°C (Figure 5A, sector 4; our unpublished results). In addition, nearly all of the msb3 msb4 cells became rounder than normal (Figure 5E), and a fraction (15–20%) were larger than normal (Figure 5E), suggesting that there is a defect in the polarization of growth. This phenotype was not significantly enhanced by deletion of MDR1, YPL249C, or both, suggesting that although all four proteins share the conserved domain, Mdr1p and Ypl249Cp may function in a pathway(s) distinct from that involving Msb3p and Msb4p.

In S. cerevisiae, the actin cytoskeleton and the septins appear to polarize independently of each other, but both are dependent on Cdc42p (Adams and Pringle, 1984; Adams et al., 1990; Ford and Pringle, 1991; Amatruda and Cooper, 1992; Cvrlková et al., 1995; Li et al., 1995; Pringle et al., 1995; Ayscough et al., 1997). To ask whether Msb3p and Msb4p are involved in the polarization of the actin cytoskeleton, the septins, or both, we stained cells of the msb3 msb4 double mutant for F-actin and for Cdc11p. Actin appeared randomly distributed in the cell cortex in most, if not all, of the large, round cells of the double mutant (Figure 6D). About half of these cells contained two or more nuclei (Figure 6E, lower two cells). Interestingly, among the cells with disorganized actin, most (65 of 75 cells scored) had patterns of Cdc11p localization that appeared at least approximately normal (Figure 6E). These results suggest that Msb3p and Msb4p are involved in the control of actin organization, but not of septin organization, by Cdc42p.
Localization of Msb3p and Msb4p and Its Dependence on Cdc42p

To explore further the apparent functional interactions among Msb3p, Msb4p, and Cdc42p, we used HA-epitope–tagged Msb3p and Msb4p to localize these proteins. Both tagged genes on high-copy plasmids (low-copy plasmids were not tested) were able to complement the phenotypes of the double mutant, suggesting that the tagged genes are functional. Initial localization experiments used diploid strains (YEF1474 and YEF1475) that were homozygous for integrated single copies of HA-MSB3 and HA-MSB4, respectively. Although immunofluorescence using anti-HA antibody allowed localization of the tagged Msb3p in some cells, the signal was very weak, and no signal was detected for the tagged Msb4p. Thus, strains YEF1474 and YEF1475 were transformed with high-copy plasmids (YEplac-3HA-MSB3 and YEplac-3HA-MSB4) containing HA-MSB3 and HA-MSB4, respectively. Immunostaining of the transformants with anti-HA antibody yielded detectable signals for both proteins. In the case of Msb3p, this signal was stronger and more consistent than, but qualitatively similar to, the signal obtained with the untransformed strain.

In the transformed strains, HA-Msb3p and HA-Msb4p displayed similar patterns of localization throughout the cell cycle. Both proteins were first observed in unbudded cells at the presumptive bud site, as indicated by the consistent association with actin staining at the same site (Figure 7, A–C, cell 1; Figure 8, A–C, cell 1). In cells with small and medium-sized buds, Msb3p and Msb4p were localized to the bud tips (Figure 7, A–C, cell 2, and D and E, cell 1). Later in the cell cycle, Msb3p and Msb4p became undetectable (Figure 7, A–C, cell 3, and D and E, cell 2; our unpublished results). The loss of the Msb3p and Msb4p signals from the bud tip occurred at approximately the same stage as the apical-to-isotropic shift (Lew and Reed, 1993) in actin organization and the extension of the mitotic spindle through the neck (Figure 7, A–C, cf. cell 2 to cell 3; Figure 7, D and E, cf. cell 1 to cell 2; our unpublished results). Among 46 cells showing localization of HA-Msb3p to the bud tip, 32 clearly had apically concentrated actin, whereas 14 appeared to have actin more isotropically arranged in the bud, and cells lacking an HA-Msb3p signal at the bud tip invariably displayed isotropically arranged actin. Near the end of the cell cycle, in cells with fully elongated spindles...
and well separated chromosome sets, HA-Msb3p and HA-
Msb4p again became detectable but were now found in a
single ring at the mother-bud neck (Figure 7, A–C, cell 4, and
D and E, cell 3; Figure 8, A–C, cell 3) that was coincident (in
24 of 24 cells scored for Msb3p) with the ring of actin
observed in such cells (Figures 7B, cell 4, and 8B, cell 3; also
see Bi et al., 1998; Lippincott and Li, 1998). Msb3p and
Msb4p appeared to remain at the division site on both
mother and daughter cells for some time after spindle break-
down and (presumably) cytokinesis (Figure 7, D and E, cell
4; our unpublished results); however, as very few unbudded
cells were observed with two patches of HA-Msb3p or HA-
Msb4p staining, these division-site remnants must disassem-
bble before the new bud site is organized. The correlation
between the localization of Msb3p and Msb4p and that of
actin (see above) and Cdc42p (Ziman et al., 1993; our unpub-
lished results; see DISCUSSION) supports the hypothesis
that Msb3p and Msb4p are involved in a pathway that links
Cdc42p to the actin cytoskeleton (as discussed further
below).

If this hypothesis is correct, it might also be predicted that
the localization of Msb3p and Msb4p would depend on
Cdc42p function. A difficulty in testing this hypothesis was
that the cdc42-1 mutation can be suppressed by multicopy
MSB3 (see above), including the multicopy HA-MSB3 as
used in the localization experiments. However, serendipi-
tously, a strain carrying a GFP-CDC42 allele (constructed for
other purposes) was found to have a temperature-sensitive-
lethal phenotype that was not suppressed by multicopy
MSB3. When this strain (YEF1517) was transformed with
YEplac-3HA-MSB3 or YEplac-3HA-MSB4 and grown at
23°C, Msb3p (Figure 8D), Msb4p (our unpublished results),

Figure 7. Immunolocalization of HA-
tagged Msb3p. Cells of strain YEF1474
harboring plasmid YEplac-3HA-MSB3
growing exponentially in SC-Leu liq-
uid medium at 23°C were triple
stained for HA-tagged Msb3p (A), ac-
tin (B), and DNA (C) or double stained
for HA-tagged Msb3p (D) and tubulin
(E). Individual cells are numbered for
reference in the text.
and actin (Figure 8E) appeared to localize normally, although some cells were misshapen even at this temperature (e.g., the upper right cell in Figure 8D). However, after a shift to 37°C for 4 h, actin appeared to be randomly distributed in the cortex of the large, round cells (Figure 8H), and no Msb3p or Msb4p localization was observed (Figure 8G; our unpublished results). Similar results were obtained when the localization of 3HA-Msb4p was examined in a cdc42-1 strain after 4 h at 37°C (our unpublished results). Thus, as predicted, the localization of Msb3p and Msb4p indeed appears to depend on Cdc42p.

Evidence for Parallel Pathways Linking Cdc42p to the Actin Cytoskeleton

If Msb3p and Msb4p are involved in linking Cdc42p to the actin cytoskeleton, why is the msb3 msb4 double mutant viable? One possibility is that there are other proteins that have overlapping function or that constitute a parallel pathway. Evidence from other studies has suggested that Bni1p, Gic1p, and Gic2p may also be targets of Cdc42p that are involved in the organization of the actin cytoskeleton (Brown et al., 1997; Chen et al., 1997a; Evangelista et al., 1997; Imamura et al., 1997; see INTRODUCTION). Thus, we asked whether mutations in these genes are synthetically lethal with msb3 and/or msb4, performing the experiments at 23°C. First, strain YEF1291 (α msb3Δ::HIS3 msb4Δ::TRP1) was crossed to strain JF16 (a bni1Δ::LEU2). Among 74 tetrads dissected, 68 produced four viable segregants, five produced three viable segregants, and one produced two viable segregants. Fourteen viable triple mutants (His+ Trp+ Leu+) were recovered (the small number reflecting the linkage of MSB3 and BNI1 on the left arm of chromosome XIV), and none of the seven inviable segregants was predicted (from the genotypes of the other segregants in the same tetrad) to be a triple mutant. Thus, the msb3, msb4, and bni1 mutations are not synthetically lethal. Next, strain YEF1269 (a msb3Δ::HIS3 msb4Δ::HIS3) was crossed to strain CCY1042-12B (a gic1Δ::LEU2 gic2Δ::TRP1), and 173 tetrads were dissected. Viable segregants were recovered that represented all of the possible double and triple mutants. However, 11% of the segregants were inviable, including all 19 that could be predicted unambiguously to harbor all four mutations. Moreover, when six viable His+ Leu+ Trp+ segregants that might have been quadruple mutants were outcrossed to strain YEF473A or YEF473B, all six proved to be triple mutants (containing msb3Δ::HIS3 or msb4Δ::HIS3 but not both). Thus, the msb3 and msb4 mutations appear to be synthetically lethal in combination with the gic1 and gic2 mutations. Finally, strain YJZ426 (a bni1Δ::HIS3) was crossed to strain CCY1042-12B, and 20 tetrads were dissected. Of the 69 viable segregants, none was a triple mutant (although the three mutations should segregate independently), and 7 of the 11 inviable segregants could be predicted to be triple mutants. Thus, the bni1 mutation appears to be synthetically lethal with the gic1 and gic2 mutations. For both the predicted msb3 msb4 gic1 gic2 quadruple mutants and the predicted bni1 gic1 gic2 triple mutants, microscopic examination of the inviable spore clones revealed microcolonies (one to a few cells) consisting almost entirely of large, round, un budded cells. Taken together, the data suggest that Msb3p/Msb4p and Bni1p may be involved in one pathway linking Cdc42p to the actin cytoskeleton, whereas Gic1p/Gic2p may function in a second, parallel pathway.

If this hypothesis is correct, enhancing one signaling pathway might compensate for a defect in the other. Indeed, high-copy MSB3 was found to suppress effectively the temperature-sensitive growth and morphological defects of the gic1 gic2 double mutant (Figure 9, A and D), and high-copy MSB4 also suppressed these defects, although somewhat less effectively (Figure 9, A and E). However, in the recip-
from those of a gic1 gic2 double mutant. Indeed, although gic1/gic1 gic2/gic2 double-mutant diploid cells appear to have a normal ellipsoidal cell shape (Figure 10, A and D), both msb3/msb3 msb4/msb4 diploids (Figure 10B) and bni1/bni1 double diploids (Figure 10C) displayed cells that are rounder than normal. In addition, mutation of MSB3 and MSB4 and mutation of BNI1 have similar effects on budding pattern. It has been shown previously that deletion of BNI1 has little or no effect on the axial budding of haploid cells but that a diploid homozygous for the bni1 deletion has a random budding pattern in which even the first bud on a daughter cell appears randomly positioned (Zahner et al., 1996; Imamura et al., 1997; Figure 10C). Similarly, msb3, msb4, and msb3 msb4 haploid displayed normal axial budding (our unpublished results), but an msb3/msb3 msb4/msb4 diploid displayed a random budding pattern (Figure 10B). (Diploid strains homozygous for deletions of MSB3 alone or of MSB4 alone displayed normal bipolar budding [our unpublished results].) Like the bni1/bni1 strains (but unlike several other mutants with defects in bipolar budding; Zahner et al., 1996; Yang et al., 1997), the msb3/msb3 msb4/msb4 strain frequently mispositioned even the first buds on daughter cells (our unpublished results). In contrast, deletion of GIC1, GIC2, or both had no obvious effect on the budding patterns of either haploid (our unpublished results) or diploid (Figure 10D) strains in the genetic background used here. Finally, examination of the Calcofluor-stained cells also revealed that both bni1/bni1 cells and msb3/msb3 msb4/msb4 cells, but not gic1/gic1 gic2/gic2 cells, produced bud scars that were heterogeneous in size (Figure 10, B–D).

Why might the cell have two parallel pathways linking Cdc42p to the actin cytoskeleton? A clue comes from the observation that the msb3 msb4 double-mutant haploids and the msb3/msb3 msb4/msb4 double-mutant diploids have more severe growth and morphological defects at 23°C than at 37°C (Figures 5A, sector 4, and 10E, sectors 2 and 6; our unpublished results). Moreover, the morphological abnormalities observed at 23°C were more pronounced in the double-mutant diploid than in the double-mutant haploid (Figure 10, F and G). Similarly, deletion of BNI1 causes more severe growth and morphological defects in a homozygous diploid than in a haploid and at low than at high temperatures (Figure 10E, sectors 3 and 7; Fares and Pringle, unpublished results). In striking contrast, a gic1 gic2 double-mutant haploid is viable at 23°C but not at temperatures above 32°C (Brown et al., 1997; Chen et al., 1997a; Figures 9A and 10E, sector 4), whereas a gic1/gic1 gic2/gic2 double-mutant diploid grew well even at 37°C (Figure 10E, sector 8). (In a different genetic background, a gic1/gic1 gic2/gic2 double-mutant diploid grew at 35°C but not at 37°C [Chen and Chan, unpublished data].) Thus, it appears that the two pathways may differ in importance in different cell types and at different growth temperatures.

**Evidence for a Cdc42p Function Distinct from Its Cdc42p–GEF Activity**

All cdc24ts alleles that have been examined are suppressed by multicopy CDC42, either alone or in combination with multicopy MSB1, CLA4, MSB3, or MSB4, genes whose products all appear to be involved in the Cdc24p–Cdc42p pathway. These observations suggest that the available cdc24ts alleles all encode products that are defective in the

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**Figure 9.** Suppression of the gic1 gic2 double mutant by multicopy MSB3 or MSB4. (A) Suspensions of strain CCY1024–19C harboring plasmid pSM217 (control), pCC1107 (MSB3), pCC1108 (MSB4), pCC904 (GIC1), or pCC967 (GIC2) were spotted on YPD plates and incubated for 2 days at the indicated temperatures. (B–E) Cells of strain CCY1024–19C containing plasmid pSM217 (B), pCC904 (C), pCC1107 (D), or pCC1108 (E) were grown to exponential phase in SD+ Ade+ Trp+ casamino acids medium at 26°C and then shifted to 37°C for 4 h before fixation and examination by DIC microscopy. B–E are printed at the same magnification.

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rocal experiment, high-copy GIC1 or GIC2 (plasmid pCC904 or pCC967) did not appear to suppress the growth and morphological defects of the msb3 msb4 double-mutant strains YEF1269 and YEF1631 (our unpublished results; see DISCUSSION).

If Msb3p/Msb4p and Bni1p indeed function in one pathway while Gic1p/Gic2p function in another, then at least some of the phenotypes of the msb3 msb4 double mutant should resemble those of a bni1 single mutant and differ
interaction with Cdc24p and/or in the GEF activity. If Cdc24p really functions only as a Cdc42p GEF, then cells lacking Cdc24p might survive if the level of Cdc42p GAP activity is reduced. To test this possibility, we generated a TRP1-marked cdc24 deletion in a diploid homozygous for deletions of both BEM3 and RGA1, yielding strain YEF1265 (Table 1). Tetrad analysis of this strain yielded only Trp− viable segregants. Thus, deletion of the two Cdc42p GAP genes cannot bypass the requirement for Cdc24p.

In a second experiment, we asked whether overexpression of other proteins involved in Cdc42p function would allow polarity establishment in the absence of Cdc24p. To this end, strain YEF1201, a cdc24 deletion strain harboring a low-copy GAL1-CDC24 plasmid, was transformed with high-copy plasmids containing CDC42 alone or CDC42 in combination with CLA4*, MSB1, or both. During growth of the resulting strains under mildly inducing conditions, Cdc24p levels were slightly greater than in wild-type cells (Figure 11A, lanes 1–4 and 9). After shift to repressing conditions for 16 h, Cdc24p was essentially undetectable in all strains (Figure 11A, lanes 5–8), indicating that overexpression of the other proteins had not stabilized Cdc24p. The presence of high-copy CDC42 alone did not prevent Cdc24p-depleted cells from losing actin polarization and arresting uniformly as large, round cells (Figure 11, B and F). However, high-copy CDC42 together with high-copy CLA4* (Figure 11, C and G), high-copy MSB1 (Figure 11, D and H), or both (Figure 11, E and I) allowed Cdc24p-depleted cells to polarize their actin cytoskeletons and cell growth. Approximately 50% of the CLA4*-containing cells became polarized and were able to produce one bud-like structure per mother cell, and nearly all of the MSB1-containing or MSB1- and CLA4*-containing cells became polarized and were able to produce multiple (up to three or four) elongated bud-like structures per cell. However, none of the strains tested was able to form colonies under the repressing conditions for CDC24 expression, suggesting that Cdc24p has at least one function in addition to activating polarity establishment through its Cdc42p GEF activity.

**DISCUSSION**

The Possibility of a Cdc24p Function Distinct from Its Cdc42p GEF Activity

Although it is well established that Cdc24p functions as an activating factor (GEF) for Cdc42p, it also seems possible that Cdc24p has one or more other targets (see INTRODUCTION). The genetic screens undertaken in this study were designed to seek such additional targets. However, as discussed below, all of the genes actually identified encode products that appear to function in the Cdc42p pathway. Nonetheless, we also obtained some evidence suggesting that Cdc24p might not function exclusively as a Cdc42p GEF. First, we observed that Cdc24p was still essential for growth in a strain in which the genes encoding the Cdc42p GAPs Bem3p and Rga1p (Zheng et al., 1994b; Stevenson et al., 1995) had been deleted. In contrast, in the S. cerevisiae Ras pathway, deletion of GAP-encoding genes rescues the inviability of mutants defective in the GEF Cdc25p (Tanaka et al., 1989, 1990a, 1990b), suggesting that the GEF is required only to counteract the activity of the GAPs. However, this argument is weakened by the possibility that Cdc24p may be
required to compensate for the relatively high intrinsic GTPase activity of Cdc42p (Zheng et al., 1994b) and by the likelihood that there are Cdc42p GAPs in addition to Bem3p and Rga1p (Bi and Pringle, 1996; Chen et al., 1997a; Bose and Lew, personal communication; Sprague, personal communication).

Thus, a more persuasive argument is provided by the observation that the presence of high-copy CDC42 together with high-copy CLA4*, high-copy MSB1, or both allowed Cdc24p-depleted cells to polarize their actin cytoskeletons and produce multiple bud-like structures, although they could not complete the cell cycle and divide. This phenotype might reflect rescue of the Cdc42p pathway, but not of another Cdc24p-dependent pathway, in these cells. However, until another Cdc24p target has actually been identified, its existence remains uncertain, and it is possible that the other domains of Cdc24p function solely in the temporal and spatial regulation of its interaction with Cdc42p. For example, the Ca²⁺-binding sites may regulate the interaction between Cdc24p/Cdc42p and Bem1p (Zheng et al., 1995), and the PH domain may serve to bind regulatory ligands or as a membranous adaptor for Cdc24p and its associated proteins (Hemmings, 1997; Aghazadeh et al., 1998; Lemmon and Ferguson, 1998; Liu et al., 1998; Rebecchi and Scarlata, 1998; Soisson et al., 1998).

**Interaction of Cla4p and Msb1p with Cdc24p and Cdc42p**

Of the three genes identified in our screens, two were the previously known CLA4 and MSB1. Overexpression of the truncated CLA4* alone, of MSB1 alone, or of CLA4* and MSB1 together could suppress the cdc24-11 mutation only when CDC42 was also overexpressed. These observations might be taken to mean that the cdc24-11 mutant is defective in each of two Cdc24p-controlled pathways, one of which involves Cdc42p and the other of which involves Cla4p and/or Msb1p. However, abundant other evidence indicates that both Cla4p and Msb1p actually function in conjunction with Cdc42p. In the case of Cla4p, we observed that multicy copy CLA4* by itself could suppress a cdc42-1 mutation but not any of several cdc24 alleles (Figure 1B). In addition, Cla4p is a member of the PAK kinase family, for which binding to and activation by the GTP-bound form of Cdc42p and its homologues are well established, although the targets of the activated PAKs themselves remain unclear (Cvrčková et al., 1995; Simon et al., 1995; Peter et al., 1996; Benton et al., 1997; Leberer et al., 1997; Sells and Chernoff, 1997; Van Aelst and D’Souza-Schorey, 1997; Eby et al., 1998; Hall, 1998; Johnson, 1999). Binding to PAK has been shown to inhibit the intrinsic GTPase activity of human Cdc42 (Manser et al., 1994). This suggests that overexpression of Cla4p*, which lacks the Cla4p kinase domain but includes its Cdc42p-binding CRIB domain, suppresses cdc42-1 or (when CDC42 is also overexpressed) cdc24-11 by inhibiting the intrinsic GTPase activity of Cdc42p and thus increasing the fraction of the protein that is in the active state. Interestingly, full-length CLA4 lacked the suppressor activities of CLA4*, suggesting that the binding of Cla4p to Cdc42p may be regulated by the kinase activity or by the presence of the kinase domain.

The evidence that Msb1p functions in conjunction with Cdc42p is less direct but nonetheless good. MSB1 was first
identified as a multicopy suppressor of the cdc24-4 mutation, which is also suppressed well by multicopy CDC42, and multicopy MSB1 also suppresses cdc42-1 at least as well as it suppresses cdc24-4 (Bender and Pringle, 1989; Figure 1B). In addition, MSB1 is a multicopy suppressor of mutations in BEM4, whose product appears to interact with Cdc42p (Mack et al., 1996), and an msb1 deletion is synthetically lethal with mutations in BEM2 (Bender and Pringle, 1991), whose product may also interact with Cdc42p (Chen et al., 1996, 1997a). Finally, Msb1p colocalizes with Cdc42p throughout the cell cycle (Bi and Pringle, unpublished results). Msb1p is a protein of 1137 amino acids for which biochemical activities or close homologues have not yet been found; it may function as a scaffold that helps assemble Cdc24p, Cdc42p, and other proteins into a functional complex. Thus, overexpression of Msb1p might suppress cdc24 and cdc42 mutations by promoting the more efficient assembly or function of such complexes or by directing them to appropriate intracellular locations.

**Parallel Pathways Linking Cdc42p to Actin Polarization**

The third gene identified in our screen was the novel MSB3. The genome sequence revealed that MSB3 has a close structural homologue, MSB4, and several lines of evidence indicate that Msb3p and Msb4p have overlapping functions in a pathway that links the activation of Cdc42p to polarization of the cytoskeleton. First, Msb3p and Msb4p colocalized with each other and with Cdc42p at a patch at the presumptive bud site, to the tip of the growing bud, and (just before division) to the mother-bud neck. (Localization of Cdc42p to the neck was not consistently visualized with anti-Cdc42p antibodies [Ziman et al., 1993] but is apparent using HA- or GFP-tagged Cdc42p [Johnson, 1999; Bi and Pringle, unpublished results].) Moreover, the localization of Msb3p and Msb4p depended on the function of Cdc42p (Figure 8, D–I), whereas Cdc42p localized normally in an msb3 msb4 strain (Bi and Pringle, unpublished results). Second, overexpression of either MSB3 or MSB4 suppressed cdc24-11 efficiently when CDC42 was also overexpressed, but each gene suppressed cdc24 alleles weakly or not at all in the absence of CDC42 overexpression. In contrast, overexpression of MSB3 by itself suppressed cdc42-1 efficiently. (The failure to observe suppression of cdc42 by multicopy MSB4 may reflect the fact that only one cdc42 allele was available for testing.) Third, although deletion of either MSB3 or MSB4 alone produced no obvious phenotype, the msb3 msb4 double mutant displayed a partial loss of cell polarization resembling that resulting from a loss of Cdc42p function. Moreover, deletion of both MSB3 and MSB4, but not deletion of either gene alone, was lethal in a gic1 gic2 background (also see below). Finally, the phenotype of the msb3 msb4 double mutant closely resembled that resulting from deletion of BN11, a gene whose product appears to function downstream of Cdc42p (and perhaps Rho1p) in a pathway(s) leading to cytoskeletal polarization (Evangelista et al., 1997; Frazier and Field, 1997; Imamura et al., 1997; Fujiwara et al., 1998; Umikawa et al., 1998; Wasserman, 1998).

Cdc42p is necessary for polarization both of the actin cytoskeleton and of the septins (Adams and Pringle, 1984; Adams et al., 1990; Li et al., 1995; Pringle et al., 1995), but polarization of actin and of the septins are independent of each other (Adams and Pringle, 1984; Ford and Pringle, 1991; Ayscough et al., 1997). Two lines of evidence suggest strongly that Msb3p and Msb4p are involved specifically in a pathway leading to actin polarization. First, the localization of Msb3p and Msb4p through the cell cycle (see above) resembles the localization of actin and associated proteins (Botstein et al., 1997; Bi et al., 1998; Lippincott and Li, 1998) more closely than it does that of the septins, which form a ring at the presumptive bud site and remain at the mother-bud neck throughout the cell cycle (Longtine et al., 1996). Second, and more convincing, the large, round cells in an msb3 msb4 double-mutant strain displayed relatively normal septin rings despite their loss of actin polarization (Figure 6, D–F).

If the Msb3p/Msb4p pathway is important for actin polarization, why do msb3 msb4 double-mutant cells survive? It appears that this is because the Msb3p/Msb4p pathway is largely redundant in function with a distinct pathway involving Gic1p and Gic2p (Figure 12). Multiple lines of evidence indicate that Gic1p and Gic2p function redundantly with each other in a pathway that is important, but not essential, for the Cdc42p-mediated polarization of the actin cytoskeleton (Brown et al., 1997; Chen et al., 1997a; Jacques et al., 1998; Richman et al., 1999). The viability of the gic1 gic2 double mutant and the observation that its phenotypes could be suppressed by overexpression of CDC42 (Brown et al., 1997; Chen et al., 1997a) had already suggested the existence of a redundant pathway, and we have shown here that overexpression of MSB3 or MSB4 can also partially suppress the gic1 gic2 double mutant. Moreover, although all double and triple mutants involving msb3, msb4, gic1, and gic2 deletions were viable, the quadruple mutant was inviable and displayed a loss-of-polarity phenotype.

A variety of other proteins have also been implicated in the communication between Cdc42p and the actin cytoskeleton (see INTRODUCTION), and it is important to determine how the functions of these other proteins relate to the pathways defined by Msb3p/Msb4p and by Gic1p/Gic2p. To date, the only clear evidence is for Bni1p, for which several observations indicate a role in the pathway involving Msb3p and Msb4p. First, the bni1 single mutant and the msb3 msb4 double mutant have similarities in the details of their phenotypes that are not shared with the gic1 gic2 double mutant (see RESULTS). These distinctions may re-
fect aspects of Msb3p/Msb4p/Bni1p pathway function that are not fully redundant with the Gic1p/Gic2p pathway, which may in turn explain why we observed no suppression of the msb3 msb4 phenotypes by overexpression of Gic1p or Gic2p (in contrast to the suppression of gic1 gic2 phenotypes by overexpression of Msb3p or Msb4p). Second, msb3 msb4 bni1 triple mutants were viable, whereas the bni1 gic1 gic2 triple mutants, like the msb3 msb4 gic1 gic2 quadruple mutants, were inviable. It is not yet clear whether Bni1p should be viewed as functioning upstream or downstream of Msb3p and Msb4p. Indeed, given the evidence that Bni1p interacts directly both with Cdc42p GTP and with components of the actin cytoskeleton (Kohno et al., 1996; Evangelista et al., 1997; Frazier and Field, 1997; Imamura et al., 1997; Fujiwara et al., 1998; Umikawa et al., 1998; Wasserman, 1998), it seems more likely that the question is meaningless because the proteins actually all function as part of a single complex. However, the specific binding interactions of Msb3p and Msb4p have not yet been defined. In particular, these proteins do not contain recognizable Cdc42p-interaction domains, and there is as yet no evidence for a direct interaction between them and Cdc42p, Bni1p, or components of the actin cytoskeleton.

The evidence that the Msb3p/Msb4p/Bni1p pathway and the Gic1p/Gic2p pathway are largely redundant in function raises the now-common question of why such redundancy has evolved. In the present case, the data suggest that the two parallel pathways may be optimized for the physiological states obtaining in different cell types and under different growth conditions. In particular, it appears that the Msb3p/Msb4p/Bni1p pathway is more important in diploids and at lower growth temperatures, whereas the Gic1p/Gic2p pathway is more important in haploids and at higher growth temperatures, and it seems likely that other variations in growth conditions would reveal other illustrations of the differential importance of the two pathways.

Possible Evolutionary Conservation of Msb3p/Msb4p Function

Both the structure of Cdc42p and its role in organizing the actin cytoskeleton have been strongly conserved during eukaryotic evolution (see INTRODUCTION). This conservation extends also to many of the apparent regulators and effectors of Cdc42p (see INTRODUCTION). In particular, proteins with similar sequence organization and significant sequence homology to Bni1p (the FH proteins) appear to be involved in linking Rho-type GTPases to the actin cytoskeleton in other fungi, nematodes, flies, and mammals, as well as in yeast (Frazier and Field, 1997; Wasserman, 1998). In this context, it seems likely that the role of Msb3p/Msb4p-type proteins in a pathway involving also an FH protein would also have been conserved. However, the available data do not allow a clear conclusion on this point. Msb3p and Msb4p are indeed members of a large protein family (see RESULTS and Figure 3). However, among the known family members, only S. pombe SpMsb3p has strong homology with Msb3p and Msb4p over the full lengths of the proteins, and the functions of SpMsb3p have not yet been investigated. In contrast, the family members from multicellular organisms, such as Pollux and tre17, like the S. cerevisiae proteins Mdr1p and Ypl249Cp, share more limited homology with Msb3p and Msb4p over only a portion of the proteins, so that the sequence similarity provides no strong indicator of homologous function. Indeed, for Mdr1p and Ypl249Cp, gene deletions produced no obvious phenotype and did not obviously enhance the phenotype caused by deletion of MSB3 and MSB4, suggesting that Mdr1p and Ypl249Cp function in another pathway(s). The available information about the proteins from multicellular organisms is also not very helpful at this time. Pollux has been reported to be both a possible cell-adhesion molecule (Zhang et al., 1996) and a calmodulin-binding protein (Xu et al., 1998), both properties that could be associated with a role in cytoskeletal organization, but its actual function remains obscure. tre17 has been identified as an oncogene by several criteria (Nakamura et al., 1992; Onno et al., 1993), but there appear to be no good clues to the origins of its oncogenicity. Nonetheless, it is intriguing that a loss of normal cell shape and polarization is a general property of cancer cells, so that a possible role for tre17 in morphogenesis is not implausible. Clearly, however, elucidation of the roles of these family members and of their shared and distinct functional domains will require further investigation.

Note Added in Proof. A recent paper by Albert and Gallwitz (J. Biol. Chem. 274, 33186–33189, 1999) showed that Msb3p can function as a GAP for several Rab proteins including Sec4p. This suggests that Cdc42p-mediated actin organization may be coupled to a late secretion function through Msb3p and Msb4p.

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Parallel Pathways for Actin Polarization


