Oligomerization Regulates the Localization of Cdc24, the Cdc42 Activator in Saccharomyces cerevisiae*

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Guanine nucleotide exchange factor activation of Rho G-proteins is critical for cytoskeletal reorganization. In the yeast Saccharomyces cerevisiae, the sole guanine nucleotide exchange factor for the Rho G-protein Cdc42p, Cdc24p, is essential for its site-specific activation. Several mammalian exchange factors have been shown to oligomerize; however, the function of this homotypic interaction is unclear. Here we show that Cdc24p forms oligomers in yeast via its catalytic DbI homology domain. Mutation of residues critical for Cdc24p oligomerization also perturbs the localization of this exchange factor yet does not alter its catalytic activity in vitro. Chemically induced oligomerization of one of these oligomerization-defective mutants partially restored its localization to the bud tip and nucleus. Furthermore, chemically induced oligomerization of wild-type Cdc24p does not affect in vitro exchange factor activity, yet it results in a decrease of activated Cdc42p in vivo and the presence of Cdc24p in the nucleus at all cell cycle stages. Together, our results suggest that Cdc24p oligomerization regulates Cdc42p activation via its localization.

Local activation of Rho G-proteins, by site-specific activation of guanine nucleotide exchange factor (GEFs), is crucial for cell polarization. Activated GTP-bound G-proteins bind to a number of effectors, which, in the budding yeast Saccharomyces cerevisiae, are critical for actin and septin organization. The yeast Rho G-protein (Cdc42p) GEF, Cdc24p, a paradigm for DbI family GEFs, is tightly regulated by a range of mechanisms, including subcellular localization, phosphorylation, and intermolecular and intramolecular interactions. Cdc24p, like virtually all DbI family GEFs, has a catalytic DbI homology (DH) domain followed by a pleckstrin homology (PH) domain. In addition to these two domains, Cdc24p also has an amino-terminal calponin homology (CH) domain and a carboxyl-terminal Phox Bem1 (PB1) domain (1, 3).

Cdc24p localization varies throughout the cell cycle and in response to mating pheromone (4–6). This GEF localizes to sites of polarized growth, i.e. at the incipient bud site during G1 phase, the bud tip at S and G2/M phases, and the mother-daughter bud neck during M phase. In addition, Cdc24p shuttles between these sites of growth and the nucleus, and this exchange factor accumulates in the nucleus during late M phase and early G1 phase via an association with the cyclin-dependent kinase inhibitor Far1p. It is likely that Cdc24p, which is sequestered in the nucleus, is unable to activate Cdc42p (4, 5). Conversely, Cdc24p, which is exported from the nucleus upon entry into the cell cycle, or upon exposure to mating pheromone, is recruited to the sites where Cdc42p is present and activates this G-protein. Cdc24p phosphorylation also varies throughout the cell cycle with phosphorylated forms accumulating at bud emergence (4, 5). Phosphorylation is thought to trigger the release of Cdc24p from the Bem1 scaffold protein (7); however, this proposal is controversial (8), and the functional significance of Cdc24p phosphorylation remains unclear.

Cdc24p binds a range of proteins either directly or indirectly, including Rsr1p, Bem1p, Cdc42p, Cla4p, Far1p, Ste4p, Ste5p, Bem4p, Tos2p, Boi2p, Sec15p, and Ste20p (8–17). Direct interactions with the first seven proteins have been shown (8–14) (17), and several of these interactions are critical for GEF localization. For example, Rsr1p, Bem1p, and Far1p are required for the initial recruitment or sustained localization of Cdc24p to the plasma membrane of the bud or mating projection tip (4, 5, 9, 18–20). In addition, association of Cdc24p with Rsr1p activates Cdc42p in vitro (18), yet in vitro GEF assays have not revealed any effect of Rsr1p (21).

A number of DbI family GEFs, including Cdc24p and Dbl, have been shown to self-associate, either via intra- or intermolecular interactions (18, 22–27). For example, for the DH domain, amino-terminal and carboxyl-terminal regions can bind one another and inhibit in vitro or in vivo activity (18, 23, 24). These associations can occur intermolecularly, yet have been suggested to occur intramolecularly. Homotypic intermolecular interactions appear to be an additional regulatory mechanism for mammalian GEFs and occur via different domains, depending on the GEF. For example, Dbl oligomerizes via its DH domain (27), whereas LARG (leukemia-associated RhoGEF) and p115 RhoGEF oligomerize via their carboxyl-terminal regions (25). This has raised the...
question whether in yeast Cdc24p oligomerizes and if so what is its regulatory function.

In this study, we show that Cdc24p oligomerizes in vivo and that the DH domain is necessary and sufficient for this homotypic interaction. Analyses of site-specific mutants suggest that the conserved region 2 (CR2) of the DH domain is necessary for Cdc24p oligomerization and localization to the bud tip and nucleus. In addition, we show that the level of Cdc24p oligomerization and localization to the bud tip and the conserved region 2 (CR2) of the DH domain is necessary for GEF activity. Analyses of site-specific mutants suggest that the DH domain is necessary and sufficient for this homotypic interaction.

TABLE 1
Yeast strains used in this study

| Strain   | Genotype                                      | Source       |
|----------|-----------------------------------------------|--------------|
| K699     | MATa, leu2-3, -112, ura3, trpl-1, ade2-1, can1-100, his3-11,15, sdc1-Δ2, GAL        | K. Nasmyth   |
| RAY950   | MATa, leu2-3, -112, ura3-52, his3-Δ209, trpl-1Δ901, by2-801, ade2, cdc24::LEU2 with pRS416GalHis6CDC24 | This study   |
| RAY1360  | RAY950 with pRS414CDC24HAFKBP instead of pRS416GalHis6CDC24 | This study   |
| RAY1773  | MATa, leu2-3, -112, ura3-52, trpl-1, ade2-1, his3-11, can1-100, GAL, bar1, cdc24::HIS3A:Klactis URA  | This study   |
| RAY1854  | RAY950 with pRS414CDC24HAFKBP, F36VHA pRS416CDC24mycFKBP, F36Vmyc, instead of pRS416GalHis6CDC24 | This study   |
| RAY1856  | RAY950 with pRS414CDC24HAFKBP, F36VGF instead of pRS416GalHis6CDC24 | This study   |
| RAY1962  | RAY950 with pRS414cd24F322A-myc-FKBP, F36VGF instead of pRS416GalHis6CDC24 | This study   |

TABLE 2
Plasmids used in this study

| Plasmid                         | Vector  | Insert                  | Source         |
|---------------------------------|---------|-------------------------|----------------|
| p414-3xmycCdc24FI               | pRS414  | 3xmycCdc24              | 11             |
| p414-3xmycCdc24FI-PH            | pRS414  | 3xmycCdc24Δ474-671      | This study     |
| p414-3xmycCdc24FI-DH            | pRS414  | 3xmycCdc24Δ280-475      | This study     |
| p414-3xmycCdc24ΔNt              | pRS414  | 3xmyCCdc24Δ-1-286       | This study     |
| p414-3xmycCdc24ΔNt-PH           | pRS414  | 3xmyCdc24Δ1-286, 474-671| This study     |
| p414-3xmycCdc24ΔNt-DH           | pRS414  | 3xmyCdc24Δ1-475         | This study     |
| p414-3xmycNdH                   | pRS414  | 3xmyCdc24Δ1-473         | This study     |
| p414-3xmycDH-3xmyc              | pRS414  | 3xmyCdc24Δ287-473,3xmyc| This study     |
| p414-3xmycCdc24DH1              | pRS414  | 3xmyCdc24Δ285A          | This study     |
| p414-3xmycCdc24DH2              | pRS414  | 3xmyCdc24Δ232A          | This study     |
| p414-3xmycCdc24DH3              | pRS414  | 3xmyCdc24Δ232A          | This study     |
| p414-3xmycCdc24DH4              | pRS414  | 3xmyCdc24Δ244A          | This study     |
| p414-3xmycCdc24DH5              | pRS414  | 3xmyCdc24Δ339A/E450I    | This study     |
| p414-3xmycCdc24DH6              | pRS414  | 3xmyCdc24Δ417A          | This study     |
| p414-3xmycCdc24DH7              | pRS414  | 3xmyCdc24Δ422G/E423G    | This study     |
| p414-3xmycCdc24DH8              | pRS414  | 3xmyCdc24Δ452G/E453G    | This study     |
| p414Cdc24HAGFP                  | pRS414  | Cdc24HAGFP              | 11             |
| p414Cdc24mycGFP                 | pRS414  | Cdc24mycGFP             | This study     |
| p414Cdc24AFKBP                   | pRS414  | Cdc24AFKBP, F36VHA      | This study     |
| p414Cdc24AFKBP                   | pRS414  | Cdc24AFKBP, F36Vmyc     | This study     |
| p414Cdc24DH3mycGFP              | pRS414  | Cdc24DH3mycGFP          | This study     |
| p414Cdc24DH4mycGFP              | pRS414  | Cdc24DH4mycGFP          | This study     |
| p414Cdc24DH5mycGFP              | pRS414  | Cdc24DH5mycGFP          | This study     |
| p414Cdc24DH6mycGFP              | pRS414  | Cdc24DH6mycGFP          | This study     |
| p414Cdc24DH7mycGFP              | pRS414  | Cdc24DH7mycGFP          | This study     |
| p414Cdc24DH8mycGFP              | pRS414  | Cdc24DH8mycGFP          | This study     |
| p414Cdc24DH9mycFKBP              | pRS414  | Cdc24DH9mycFKBP         | This study     |
| pRS425TPISHmyc                    | pRS425  | Sph1myc                 | 29             |
| p177Cdc24                       | p177ag   | Cdc24                   | This study     |
| pc4MV2Y2                        | pGEX-3X  | FKBP, F36V              | ARIAD pharmaceutical |
| pGSTCdc24                       | pGEX-6P-2| Cdc24                   | This study     |
| pGSTCdc24DH                     | pGEX-6P-2| Cdc24DH                 | This study     |
| pGSTCdc24DHPH                   | pGEX-6P-2| Cdc24DHPH               | This study     |
| pGSTCdc24DHPH-DH3               | pGEX-6P-2| Cdc24DHPH-DH3           | This study     |
| pGSTCdc24DHPH-DH5               | pGEX-6P-2| Cdc24DHPH-DH5           | This study     |
| pGSTCdc24DHPH-DH8               | pGEX-6P-2| Cdc24DHPH-DH8           | This study     |
| pGSTCdc24DHPH-FKBP              | pGEX-6P-2| Cdc24DHPH-FKBP          | This study     |
| pMBPCdc24DHPH                   | pMal-c2   | Cdc24DHPH               | This study     |
| pMBPCdc24                       | pMal-c2   | Cdc24                  | 36             |
| pHis-cdc24                       | pTrcB    | Cdc24                   | This study     |
| pGST-CRIB                       | pGEX-6P-2| CRIB[TTS20]             | 36             |

EXPERIMENTAL PROCEDURES

Strains and Plasmids—Standard techniques and media were used for yeast growth and genetic manipulation (28). Strains used in this study are described in Table 1. A 3xHA epitope-tagged Cdc24p fusion was constructed using an Agel-linearized pDM1-Cdc24-3xHA gene replacement cassette in either RAY819 or RAY1599. pDM1-Cdc24-3xHA (a gift from D. McCusker) was constructed by cloning a BamHI-linearized tagged Cdc24p fusion was constructed using an AgeI-linearized pGST-CRIB pGEX 6P-2 CRIB STE20 (36). Strains and Plasmids—Standard techniques and media were used for yeast growth and genetic manipulation (28). Strains used in this study are described in Table 1. A 3xHA epitope-tagged Cdc24p fusion was constructed using an Agel-linearized pDM1-Cdc24-3xHA gene replacement cassette in either RAY819 or RAY1599. pDM1-Cdc24-3xHA (a gift from D. McCusker) was constructed by cloning a BamHI/Sall fragment from pGEX4TCDC24 that contained the 382 carboxyl-terminal amino acids of Cdc24p into YIplac211-3xHA resulting in the carboxyl-terminal portion of Cdc24p fused to 3xHA followed by a URA3. Cdc24 mutants and FKBP fusions were transformed in RAY950, which were then grown in the presence of glucose to repress GalHis6Cdc24, and the loss of this pRS416 URA3 plasmid was confirmed by its inability to grow on media lacking uracil and by PCR.

Plasmids used in this study are listed in Table 2. p414-3xmycCdc24 contains the CDC24 open reading frame, including
### TABLE 3
Primers used in this study

| Primers | Mutation | Restriction site |
|---------|----------|------------------|
| gttgtagtatattatgcagcaaa | E285A/F286M | NcoI |
| gattatcctcgctgccatacatagcat | V280G/K281Y | BstEII |
| cattacgagatagtgcgrcaaaagaaaaa | V280G/K281Y | BstEII |
| cggagaattctgcacct | P463Q/M464V | NcoI |
| cggagaattctgcacct | P463Q/M464V | NcoI |
| cggagaattctgcacct | P463Q/M464V | NcoI |
| gatctgatagttcgacagttcgact | E285A | StuI |
| gatctgatagttcgacagttcgact | E285A | StuI |
| gatctgatagttcgacagttcgact | E285A | StuI |
| gatctgatagttcgacagttcgact | E285A | StuI |
| gatctgatagttcgacagttcgact | E285A | StuI |
| gatctgatagttcgacagttcgact | E285A | StuI |
| gatctgatagttcgacagttcgact | E285A | StuI |

The 258 bp upstream of the ATG, 10 unique restriction sites in CDC24 (10), and a 3xmyc epitope followed by an am NcoI site prior to the ATG, generated by PCR. The 3xmycCdc24 deletion mutants were generated by the creation of silent restriction sites using site-directed mutagenesis (Table 3; primers 1–8) with the Pfu polymerase (Promega) and the DpnI method (30, digestion and religation.

To generate p414-3xmycCdc24DH-3xmyc, the CDC24 promoter together with 3xmycCdc24DH was amplified using p414-3xmycCdc24DH as template and primers 23 and 25 primers yielding 3xmycCdc24DH3, p414-3xmycCdc24DH5, and p414-3xmycCdc24DH8 were subcloned using KpnI/SalI into p414Cdc24mycCFKBPmGFP, and similarly the DH3 mutation was subcloned into p414Cdc24mycCFKBPmGFP.

For in vitro binding with full-length Cdc24p, a 2.6-kb BamHI/SalI CDC24 fragment from pEG(kt)CDC24 was cloned into pGEX-3X yielding pGSTCDC24. A 1.1-kb EcoRI/SalI CDC24 fragment encompassing the DHPH domains (amino acids 285–681) from p414-3xmycCdc24 was cloned into pMal-C2 resulting in pMBPcDC24DH. pGSTCdc24DH was generated by PCR amplification of p414-3xmycCdc24DH (primers 26 and 36), digestion with BamHI/SalI, and cloning into pGEX-3X.

DH domain point mutations were generated by site-directed mutagenesis and the addition of a silent restriction site (Table 3, primers 9–22). All mutations were confirmed by sequencing (ABI PRISM big-dye terminator cycle sequencing kit). For Cdc24 DH mutant localization, DH domain mutations DH3, DH5, and DH8 were subcloned using KpnI/Sall into p414Cdc24mycGFP, and similarly the DH3 mutation was subcloned into p414Cdc24mycCFKBPmGFP.

**Cdc24 Exchange Factor Oligomerization**
of p414Cdc24HAGFP using site-directed mutagenesis primers 27 and 28, resulting in p414BSCdc24HAGFP. Subsequently, the FKBP$_2$F36V domain was PCR-amplified from pC4Mvf2E (ARIAD Pharmaceuticals, Inc.) (either 30 and 31 or 29 and 31 primers) resulting in Myr-FKBP$_2$F36V and FKBP$_2$F36V, respectively. These PCR fragments were cloned into the appropriately digested p414BSCdc24HAGFP plasmid, yielding p414MyrFKBPcdc24HAGFP and p414FKBPcdc24-HAGFP. p414Cdc24mycGFP was constructed using the PCR primers 37 and 42 and p414Cdc24HAGFP as a template. The PCR fragment containing the 3’ portion of Cdc24 fused to the myc epitope tag was cloned in p414Cdc24HAGFP, yielding p414Cdc24mycGFP. For the carboxy-terminal FKBP fusions, a unique ClaI site was inserted, using primers 38 and 39, between the epitope tag and GFP, in p414Cdc24HAGFP and p414Cdc24-mycGFP, resulting in p414Cdc24HACGFP and p414Cdc24mcyC-GFP. To construct HA- and myc-tagged Cdc24FKBP fusions, FKBP was PCR-amplified from pC4Mvf2E using primer pairs 32 and 33 or 32 and 34. The resulting FKBP-HA and FKBP-myc PCR fragments were cloned into CiaI/NotI-digested p414Cdc24-HACGFP and p414Cdc24mcyCGFP, respectively, yielding p414Cdc24HAFKBP-HA and p414Cdc24mcyFKBP-myc. To generate pGSTCdc24DHPHFKBP, FKBP was PCR-amplified (primers 26 and 43) from p414Cdc24mcyFKBP-myc, digested with Sall/NotI, and cloned into pGSTCdc24DHPH. For the carboxy-terminal FKBP fusion, Ala-206 was mutated to Lys using site-directed mutagenesis primers 40 and 41, as this mutation has been shown to block the inherent dimerization of GFP (31), yielding p414MyrFCDC24HAGFP and p414FKBPcdc24-HAGFP. For the carboxyl-terminal FKBP fusions, a unique HindIII site was inserted, using primers 37 and 42, and p414Cdc24HAGFP as a template. The PCR fragment containing the 3’ portion of Cdc24 fused to the myc epitope tag was cloned in p414Cdc24HAGFP, yielding p414Cdc24mycGFP. For the carboxy-terminal FKBP fusion, the Myc epitope tag and GFP in p414Cdc24HAGFP and p414Cdc24-mycGFP were rebound to the respective resin. Sequence Alignments and Structure Modeling—Sequence alignments were carried out using the BLAST algorithm (32). Structural model threading was carried out using a web-based Modeller program (33, 34) with the Tiam1, Rac1 Protein Data Bank file 1FOE (35).

Protein Binding Assays—Pulldowns were carried out as described previously (11) with the following modifications. Yeast cells (~2 x 10$^8$) from logarithmically growing cultures were harvested by centrifugation and lysed by agitation with glass beads (Sigma) in buffer A (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.1% Triton X-100, 1 mM PMSF, and protease inhibitor mixture; Roche Applied Science) cross-linked with dimethyl pimelimidate (Sigma) or anti-HA resin from Vector Laboratories) in buffer A for 3 h. Resin was washed five times in buffer A and analyzed by SDS-PAGE, transferred to a nitrocellulose membrane (American Biosciences), and probed with mouse monoclonal antibody HA11 against HA (1:1000 dilution; Covance) or mouse monoclonal antibody 9E10 against myc (1:1000 dilution; Covance). Immunoblots were visualized by enhanced chemiluminescence (luminol-coumaric acid) on a Fuji-Las3000. Equal amounts of cells were used in each experiment, and equal amounts of protein from the 10,000 x g supernatants was confirmed by Ponceau S staining of nitrocellulose membranes. Pulldowns of cells expressing Cdc24FKBP fusions were carried out as described above except that extracts were prepared with 0.5 x yeast buster reagent (Calbiochem) following the manufacturer’s instructions. For cells grown in the presence of AP20187 (1 µM), all subsequent steps were carried out with buffers containing 0.1 µM AP20187. To examine the effect of induced oligomerization in vitro, cell extract 10,000 x g supernatants were treated with 0.1 µM AP20187 for 15 min at 4 °C prior to pulldowns. In vitro coupled transcription-translation was carried out using a Quick TnT rabbit reticulocyte lysate system (Promega) and 5 µCi of [35S]methionine (ICN 1175 Ci/mmol) using either pT7CDC24 (36) or T7-luciferase, following the manufacturer’s instructions. GST and MBP fusion proteins were expressed in E. coli BL21 cells grown at 30 °C and induced with isopropyl-1-thio-β-D-galactopyranoside for 4 h. Cells expressing GST or MBP fusions were lysed in buffer B (phosphate-buffered saline, protease inhibitor mixture (Roche Applied Science), 1 mM PMSF, 0.1% Triton X-100, and 1 mM EDTA) by sonication followed by snap freezing and thawing. Extracts were clarified by two centrifugations (10,000 x g for 10 min), and fusion proteins were isolated using glutathione-Sepharose 4B resin (Amer- sham Biosciences) or amylase resin (New England Biolabs). Protein concentrations were estimated by comparing intensities of bands on Coomassie-stained SDS-polyacrylamide gels with bovine serum albumin (Sigma) as a standard. GSTCdc24 was washed with the same buffer containing 250 mM and then 500 mM NaCl. GSTCdc24 was eluted with buffer C (10 mM Tris-Cl, pH 8, 10 mM glutathione). After extensive dialysis using a Centricon YM30 (Amicon), protein-containing fractions were reloaded to the respective resin. For [35S]Cdc24 binding to GSTCdc24 or GST GSH-Sepharose resin-bound proteins (~200 ng of protein) were incubated with radiolabeled protein in buffer D (50 mM HEPES, pH 7.6, 50 mM NaCl, 5 mM MgCl$_2$, 0.1% Triton X-100) for 3 h at 4 °C. Resin samples were then washed five times with 100 µl of buffer E (50 mM Tris-Cl, pH 8, 150 mM NaCl, 1 mM PMSF, and protease inhibitor mixture; Roche Applied Science) at 4 °C. Proteins were eluted with SDS-PAGE sample buffer and analyzed by SDS-PAGE followed by Coomassie Blue staining and autoradiography. A Fuji BAS1000 PhosphorImager was used for quantification based upon two input amounts.

For Cdc24DHPH binding to Cdc24DH expressed in yeast, supernatants (10,000 x g) of extracts from yeast cells (~2 x 10$^8$) expressing 3xmycCdc24DH-3xmyc in buffer A were incubated with either MBP or MBPCdc24DHPH (~200 ng of protein) bound to amylose resin for 2 h at 4 °C. For binding of bacterially expressed His$_6$Flag2xmyc-Cdc24DH to GSTCdc24DHPH and GSTCdc24DH, 10,000 x g supernatants of bacterial cells expressing His$_6$FLAG2xmyc-Cdc24DH in buffer F (phosphate-buffered saline, protease inhibitor mixture (Roche Applied Science), 1 mM PMSF, and 0.1% Triton X-100)
were incubated with GST, GST-Cdc24DHPH, or GST-Cdc24DH bound to GSH-agarose (~200 ng of protein) for 2 h at 4 °C. Resin samples were then washed five times with 10 volumes of the respective buffer. Proteins were eluted with SDS-PAGE sample buffer, analyzed by SDS-PAGE, transferred to nitrocellulose, probed with mouse monoclonal antibody 9E10 against myc (1:1000 dilution; Covance) for experiments with 3×mycCdc24DHPH-3×myc or rabbit polyclonal sera against the FLAG epitope (1:1000; Santa Cruz) for experiments with His6-Flag2×myc-Cdc24DH and visualized by enhanced chemiluminescence (luminol-coumaric acid) on a Fuji-Las3000. Equal amounts of cells were used in each experiment, and equal amounts of protein from the 10,000 × g supernatants was confirmed by Ponceau S staining of nitrocellulose membranes.

GST-CRIB pulldown experiments were carried out essentially as described (37) with GST-CRIB-GSH-agarose (~400 ng of protein). Cell extract 10,000 × g supernatants were also incubated with 0.1 μM AP20187 at 4 °C for 1 h prior to incubation with GST-CRIB resin. Cell extract 10,000 × g supernatants were loaded with GTPγS to determine the maximum amount of Cdc42-GTP. EDTA was added (20 mM final concentration) to the 10,000 × g supernatants, and subsequently GTPγS (190 μM final concentration) was added, and the mixture was incubated at room temperature for 15 min. MgCl2 was then added (107 mM final concentration), and the samples were chilled to 4 °C. Proteins were eluted from GST-CRIB resin in SDS-PAGE sample buffer, analyzed by SDS-PAGE, transferred to nitrocellulose, probed with rabbit anti-Cdc42 (36) (1:1000 dilution), and visualized by enhanced chemiluminescence (luminol-coumaric acid) on a Fuji-Las3000. Equal amounts of cells were used in each experiment, and equal amounts of protein from 10,000 × g supernatants was confirmed by Ponceau S staining of nitrocellulose membranes.

Guanine Nucleotide Exchange Factor Assays—GST and MBP fusion proteins were expressed in E. coli BL21 cells grown at 30 °C and induced with isopropyl-1-thio-β-D-galactopyranoside for 4 h. Cells expressing MBP-Cdc42 and GST fusion were lysed in buffer G (20 mM Tris-Cl, pH 7.5, 100 mM NaCl, 2.5 mM MgCl2, 1 mM DTT, 10 μM GDP, and 1 mM PMSF) (38) and buffer H (50 mM Tris-Cl, pH 7.5, 100 mM NaCl, 2.5 mM MgCl2, 1 mM DTT, 1 mM PMSF, 10% glycerol, and Boehringer protease inhibitor mixture), respectively, by sonication followed by snap freezing and thawing. Extracts were clarified by centrifugation (4,000 × g for 10 min), and fusion proteins were isolated using amylose resin or glutathione-Sepharose 4B resin. MBP-Cdc42 resin was washed extensively with buffer G and MBP-Cdc42 was eluted with same buffer G containing 10 mM maltose. GST-DHPH and GST-DHPH-FKBP resin were washed extensively in buffer H and successively with buffer H containing 250 mM potassium acetate and 500 mM potassium acetate. After an additional wash in buffer H, fusion proteins were eluted with buffer I (50 mM Tris-Cl, pH 8, 100 mM NaCl, 2.5 mM MgCl2, 1 mM DTT, 1 mM PMSF, 10% glycerol, and 10 mM glutathione). Protein concentrations were estimated by comparing intensities of bands on Coomassie-stained SDS-polyacrylamide gels and by Bradford protein assays with bovine serum albumin as a standard (39). To remove GST, DHPH and DHPH-FKBP fusions were incubated with PreScission protease (Amersham Biosciences, 20 units) for 24 h at 24 °C. Subsequently, proteins were concentrated using a Centricon YM30 (Millipore).

Guanine nucleotide exchange factor assays were carried out in a 25-μl volume with 18 μl of buffer L1 (25 mM Tris-Cl, pH 7.5, 200 mM (NH4)2SO4, 5 mM MgCl2, 2 mM DTT, 1 mM EDTA, 5 μM GTP, Boehringer protease inhibitor mixture), which contained 3–6 μCi of [α-32P]GTP. Each reaction contained ~40 pmol of MBP-Cdc42 (or MBP as control) and ~10 pmol of GST-DHPH, GST-DHPH-FKBP, DHPH, or DHPH-FKBP. For chemically induced oligomerization, DHPH-FKBP was incubated with 20 μM AP20187 for 30 min at 4 °C prior to addition of buffer L1 and Cdc42 (final AP20187 concentration 4 μM). GEF assays were incubated at 22 °C for 0, 2, 5, 10, 15, and 30 min and quenched with the addition of 450 μl of chilled buffer L2 (25 mM Tris-Cl, pH 7.5, 5 mM MgCl2, 2 mM DTT, 1 mM EDTA, 1 mM GTP, and Boehringer protease inhibitor mixture) on ice.
FIGURE 2. The DH domain is necessary and sufficient for Cdc24p association in yeast. A, schematic representation of different myc-tagged Cdc24p constructs. B–F, binding of indicated 3xmyc-Cdc24p constructs with Cdc24-3xHAp carried out as described in Fig. 1A. Co-IP, co-immunoprecipitation; FL, full length.
Samples were then filtered on prewetted 0.45-μm HA filters (Millipore) that were then washed with five times with 2 ml of buffer L3 (25 mm Tris-Cl, pH 7.5, 5 mm MgCl₂, 2 mm DTT, 1 mm EDTA, and 10 μM GTP). Filters were dried, and radioactivity was counted with Biofluor scintillation fluid (PerkinElmer Life Sciences) in a Beckman LS 6500 scintillation counter.

Localization of Cdc24p—Strains with Cdc24HAGFP, Cdc24HAKBPGFP, or Cdc24mycFKBPGFP as the sole copy or wild-type strains with Cdc24mycGFP, Cdc24-DH3mycGFP, Cdc24-DH5mycGFP, or Cdc24-DH8mycGFP were imaged on a Zeiss LSM510 meta inverted confocal microscope using a Plan-Apo 63× NA 1.4 objective and 488-nm LASER excitation as described (4). Both differential interference contrast and fluorescence images of random field of views were captured, and cells with GFP fluorescence localized to the bud, mother-daughter bud neck, or nucleus were counted. Based upon differential interference contrast images, cells with GFP fluorescence were counted in the following categories: stage 1a, cells with GFP fluorescence localized to the bud, mother-wall; stage 2, small budded (<1/8 diameter of mother cell); stage 2, small budded (≤½ radius of mother cell); and stage 3, dividing cells with mother and daughter cells equal size.

RESULTS

Cdc24p Forms Oligomers—To determine whether Cdc24p binds Cdc24p in vivo, we used a strain that expressed two different epitope-tagged Cdc24 proteins, Cdc24-3xHAp and 3xmyc-Cdc24p. As a control we used a strain in which only triple myc-tagged Cdc24p was expressed. Both tagged versions of Cdc24p were expressed via the Cdc24 promoter with the HA fusion replacing the genomic copy and the myc fusion on a centromeric plasmid. The HA-tagged Cdc24p was pulled down using an anti-HA antibody, and bound 3xmyc-Cdc24p was detected by immunoblot probed with an anti-myc antibody. As illustrated in Fig. 1A, 3xmyc-Cdc24p bound Cdc24p-3xHAp. We also observed lower molecular weight bands in the α-myc probed blot that were likely to be 3xmyc-Cdc24p degradation products, as they were only observed in strains expressing 3xmyc-Cdc24p. Their apparent sizes (∼80, ∼65, and ∼60 kDa) indicated that these fragments contain the DH domain. These degradation fragments bound Cdc24p-3xHAp suggesting that the carboxyl terminus of Cdc24p is not necessary for this binding. We examined if this binding was specific using as a control another yeast protein Sph1-αmyc. Fig. 1A shows that Sph1-αmyc does not bind Cdc24p, confirming the specificity of the Cdc24p-Cdc24p interaction. An average of 3 ± 1% (n = 3) of the total 3xmyc-Cdc24p bound to Cdc24p-3xHAp; however, this value is an underestimate as each epitope-tagged protein can associate with itself or the other tagged fusion. This level of oligomerization is consistent with the notion that Cdc24p oligomers are dynamic. To determine whether this association reflects direct binding as opposed to a protein complex containing many Cdc24p molecules, we examined whether Cdc24p expressed in bacteria could bind to rabbit reticulocyte translated [35S]Cdc24p. Immobilized GST-Cdc24, but not GST alone can bind [35S]Cdc24p (Fig. 1B). This interaction was specific as immobilized GST-Cdc24 did not bind [35S]luciferase. Together these data establish that Cdc24p oligomerizes.

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FIGURE 3. The Cdc24p DH domain oligomerizes in the absence of other yeast proteins. A, supernatant from bacteria expressing Cdc24p, 3xmycDH-3xmyc was either analyzed by SDS-PAGE (Input, 1% directly or following incubation with amylase resin-bound MBP or MBP-DHPH (Pull down). Immunoblots were probed with anti-myc sera. B, supernatants from bacteria expressing Cdc24p, HisFFlag2xmycDH were either analyzed by SDS-PAGE (Input) directly or following incubation with indicated GST-agarose (Pull down). Immunoblots were probed with anti-FLAG sera.

The Cdc24p Dbl Homology Domain Is Necessary and Sufficient for Oligomerization—To determine the region of Cdc24p that is required for oligomerization, we generated amino-terminal and carboxyl-terminal deletions of 3xmyc-Cdc24p (Fig. 2A) and examined pulldowns with Cdc24p-3xHAp. Deletion of the amino-terminal 280 amino acids of Cdc24p, including the CH domain, did not abolish Cdc24p oligomerization (Fig. 2B). Similarly, deletion of the PH domain in the full-length Cdc24p or in a construct lacking the amino-terminal 280 amino acids had little to no effect on Cdc24p binding (Fig. 2, B and D). Indeed the amino-terminal 470 amino acids of Cdc24p, which include the DH domain, bound to Cdc24p, indicating that the PB1 domain at the carboxyl terminus is not required for oligomerization (Fig. 2C). The DH domain is the only region, which is common to these constructs. Cdc24p lacking the DH domain did not bind Cdc24p, and similarly a carboxyl-terminal 375 amino acid fragment lacking the CH and DH domains did not bind Cdc24p (Fig. 2, E and F). These results also confirm the specificity of the Cdc24p-Cdc24p interaction. Furthermore, the DH domain was sufficient to bind Cdc24p-3xHAp in yeast (Fig. 2F), suggesting that the DH domain alone is sufficient for oligomerization. Consistent with this result, the DH domain expressed in yeast bound an MBP-DHPH domain fusion (Fig. 3A). The DH domain expressed in bacteria bound both GST-DH and GST-DHPH domain fusions (Fig. 3B), with the binding of the latter being somewhat reduced. Together these results indicate that the DH domain is necessary and sufficient for Cdc24p oligomerization.

We next examined if conserved residues required for catalytic activity and G-protein binding are necessary for exchange factor oligomerization. The Cdc24p DH domain, modeled onto the structure of the Tiam1 DH domain, has a flattened elongated helical bundle with six major helical axes (Fig. 4A) (35, 40) similar to the Dbs DH domain (40). DH domains are characterized by three conserved regions (CR1–3), which range in length...
from 21 to 28 amino acids (Fig. 4B). CR1, which is composed principally of α-helix 1 and CR3, which includes the α-helix 2 together with the carboxyl-terminal end of α-helix 3 and α-helix 6, forms an exposed surface, which constitutes the major contact sites to the GTPase (35, 40). CR2, which is composed principally of α-helix 5, is thought to stabilize the helical bundle and is on the opposite side of the bundle with respect to CR1 and CR3. We mutagenized residues that are conserved in most RhoGEFs as follows: E285A (DH1) in CR1; F322A (DH3), Q332G (DH4), and L339A/E340A (DH5) in CR2; Y417A (DH6) and K422G/E423G (DH7) in CR3; and N452G/E453G (DH8) in the 6th helix. The crystal structure of Dbs-Cdc42 suggests that equivalent residues mutated in Cdc24p DH1, DH7, and DH8 make contacts with Cdc42p (40). The Dbl mutations equivalent to DH3 and DH8 substantially affected GEF activity (41).

Strikingly, DH1, DH3, DH4, DH6, and DH7 mutants were functional in vivo, whereas only DH5 and DH8 were inviable (Fig. 4D). DH1 and DH6 were unaffected in their ability to oligomerize with wild-type Cdc24p (Fig. 4C). The oligomerization defects of the other DH domain mutants could be divided into three classes with DH4 and DH8 showing a reduction in oligomerization of ~20%, DH3 and DH7 exhibiting ~2-fold reduction in oligomerization, and DH5 showing a substantial defect with an ~10-fold reduction (Fig. 4C). We also carried out control pulldowns with anti-HA antibody using strains, which expressed only the myc-tagged Cdc24p DH mutants, and no binding was observed with any mutant. In contrast to Cdc24p DH4, the equivalent Dbl mutant is oligomerization-deficient, whereas the Dbl mutant equivalent to DH5 had little effect on oligomerization (27). As both mutations that substantially reduced oligomerization (DH3 and DH5) are in the CR2, our results are consistent with studies on Dbl oligomerization, in which the CR2 is important for DH-DH interactions.

Cdc24p Oligomerization Is Necessary for Its Nuclear Accumulation—As Cdc24p shuttles between the sites of growth and nucleus (4–6, 42), we examined whether the level of Cdc24p oligomers is altered when this GEF accumulates in the nucleus. The level of Cdc24p oligomers in a cdc28 temperature-sensitive strain was unaffected at the nonpermissive temperature in which Cdc24p accumulates in the nucleus as cells arrest at G1 (4) (a 5-fold decrease in the percentage of budded cells) (Fig. 5). These results are consistent with Cdc24p oligomers being present in the nucleus.

We examined whether the DH mutants representing the three classes of oligomerization defects could localize to the nucleus. To determine the localization of Cdc24p mutants DH3, DH5, and DH8, we examined wild-type cells expressing GFP fusions as DH5 and DH8 mutants were inviable as the sole copy. These strains grew similarly to the wild type, and localization of DH8 GFP fusion was indistinguishable from the wild-type Cdc24p GFP fusion (Fig. 6, A and B). In wild-type cells prior to bud emergence (Fig. 6B, stage 1a), essentially all cells have Cdc24-GFP localized to the nucleus (Fig. 6, A and B). Strikingly, the percentage of cells with Cdc24p observed in the nucleus was reduced by ~30% with the DH3 GFP fusion and ~50% with the DH5 GFP fusion (Fig. 6, A and B). All three mutants exhibited a decrease in the number of cells with Cdc24p in the nucleus following bud emergence (Fig. 6B, stage 1b). The DH3 fusion was observed in the nucleus in dividing cells similar to the wild type, whereas the DH5 fusion did not accumulate in the nucleus of dividing cells. Quantitation of the percentage of cells with the different GFP fusions localized to the bud tip revealed that both DH3 and DH5 mutants were somewhat defective in localization to small and large buds. These results are consistent with the idea that Cdc24p oligomerization is critical for its localization, both to the nucleus and the bud tip. Given that the Cdc24p DH3 mutant was viable as the sole Cdc24p copy and could localize to the nucleus and bud tip, albeit with reduced efficiency, we expected this mutant to be able to activate Cdc42p in vivo. We used the CRIB pulldown method to measure the level of Cdc42p-GTP in cells expressing Cdc24p-DH3 as the sole Cdc24p copy. Fig. 6C shows that the levels of Cdc42p-GTP were similar in cdc24-DH3 and wild-type cells suggesting that a 2-fold decrease in oligomerization does not substantially alter Cdc42 activation in vivo.

Cdc24p Oligomerization Regulates Cdc42p Activation in Vivo—To investigate the effect of Cdc24p oligomerization, we used a regulated oligomerization system, which enables drug-induced multimerization (43). We generated fusions of epitope-tagged Cdc24p with two modified FKBP domains in which oligomerization can be induced by a synthetic, cell-permeant, small bivalent molecule. Two copies of the FKBP domain carrying an F36V mutant were used to favor the formation of higher order oligomers with the synthetic oligomerization inducer AP20187, which binds specifically to this modified FKBP domain without substantially affecting the yeast FK506-
Cdc24 Exchange Factor Oligomerization

A

B

C

FIGURE 6. Cdc24 mutants defective in oligomerization are defective in localizing to the nucleus and bud tip. A, confocal fluorescence images of wild-type (WT) yeast cells expressing wild-type or mutant Cdc24p fused to GFP. B, quantitation of percentage of cells with nuclear and bud tip localized Cdc24p in cells. Schematic representation of cell categories corresponding to G1 (stage 1a), G1/S (1b), G2/M (stage 2), and M (stage 3) cell cycle phases with Cdc24p in green. Localization of Cdc24-GFP in wild-type yeast cells (n = 50–100 per cell cycle stage) expressing Cdc24-GFP (white bars), Cdc24-DH8-GFP (black bars), Cdc24-DH5-GFP (dark gray bars), and Cdc24-DH3-GFP (light gray bars) was quantitated. C, yeast strains expressing as sole copy wild-type Cdc24p or Cdc24-DH3p were analyzed by SDS-PAGE prior to (Ex) and following incubation with GST-CRIB resin (Pull downs), and immunoblots were probed with anti-Cdc42 sera.

sensitive proline rotamase (Fpr1p). This regulated oligomerization system has been used successfully in S. cerevisiae to induce Apg16p oligomerization and restore autophagy (44). Both Cdc24-HA-FKBP-HA and Cdc24-myc-FKBP-myc fusions were functional as the sole copy of Cdc24p in yeast, and these strains grew similar to wild-type cells at 25, 30, and 37 °C (data not shown). When yeast cells were grown in the presence of 1 μM AP20187, there was a substantial increase in the amount of Cdc24-myc-FKBP-myc bound (Fig. 7A), indicating that the majority of Cdc24p was oligomeric (>90% by quantitation). Incubation of cell extracts with AP20187 for 15 min at 4 °C did not increase the amount of Cdc24p oligomers, suggesting that the FKBP domains may not be accessible to AP20187 during this short incubation at 4 °C. AP20187 did not affect cell growth in strains in which Cdc24-FKBP was the sole copy (RAY1854) in liquid or solid media, bud site selection, nor actin cytoskeleton organization (data not shown).

We next examined if induced oligomerization of Cdc24p affected Cdc42p activation in vivo using the CRIB pulldown method to measure the level of Cdc42-GTP. Cells expressing Cdc24-HA-FKBP-HA as the sole Cdc24p copy were grown either in the presence or absence of 1 μM AP20187, and Fig. 7B shows a specific decrease in the amount of Cdc42-GTP when cells were grown in the presence of AP20187. Quantitation of four independent experiments, normalized to the maximum level of activated Cdc42p, indicated that ~30% of the Cdc42p was activated in the absence of AP20187 with little effect of AP20187 added to cell extracts. In contrast, a 2-fold reduction in the level of activated Cdc42p was observed when cells were grown in the presence of AP20187. It is unlikely that this effect is because of an altered accessibility of the G-protein to the oligomerized exchange factor as similar results were observed with amino-terminal FKBP Cdc24p fusions (data not shown). These results suggest that oligomerization may regulate Cdc24p GEF activity, either by altering its distribution or affecting its catalytic activity.

To determine whether oligomerization affects Cdc24 GEF activity, we carried out exchange factor assays in vitro with wild-type and mutant DHPH domains in addition to a DHPH-FKBP fusion in which oligomerization could be induced. MBP-Cdc42, GST-DHPH, and GST-DHPH-FKBP were expressed and purified from bacteria (Fig. 8A, lanes 1–3). MBP-Cdc42 was purified in the presence of GDP and was able to bind [α-32P]GTP (Fig. 8B, gray diamond). Addition of GST-DHPH or GST-DHPH-FKBP resulted in an increase in GDP-GTP exchange by 2–3-fold, which was most evident at the 2- and 5-min incubation times (Fig. 8B, black triangle and gray box). These results are consistent with a previous report of Cdc24 exchange factor activity stimulation of ~3-fold (with [35S]GTPγS) using GST-Cdc24 and GST-Cdc42 from baculovirus (45). The GEF activity we observed required both Cdc24p and Cdc42p, as no significant GTP binding was detected when MBP replaced MBP-Cdc42, and no stimulation of GTP binding was observed when GST replaced GST-DPHH. In addition, we did not observe an increase in GDP-GTP exchange when heat-inactivated GST-DPHH was used. To rule out interfering effects of the adjacent GST domain, we cleaved off the GST moiety by digestion with PreScission protease, resulting in DPHH and DPHH-FKBP proteins (Fig. 8A, lanes 4 and 5).

We examined the ability of the DH mutants DH3, DH5, and DH8 to stimulate Cdc42p GDP-GTP exchange. Fig. 8C shows
that each mutant protein stimulated GDP-GTP exchange to a similar extent as the wild-type DHPH protein, with a 2–3-fold increase in GTP binding for mutant and wild-type DHPH compared with MBP-Cdc42 alone. Together our results indicate that oligomerization does not directly affect Cdc24p GEF activity.

To determine whether oligomerization affects Cdc24p localization, we examined cells expressing Cdc24-FA-GFP or Cdc24-FA-FKBP-GFP as the sole copy. These strains grew similarly, and the localization of these two Cdc24p fusions was indistinguishable (Fig. 9A). Cdc24p was localized to the nucleus prior to bud emergence (Fig. 9B, stage 1a), to the bud tip upon bud emergence (Fig. 9B, stage 1b), at the bud plasma membrane as the bud increased in size (Fig. 9B, stage 2), and to the septum and both nuclei during cell division (Fig. 9B, stage 3). However, this GEF was observed in the nucleus at all cell cycle stages when Cdc24-FA-FKBP-GFP cells were grown in the presence of AP20187 (Fig. 9, B and C). After bud emergence, 75% of the cells grown in the presence of AP20187 had Cdc24p localized to their nuclei compared with 25% in its absence. At this stage, essentially all the cells had Cdc24p (in the presence and absence of AP20187) localized to the bud tip. Strikingly, in cells with larger buds (stage 2) in presence of AP20187, 40% of cells had Cdc24p localized to the nucleus, whereas in the absence of AP20187, Cdc24p was not observed in the nucleus. At stage 2, ~90% of the cells had Cdc24p localized to the bud plasma membrane in the presence or absence of AP20187. In cells undergoing cytokinesis, there was no difference in the distribution of Cdc24p in the presence or absence of AP20187 with similar percentages of cells having Cdc24p localized to the nucleus and cell division sites. These results suggest that oligomeric Cdc24p can undergo nucleocytoplasmic shuttling, however, export of nuclear oligomeric Cdc24p is reduced, resulting in nuclear Cdc24p at all cell cycle stages. This reduction in nuclear export is likely to account for the decrease in active Cdc24p.

To determine whether chemically induced oligomerization could restore the localization defect of the Cdc24p DH3 mutant, we fused FKBP to the carboxyl terminus to Cdc24p in Cdc24-DH3-GFP and examined cells expressing this fusion as the sole Cdc24p copy. This strain grew normally, and the localization of Cdc24-DH3-FKBP-GFP, in the absence of AP20187, was similar to that of Cdc24-DH3-GFP in wild-type cells (Fig.
Cdc24 Exchange Factor Oligomerization

A

MBP-Cdc42

B

MBP-Cdc42 + GST-DPH
MBP-Cdc42 + GST-DPH-FKBP
MBP
MBP + GST-DPH

C

D

MBP-Cdc42 + DHP4-FKBP
MBP-Cdc42 + DHP4-FKBP + AP20187
MBP

increase in G2/M (stage 2) cells. These results indicate that the determination by scintillation counting. Each determination was carried out in duplicate with standard deviation indicated.

Each experiment (3–9 pmol of Cdc42 for guanine nucleotide exchange factor activity as described above. The amount of MBP-Cdc42 factor activity of wild-type DHPH and DHPH mutants. Following cleavage of GST moiety using PreScission protease, the indicated DHPH proteins were assayed for guanine nucleotide exchange factor activity. Together these results suggest that Cdc24p oligomerization is important for the correct localization of this exchange factor and may down-regulate this GEF by increasing nuclear sequestration.

Cdc24p oligomers are dynamic with their formation or stability dependent on the cell cycle stage, Cdc24p localization, Cdc24p association with additional proteins such as Cdc42p, Bem1p, or Rsr1p, or phosphorylation state. Blocking cells in G1 phase in a cdc28 temperature-sensitive mutant did not dramatically alter the level of oligomers, suggesting that Cdc24p oligomers are present in G1 cells. We consider it unlikely that oligomer formation is strictly dependent on association with Bem1p or Rsr1p as truncation mutants lacking domains, which are necessary for binding these proteins, still oligomerized. Furthermore, Cdc24p truncations lacking the amino-terminal 280 amino acids, which do not accumulate in the nucleus (4–6), nonetheless oligomerized, suggesting that Cdc24p does not need to localize to the nucleus to form oligomers. A Cdc24p truncation, which lacks the carboxyl-terminal 380 amino acids, including the PB1 and PH domains, was able to bind wild-type Cdc24, albeit at a lower level compared with wild-type Cdc24p oligomerization. Such a carboxyl-terminal truncation does not localize to bud tips or sites of cell division (4–6), suggesting that Cdc24p oligomer stability requires its cortical localization. Cdc24p oligomerization was also observed in vitro, indicating that other yeast proteins and phosphorylation are not critical for this multimeric state.

Similar to Dbl and RasGRF (22, 27), the DH domain of Cdc24p is necessary and sufficient for oligomerization. This contrasts the oligomerization observed with the exchange factors α-Pix, p115 RhoGEF, PDZ-RhoGEF (PRG), and LARG, which requires the carboxyl-terminal region of the respective GEF (25, 26, 46). Previously, genetic and two-hybrid studies have suggested that the PB1 domain and an amino-terminal PB1-like region (18) interact intramolecularly, yet such interactions are not important for oligomerization as mutants lacking these regions still oligomerize. Nonetheless, the relationship between Cdc24p intra- and intermolecular interactions remains unclear, and it will be necessary to observe such interactions in intact cells to assess how they affect one another.

Our analyses of the importance of the different conserved regions in the DH domain indicate that CR2 is critical for oligomerization. Crystallographic studies with Dbs revealed that the equivalent residues mutated in Cdc24p CR1 (DH1) and CR3 (DH7) make contacts with Cdc42p (40). The former mutant oligomerizes similar to wild-type Cdc24p, whereas the latter is 2-fold reduced. Surprisingly, the Cdc24p DH4 mutant (Db1 equivalent H556A) is hardly affected in its oligomerization, whereas this Dbl mutant is oligomerization-deficient (27).

**DISCUSSION**

We show that Cdc24p forms oligomers in vivo via the catalytic DH domain. This homotypic interaction requires the conserved region 2 of the catalytic DH domain, and mutation of two residues in CR2 (DH5), which dramatically reduces Cdc24p oligomerization, renders this exchange factor non-functional in vivo. Neither these DH5 mutations nor the DH3 mutation altered Cdc24p in vitro guanine nucleotide exchange factor activity. G1 cell cycle arrest, which results in nuclear accumulation of this exchange factor, did not alter Cdc24p oligomerization levels. Both the DH3 and DH5 mutations, which reduced Cdc24p oligomerization, also resulted in a reduction in Cdc24p localization to the nucleus and the bud tip. The localization of the Cdc24p DH3 mutant to the nucleus and bud tip was partially restored upon chemically induced oligomerization. Chemically induced oligomerization of wild-type Cdc24p resulted in a decrease in Cdc42p activation and the accumulation of Cdc24p in the nucleus at all cell cycle stages. In vitro, induced oligomerization of the DHPH domain did not affect guanine nucleotide exchange factor activity. Together these results suggest that Cdc24p oligomerization is important for the correct localization of this exchange factor and may down-regulate this GEF by increasing nuclear sequestration.
Cdc24p DH3 mutant (Dbl equivalent F546A) is somewhat defective in oligomerization, whereas the DH5 mutant (Dbl equivalent E565A) is substantially defective in oligomerization. In Dbl, the former mutant (F546A) is defective in oligomerization, whereas the latter Dbl mutant (E565A) had little effect (27). This Cdc24p DH5 mutant is also nonfunctional in vivo, raising the attractive possibility that oligomerization is necessary for in vivo function.

In vitro GEF assays indicate that Cdc24p DH3 and DH5 are both able to stimulate Cdc42p GDP-GTP exchange. Strikingly, Cdc24p DH3 and DH5 were defective in localizing to both the nucleus and the bud tip. Furthermore, chemically induced oligomerization of Cdc24p DH3 mutant partially restored localization to both the nucleus and the bud tip, indicating that the DH3 localization defect is in part due to a reduction in oligomerization. It is difficult to relate the levels of Cdc24p oligomer observed by co-immunopurification...
to the localization defect observed in Cdc24p DH3 and DH5 cells. It is possible that the level of Cdc24p oligomers determined by co-immunopurification substantially underestimates the amount of oligomer in the cell, that these DH domain mutants affect other functions in addition to oligomerization, or that the localization of nonoligomeric Cdc24p is dependent on Cdc24p oligomers. Presently we are unable to distinguish between these possibilities. Irrespective, truncation mutants, which do not localize to the nucleus or bud tip, can nonetheless oligomerize. Taken together these results suggest that Cdc24p oligomerization is necessary for its localization. Furthermore, the Cdc24-m1p mutant, which is defective in Far1 binding and nuclear localization, still forms oligomers. Our results are consistent with studies on DbI oligomerization, in which the CR2 region, which is opposite the Rho G-protein interaction site, is important for DH domain-DH domain interactions.

Our studies do not address the size of Cdc24p oligomers, specifically whether dimers, trimers, tetrarsers, or higher order multimers of Cdc24p exist. Certainly the existence of higher order Cdc24p multimers, if correctly localized, could result in increased local concentrations of activated Cdc42p.

We examined the effect of constitutive Cdc24p oligomerization using two carboxyl-terminal fused FKBP domains, and we observed a decrease in the level of activated Cdc42p when cells were grown in the presence of the homo bi-functional dimerizer AP20187. Examination of the cellular distribution of these chemically induced Cdc24p oligomers revealed an increase in the number of cells with nuclear localized Cdc24p, most apparent in cells with small and medium sized buds. These results suggest that in wild-type cells Cdc24p can associate to form oligomers, which then dissociate for efficient nuclear export.

Recently Baumeister et al. (42) have shown that chemically induced dimerization of the Dbs DPH domain is sufficient to alter its subcellular distribution and drive it to the plasma membrane. This effect is presumably because of an increase in avidity of the PH domain interactions with the phosphoinositide phosphatidylinositol 4,5-bisphosphate. However the authors were unable to detect an increase in Cdc42p activation upon dimerization of Dbs DPH domain. Similarly, a DbI mutant that is defective for oligomerization does not affect in vitro GEF activity (27). Our observations that mutants that are oligomerization-defective and chemically induced Cdc24p oligomerization both affect the localization of this exchange factor are consistent with this recent study on Dbs (42). Oligomerization-defective or constitutively oligomerized Cdc24p mutants catalyzed Cdc42p GDP-GTP exchange in vitro similar to wild-type Cdc24p. Therefore, we consider it unlikely that the decrease in activated Cdc42p observed in vivo is because of changes in exchange factor activity upon oligomerization, but rather nuclear sequestration.

Interestingly, two ring finger domain-containing proteins, which associate with Cdc24p, also appear to oligomerize. Ste5p has been shown to oligomerize by interallelic complementation, two-hybrid analysis, and pulldowns (47–51). The majority of both Ste5p (48) and Cdc24p appears to be monomeric in cells. Ste5p oligomerization affects its nuclear shuttling, recruitment to the plasma membrane, and activation (48). In contrast to our results with Cdc24p oligomers, which are more efficiently retained in the nucleus, Ste5p oligomers are more efficiently exported from the nucleus and more efficiently recruited to the plasma membrane (48). It is likely that Cdc24p binds oligomeric Ste5p (14). We favor the possibility that oligomeric Cdc24p binds Far1p, and this complex is retained in the nucleus, whereas monomeric Cdc24p binds oligomeric Ste5p, and this complex is efficiently exported from the nucleus. It also possible that Far1p oligomerizes, as artificial dimerization of Far1p can activate Cdc24p. However, Far1p dimers have not been detected in vivo (19). Further work will be needed to determine whether Cdc24p, Ste5p, and Far1p oligomerization regulates the association of these three proteins.

Oligomerization of Dbl family exchange factors is conserved between yeast and mammalian GEFs. Both yeast and mammalian exchange factors such as DbI associate via their catalytic DH domain, and the conserved region 2 of the DH domain is required for this homotypic interaction. Our results suggest that oligomerization of DbI family exchange factors may be important for regulating their localization and hence activity.

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