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Cdc42p GTPase Is Involved in Controlling Polarized Cell Growth in *Schizosaccharomyces pombe*

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Cdc42p is a highly conserved low-molecular-weight GTPase that is involved in controlling cellular morphogenesis. We have isolated the Cdc42p homolog from the fission yeast *Schizosaccharomyces pombe* by its ability to complement the *Saccharomyces cerevisiae cdc42-1*^{ts} mutation. *S. pombe* Cdc42p is 85% identical in predicted amino acid sequence to *S. cerevisiae* Cdc42p and 83% identical to the human Cdc42p homolog. The Cdc42p protein fractionates to both soluble and particulate fractions, suggesting that it exists in two cellular pools. We have disrupted the *cdc42*$^{+}$ gene and shown that it is essential for growth. The *cdc42* null phenotype is an arrest as small, round, dense cells. In addition, we have generated three site-specific mutations, G12V, Q61L, and D118A, in the Cdc42p GTP-binding domains that correspond to dominant-lethal mutations in *S. cerevisiae CDC42*. In contrast to the *S. cerevisiae cdc42* mutations, the *S. pombe cdc42* mutant alleles were not lethal when overexpressed. However, the *cdc42* mutants did exhibit an abnormal morphological phenotype of large, misshapen cells, suggesting that *S. pombe* Cdc42p is involved in controlling polarized cell growth.

Cells generate and maintain characteristic shapes as they grow and divide (25). The rod-shaped fission yeast cell and the ellipsoidal budding yeast cell accomplish this by directing the insertion of new material to specific regions of their cell surfaces in a spatial and temporal pattern of growth that is precisely coordinated with the cell division cycle. Although a switch point for polarized growth is present in both yeasts, the manifestations of polarized growth are different between these distantly related yeasts. In the budding yeast *Saccharomyces cerevisiae*, growth is unidirectional during the cell cycle, with the majority of growth being directed from the mother cell into the emerging daughter cell (6, 7, 28). After cytokinesis and cell septation, however, the undersized new daughter cell switches to isotropic growth in order to attain the proper size to initiate the next round of cell division. Over 20 genes that are involved in producing a daughter cell by budding have been identified (6, 7, 25). In contrast, the fission yeast *Schizosaccharomyces pombe* exhibits both unidirectional and bidirectional growth during its cell cycle (15). Initially, growth is localized to one end of the cylindrical fission yeast cell. About one-third of the way through the cell cycle, growth switches to bidirectional incorporation of new material at both ends of the cell, resulting in an elongated cell that then divides by septation. To date, genes involved in controlling this process in *S. pombe* have not been characterized at the molecular level. It is of interest to determine whether the protein mechanisms involved in generating directional growth in both of these yeasts are similar in structure and/or function.

Cdc42p is one of the proteins involved in controlling polarized cell growth in *S. cerevisiae* (16). The *cdc42-1*^{ts} mutant is unable to form buds at the restrictive temperature, but nuclear division and nonlocalized cell growth continue, resulting in large, round, multinucleate cells (1). *S. cerevisiae CDC42* encodes a 21-kDa protein that belongs to the Rho/Rac subgroup of the Ras superfamily of GTPases (17). These proteins are believed to act as molecular switches by virtue of their ability to exist in two forms, an active GTP-bound form and an inactive GDP-bound form (4, 11). The human Cdc42p homolog, which is 80% identical to *S. cerevisiae* Cdc42p in predicted amino acid sequence, is able to complement the *S. cerevisiae cdc42-1*^{ts} mutation, indicating both functional and structural homology (24, 36). We previously generated site-specific mutations in the GTP-binding domains of *S. cerevisiae* Cdc42p that were analogous to dominant transforming mutations in *ras* (37). These *cdc42* mutations gave a dominant-lethal phenotype in *S. cerevisiae*, resulting in cells with abnormal growth and morphological properties.

To study the control of polarized cell growth in *S. pombe*, we have isolated the *S. pombe cdc42*$^{+}$ homolog by functional complementation of the *S. cerevisiae cdc42-1*^{ts} mutation, using an *S. pombe* cDNA library. DNA sequence analysis of the cDNA and genomic *cdc42*$^{+}$ revealed that the gene contained two introns and that its predicted amino acid sequence was 85% identical to the *S. cerevisiae* Cdc42p sequence and 83% identical to the human Cdc42p sequence. Gene disruption experiments and site-directed mutagenesis experiments showed that the gene was essential and that it played a critical role in controlling cell growth in *S. pombe*. The morphological phenotypes of *S. pombe cdc42* mutants were different from those of analogous *S. cerevisiae* mutants, suggesting different requirements for Cdc42p function between the two cell division strategies. However, these results do support a conserved role for Cdc42p in controlling cell growth.

MATERIALS AND METHODS

Reagents. Enzymes, M13 dideoxy sequencing and mutagenesis kits, and other reagents were obtained from standard commercial sources and used as specified by the suppliers. [^{32}P]dCTP was obtained from Amersham Corp. (Arlington Heights, Ill.). Calcofluor (fluorescent brightener), 4′,6-diamidino-2-phenylindole (DAPI), and horseradish peroxidase-

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conjugated goat anti-rabbit secondary antibodies were obtained from Sigma Chemical Co. (St. Louis, Mo.). Anti-yeast actin was a gift from Sue Lillie (The University of Michigan, Ann Arbor).

Media, strains, and microbiological techniques. Methods for the growth and genetic manipulation of *S. pombe* and *S. cerevisiae* have been described elsewhere (23, 34). *S. pombe* strains used were ED665 (*h⁻ ade6-M210 leu1-32 ura4-D18*), ED668 (*h⁺ ade6-M216 leu1-32 ura4-D18*) (both provided by P. Fantes), and PM1 (*h⁺/h⁻ ade6-M210/ade6-M216 leu1-32/leu1-32 ura4-D18/ura4-D18* (constructed by crossing ED665 with ED668). The *S. cerevisiae* strain used was DJTD2-16A (*MAT*a *cdc42-1*^{ts} *ura3 his4 leu2 trp1 gal2*) (17); permissive and restrictive temperatures for this strain were 23 and 37°C, respectively. *Escherichia coli* HB101 and SURE (32) were used as plasmid hosts, and *E. coli* CJ236 and MV1190 (32) were used in site-directed mutagenesis experiments. Thiamine (2 μM) was added to *S. pombe* growth medium to repress transcription from the *nmt1*⁺ promoter.

Plasmids, libraries, and DNA manipulations. Standard procedures were used for recombinant DNA manipulations (32), *E. coli* and yeast transformations (23, 32, 34), and colony hybridizations (32). DNA sequencing was performing by the dideoxy-chain termination method (33) with a Sequenase sequencing kit (United States Biochemical Corp., Cleveland, Ohio), and both strands of the cDNA and genomic clones were determined (see Fig. 2A). Probes for colony hybridization and DNA-DNA blot hybridizations were generated by using [^{32}P]CTP and a Pharmacia Oligo-labelling kit (Pharmacia LKB Biotechnology, Inc., Piscataway, N.J.). Site-directed mutagenesis was performed with the MUTA-GENE kit (Bio-Rad Laboratories, Richmond, Calif.), using the *cdc42*⁺ cDNA in M13mp19 as the starting template. The mutagenic oligonucleotides were GGAGACGTTGCTGTAGG(GGT to GTT [G12V]), CCGCTGGTCTGGAGG (CAG to CTG [Q61L]), and CCAAATTGCTTTACG (GAT to GCT [D118A]). The entire coding region sequence of each mutant gene was confirmed.

Plasmids pDB20, pREP1, pREP2, pTZ18U, and YEp351 (*CDC42*) have been described elsewhere (3, 21, 30, 37). The pDB20-based cDNA library (9) was provided by J. Fikes, and the pWH5-based genomic library (36a) was provided by P. Young. The 1.0-kb *Hin*dIII cDNA fragment containing *cdc42*⁺ was inserted into the unique *Hin*dIII sites of pTZ18U and M13mp19 for DNA sequencing and site-directed mutagenesis experiments (18). To conditionally express the mutant and wild-type alleles, the *Hin*dIII cDNA fragment was blunt ended by using the Klenow fragment of DNA polymerase I and inserted into the unique *Sma*I site of pREP1 and pREP2 (21), which places expression of *cdc42*⁺ under the control of the thiamine-repressible *nmt1*⁺ promoter.

To disrupt the *S. pombe cdc42*⁺ gene with the *ura4*⁺ gene, the 2.7-kb genomic *Xba*I fragment containing a centrally placed *cdc42*⁺ was first inserted into pTZ18U. The 1.8-kb *Hin*dIII fragment of pREP2 containing the *ura4*⁺ gene was blunt ended by using the Klenow fragment of DNA polymerase I and inserted into the unique *Aat*II site of pTZ18U (*cdc42*⁺). The new 4.5-kb *Xba*I fragment containing *cdc42::ura4*⁺ was then used to transform the diploid strain PM1 to Ura⁺. Stable Ura⁺ transformants were subsequently analyzed by DNA-DNA hybridization and tetrad analysis to verify the proper replacement of a wild-type *cdc42*⁺ allele (see Results).

Immunological, photomicroscopy, and density centrifugation methods. For immunoblots of plasmid-containing cells, cells were grown at 30°C to mid-log phase in leucine-deficient minimal media, washed twice with water, resuspended in lysis buffer (0.8 M sorbitol, 1 mM EDTA, 10 mM morpholinepropanesulfonic acid [MOPS], pH 7) with protease inhibitors (0.5 mM phenylmethylsulfonyl fluoride in isopropanol; 1:1,000 dilutions of a 1-mg/ml stock of aprotinin in water, a 1-mg/ml stock of *N*-tosyl-L-phenylalanine chloromethyl ketone in 95% ethanol, a 1-mg/ml stock of leupeptin in water, and a 1-mg/ml stock of pepstatin in methanol), and lysed by vortexing with acid-washed glass beads. Equal amounts of protein were diluted 1:2 in sodium dodecyl sulfate (SDS) lysis buffer (19) containing 40% β-mercaptoethanol, heated at 100°C for 5 min, and separated on an SDS–15% polyacrylamide gel, and protein was transferred to nitrocellulose paper (Schleicher & Schuell, Keene, N.H.). The gels shown in Fig. 6 and 8 were run on different gel systems with different resolving power; the gel in Fig. 6 does not adequately resolve the multiple bands observed in Fig. 8. Affinity-purified anti-Cdc42p antibodies were prepared as previously described (37) and used at 1:500 dilution. Immunoblots were developed by using enhanced chemiluminescence protocols (Amersham).

Cell fractionation experiments were performed by using techniques described by Goud et al. (10). Briefly, ED668 cells were grown at 30°C to an optical density at 595 nm (OD_{595}) of ~0.5, washed twice with water, resuspended in lysis buffer with protease inhibitors, and lysed by vortexing with acid-washed glass beads. Cells lysates were spun at 500 × *g* for 5 min at 4°C, and the pellets were washed once and resuspended in the same volume of lysis buffer as the supernatants. The 500 × *g* supernatants were then spun at 10,000 × *g* for 10 min at 4°C, and the pellets were resuspended in the same volume of lysis buffer. Equal volumes of each fraction were loaded onto an SDS–15% polyacrylamide gel (see above).

Methods for the formaldehyde fixation of cells, DAPI staining of nuclei, immunofluorescence techniques, and fluorescence-activated cell sorting (FACS) analysis of propidium iodide-stained DNA have been described elsewhere (29, 35). Photomicrographs were obtained with an Olympus BH-2 epifluorescence microscope equipped with Hoffman modulation contrast optics.

To determine the density of *cdc42* null mutants, we used the protocol of Novick et al. (26) for density gradient centrifugation, using Ludox gradients. Briefly, we constructed a haploid strain that had the *cdc42::ura4*⁺ disruption allele complemented by wild-type *cdc42*⁺ on a plasmid. These plasmid-containing cells, as well as wild-type cells containing the same plasmid, were grown under nonselective conditions leading to plasmid loss. After approximately 20 generations of growth under these conditions, 16 OD_{595} units of a 50:50 mixture of wild-type and mutant cells (~1.6×10^8 cells) was spun down, washed with water, and layered on the top of a 60% (vol/vol) Ludox gradient, which contained Edinburgh minimal medium salts, in a 50-ml Corex tube. Samples were spun at 22,000 × *g* for 20 min at 4°C. Two distinct, visible bands of cells were observed in the gradient, and 0.1-ml fractions were collected through the bands. The less dense band of cells corresponded to wild-type cells run alone on a separate gradient, and the more dense band of cells corresponded to mutant cells run alone on a separate gradient. The OD_{595}s of the fractions were measured and the sizes of the cells were measured with an ocular micrometer.

Nucleotide sequence accession number. The sequence data shown in Fig. 2B are available from GenBank under accession number L25677.

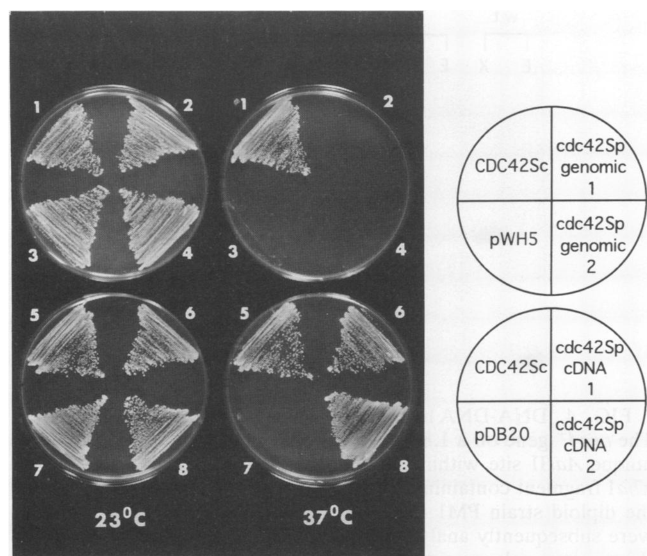

FIG. 1. Complementation of *S. cerevisiae cdc42-1*^{ts} by *S. pombe cdc42*$^+$. *S. cerevisiae* DJTD2-16A was transformed with the indicated plasmids, and individual transformants were then incubated at the *cdc42-1*^{ts} permissive and restrictive temperatures of 23 and 37°C, respectively. Plasmids are as follows: 1, YEp351(*CDC42Sc*); 2, pWH5(*cdc42*$^+$)-1; 3, pWH5; 4, pWH5(*cdc42*$^+$)-2; 5, YEp351 (*CDC42Sc*); 6, pDB20(*cdc42*$^+$)-1; 7, pDB20; 8, pDB20(*cdc42*$^+$)-2. CDC42Sc is *S. cerevisiae* Cdc42p; cdc42Sp is *S. pombe* Cdc42p.

RESULTS

Isolation and characterization of *cdc42*$^+$ cDNA and genomic clones. We reasoned that since a human cDNA encoding the human Cdc42p can complement the *S. cerevisiae cdc42-1*^{ts} mutation (24, 36), then an *S. pombe* cDNA encoding the *S. pombe* Cdc42p may also complement. Therefore, we transformed strain DJTD2-16A with a pDB20-based *S. pombe* cDNA library (9), selecting for Ura$^+$ Ts$^+$ transformants at 37°C. Of the 30 Ura$^+$ Ts$^+$ transformants obtained, 11 contained an unstable plasmid. Plasmids from these 11 transformants were recovered into *E. coli* and used to retransform DJTD2-16A. Two plasmids, pDB20(*cdc42*$^+$)-1 and pDB20 (*cdc42*$^+$)-2, were able to retransform DJTD2-16A to Ts$^+$ (Fig. 1) and had identical restriction maps. DNA sequence analysis of the cDNA insert identified an open reading frame that represented the *S. pombe* Cdc42p homolog (see below). In addition, the *cdc42*$^+$ cDNA could rescue a *S. cerevisiae cdc42* null mutant (data not shown), indicating that *S. cerevisiae* Cdc42p function can be completely provided by *S. pombe* Cdc42p.

The genomic *cdc42*$^+$ was isolated by colony hybridization using the cDNA as a probe. We screened a pWH5-based *S. pombe* genomic library transformed into *E. coli*; screening of 10^4 *E. coli* colonies yielded six positive clones, two of which were purified by two further rounds of colony hybridization. These clones contained 5-kb inserts with four internal *Hin*dIII fragments, the largest of which contained both an *Aat*II and a *Kpn*I site, sites that are present in the cDNA isolate. DNA sequence analysis showed that this fragment encompassed the *cdc42*$^+$ open reading frame. This fragment, however, was unable to complement the *S. cerevisiae cdc42-1*^{ts} allele (Fig. 1), possibly because of incompatible promoter or intron-splicing sequences.

The *cdc42*$^+$ cDNA contained an open reading frame that

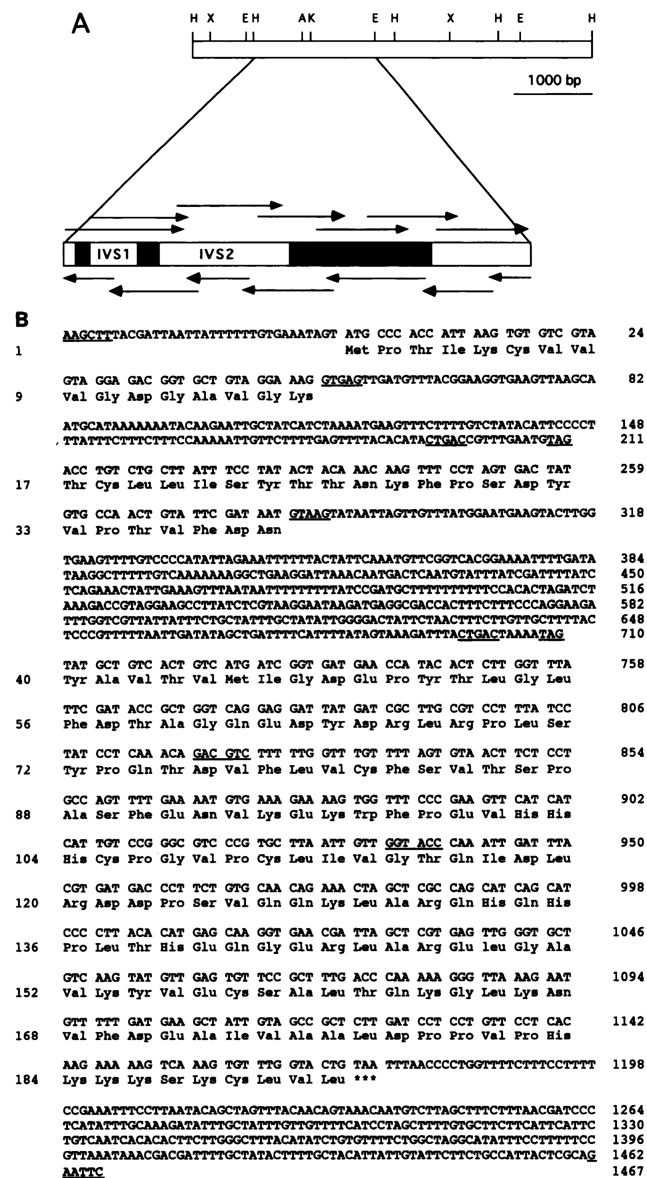

FIG. 2. Restriction map and DNA and amino acid sequences of *cdc42*$^+$. (A) Restriction map and DNA sequencing strategy. Restriction sites: *Aat*II (A), *Eco*RI (E), *Hin*dIII (H), *Kpn*I (K), and *Xba*I (X). Arrows indicate the direction and extent of individual DNA sequencing runs. IVS1 and IVS2, intervening sequences 1 and 2; shaded boxes, protein-coding sequences. (B) Nucleotide and predicted amino acid sequences of *cdc42*$^+$. Nucleotide sequences are numbered on the right, and amino acid sequences are numbered on the left. Underlined nucleotide sequences within the two introns (nucleotides 49 to 211 and 281 to 710) represent consensus splicing signals. Underlined sequences elsewhere are restriction sites for *Hin*dIII (nucleotides −34 to −29), *Aat*II (nucleotides 819 to 824), *Kpn*I (nucleotides 933 to 938), and *Eco*RI (nucleotides 1462 to 1467).

could encode a protein of 192 amino acids (Fig. 2B). This protein was 85% identical (89% related) in predicted amino acid sequence to *S. cerevisiae* Cdc42p and 83% identical (90% related) to human Cdc42p (Fig. 3). The sequence of the open reading frame was also in agreement with that of a cDNA clone previously isolated (8). However, we observed several nucleotide differences within the 3′ untranslated

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Cdc42Sc   M Q T L K C V V V G D G A V G K T C L L I S Y T T N Q F
Cdc42Sp   * P * ~ * * * * * * * * * * * * * * * * * * * * * * K *
Cdc42Hs   * * * ~ * * * * * * * * * * * * * * * * * * * * * * K *

Cdc42Sc   P A D Y V P T V F D N Y A V T V M I G D E P Y T L G L F
Cdc42Sp   * S * * * * * * * * * * * * * * * * * * * * * * * * * *
Cdc42Hs   * S ~ * * * * * * * * * * * * * * * * G * * * * * * * *

Cdc42Sc   D T A G Q E D Y D R L R P L S Y P S T D V F L V C F S V
Cdc42Sp   * * * * * * * * * * * * * * * * * Q * * * * * * * * * *
Cdc42Hs   * * * * * * * * * * * * * * * * * Q * * * * * * * * * *

Cdc42Sc   I S P P S F E N V K E K W F P E V H H H C P G V P C L V
Cdc42Sp   T * * A * * * * * * * * * * * * * * * * * * * * * * * ~
Cdc42Hs   ~ * * S * * * * * * * * * V * * ~ T * * * * K T * F * ~

Cdc42Sc   V G T Q I D L R D D K V I I E K L Q R Q R L R P I T S E
Cdc42Sp   * * * * * * * * * * P S ~ Q Q * * A * * H Q H * ~ * H *
Cdc42Hs   * * * * * * * * * * P S T * * * * A ~ N ~ Q ~ * * * P *

Cdc42Sc   Q G S R L A R E L K A V K Y V E C S A L T Q R G L K N V
Cdc42Sp   * * E * * * * * * G * * * * * * * * * * * * ~ * * * * *
Cdc42Hs   T ~ E ~ * * * ~ * * * * * * * * * * * * * * ~ * * * * *

Cdc42Sc   F D E A I V A A L E P P V I   K K S K K C T I L
Cdc42Sp   * * * * * * * * * ~ * * * P H * * K S * * L ~ *
Cdc42Hs   * * * * * ~ * * * * * * E P   * * * ~ ~ * V ~ *
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FIG. 3. Predicted amino acid sequence comparison between Cdc42 proteins. Comparisons are to *S. cerevisiae* Cdc42p (Cdc42Sc) sequences (16). The sequence of human Cdc42p (Cdc42Hs) is as published elsewhere (36). Cdc42Sp is the *S. pombe* sequence. The one-letter amino acid code is used. *, identical amino acids; ~, related amino acids. Groups of related amino acids are as follows: leucine, isoleucine, valine, alanine, and glycine; serine and threonine; aspartate and glutamate; asparagine and glutamine; lysine and arginine.

wt 1 2 3

E X E X E X E X

4.7

3.3

2.9

1.5

FIG. 4. DNA-DNA hybridization analysis of $cdc42^+$ disruptions. The $ura4^+$ gene on a 1.8-kb *Hin*dIII fragment was inserted into the unique *Aat*II site within the coding region of $cdc42^+$. The 4.7-kb *Xba*I fragment containing $cdc42::ura4^+$ was then used to transform the diploid strain PM1 to Ura^+. Three stable Ura^+ transformants were subsequently analyzed by DNA-DNA hybridization to verify the proper replacement of a wild-type (wt) $cdc42^+$ allele by the $cdc42::ura4^+$ allele. In a diploid strain, replacement of one chromosomal copy of $cdc42^+$ by $cdc42::ura4^+$ should result in two fragments, one the size of the original chromosomal copy and another 1.8 kb larger. All three Ura^+ transformants displayed this pattern, indicating that the replacement had occurred at the correct location. The ^{32}P-labeled probe was the 1.0-kb *Hin*dIII $cdc42^+$ cDNA fragment. The sizes of the fragments visualized are indicated in kilobase pairs. Restriction enzymes used were *Eco*RI (E) and *Xba*I (X).

region: addition of a C at nucleotide 1180, addition of a G instead of an A at nucleotide 1295, and loss of a G at nucleotide 1462. DNA sequence analysis of the genomic clone revealed that the N-terminal half of the $cdc42^+$ open reading frame contained two introns both located within conserved GTP-binding domains (Fig. 2B). The second intron of 430 bp belongs to a rare class of large *S. pombe* introns; 89% of recorded introns are less than 250 bp long, and 68% are less than 80 bp long (27).

Disruption of $cdc42^+$ and the null phenotype. We disrupted a plasmid-borne $cdc42^+$ gene by inserting the $ura4^+$ gene at the unique *Aat*II site located in the $cdc42^+$ coding region (Fig. 4). This gene disruption was then used to replace one copy of the $cdc42^+$ gene in the diploid strain PM1. DNA-DNA hybridization analysis confirmed that three of four Ura^+ transformants contained a single copy of the disrupted allele at the $cdc42^+$ locus (Fig. 4). We analyzed 75 tetrads from these three disrupted strains; 41 tetrads segregated one live spore, and 34 segregated two live spores. No tetrad gave rise to three or more live spores, and all of the live spores were Ura^-, indicating that they did not harbor the $ura4^+$-marked disruption. Microscopic examination of the dead spores revealed that either they failed to germinate or they formed a germ tube or two small round cells. This result indicates that $cdc42^+$ is an essential gene.

To determine the *cdc42* null phenotype, we constructed a haploid strain that had the $cdc42::ura4^+$ disruption allele complemented by wild-type $cdc42^+$ on a plasmid. These plasmid-containing cells, as well as wild-type cells containing the same plasmid, were grown under nonselective conditions leading to plasmid loss. After approximately 20 generations of growth under these conditions, we observed a significant increase in small, round cells within the culture of cells containing the $cdc42::ura4^+$ allele (Fig. 5A); no such cells were found in the wild-type culture. We measured the length of cells within the population and found that approximately 50% of the cells from the $cdc42::ura4^+$ culture were smaller than cells from a wild-type culture (Fig. 5B), with 10 to 15% of these appearing spherical.

To determine whether macromolecular synthesis was still occurring in these small, round cells, we determined their density in Ludox gradients (Fig. 5C). Wild-type cells and mutant cells as well as a 50:50 mixture of wild-type and mutant cells were centrifuged separately in 60% Ludox gradients. Two separate distinct bands of cells were observed in the mixture experiment, with the small, round cells corresponding to the more dense cells (Fig. 5C). Wild-type cells of all sizes were less dense than these small, round cells. This result suggests that macromolecular syntheses can continue in these small, round cells. These cells were uninucleate, as measured by DAPI staining (data not shown). In addition, FACS analysis of propidium iodide-stained wild-type and mutant cells showed no significant differences in DNA content (data not shown). These results suggest that loss of Cdc42p function results in a cell cycle block with a continuation of macromolecular syntheses but a loss of incorporation of new material into enlarging cells.

Generation of site-specific mutations in $cdc42^+$. To observe the phenotype of a constitutively active Cdc42p, we generated three site-specific mutations in its GTP-binding and hydrolysis domains (Fig. 6A). The analogous *S. cerevisiae* *cdc42* mutations, G12V, Q61L, and D118A, have dominant-lethal phenotypes causing arrest as either enlarged, irregularly shaped cells with multiple misshapen buds (dominant-active G12V and Q61L) or large, spherical, unbudded cells (dominant-negative D118A) (37). We generated the same three mutations in $cdc42^+$ and expressed them in a wild-type $cdc42^+$ background under the control of the thiamine-repressible $nmt1^+$ promoter. Growth of plasmid-containing cells in medium lacking thiamine resulted in overproduction of the mutant proteins (Fig. 6B). Overproduction of wild-type Cdc42p did not result in any morphological abnormalities (data not shown).

Plasmid-containing cells grown under repressing condi-

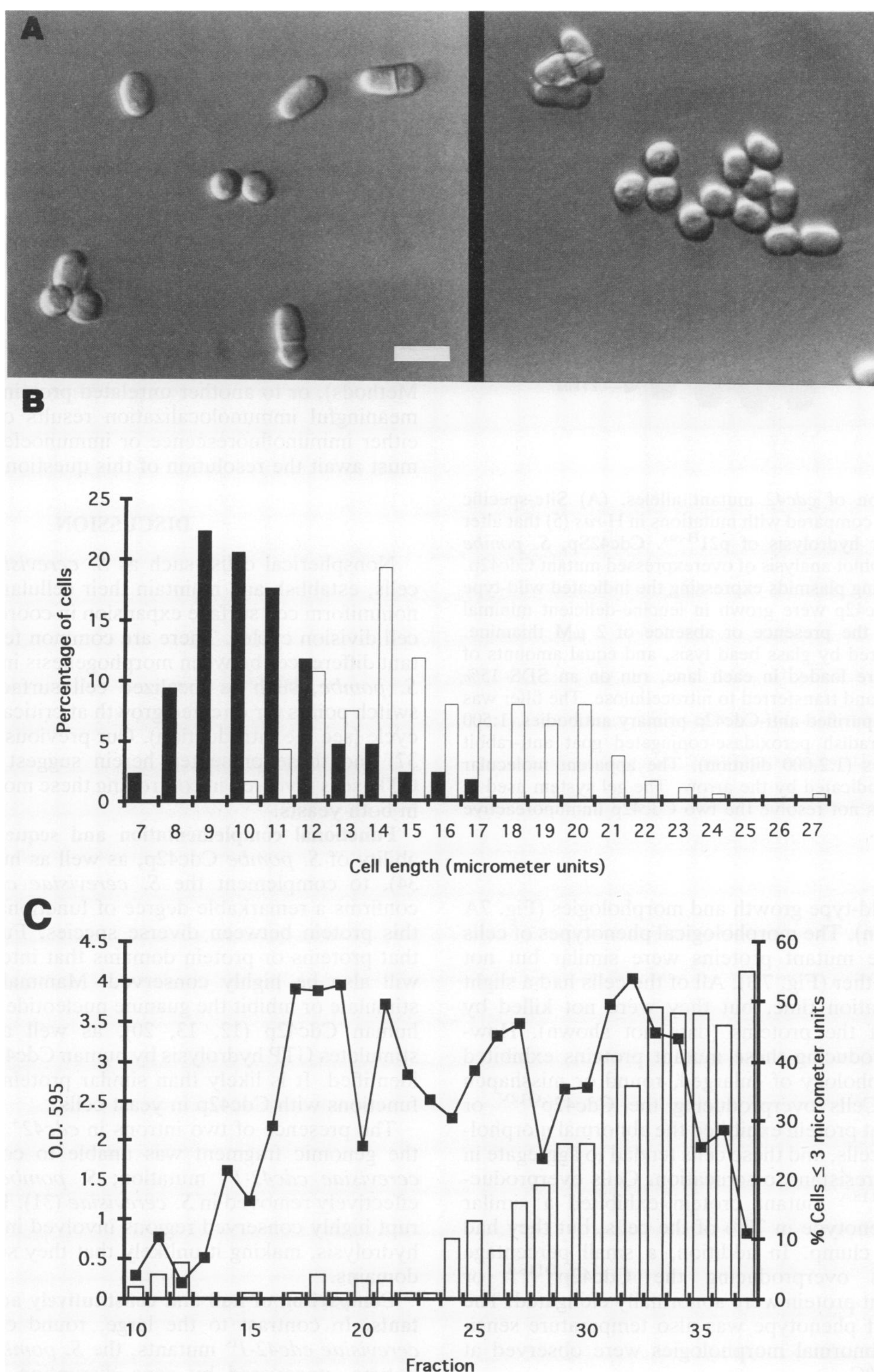

FIG. 5. The *cdc42* null phenotype. Haploid cells containing the *cdc42::ura4*$^+$ allele complemented by pREP2(*cdc42*$^+$) were picked from a leucine-deficient minimal medium selective plate into nonselective complex medium containing leucine and adenine and allowed to grow for approximately 20 generations. Cells were fixed with formaldehyde and observed microscopically. (A) Photomicrograph of *cdc42::ura4*$^+$ cells grown under plasmid-nonselective conditions. Size bar is 10 μm. (B) Graph of percentage of cells versus cell length. Solid bars, *cdc42::ura4*$^+$ cells (n = 170 cells); open bars, wild-type cells (n = 180 cells). Cell lengths were measured with an ocular micrometer and reported in ocular micrometer units; 1 micrometer unit equals 0.78 μm. (C) Graph of cell lengths and OD_{595} versus fractions from a Ludox density centrifugation gradient. A 50:50 mixture of wild-type and mutant cells was loaded onto the gradient. This mixture of cells gave two distinct bands after centrifugation. Open bars, percentage of cells measuring ≤3 micrometer units (n = 100 cells per fraction; 1 micrometer unit equals 1.96 μm); closed squares, OD_{595}, which represents total cells in the population.

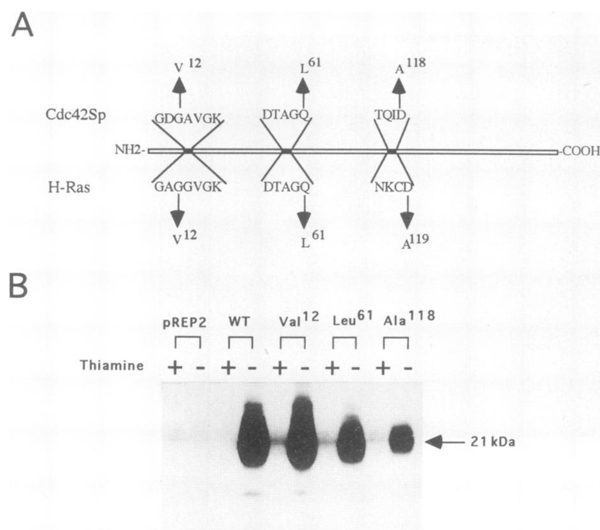

FIG. 6. Generation of *cdc42* mutant alleles. (A) Site-specific mutations in $cdc42^+$ compared with mutations in H-*ras* (5) that alter the GTP binding or hydrolysis of $p21^{H\text{-}ras}$. Cdc42Sp, *S. pombe* Cdc42p. (B) Immunoblot analysis of overexpressed mutant Cdc42p. ED668 cells containing plasmids expressing the indicated wild-type (WT) or mutant Cdc42p were grown in leucine-deficient minimal medium at 30°C in the presence or absence of 2 μM thiamine. Lysates were prepared by glass bead lysis, and equal amounts of protein (~5 μg) were loaded in each lane, run on an SDS–15% polyacrylamide gel, and transferred to nitrocellulose. The filter was probed with affinity-purified anti-Cdc42p primary antibodies (1:500 dilution) and horseradish peroxidase-conjugated goat anti-rabbit secondary antibodies (1:2,000 dilution). The apparent molecular mass of Cdc42p is indicated by the arrow. The gel system used in this experiment does not resolve the two Cdc42p immunoreactive bands seen in Fig. 8.

tions exhibited wild-type growth and morphologies (Fig. 7A and data not shown). The morphological phenotypes of cells overproducing the mutant proteins were similar but not identical to each other (Fig. 7B). All of the cells had a slight increase in generation time, but they were not killed by overproduction of the proteins (data not shown). However, cells overproducing these mutant proteins exhibited an abnormal morphology of enlarged, round or misshapen cells (Fig. 7B). Cells overproducing the $Cdc42p^{G12V}$ or $Cdc42p^{Q61L}$ mutant protein exhibited the abnormal morphology in 90% of the cells, and these cells tended to aggregate in clumps that were resistant to sonication. Cells overproducing the $Cdc42p^{D118A}$ mutant protein exhibited a similar morphological phenotype in 70% of the cells, but they had less tendency to clump. In addition, a small percentage (<10%) of cells overproducing the $Cdc42p^{D118A}$ or $Cdc42p^{Q61L}$ mutant protein were abnormally elongated. The $cdc42^{D118A}$ mutant phenotype was also temperature sensitive; cells with abnormal morphologies were observed at 23°C but not at 30°C.

Cells overproducing these mutant proteins could still undergo cytokinesis, as evidenced by the presence of septa (Fig. 7B). Immunofluorescence localization of actin within the cells revealed a random, punctuate distribution (Fig. 7D), in contrast to wild-type cells, in which actin localizes to the septa and the growing ends of cells (Fig. 7C). In addition, these cells were uninucleate (data not shown).

Localization of Cdc42p by cell fractionation. *S. cerevisiae* Cdc42p runs as a single protein band on SDS–15% polyacrylamide gels and fractionates to both soluble and particulate fractions at 10,000 × *g*, suggesting that it exists in both cytosolic and membrane-bound pools (39). Fractionation of *S. pombe* cell lysates revealed the presence of two immunoreactive bands, one of which comigrates with *S. cerevisiae* Cdc42p and both of which were amplified upon overexpression of $cdc42^+$ (Fig. 8 and data not shown). Both bands fractionated to soluble and particulate fractions at 10,000 × *g*, suggesting that their gross cellular distribution is similar to that of *S. cerevisiae* Cdc42p. While it is likely that both immunoreactive bands are different processed forms of *S. pombe* Cdc42p, we cannot rule out the possibility that one of the immunoreactive bands corresponds to a proteolytic product, an unlikely possibility because of the lack of proteolysis of *S. cerevisiae* Cdc42p in the same lysis buffer containing a variety of protease inhibitors (see Materials and Methods), or to another unrelated protein. For this reason, meaningful immunolocalization results obtained by using either immunofluorescence or immunoelectron microscopy must await the resolution of this question.

DISCUSSION

Nonspherical cells, such as *S. cerevisiae* and *S. pombe* cells, establish and maintain their cellular morphologies by nonuniform cell surface expansion in coordination with their cell division cycles. There are common features and important differences between morphogenesis in *S. cerevisiae* and *S. pombe*, such as localized cell surface expansion and switch points for directed growth at critical points in the cell cycle (see the introduction). Our previous results (1, 16, 17, 37) and those presented herein suggest that the Cdc42p GTPase is involved in controlling these morphogenetic event in both yeasts.

Functional complementation and sequence analysis. The ability of *S. pombe* Cdc42p, as well as human Cdc42p (24, 34), to complement the *S. cerevisiae* $cdc42\text{-}1^{ts}$ mutation confirms a remarkable degree of functional conservation of this protein between diverse species. Further, it suggests that proteins or protein domains that interact with Cdc42p will also be highly conserved. Mammalian proteins that stimulate or inhibit the guanine nucleotide exchange rate for human Cdc42p (12, 13, 20), as well as a protein that stimulates GTP hydrolysis by human Cdc42p (14), have been identified. It is likely than similar proteins perform similar functions with Cdc42p in yeast cells.

The presence of two introns in $cdc42^+$ may explain why the genomic fragment was unable to complement the *S. cerevisiae* $cdc42\text{-}1^{ts}$ mutation; *S. pombe* introns are not effectively removed in *S. cerevisiae* (31). Both introns interrupt highly conserved regions involved in GTP binding and hydrolysis, making it unlikely that they separate functional domains.

Comparison of null and constitutively activated *cdc42* mutants. In contrast to the large, round cells seen with *S. cerevisiae* $cdc42\text{-}1^{ts}$ mutants, the *S. pombe cdc42* null phenotype, uncovered by gene disruption and plasmid loss experiments, appears to be an arrest as small, round, dense cells. A more precise determination of the null phenotype, however, must await the generation of conditional-lethal alleles of $cdc42^+$. The apparent disparity between the *S. cerevisiae* and *S. pombe cdc42* null phenotypes suggests that mechanisms for incorporation of new cell surface material in these organisms are different. Cdc42p seems to be required for incorporation of new cell surface material in *S. pombe*, whereas there is a Cdc42p-independent mechanism for iso-

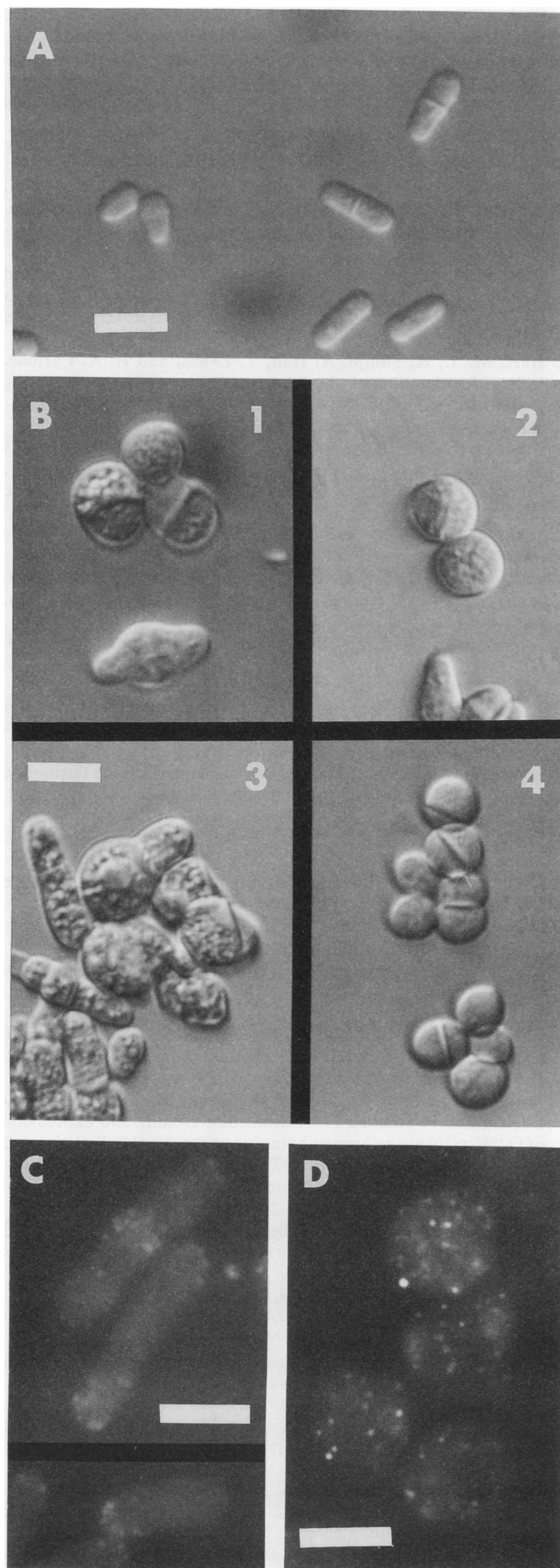

FIG. 7. Cellular morphologies of cells overexpressing Cdc42p. (A) Photomicrograph of *S. pombe* cells containing pREP2 (*cdc42*^{G12V}) grown under repressing conditions; size bar is 10 μm. (B) Photomicrographs of cells overproducing mutant Cdc42p. 1, *cdc42*^{G12V} cells; 2, *cdc42*^{D118A} cells; 3, *cdc42*^{Q61L} cells; 4, *cdc42*^{D118A} cells. Size bar is 10 μm. (C) Immunofluorescence localization of actin in wild-type cells. Size bar is 5 μm. Affinity-purified antiactin antibodies were used at 1:10 dilution. (D) Immunofluorescence localization of actin in cells overproducing the *cdc42*^{G12V} mutant protein. Size bar is 5 μm. Affinity-purified antiactin antibodies were used at 1:10 dilution.

tropic incorporation of new cell surface material in *S. cerevisiae*. The fact that the nuclear cycle can proceed in *S. cerevisiae cdc42* null mutants, evidenced by multinucleate cells (1), but does not in *S. pombe cdc42* null mutants suggests that *S. pombe* Cdc42p is required for an early step in the cell cycle upon which the nuclear cycle is dependent, while the nuclear and budding cycles in *S. cerevisiae* are independent pathways within the cell cycle.

The G12V, Q61L, and D119A mutations in Ras-like GTPases are predicted to result in a constitutively activated phenotype either by preventing the hydrolysis of bound GTP to GDP or by increasing the GDP dissociation rate of the protein (4, 11). These mutations in mammalian Ras are dominant, resulting in proteins that cause focus formation and morphological changes when introduced into cells in vitro (2). In *S. cerevisiae*, these *cdc42* mutations are dominant lethal, resulting in enlarged cells with abnormal morphologies and growth characteristics (37). However, the *S. cerevisiae cdc42*^{G12V} and *cdc42*^{Q61L} mutants displayed a morphological phenotype indicative of a constitutively activated Cdc42p, while the *cdc42*^{D118A} mutant phenotype indicated a loss of function phenotype (37) and was temperature sensitive (38).

In contrast, we found that analogous *S. pombe cdc42* mutants were not dominant lethal, and all three mutations had similar effects on cellular morphology. The *cdc42*^{D118A} mutant phenotype was also temperature sensitive. The growth characteristics of cells expressing these mutant proteins were altered from directional end elongation growth to

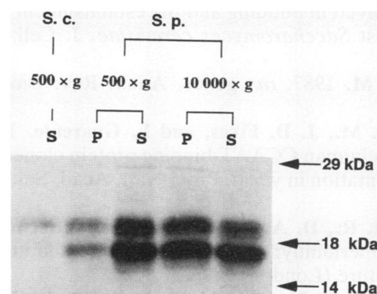

FIG. 8. Immunoblot analysis of fractionated *S. pombe* (S.p.) cell lysates. ED668 cells were grown at 30°C to mid-log phase and lysed with glass beads. Extracts from *S. cerevisiae* (S.c.) strains were prepared in the same manner. Protein extracts were spun at 500 × *g* to produce pellet (P) and supernatant (S) fractions; the supernatant fraction was subsequently spun at 10,000 × *g*. Equal volumes of protein extracts were run on an SDS–15% polyacrylamide gel and transferred to nitrocellulose, which was probed with affinity-purified anti-Cdc42p primary antibodies (1:500) and horseradish peroxidase-conjugated goat anti-rabbit secondary antibodies (1:2,000). This result is indicative of two independent experiments.

isotropic growth, resulting in large, round or misshapen cells. The morphological phenotype of these mutants is consistent with an overexpressed, constitutively active Cdc42p localizing cell surface deposition inappropriately. This result suggests that Cdc42p is involved in controlling directional cell growth. The increase in size of these cells also suggests some alteration in the size control mechanism intrinsic to cell division cycle control in *S. pombe*. Overexpression of mutant Cdc42p in both *S. cerevisiae* (37) and *S. pombe* (Fig. 7D) results in the disruption of normal actin distribution, suggesting that Cdc42p functions in an actin-mediated pathway in both organisms. Previous genetic and immunolocalization studies in both yeasts suggested that actin is involved in generating polarized growth (7, 15).

The differences in lethality of *S. cerevisiae* and *S. pombe cdc42* mutations may reflect a fundamental difference between the morphology and cell growth of the two yeasts. Because of the budding mechanism, *S. cerevisiae* cells require proper organization of the bud site and directed cell surface growth to form daughter cells. Therefore, perturbation of budding and directed cell growth by a constitutively active or inactive Cdc42p is a lethal event. *S. pombe* cells, however, seem to be able to divide even if the spatial pattern of cell surface expansion is disturbed, as long as the cells can still undergo mitosis and form a septum. These results underscore the need to study cellular morphogenesis in a variety of experimentally tractable organisms because even though the protein machinery involved in controlling morphogenesis, such as Cdc42p, may be highly conserved, the underlying mechanisms of directed cell growth may be different.

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