Molecular Characterization of CDC42, a Saccharomyces cerevisiae Gene Involved in the Development of Cell Polarity

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Abstract. The Saccharomyces cerevisiae CDC42 gene product is involved in the morphogenetic events of the cell division cycle; temperature-sensitive cdc42 mutants are unable to form buds and display delocalized cell-surface deposition at the restrictive temperature (Adams, A. E. M., D. I. Johnson, R. M. Longnecker, B. F. Sloat, and J. R. Pringle. 1990. J. Cell Biol. 111:131–142). To begin a molecular analysis of CDC42 function, we have isolated the CDC42 gene from a yeast genomic DNA library. The use of the cloned DNA to create a deletion of CDC42 confirmed that the gene is essential. Overexpression of CDC42 under control of the GAL10 promoter was not grossly deleterious to cell growth but did perturb the normal pattern of selection of budding sites. Determination of the DNA and predicted amino acid sequences of CDC42 revealed a high degree of similarity in amino acid sequence to the ras and rho (Madaule, P., R. Axel, and A. M. Myers. 1987. Proc. Natl. Acad. Sci. 84:779–783) families of gene products. The similarities to ras proteins (~40% identical or related amino acids overall) were most pronounced in the regions that have been implicated in GTP binding and hydrolysis and in the COOH-terminal modifications leading to membrane association, suggesting that CDC42 function also involves these biochemical properties. The similarities to the rho proteins (~60% identical or related amino acids overall) were more widely distributed through the coding region, suggesting more extensive similarities in as yet undefined biochemical properties and functions.

The Saccharomyces cerevisiae CDC24, CDC42, and CDC43 gene products play critical roles in the establishment of cell polarity, the localization of secretion and cell-surface deposition, and the development of normal cell shape (Hartwell et al., 1974; Sloat and Pringle, 1978; Field and Schekman, 1980; Sloat et al., 1981; Pringle and Hartwell, 1981; Pringle et al., 1986; Adams et al., 1990). Yeast strains carrying temperature-sensitive lethal mutations in these genes have essentially identical morphological phenotypes. At permissive temperatures, the mutants grow and bud normally; at restrictive temperatures, the nuclear cycle continues but bud formation is blocked. Cell mass and volume continue to increase, resulting in greatly enlarged, unbudded cells. The cytoplasmic actin network appears disorganized (Adams and Pringle, 1984; Adams et al., 1990), and chitin and other cell surface materials appear to be deposited randomly or uniformly throughout the enlarging cell walls, in contrast to their normal highly localized patterns of deposition. In addition, some temperature-sensitive cdc24 mutants show abnormal positioning of budding sites when grown at permissive temperatures, suggesting that the CDC24 gene product is in the initial selection and organization of the budding site (Sloat et al., 1981). Another cdc24 mutant was identified among a collection of calcium-sensitive mutants, suggesting a possible interaction of the gene product with calcium (Ohya et al., 1986a,b). The CDC24 gene has been cloned (Coleman et al., 1986; Ohya et al., 1986a) and sequenced (Miyamoto et al., 1987). The predicted gene product contains two putative Ca$^{2+}$-binding domains.

As a step in the further analysis of this system, we have begun a molecular characterization of the CDC42 gene and its product. We report here the isolation and sequence analysis of CDC42, as well as the phenotypes associated with its deletion or overexpression. Remarkably, the predicted amino acid sequence of the CDC42 protein is strikingly similar to the ras (Capon et al., 1983; Powers et al., 1984; Tatchell, 1986) and rho (Madaule and Axel, 1985; Madaule et al., 1987; Anderson and Lacal, 1987) families of gene products from yeast and larger eukaryotes.

Materials and Methods

Reagents

Enzymes, M13 dideoxy sequencing kits, and other reagents were obtained from standard commercial sources and used according to the suppliers' specifications. [35S]-dATP was obtained from Amersham Corp. (Arlington Heights, IL) and [32P]-dATP was obtained from ICN Biomedicals, Inc. (Irvine, CA). Calcofluor White M2R New was a gift from American Cyanamid Co. (Bound Brook, NJ).
Media, Growth Conditions, Strains, and Plasmids

Conditions for the growth and maintenance of bacterial and yeast strains have been described (Maniatis et al., 1982; Lilley and Pringle, 1980; Sherman et al., 1986). The permissive and restrictive temperatures for growth of temperature-sensitive mutants were 23 and 36°C, respectively. Escherichia coli strain HB101 was routinely used as a plasmid host. The S. cerevisiae strains used were C276, MA10/MATA gal2/gal2 prototrophic, and C276-4A, MA10a gal2 prototrophic (Wilkinson and Pringle, 1974; JPT163BD5-5C, MA10a cdc42-1 gal2) (Adams et al., 1990; Adams, A., and J. R. Pringle, unpublished results); TD4, MA10a ura3 his4 leu2 trpl gal2, and TD1, MA10a ura3 his4 trpl gal2 (both provided by G. Pink, Whitehead Institute, Cambridge, MA); DJTD2-16D, MA10a cdc42-1 urn3 his4 leu2 trpl gal2 (both constructed by crossing JPT163BD5-5C to TD4); DJTD2-16A, MA10a cdc42-1 urn3 his4 leu2 trpl gal2 (both constructed by crossing JPT163BD5-5C to TD1); DJTD2-16D, MA10a cdc42-1urn3 his4 leu2 trpl gal2 (both constructed by mating DJTD2-16A to TD1); DJMD2-7C, MA10a cdc42-1urn3 his4 leu2 gal2 RDN1-Leu2 (Johnson et al., 1987); YEpD4-30B, MA10a ura3 asp5 his3 i5v leu2 GAL2 (Johnson et al., 1987); DJMD22-3B MA10a cdc42-1 his4 leu2 trpl GAL2 (constructed by crossing DJMD4-30B to DJTD2-16D); and, DJDI, MA10a/MATA cdc42-1/cdc42-1 urn3 his4 leu2 trpl GAL2/gal2 (constructed by mating DJMD22-3B to DJTD2-16D).

Plasmids pBR322, YEp24, YRp7, YIp5, and YEp51 have been described elsewhere (Maniatis et al., 1982; Botstein et al., 1979; Bouch et al., 1983). The yeast-E. coli shuttle plasmid YEp03 contains the URA3 selectable yeast marker and the 2-µ plasmid origin of replication (Lilley, S., and J. R. Pringle, unpublished results). The yeast genomic DNA library in plasmid YEp24 (provided by D. Botstein, Genentech, South San Francisco, CA) contains fragments produced by partial Sau 3A digestion of DNA from S. cerevisiae strain DBY939 (Carlson and Botstein, 1982).

DNA and RNA Manipulations

Standard procedures were used for recombinant DNA manipulations (Maniatis et al., 1982), E. coli and yeast transformations (Maniatis et al., 1982; Lilley and Pringle, 1980; Lilley, S., and J. R. Pringle, unpublished results), and the 2-µ plasmid origin of replication (Lilley, S., and J. R. Pringle, unpublished results). The yeast genomic DNA library in plasmid YEp24 (provided by D. Botstein, Genentech, South San Francisco, CA) contains fragments produced by partial Sau 3A digestion of DNA from S. cerevisiae strain DBY939 (Carlson and Botstein, 1982).

Computer Programs

DNA sequences were analyzed on an IBM-compatible computer using the Pustell sequence analysis programs (International Biotechnologies, Inc.).

Figure 1. (A) Restriction maps of the CDC42 region and of the inserts of plasmids discussed in the text. YeEp(42)1 and YeEp(42)2 were primary isolates from the YeEp24 library. pBR(42)1 was constructed by inserting the 3.7-kb Bam HI-Sal I fragment from YeEp(42)1 into Bam HI/Sal I-digested pBR322. YeRp(42)1 was constructed by inserting the 1.1-kb TRP1/ARS1 Eco RI fragment from YeEp(42)1 into the Eco RI site of pBR322. YeRp(42)2 was constructed by deleting a 3.3-kb Ava I fragment from pBR(42)1 (using a site in the vector) and then inserting the TRP1/ARS1 fragment as just described. pBR(42)3 was constructed from pBR(42)1 by deleting DNA to the left of the ScA site and to the right of the Nde I site using restriction sites within the vector. YeRp(42)3 was constructed by inserting the TRP1/ARS1 fragment into pBR24 as just described. The cdc42-complementing activity of each plasmid capable of replicating in yeast was determined by streaking plasmid-containing DJTD2-16D cells onto YEPlD plates at 36°C; + indicates essentially uniform growth at 36°C, − indicates no growth at 36°C. Restriction sites are indicated: A, Ava I, B, Bam HI, E, Eco RI, H, Hind III, P, Pvu II, V, XhoI, S, Sal I, Sc, Sca I, Sp, Spe I, X, Xba I, Xh, Xho I. All sites are shown for each enzyme. (B) Expanded maps of the cdc42-complementing Sca I-Nde I region and of the inserts of additional plasmids. Restriction sites are indicated as in A except that some but not all Rsa I (R) and Hind I (Hf) sites are also shown. YeEp(42)3 was constructed by inserting the 1.1-kb Hpa I-Xba I fragment from pBR(42)1 into Sma I/Nhe I-digested YeEp24. YeEp(42)4 was constructed by inserting the 0.5-kb Rsa I fragment from pBR(42)3 into Pvu II-digested YeEp24. YeEp(42)5 was constructed by inserting the 0.6-kb Hind I fragment from pBR(42)3 into Pvu II-digested YeEp24. Plasmids were tested for cdc42-complementing activity as described in A; +/− indicates that most cells failed to grow at 36°C but that 7%−9% of the colonies grew. Arrows and associated numbers indicate the directions and lengths (in codons) of the ATP-initiated open reading frames revealed by sequencing (see text).
New Haven, CT). Amino acid sequence similarities were determined using the Microgenie™ sequence-analysis programs (Beckman Instruments, Inc., Fullerton, CA).

**Visualization of Chitin Rings**

Plasmid-containing yeast cells were grown under conditions selective for the plasmid, with 3% glucose or 2% galactose as the sole carbon source. Chitin rings were visualized by fluorescence microscopy after staining cells in 0.1% Calcofluor for 3 min and washing in distilled water (Sloat and Pringle, 1978).

**Results**

**Isolation and Identification of CDC42**

Plasmids that complemented the temperature-sensitive cdc42-1 mutation in strain DJTD2-16D were isolated from a yeast genomic-DNA library in the URA3-containing plasmid YEp24. 24 primary Ura* Ts* transformants were obtained. From each transformant, a plasmid was recovered into E. coli that could retransform DJTD2-16D to Ura* Ts*. The Ura* and Ts* phenotypes of these transformants cosegregated after growth on nonselective media (data not shown), indicating that the complementation of cdc42-1 was due to the autonomously replicating recombinant plasmids. Restriction enzyme analyses and DNA-DNA blot hybridization experiments (data not shown) indicated that all 24 plasmids contained overlapping regions of DNA. Several representative plasmids that were examined in more detail shared a common 2.7-kb region of DNA (Fig. 1 A). DNA-DNA blot hybridization experiments using total yeast DNA and a probe derived from one of these plasmids revealed only the fragments expected if the cloned DNA was derived without rearrangement from contiguous chromosomal DNA that was single copy in the haploid genome (Fig. 2 A, lanes 1-5). The observation that all 24 complementing plasmids contained overlapping DNA inserts suggested that the CDC42

Figure 2. (A and B) DNA–DNA blot hybridization analyses of chromosomal DNA from parental and transformed strains. After digestion with the indicated restriction enzymes, DNA fragments were separated and hybridized to radioactively labeled pBR(42) as described in Materials and Methods. The sizes of the fragments visualized are indicated in kilobase pairs. (A) Total DNA from strain TD4 (lanes 1-5) and from the same strain after integration of a plasmid containing cdc42-complementing sequences and the URA3 gene (see text; lane 6) was digested with Eco RI (lane 1), Eco RI + Xba I (lane 2), Eco RI + Pvu I (lane 3), Eco RI + Xho I (lane 4), or Eco RI + Bam HI (lanes 5 and 6). As the integrated vector sequence contains a single Bam HI site, the replacement of the original 4.3-kb Eco RI fragment (lane 5) by two new Eco RI/Bam HI fragments in the transformant (lane 6) indicates that the integration had occurred at the chromosomal site homologous to the cdc42-complementing DNA. (B) Total DNA from strain DJID7-1 (lanes 1 and 2) and from the same strain after integration of a fragment in which cdc42-complementing DNA had been replaced by URA3 (see text; lanes 3 and 4) was digested with Eco RI (lanes 1 and 2) or Eco RI + Xba I (lanes 3 and 4). The URA3 fragment used contained an Eco RI site immediately adjacent to the Spe I site used in the cloning. Thus, integration of the hybrid fragment at the chromosomal site homologous to the cdc42-complementing DNA would result in the loss of the chromosomal Xba I site but the addition of a new Eco RI site at nearly the same location. Therefore, digestion of DNA from the transformant with Eco RI should yield two new fragments of about the same sizes as those generated by an Eco RI + Xba I digestion of the parental DNA, together with the original 4.3-kb Eco RI fragment (from the chromosome not involved in the integration event). Digestion of DNA from the transformant with Eco RI + Xba I should yield doublet bands at the positions of the two new bands in the Eco RI digest. The results shown conform to these predictions. (C) Analyses of mRNA transcripts encoded by the cdc42-complementing region. Poly(A)+-RNA from strain C276 (20 μg/lane in lanes 1 and 2; 10 μg/lane in lanes 3 and 4) was separated and hybridized to radioactive probes as described in Materials and Methods. The probe for lanes 1 and 2 was pBR(42); autoradiography was for 9.5 (lane 1) and 140 h (lane 2). The probes for lanes 3 and 4 were single-strand DNAs prepared by primer extension in the presence of 32P-dATP on templates of M13mpl8 (lane 3) and M13mpl9 (lane 4) into which the cdc42-complementing Sca I-Xba I fragment had been cloned using the Sma I and Xba I sites of the vectors.
the plasmid integrated at the chromosomal site homologous to pBR(42)l was inserted into the Sal I site of the DJID7-1C. 80 of 84 four-spore tetrads were parental di-

These transformants were crossed to the cdc42-complementing DNA (Fig. 2A, lanes 5 and 6). These transformants were crossed to the cdc42-complementing strain DJMD2-7C. 80 of 84 four-spore tetrads were parental di-

gene itself, rather than a plasmid borne suppressor, had been cloned. This hypothesis was supported by the observations that cdc42 maps to chromosome XII and that the cloned DNA hybridized to the chromosome XII band after or-

clone. This hypothesis was supported by the observations that the integrated URA3 gene and a

gate between the integrated URA3 gene and a

of the constitutive expression of certain promoters (Struhl, 1986). These data sequence are available from EMBL/Gen-

Bank/DDBJ under accession number X51906.

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Figure 3. Nucleotide sequence of the cdc42 region and predicted amino acid sequence of the cdc42 product. The cdc42 open reading frame was identified as described in the text. The nucleotide sequence is numbered relative to the A of the putative initia-
termination codon. The Xho I, Hpa I, and Pvu I sites (underlined) are located at positions -525 to -520, -67 to -62, and +192 to +197, respectively. The TTGLeu codon altered by site directed mutagenesis is boxed. Multiple inframe stop codons (.) are present within the 40-bp 5' to the putative initiator ATG and within the 30-bp 3' to the putative termi-

nation codon. Possible TATA promoter sequences (broken underlines) are present at positions -112 to -107 and -96 to -91. In addition, a 17-bp stretch of poly(AD-dT) is present at position -510 to -494. Similar stretches of poly(A-

dT) have been implicated in the constitutive expression of certain promoters (Struhl, 1986). These data sequence are available from EMBL/Gen-

Bank/DDBJ under accession number X51906.
Ura⁻ Ts⁻ : 2 dead, showing that the insertion of URA3 had produced a lethal mutation that was at or near the CDC42 locus. Microscopic examination of the dead spore clones showed that they had undergone several rounds of cell division before arresting as large, unbudded cells (like the cdc42-1 ts mutant). Moreover, the Ts⁻ phenotype of the original diploid cells transformed with plasmid YEp(42)3 yielded cdc42-1 ts mutant. However, two control plasmids that had been generated using the same procedure, but without the mutation and inserted into Sal I/Hind III- or Sca I-Xba I sites in M13mpl9, were completely sequenced using this strategy (Fig. 1 B) and no TACTAAC consensus splicing sequence (Lanford et al., 1984), suggesting an absence of introns. The various data presented above suggested strongly that the 191 codon ORF was the CDC42 gene, but did not completely eliminate the possibility that the 100 codon ORF on the other strand was responsible for CDC42 activity. To settle this point, we used site-directed mutagenesis to change the TTG Lue codon at nucleotides 208-210 of the 191 codon ORF (see Fig. 3) to a TAG nonsense codon (Fig. 4). The corresponding change in the 100 codon ORF was from ACA™ to ACT™ at codon 2; as both of these threonine codons are used frequently (Bennetzen and Hall, 1982), this change presumably would not affect the expression of the hypothetical 100 amino acid gene product. DNA sequence analyses showed the absence of any other nucleotide changes within the 191 codon or 100 codon ORFs. After mutagenesis and sequence analysis, the Xho I-Hind III fragment containing the CDC42 region (see Fig. 1; the Hind III site is next to the Xba I site in M13mpl9) was isolated from phage with and without the mutation and inserted into Sal I/Hind III-digested plasmid YEp103. The resulting plasmids that contained the mutation were unable to complement the cdc42-1 mutation in strain DJTD2-16D. In contrast, two control plasmids that had been generated using the same procedure, but

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Figure 4. DNA sequence of the region altered by site-directed mutagenesis. Mutagenesis was performed as described in Materials and Methods using M13mpl9 containing the 1.8-kb Sca I-Xba I fragment (see text) as template. The column of letters is the wild-type CDC42 sequence; the T to A transversion produced by mutagenesis is also indicated. Sequence 1 is a control template that was not altered by the mutagenesis; note that the uppermost two bands in the G lane are "ghost bands" that were not present in other sequencing runs on control templates. Sequence 2 is of a template containing the mutation. The dideoxy nucleotide used in each sequencing reaction is indicated above the corresponding lane.

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Analysis of mRNA Transcripts

RNA-DNA blot hybridization using a probe spanning the cdc42-complementing region revealed only one major transcript (Fig. 2 C, lane 1) ~1 kb long (as judged by its comigration with the URA3 transcript; data not shown). A 14-fold longer exposure of the autoradiogram revealed a faint band corresponding to a transcript of ~3 kb, but did not reveal any additional transcripts of lower molecular weight (Fig. 2 C, lane 2; see Discussion). Hybridization of the ~1-kb transcript to one of two single-strand probes (Fig. 2 C, lanes 3 and 4) indicated that it is transcribed from left to right as shown in Fig. 1 B.

Analysis of CDC42 Nucleotide and Predicted Amino Acid Sequences

The 1.8-kb Sca I-Xba I fragment from pBR(42)1 was inserted into M13mpl8 and M13mpl9 that had been digested with Sma I and Xba I. The same gene, but did not reveal any additional transcripts of lower molecular weight (Fig. 2 C, lane 2; see Discussion). Hybridization of the ~1-kb transcript to one of two single-strand probes (Fig. 2 C, lanes 3 and 4) indicated that it is transcribed from left to right as shown in Fig. 1 B.

1. Abbreviation used in this paper: ORF, open reading frame.
The human rho nucleotide-binding site, are replaced by Thr and Gln in amino acids with molecular weight of 21,356 and a net charge of +2 (Fig. 3). Comparison of the amino acid sequence to the human H-ras product and members of the ras and rho families of gene products (Fig. 5). For example, the one letter amino acid code is used. Numbering corresponds to the human H-ras amino acid sequence. Dashes and asterisks indicate amino acids identical and related, respectively, to those of the CDC42 gene product. (Amino acids considered related were aspartate and glutamate; isoleucine, leucine, valine, and alanine; lysine and arginine; asparagine and glutamine; and serine and threonine.) Gaps were introduced by eye to maximize the amino acid similarities. Overlines indicate the regions for which peptide sequence data are available for the mammalian protein G25K (see text). Note that the amino-terminal sequence of the human rho protein is missing because the available sequence was derived from an incomplete cDNA.

Figure 5. Similarities between the predicted amino acid sequences of the CDC42 gene product, the S. cerevisiae RHO1 and RHO2 gene products (Madaule et al., 1987), the human rho gene product (Madaule and Axel, 1985), and the human H-ras gene product (Capon et al., 1983). The one letter amino acid code is used. Numbering corresponds to the human H-ras amino acid sequence. Dashes and asterisks indicate amino acids identical and related, respectively, to those of the CDC42 gene product. (Amino acids considered related were aspartate and glutamate; isoleucine, leucine, valine, and alanine; lysine and arginine; asparagine and glutamine; and serine and threonine.) Gaps were introduced by eye to maximize the amino acid similarities. Overlines indicate the regions for which peptide sequence data are available for the mammalian protein G25K (see text). Note that the amino-terminal sequence of the human rho protein is missing because the available sequence was derived from an incomplete cDNA.

did not contain the mutation, retained cdc42-complementing activity. Thus, it seems clear that the 191 codon ORF encodes the CDC42 gene product.

The predicted CDC42 product is a polypeptide of 191 amino acids with molecular weight of 21,356 and a net charge of +2 (Fig. 3). Comparison of the amino acid sequence with the National Biomedical Research Foundation protein database revealed a high degree of similarity between the CDC42 product and members of the ras and rho families of gene products (Fig. 5). For example, the CDC42 product is 30% identical (40% identical or related) in predicted amino acid sequence to the human H-ras product and comparably similar to the yeast RAS1 and RAS2 gene products. Much of this similarity is in the regions of the ras proteins that have been implicated in GTP binding and hydrolysis (residues 5-21, 28, 57-64, 114-120, and 143-148; Dever et al., 1987; de Vos et al., 1988) and the COOH-terminal modifications leading to membrane association (residues 186-189; Clarke et al., 1988; Hancock et al., 1989; Schafer et al., 1989). Interestingly, however, the highly conserved Asn and Lys residues at positions 116 and 117 of ras, which have been implicated in the guanine specificity of the nucleotide-binding site, are replaced by Thr and Gin in CDC42 (see also below). The similarities of CDC42 to the rho proteins are more extensive and more widely distributed through the coding regions. In particular, the CDC42 product is ~53% identical (~58% identical or related) to the human and Aplysia rho gene products, 52% identical (61% identical or related) to the S. cerevisiae RHO1 gene product, and 37% identical (51% identical or related) to the S. cerevisiae RHO2 gene product, which is itself 53% identical (66% identical or related) to the S. cerevisiae RHO1 gene product. Although the available data are still limited, it appears that the closest homologue to CDC42 may be "G25K," a 25-kD GTP-binding protein that has been purified from several mammalian tissues (Evans et al., 1986; Waldo et al., 1987; Polakis et al., 1989). Of the four peptides for which sequence data are available, three are very similar to regions in the mammalian protein G25K (see text). Note that the amino-terminal sequence of the human rho protein is missing because the available sequence was derived from an incomplete cDNA.
Expression of CDC42 under GAL10-promoter Control

Inspection of the CDC42 sequence revealed that the Hpa I site is at position -67 to -62 relative to the putative initiator ATG codon (Fig. 3). This suggested that the lack of complementation and unusual marker-rescue results obtained with the Hpa I-Xba I fragment in plasmid YEp(42)3 (see above) might have reflected the presence of only an incomplete promoter in this fragment, a possibility consistent with the observation of potential TATA promoter sequences at positions -112 to -107 and -96 to -91 (see Fig. 3). To explore this possibility, the Hpa I-Xba I fragment from plasmid pBR(42)l was inserted into the Sal I site of plasmid YEpl51 so that the 191 codon ORF should be under the control of the yeast galactose-inducible GAL10 promoter in the resulting shuttle plasmid. This plasmid, YEpl51(42), complemented the cdc42-1 mutation in strain DJMD22-3B when cells were grown on either 2% glucose or 2% galactose as the sole carbon source, as judged by the cells' ability to grow at a normal rate at 36°C. These results supported our interpretation of the results obtained with plasmid YEp(42)3 (see Discussion). However, even at 23°C, YEpl51(42)-containing cells growing on either carbon source displayed abnormalities in their budding patterns, as revealed by the staining of bud scars with Calcofluor. In contrast to the normal unipolar budding pattern of haploid cells and bipolar budding pattern of diploid cells (Fig. 6A and C; Sloat et al., 1981), we observed apparently random budding patterns in ~75% of YEpl51(42)-containing DJMD22-3B and DJDI cells grown under derepressing conditions (2% galactose) and ~35% of YEpl51(42)-containing DJMD22-3B cells grown under repressing conditions (2% glucose) (Fig. 6, B and D). (Note that only cells with two or more bud scars could be included in these counts; 100 such cells were counted in each case.) Only ~10% of the haploid or diploid cells containing YEpl51 itself exhibited such budding patterns when growing on either galactose or glucose. However, similar abnormalities of budding pattern were also observed in ~50% of DJTD2-16D cells containing the CDC42 gene on a 3.7-kb Bam H1-Sal I fragment (Fig. 1A) inserted into the high-copy number plasmid YEpl03.

Discussion

The CDC42 gene product is involved in the morphogenetic steps of the yeast cell cycle. To begin exploring CDC42 function at the molecular level, we have isolated and sequenced this gene. All cdc42-complementing clones that we analyzed...
contained overlapping DNA segments, suggesting that the bona fide CDC42 gene had been isolated. This suggestion was confirmed by the findings that the isolated DNA segment could direct integration to the CDC42 locus and that deletion of this segment yielded a lethal mutation that failed to complement the cdc42-1\textsuperscript{a} mutation. Subcloning localized CDC42 to a 1.6-kb segment of DNA, the sequencing of which revealed several ORFs. The longest of these was identified as CDC42 by several lines of evidence, including the results of transposon and site-directed mutagenesis, the demonstration that a plasmid in which this ORF should be expressed under GAL10 control could complement a cdc42-1\textsuperscript{a} mutation, and the demonstration that the putative CDC42 mRNA was transcribed from the strand appropriate for this ORF. This mRNA was somewhat longer than expected from the size of the ORF, and it is conceivable that the actual CDC42 transcript is a lower abundance species that we did not detect. However, it seems more likely that the CDC42 transcript has unusually extensive untranslated regions at the 5' end, the 3' end, or both. Further work will be necessary to resolve these issues.

We obtained rather confusing results when we attempted to use recombination-mediated marker rescue (Patterson et al., 1986) to confirm that the bona fide CDC42 gene had been isolated and to localize the cdc42-1\textsuperscript{a} mutation. Plasmid YEp(42)3 (Fig. 1 B) failed to complement the cdc42-1\textsuperscript{a} mutation but yielded Ts\textsuperscript{+} recombinant subclones at the surprisingly high frequency of \(~10\times\). As the insert in YEp(42)3 spans the entire CDC42 ORF, the failure of complementation presumably results from the absence of an adequate promoter in the 64 bp of sequence 5' to the initiator ATG in this clone. This hypothesis is supported by the observation that the same fragment could complement the cdc42-1\textsuperscript{a} mutation when linked to the GAL10 promoter. Interestingly, this complementation was observed both during growth on galactose and during growth on glucose, which should repress transcription from the GAL10 promoter. Perhaps only a small amount of CDC42 mRNA is required and repression of the particular promoter construction used here (the GAL10 promoter plus 64 bp of CDC42 upstream sequence) is incomplete during growth on glucose. In any event, similar effects have been observed by others (Broach, J., personal communication).

The Ts\textsuperscript{+} subclones observed with YEp(42)3 apparently resulted from integration of the entire plasmid. It is clear that such integration could generate a complete, normal copy of the gene, but it is unclear why the event occurred in such high frequency. Perhaps the DNA segment contains a hot spot for recombination, or the production of some normal CDC42 product (from a partially functional promoter in YEp(42)3) allows the cells to survive long enough at restrictive temperature that the chances of rescue by plasmid integration are increased. Alternatively, there could be selection for such integrants even during growth at the nominally “permissive” temperature (see Adams et al., 1990).

In contrast, neither plasmid YEp(42)4 nor plasmid YEp(42)5 (Fig. 1 B) yielded Ts\textsuperscript{+} recombinant transformants at a frequency detectably above control values, although the inserts of these plasmids, taken together, span the entire CDC42 coding region. A possible explanation for this result is that the cdc42-1 allele actually contains two mutations, such that neither plasmid's insert contains the wild-type information corresponding to both mutant sites. This hypothesis is consistent with the observation that only the one cdc42\textsuperscript{a} mutant was isolated in a search that yielded multiple independent mutants for each of the related genes CDC24 and CDC43 (Adams et al., 1990). Sequencing of the cdc42-1 allele should resolve this point.

Our results have provided some additional information about CDC42 function. First, the finding that overexpression of CDC42 can lead to mislocalization of budding sites suggests that the CDC42 product is involved in the initial selection of the budding site as well as in the subsequent emergence of the bud. Interestingly, the same inference has been made about the CDC24 product on the basis of mutants that display a similar mislocalization of budding sites (Sloat et al., 1981). In addition, deletion of RSRI (another ras-related gene that seems to be involved in bud emergence) produces a similar mislocalization of budding sites (Bender and Pringle, 1989), and overproduction of ABP (actin-binding protein) 85 leads to a somewhat different abnormal pattern of budding (Drubin et al., 1988). It seems possible that these proteins are involved in marking the site at which bud emergence should occur, and that mislocalization of an overproduced or abnormal protein can thus perturb the normal budding pattern. However, it also remains possible that the mislocalization of budding sites in these situations simply reflects a less specific cellular pathology (Hayashibe, 1975; Thompson and Wheals, 1980).

Second, the finding that the predicted CDC42 protein has substantial sequence similarity to the ras and rho families of proteins provides important, though limited, clues to its function at the molecular level. Much of this similarity is in the regions that have been implicated in the binding and hydrolysis of GTP. In this regard, it is relevant that the Aplysia rho gene product has been shown to bind and hydrolyze GTP after expression in E. coli (Anderson and Lacal, 1987), and that the G25K protein (which shares with CDC42 an unusual feature in the putative GTP-binding site: see Results) binds guanine nucleotides avidly (Evans et al., 1986; Waldo et al., 1987; Polakis et al., 1988). If the CDC42 product indeed also has GTP binding and hydrolysis activities (a point to be tested as soon as CDC42-specific antibodies are available), it is likely also to be involved in signal transduction of some sort. However, it is important to note that this biochemical motif has apparently been adapted to a wide variety of purposes in yeast. Confirmed or suspected GTP-binding/hydrolyzing proteins identified to date include the RAS1 and RAS2 products, implicated in the control of adenylate cyclase and hence (via the cAMP-dependent protein kinase) in the overall coordination of cell growth and macromolecular synthesis (Powers et al., 1984; Tatchell, 1986); the GST\textsuperscript{p} product, which may be involved in the Gl-to-S phase transition (Kikuuchi et al., 1988); the YPT1 product (Gallwitz et al., 1983), which appears to be localized to the Golgi apparatus (Segev et al., 1988) and is implicated in the control of secretion and Ca\textsuperscript{2+} flux (Segev and Botstein, 1987; Wagner et al., 1987); the SEC4 product, which is involved in post-Golgi apparatus events of the secretory pathway (Salminen and Novick, 1987; Goud et al., 1988); the SCG1/GPA1 product, which appears to be involved in the pheromone response pathway (Dietzel and Kurjan, 1987; Miyajima et al., 1987); the GPA2 product, which may be involved in regulating cAMP levels (Nakafuku et al., 1988); the CIN4 product, which appears to be involved in ensuring normal chromosome transmission dur-
ing mitosis (Stearns, T., personal communication); the ARF1 and ARF2 gene products, which are similar to mammalian ADP-ribosylation factor (Sewell and Kahn, 1988); and the RHO1 and RHO2 products, whose function(s) are unknown but apparently distinct from those of the RAS1 and RAS2 products (Madaule et al., 1987). There is no reason to think that this list is complete.

Thus, the critical step in elucidating CDC42 function is likely to be determining what signals are transduced, and by means of what downstream effectors. On these points we have as yet few clues. The extensive sequence similarities between the CDC42 protein and the Rho gene products of yeast and animals suggests that there may be common aspects of function beyond GTP binding and hydrolysis. However, this clue is not too helpful until more is known of the function of the other Rho gene products. The similarity ofcdc42 mutants tocdc42 mutants and the evidence that the CDC42 product is a Ca2+-interactive protein (Ohya et al., 1986a,b; Miyamoto et al., 1987) suggest that signal transduction by the CDC42 protein may also involve Ca2+, but there are no clues yet as to the nature of such possible involvement. Finally, the observation that the CDC42 protein has COOH-terminal sequences similar to those at which ras and related proteins become modified by hydrophobic prostatic groups (Clarke et al., 1988; Goodman et al., 1988; Molenaar et al., 1988; Hancock et al., 1989; Schafer et al., 1989) suggests that the CDC42 product also may be modified as a mechanism for attachment to the cell membrane. This is an attractive notion if, as suggested above, one function of the CDC42 product is to help mark the site on the cell surface at which the new bud should emerge.

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Note Added in Proof. Since submission of this paper, we have learned that since submission of this paper, we have learned that the predicted product of the human cdc42 gene (Didsbury, J., R. F. Weber, M. G. Bokoch, T. Evans, and G. B. Kohlhaw. 1986. Three-dimensional structure of an oncogene protein: catalytic domain of human c-h-ras p21. Science (Wash. DC). 239:888-893.

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