

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 involved 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 Ca²⁺-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* product 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).

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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 2% 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 ob-

tained. 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*

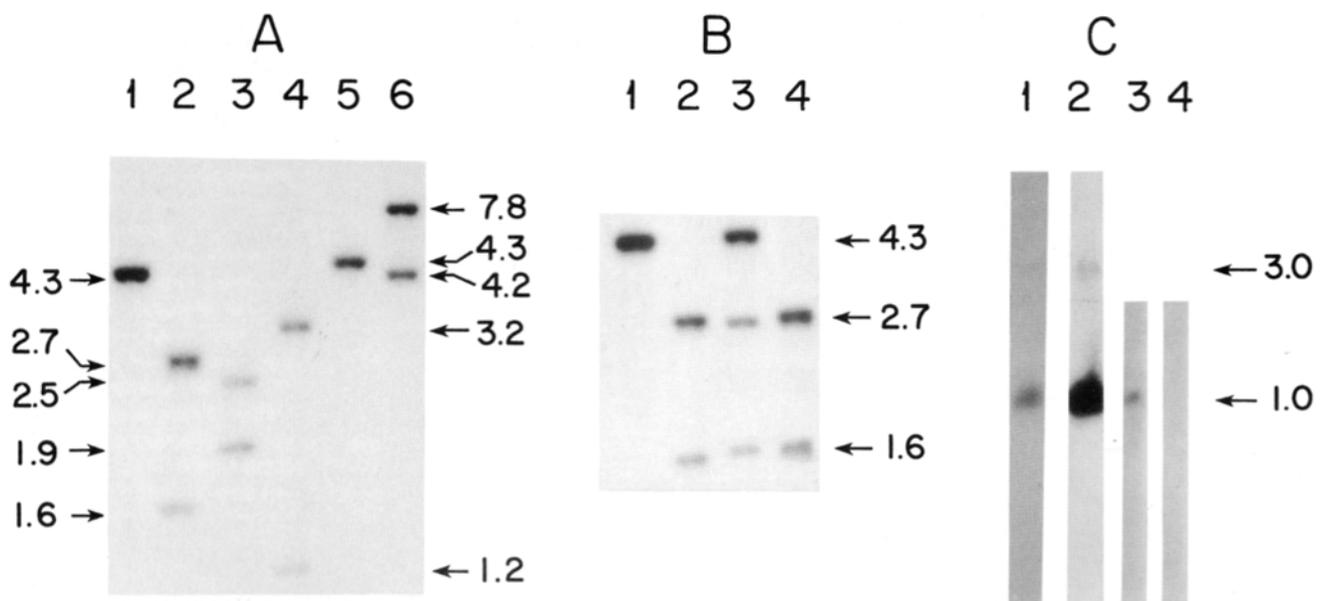


Figure 2. (A and B) DNA-DNA blot hybridization analyses of chromosomal DNA from parental and transformed strains. After digestion with the indicated restriction enzymes, DNA fragments were separated and hybridized to radioactively labeled pBR(42)1 as described in Materials and Methods. The sizes of the 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 3) or Eco RI + Xba I (lanes 2 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)3; 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 M13mp18 (lane 3) and M13mp19 (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.

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                                GCC GAACTCAA AGGTAATTT CGTAAAAAC -701
AATCATCTAC GGTATAAATA ACAATTTAAT TTACGTCTCT TTGAAAATG CTGCTGTAGC TCAGTGGTTA -631
GAGCTTCGTG CTTATAGCAA CATTGGGTTT GCGAAGTTT TGTGCCAAAG ACCTTTCAA CAGGCCTTTA -561
AAAGCAACGC GACCCGTCGT GGGTCAATC CCCACCTCGA GCACTTTCTC TTTTTTTTTT AAAAAAAGTT -491
GCATTATTC TATCAACTGT TGTAGCTACG TTACACCTAA TTTTGTATTG TAGACTGTTA GATAATCCAC -421
ATATACAGGT AAATACGGCA CATTGTCTTC TAAACATGGC ATTAAGATG TCTTCCACCG TCGATTCAAG -351
GGTCATTCAA TACGGGTAAT ACTGCTTCCA TTGGGCCCTT CCAAATGAAA CAAGATACCG GAAAAAGTAA -281
AGTGATAAAA AAAAGCAAAC AAGGTTGATT GATCGATGAT AGACGCGATT CTTGGAAGG GCAAGAGCAG -211
TGTTTTGATA AATAGGTTCC AGGTGTACCG AGATATACTG ATTATCCTTC TCTGTCATTC TTCACTTTTT -141
GCAATAGGTT TCCTTTGTCG CAAAAAATA TAATTCGTTAT TTATTATACT ATTCTATTTT CCGTGGGAGA -71

TAGGTTAACA AACGAATTAG AGAAGCAAAA CTCATAAAAC AAGAAATAAA CGTATTAGGT GTTCCACAAA -1
ATG CAA ACG CTA AAG TGT GTT GTT GTC GGT GAT GGT GCT GTT GGG AAA ACG TGC CTT CTA 60
Met Gln Thr Leu Lys Cys Val Val Val Gly Asp Gly Ala Val Gly Lys Thr Cys Leu Leu
ATC TCC TAT ACA ACG AAT CAA TTT CCA GCC GAC TAT GTT CCA ACA GTG TTC GAT AAC TAT 120
Ile Ser Tyr Thr Thr Asn Gln Phe Pro Ala Asp Tyr Val Pro Thr Val Phe Asp Asn Tyr
GCG GTG ACT GTG ATG ATT GGT GAT GAA CCA TAT ACG TTA GGT TTG TTT GAT ACG GCC GGT 180
Ala Val Thr Val Met Ile Gly Asp Glu Pro Tyr Thr Leu Gly Leu Phe Asp Thr Ala Gly
CAA GAA GAT TAC GAT CGA TTG AGA CCC TTG TCA TAT CCT TCT ACT GAT GTA TTT TTG GTT 240
Gln Glu Asp Tyr Asp Arg Leu Arg Pro Leu Ser Tyr Pro Ser Thr Asp Val Phe Leu Val
TGT TTC AGT GTT ATT TCC CCA CGC TGT TTT GAA AAC GTT AAA GAA AAA TGG TTC CCT GAA 300
Cys Phe Ser Val Ile Ser Pro Pro Ser Phe Glu Asn Val Lys Glu Lys Trp Phe Pro Glu
GTA CAT CAC CAT TGT CCA GGT GTA CCA TGC CTG GTC GTC GGT ACG CAG ATT GAT CTA AGG 360
Val His His His Cys Pro Gly Val Pro Cys Leu Val Val Gly Thr Gln Ile Asp Leu Arg
GAT GAC AAG GTA ATC ATC GAG AAG TTG GAA AGA CAA AGA TTA CGT CCG ATT ACA TCA GAA 420
Asp Asp Lys Val Ile Ile Glu Lys Leu Gln Arg Gln Arg Leu Arg Pro Ile Thr Ser Glu
CAA GGT TCC AGG TTA GCA AGA GAA CTG AAA GCA GTA AAA TAT GTC GAG TGT TCG GCA CTA 480
Gln Gly Ser Arg Leu Ala Arg Glu Leu Lys Ala Val Lys Tyr Val Glu Cys Ser Ala Leu
ACA CAA CGC GGT TTG AAG AAT GTA TTC GAT GAA GCT ATC GTG GCC GCC TTG GAG CCT CCT 540
Thr Gln Arg Gly Leu Lys Asn Val Phe Asp Glu Ala Ile Val Ala Ala Leu Glu Pro Pro
GTT ATC AAG AAA AGT AAA AAA TGT ACA ATT TTG TAG 576
Val Ile Lys Lys Ser Lys Lys Cys Thr Ile Leu End
TCATATTAGT ATATGCCAT CTTTCTTAA TCTATATCTA AAATTAACCT ATATATACAC CTTCTATCC 646
TTTTATTATC GTTGTATTTC ATTTTTCGGC TTTGCTCAGA GTTGATCTC TTGTTTCTT GCGCCAGGAT 716
CATCCGCTCG TTCAAGCGCG AGTAGTGCAA AAAAGTCGAG CCGTCAAGCC CCAAACGTTT AATACTTCCA 786
TGTTCTTCAA CGCTAAACGC GACATTTGCG AGTCCTACCA CGTCTTCTCC GTTACGGGGC AAAAAACCT 856
CTTAACATCA AAAATTTGCG CTTTCTTGC TTTTCTTGA AATATATTCC TTGCATAGAC ACGGTATGCA 926
TATGAAACAT

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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 initiator 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 TTG^{Leu} 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 termination 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(dA-dT) is present at position -510 to -494. Similar stretches of poly(dA-dT) have been implicated in the constitutive expression of certain promoters (Struhl, 1986). These sequence data are available from EMBL/GenBank/DBJ under accession number X51906.

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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 orthogonal field alternation gel electrophoresis (Johnson et al., 1987). To test further the identity of the cloned DNA, we integrated a plasmid containing *cdc42*-complementing sequences and the yeast selectable marker *URA3* into a *CDC42*⁺ yeast strain and then determined the meiotic linkage between the integrated *URA3* gene and a *cdc42* mutation. The 2.5-kb Xho I-Sal I fragment from plasmid pBR(42)1 was inserted into the Sal I site of the *URA3*-containing plasmid YIp5 (which cannot replicate autonomously in yeast). The resulting plasmid was linearized within the insert at the unique Xba I site and transformed into strain TD4, selecting for Ura⁺. Two stable Ura⁺ transformants were shown by DNA-DNA blot hybridization to have the plasmid integrated at the chromosomal site homologous to the *cdc42*-complementing DNA (Fig. 2 A, lanes 5 and 6). These transformants were crossed to the *cdc42-1* strain DJMD2-7C. 80 of 84 four-spore tetrads were parental di-

types (2 Ura⁺ Ts⁺: 2 Ura⁻ Ts⁻); the remaining four tetrads segregated 3 Ts⁺: 1 Ts⁻. Thus, integration had indeed occurred at the *CDC42* locus.

The recessive, temperature-sensitive lethal phenotype of the *cdc42-1* mutation suggests that the *CDC42* gene product is essential for vegetative growth. To test this conclusion and complete the identification of the cloned DNA, we generated a deletion mutation by replacing the *cdc42*-complementing region with the *URA3* gene. The DNA between the unique Hpa I and Xba I sites in plasmid pBR(42)1 (Fig. 1 A) was replaced with the *URA3*-containing Sma I-Spe I fragment from YE24. A 2.5-kb Xho I-Spe I fragment, which contained the *URA3* gene flanked on both sides by DNA from the *cdc42*-complementing region, was excised from the resulting plasmid and used to transform the *CDC42/cdc42* heterozygous diploid strain DJID7-1 to Ura⁺. Two stable Ura⁺ Ts⁻ transformants were shown by DNA-DNA blot hybridization to have sustained fragment replacement by the transforming sequences at the site homologous to the *cdc42*-complementing DNA (Fig. 2 B). Dissection of 22 tetrads from these transformants yielded exclusively a segregation of 2 live,

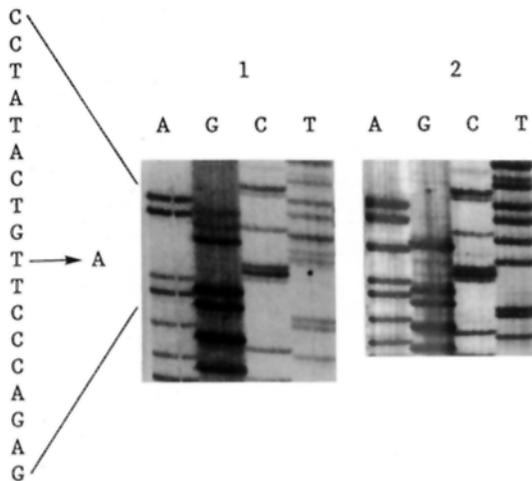


Figure 4. DNA sequence of the region altered by site-directed mutagenesis. Mutagenesis was performed as described in Materials and Methods using M13mpl9 containing the 1.8-kb Sca I-Xba I fragment (see text) as template. The column of letters is the wild-type *CDC42* sequence; the T to A transversion produced by mutagenesis is also indicated. Sequence 1 is of 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.

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^s* mutant). Moreover, the Ts⁻ phenotype of the original diploid transformants showed that the new lethal mutation failed to complement the *cdc42-1^s* mutation, providing further evidence that the bona fide *CDC42* gene had been cloned.

Localization of *CDC42* on the Complementing DNA

To localize the *CDC42* gene, subclones were constructed as described in Fig. 1 A. Complementation of the *cdc42-1* mutation by plasmids YRp(42)1 and YRp(42)3, but not by YRp(42)2, indicated that *CDC42* lies within the 1.6-kb Sca I-Nde I region. Subclones derived from this region all failed to complement the *cdc42-1* mutation (Fig. 1 B). However, *cdc42-1* cells transformed with plasmid YEp(42)3 yielded Ts⁺ subclones at a frequency of $\sim 10^{-1}$ when plated at 36°C. These subclones were stably Ts⁺ and Ura⁺ when grown under nonselective conditions, suggesting that integration of the plasmid had generated a complete, Ts⁺ copy of *CDC42* from the complete, but mutant, copy originally in the chromosome and an incomplete, but otherwise wild-type, copy in the plasmid. Although the frequency of integration seemed surprisingly high (see Discussion), this result suggested strongly that the *cdc42-1* mutation lies to the right of the Hpa I site. In contrast, *cdc42-1* cells transformed with either plasmid YEp(42)4 or YEp(42)5 yielded Ts⁺ subclones at frequencies no greater than those observed with the control plasmid YEp24, indicating that recombination-mediated marker rescue could not occur. This result was surprising in view of the data (presented below) suggesting that these two

fragments span the entire *CDC42* coding region (see Discussion).

Localization of *CDC42* was also attempted by determining the distribution of sites at which transposon insertion could inactivate *cdc42*-complementing activity. Transposon-containing plasmids were collected and analyzed as described in Materials and Methods, then tested for *cdc42*-complementing activity in strain DJTD2-16D. The results (Fig. 1 B) suggested that the *CDC42* gene occupied a ~ 0.9 -kb region lying mostly between the Hpa I and Nde I sites.

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. These phages and appropriate deletion derivatives (see Materials and Methods) were then used in dideoxy-sequencing reactions. Both strands of the *cdc42*-complementing region between the Sca I and Nde I sites (Fig. 1 B) were completely sequenced using this strategy (Fig. 3). This analysis revealed the presence of eight ATG-initiated open reading frames (ORFs)¹ of ≥ 40 codons (Fig. 1 B) and no TACTAAC consensus splicing sequence (Langford 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^{Leu} 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^{Thr} to ACT^{Thr} 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

1. Abbreviation used in this paper: ORF, open reading frame.

CDC42		M Q T L K C V V V G D G A V G K T C L L I S Y
RHO1	M S Q Q V G N S I R R - L - * - - - - C - - - - - V F	
RHO2	M S E K A V R R - L - * * - - - C - - - S - - Y V F	
Human rho		
Human H-ras	- T E Y - L - - - - A - G - - - * A - T - Q L	23
CDC42		T I N Q F P A D Y V P T V F D N Y A V T V M I G D E P Y
RHO1	* K G - - - E V - - - - - * - - - * D - E * D G R R V	
RHO2	- L G K - - E Q - H - - - - - * - - - * T D C R * D G I K V	
Human rho	E - - E V - - - - - * - - - * D * E * D G K Q V	
Human H-ras	I Q - H - V D * - D - - * E - S - R K Q - V - D G - T C	51
CDC42		T L G L F D T A G Q E D Y D R L R P L S Y P S T D V F L
RHO1	E - A - W - - - - - - - - - - - - - - - D * N - V -	
RHO2	* - T - W - - - - - - * - - - - - F - - S K A - * I -	
Human rho	E - A - W - - - - - - - - - - - - - - - D - - - I -	
Human H-ras	L - D * L - - - - - - * - S A M - D Q Y M R * G * G - -	79
CDC42		V C F S V I S P P S F E N V K E K W F P E V H H H C P G
RHO1	* - - - * D L - D - L - - - Q - - - I A - - L - F - Q -	
RHO2	* G - A - D N F E - L I - * * T - - A D - * L R Y - - D	
Human rho	M - - - - D - D - L - - * P - - - V - - - K - F - - N	
Human H-ras	C V - A * N N T K - - - D * H Q Y R E Q I K R V K D S D	107
CDC42		V P C L V V G T Q I D L R D D K V I I E K L Q R Q R L
RHO1	- - I * * - - C K * - - - N - P Q T - - Q - R Q E G Q	
RHO2	* - I * * - - L K K - - - Q * A H F K - N * T	
Human rho	- - I * * - - A N K K - - - S - E H * R T E - A - M * Q	
Human H-ras	D - - M * * - - N K C - - A A R T * E S * - A - D -	133
CDC42		R P I T S E Q G S R L A R E L K A V K Y V E C S A L T Q
RHO1	Q - * - - Q E - Q S * - D Q * G - T G - Y - - - K - G	
RHO2	E M * P I - D A K Q * - - A * G - K - M - - - - - G	
Human rho	E - * R * * D - R A M - V R * Q - Y D - * - - - K - X	
Human H-ras	- - - S Y G * P - * - T - - K - R	149
CDC42		R G L K N V F D E A I V A A L E P P V I
RHO1	Y - * * E - - * A - T R - S - M G K S K T N G K A K	
RHO2	E - * D D - - * V - T R T S - L M K	
Human rho	E - * * E - - * T - T R - - - Q K R Y	
Human H-ras	Q - * E D * - Y T * * R E * R Q H K L R K L N P P D	175
CDC42		K̄ K S K K C T I L
RHO1	K N T T E - - K - - - V * -	
RHO2	- E P G A N C - I - -	
Human rho	G S Q N G C I N C - K * -	
Human H-ras	E S G P G C M - C - - V * S	189

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 Gln 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 is available, three are very similar to regions in the *CDC42* product (overlined in Fig. 5): p4 is identical in 19/20 positions to residues 47–66 of *CDC42*; p2 is identical in 10/12 (and similar in 1/12) positions to residues 109–120 of *CDC42*; and p1 is identical in 14/17 (and similar in 1/17) positions to residues 167–183 of *CDC42*. It is worthy of special note that G25K, like *CDC42*, has Thr-Gln instead of Asn-Lys in the putative guanine specificity region (see above and Discussion).

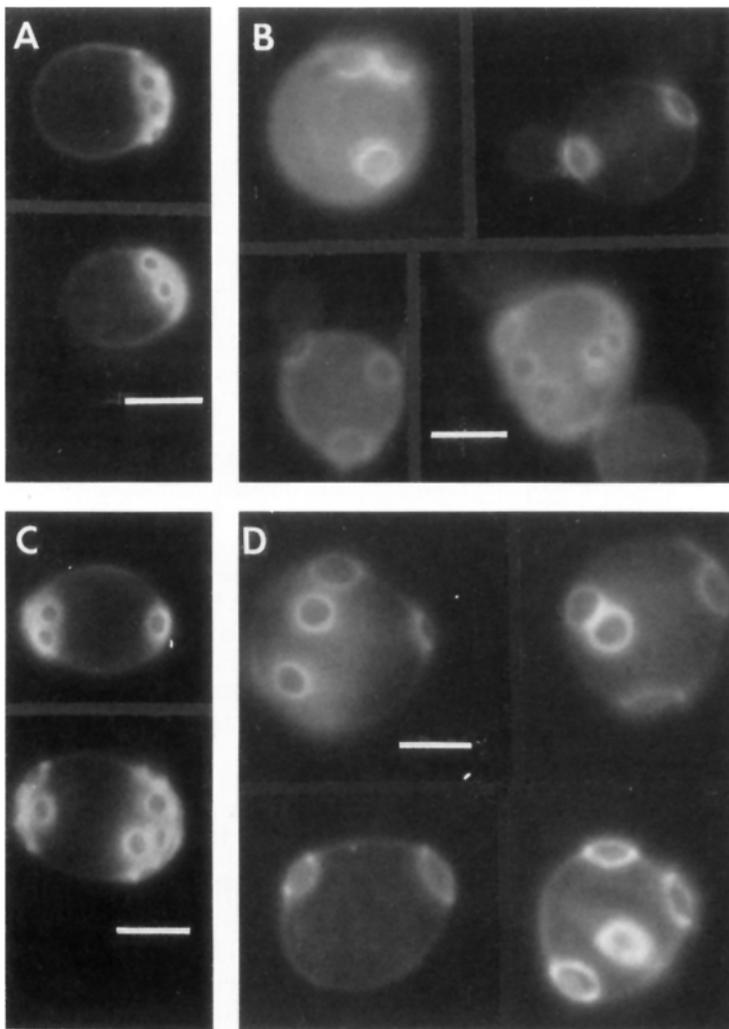


Figure 6. Budding patterns of plasmid-containing yeast cells. Cells were grown on 2% galactose as the sole carbon source and stained with Calcofluor to reveal bud scars as described in Materials and Methods. (A and B) Haploid strain DJMD22-3B containing (A) control plasmid YEp51 or (B) plasmid YEp51(42). (C and D) Diploid strain DJD1 containing (C) plasmid YEp51 or (D) plasmid YEp51(42). Bars, 4 μ m.

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)1 was inserted into the Sal I site of plasmid YEp51 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, YEp51(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, YEp51(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. 6 A and C; Sloat et al., 1981), we observed apparently random budding patterns in $\sim 75\%$ of YEp51(42)-containing DJMD22-3B and DJD1 cells grown under derepressing conditions (2% galactose) and $\sim 35\%$ of YEp51(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 $\sim 10\%$ of the haploid or diploid cells containing YEp51 itself exhibited such budding patterns when growing on either galactose or glucose. However, similar abnormalities of budding pattern were also observed in $\sim 50\%$ of DJTD2-16D cells containing the *CDC42* gene on a 3.7-kb Bam HI-Sal I fragment (Fig. 1 A) inserted into the high-copy number plasmid YEp103.

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^{ts}* 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^{ts}* 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^{ts}* mutation. Plasmid YEp(42)3 (Fig. 1 B) failed to complement the *cdc42-1^{ts}* mutation but yielded Ts⁺ recombinant subclones at the surprisingly high frequency of ~10%. 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^{ts}* 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⁺ 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⁺ 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^{ts}* 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 *RSR1* (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 *GST1* product, which may be involved in the G1-to-S phase transition (Kikuchi 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 Ca²⁺ 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* product 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 of *cdc42* mutants to *cdc24* mutants and the evidence that the *CDC24* product is a Ca^{2+} -interactive protein (Ohya et al., 1986a,b; Miyamoto et al., 1987) suggest that signal transduction by the *CDC42* product may also involve Ca^{2+} , but there are no clues yet as to the nature of such possible involvement. Finally, the observation that the *CDC42* product has COOH-terminal sequences similar to those at which *ras* and related proteins become modified by hydrophobic prosthetic 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 the predicted product of the human *rac1* gene (Didsbury, J., R. F. Weber, G. M. Bokoch, T. Evans, and R. Snyderman. 1989. *J. Biol. Chem.* 264:16378-16382) is ~70% identical to that of *CDC42*. In addition, two apparently nonidentical versions of "G25K" (see text) have now been cloned; their sequences are ~80% identical (~86% including related amino acids) to the *CDC42* product (Polakis, P., personal communication; Cerione, R., personal communication).

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