The *Saccharomyces cerevisiae* Cdc42p GTPase is localized to the plasma membrane and involved in signal transduction mechanisms controlling cell polarity. The mechanisms of action of the dominant negative *cdc42*<sup>D118A</sup> mutant and the lethal, gain of function *cdc42*<sup>G12V</sup> mutant were examined. Cdc42<sup>D118A</sup>p and its guanine-nucleotide exchange factor Cdc24p displayed a temperature-dependent interaction in the two-hybrid system, which correlated with the temperature dependence of the *cdc42*<sup>D118A</sup> phenotype and supported a Cdc24p sequestration model for the mechanism of *cdc42*<sup>D118A</sup> action. Five *cdc42* mutations were isolated that led to decreased interactions with Cdc24p. The isolation of one mutation (V44A) correlated with the observations that the T35A effector domain mutation could interfere with Cdc42<sup>D118A,C188P</sup>p-Cdc24p interactions and could suppress the *cdc42*<sup>D118A</sup> mutation, suggesting that Cdc24p may interact with Cdc42p through its effector domain. The *cdc42*<sup>G12V</sup> mutant phenotypes were suppressed by the intragenic T35A and K183–187Q mutations and in *skm1Δ* and *cla4Δ* cells but not *ste20Δ* cells, suggesting that the mechanism of *cdc42*<sup>G12V</sup> action is through the Skm1p and Cla4p protein kinases at the plasma membrane. Two intragenic suppressors of *cdc42*<sup>G12V</sup> were also identified that displayed a dominant negative phenotype at 16 °C, which was not suppressed by overexpression of Cdc24p, suggesting an alternate mechanism of action for these dominant negative mutations.

The establishment of cell polarity is crucial for the control of many cellular and developmental processes, such as the generation of cell shape, the intracellular movement of organelles, and the secretion and deposition of new cell surface constituents (1). Polarized growth in the yeast *Saccharomyces cerevisiae* occurs in response to both internal and external signals, resulting in different morphological structures (2–5). The mechanics of cell polarity initiation during the mitotic cell cycle can be divided into three sequential phases: (i) nonrandom bud site selection; (ii) organization of proteins at the bud site; and (iii) bud emergence and polarized growth. Genetic and biochemical studies have identified over 25 proteins, including several GTPases and components of the actin cytoskeleton, that are involved in the regulation of the cell polarity pathway in *S. cerevisiae* (1, 6, 7).

At least six members of the Ras superfamily of GTPases (Rsr1p/Bud1p, Cdc42p, Rho1p, Rho2p, Rho3p, and Rho4p) are involved in controlling cell polarity in *S. cerevisiae*. These proteins are active when in the GDP-bound state and inactive in the GTP-bound state (8, 9). The activity of these GTPases is controlled by regulatory proteins, such as guanine-nucleotide exchange factors, GTPase-activating proteins, and guanine-nucleotide dissociation inhibitors, as well as by the intracellular localization of the GTPase. Rsr1p/Bud1p is a member of the Ras subfamily and is responsible for bud site selection at one of the two cell poles, but it is not required for bud emergence or polarized cell growth (10–12). Cdc42p is a member of the Rho/Rac subfamily and is involved in bud site selection, bud emergence, polarized growth, and cytokinesis (13–16). The Rho proteins have been implicated in bud formation, actin reorganization, polarized growth, and activation of β-glucan synthesis (17–23).

Highly conserved (80–85% identical) functional homologs of *S. cerevisiae* Cdc42p have been characterized in *Schizosaccharomyces pombe* (24, 25), *Caenorhabditis elegans* (26), *Drosophila melanogaster* (27), and *Homo sapiens* (28, 29), suggesting that Cdc42p may have conserved functions in these other eukaryotes. Analyses of the morphological phenotypes of dominant lethal *S. cerevisiae* cdc42 alleles indicated that Cdc42p functions in bud emergence and the subsequent polarized cell growth and cytokinesis (16). These data included the observation that the *cdc42*<sup>G12V</sup> mutation resulted in dominant lethality and large, multibudded cells, suggesting that the mutant protein was activated (GTP-bound) and constitutively interacting with downstream effectors of the pathway. These effectors may include Cla4p, Ste20p, and/or Skm1p, three *S. cerevisiae* members of the Pak family of protein kinases that interact with GTP-bound Cdc42p (25, 30–34). In contrast, the *cdc42*<sup>D118A</sup> mutant exhibited a temperature-dependent, dominant negative phenotype, suggesting that Cdc42<sup>D118A</sup>p was inactive (GDP-bound) but could bind and sequester a cellular factor necessary for the budding process (16, 35). A candidate for this cellular factor was Cdc24p due to its ability to multicycopress the *cdc42*<sup>D118A</sup> mutation and because a *cdc24*<sup>Δ</sup> *cdc24*<sup>A2</sup> double mutant displayed synthetic lethality (35). In addition, Cdc24p showed limited amino acid sequence similarity with the Dbl proto-oncoprotein, which acts as a guanine-nucleotide exchange factor for human Cdc42p (36), and biochemical evidence indicated that Cdc24p catalyzes guanine-nucleotide exchange on Cdc42p in vitro (37).
In localization studies, S. cerevisiae Cdc42p was found to be targeted to the plasma membrane in the vicinity of secretory vesicles that are found at the site of bud emergence, to the tips and sides of enlarging buds, and to the tips of mating projections. The polybasic domain of four lysine residues that is next to the prenylated Cys residue is another possible localization site of Cdc42p away from endogenous Cdc42p due to sequestration of Cdc24p away from endogenous Cdc42p and suggest that the nature of the C188S mutation is nonfunctional because the CDC42-D118A lethal mutations (16). However, whether geranylgeranylation is necessary and sufficient for Cdc42p targeting to the sites of polarized growth is unknown. The polybasic domain of four lysine residues that is next to the prenylated Cys residue is another possible localization determinant. Similar domains in the K-Ras protein are important for membrane targeting; altering these Lys residues to Gln results in deocalized K-Ras proteins (40, 41).

To determine the mechanisms of action of the cdc42G12V and cdc42D118A mutations, the interactions between Cdc42p and Cdc24p were examined in the yeast two-hybrid protein system, and extragenic and intragenic suppressors of the cdc42G12V and Cdc24p were examined in the yeast two-hybrid protein system (Amersham Corp.) or Renaissance system (NEN Life Science Products). Horseradish peroxidase-conjugated goat anti-rabbit IgG, protease inhibitors (phenylmethylsulfonyl fluoride, N-tosyl-l-phenylalanine chloromethyl ketone, aprotinin, leupeptin, and pepstatin), and glass beads (425–600 μm) were obtained from Sigma. Cdc42p-specific antibodies were isolated and purified as described previously (16).

Protein determinations were performed using the Bio-Rad protein assay kit using bovine serum albumin as the standard, and immunoblots were developed using either the Enhanced Chemiluminescence (ECL) system (Amersham Corp.) or Renaissance system (NEN Life Science Products).

Plasmids and DNA Manipulations—Standard procedures were used for recombinant DNA manipulations (42) and plasmid isolation from Escherichia coli (45). Sequencing was either by the dideoxy chain termination method (46) with the U.S. Biochemical Corp. Sequenase sequencing kit or through automated sequencing at the Vermont Cancer Center DNA Sequencing Facility. Site-directed mutagenesis was performed with the MUTAGENE kit (Bio-Rad). Plasmids pBM272 (47), pRS306 and pRS315 (48), pRS425 (49), pJDL25 (50), pPGK (51), pAS1-CYH2 (52), pGAD2F (53), pRS315(CDC42-B) (35), and pYEp51(cDC42) (54), pGAD424, pRS315(CDC42), pRS315(cdc42G12V), pRS315(cdc42D118A), pRS315(cdc42D118A(C188S)), pGAL-cdc42G12V, and pGAL-cdc42D118A (16) have been previously described. Plasmid pRS315(GAL1/10) was constructed by blunt-ending the 865-base pair EcoRI-HindIII fragment from plasmid M272 containing the divergent GAL1/10 promoters with the plasmid promoter from plasmid pPGK on a XhoI plus Sall fragment into the unique SalI site of pRS315. Plasmid pPGK2 was constructed by inserting the PGK promoter from plasmid pPGK on an XhoI plus SalI fragment into the unique SalI site of a derivative of pRS425, which had the BamHI to HindIII fragment from its multiple cloning site removed.2 Plasmid pPGK2, which has the unique EcoRI site of pPGK2 removed, was constructed by cleaving plasmid pPGK2 with EcoRI, blunting-end with S1 nuclease, and religating with T4 DNA ligase. Plasmids pPGK2-CDC42 and pPGK2-CDC42 were constructed by inserting a PCR-generated CDC42 gene contained on a BamHI plus HindIII fragment into either pPGK2 or pPGK2E that had been digested with BamHI plus HindIII. pGAD2F-CDC24 was constructed by inserting the ~4-kilobase pair BamHI plus HindIII fragment from pRS315(CDC24-B), which was blunt-ended with the Klenow fragment of DNA polymerase, into pGAD2F that had been digested with BamHI and blunt-ended with the Klenow fragment of DNA polymerase.

1 The abbreviations used are: PCR, polymerase chain reaction; X-gal, 5-bromo-4-chloro-3-indolyl-β-D-galactoside.

2 P. Miller and D. I. Johnson, unpublished results.

---

### Analysis of cdc42G12V and cdc42D118A Mutations

#### TABLE I

| Strain | Genotype | Source |
|--------|----------|--------|
| DJTD2–16A | MATa cdc42–1 his4 leu2 trpl ura3 | Ref. 13 |
| DJD6–11 | MATa/MATα cdc42Δ::TRP1+ his3Δ200/Δ1 his4+ trp1 ura3+ can1+ lys2–801/lys2–801 | Ref. 15 |
| W303–1A | MATa his3–11, 5 leu2–3, 112 trpl Δ1 ade2–101 ura3–1 can1–100 | J. Kurjan |
| Y763 | MATa ade2 his3 trpl Δ1 ade2-100 cdc42–101 lys2–801 trp1–901 ura3–112 gal2–542 gal80–538 | D. Beach |
| HFC7 | URA3::C188S-GAL1 lacZ | |
| RAK63 | MAD2-206::ADE2 his3Δ200 ura3-101 lys2-801 trp1-901 ura3-112 gal2-542 gal80-538 | D. Beach |
| HD1 | MAD2-206::ADE2-trp1-1 ura3-1 can1-100 lacZ | This study |
| HD2 | MAD2-206::ADE2-trp1-1 ura3-1 can1-100 lacZ | This study |
| HD2–1-2B | MAD2-206::ADE2-trp1-1 ura3-1 can1-100 lacZ | This study |
| HD2–1-7B | MAD2-206::ADE2-trp1-1 ura3-1 can1-100 lacZ | This study |
| HD2–1-4D | MAD2-206::ADE2-trp1-1 ura3-1 can1-100 lacZ | This study |
| TRY1 | MAD2-206::ADE2-trp1-1 ura3-1 can1-100 lacZ | This study |
| TRY1–1A | MAD2-206::ADE2-trp1-1 ura3-1 can1-100 lacZ | This study |
| TRY1–6B | MAD2-206::ADE2-trp1-1 ura3-1 can1-100 lacZ | This study |
| TRY2 | MAD2-206::ADE2-trp1-1 ura3-1 can1-100 lacZ | This study |
| TRY2–13B | MAD2-206::ADE2-trp1-1 ura3-1 can1-100 lacZ | This study |

* HD2 was generated by mating W303–1A with Y763. HD2–1 was generated by integrating the skm1::HIS3 fragment into HD2. TRY2 was generated by mating RAK63 with HD2–1-6D. TRY1 was generated by integrating the clst::TRP1 fragment into TRY2. HD2–1-2B and HD2–1-6D are congenic strains derived from HD2–1.

---

* This study

---

[1] The abbreviations used are: PCR, polymerase chain reaction; X-gal, 5-bromo-4-chloro-3-indolyl-β-D-galactoside.

---

[2] P. Miller and D. I. Johnson, unpublished results.
SKM1 (Ref. 32; GenBank\textsuperscript{TM} accession number X69322) was isolated from W303–1A genomic DNA by PCR using the 5′-primer TCC-CGCCGATCATGAGGGCGGCTAAAAAG (underlined sequence is a NdeI site and contains the SKM1 start codon; double underlined sequence is a BamHI site) and the 3′-primer GCCGGGATCATGCAGCAGGTAACCGCAGAAGCG (underlined sequence is a XbaI site; double underlined sequence is a XhoI site). The PCR genomic DNA was digested with BamHI and inserted into Smal plus XhoI-digested pTZ18U (54). To generate a skmt1::HIS3 disruption, the Smal-XhoI fragment containing HIS3 from plasmid pSK1215 was blunt-ended with Klenow fragment and inserted into the blunt-ended unique StuI site at +241 of the SKM1 coding region in pTZ18U(SKM1). The resulting plasmid was digested with Smal plus XbaI, releasing a 3.86-kilobase pair skmt1::HIS3 fragment that was used to transform the diploid strain HD2 to His\textsuperscript{a}. Sporulation and tetrad analysis of stable His\textsuperscript{a} transformants yielded 

**PCR and Site-directed Mutagenesis**—The PCR mutagenesis protocol was based on the Zhou et al. (55) protocol previously described. Plasmid pRS15(cdc2\textsuperscript{42}, His\textsuperscript{a}) was amplified under essentially standard reaction conditions (reaction volume was 200 µl; reaction conditions were 94 °C for 1 min, 50 °C for 2 min, and 72 °C for 2 min) and was used to transform the diploid strain HD2 to His\textsuperscript{a}. The mutagenic primer was used as the 3′-primer in the PCR reaction with the same 5′-primer used in the PCR mutagenesis reactions (see above). Primers were used at a final concentration of 0.1 µM in a final reaction volume of 50 µl. The PCR cycling parameters were as described above. The mutant genes were sequenced to confirm the presence of the desired mutation(s) and the absence of any spurious mutations.

**Two-hybrid Protein Interactions**—The two-hybrid interaction methodology has been described (44, 53). The H7F7c transformants containing pGAD2F(CDC24) and pAS1-CYH2(cdc2\textsuperscript{42} D118A) were selected on SC-Leu-Trp media at 23 °C and then tested at various temperatures. Colonies were transferred to nitrocellulose paper, permeabilized in liquid nitrogen, and incubated at 30 °C on 3MM Whatman paper pre-soaked with 0.5 ml/nch\textsuperscript{2} Z buffer (56) containing 1 mg/ml 5-bromo-4-chloro-3-indolyl-β-galactoside (X-gal). Plasmid DNA from selected colonies containing pAS1-CYH2(cdc2\textsuperscript{42} D118A) was recovered by electroporation and sequenced to confirm the presence of the desired mutation(s) and the absence of any spurious mutations. Sequencing was performed in triplicate, and specific activities were calculated as described (56).

**Selection of Intragenic Suppressors of the cdc2\textsuperscript{42} D118A Mutant**—A PCR mutagenesis approach was taken to identify intragenic mutations that suppress the cdc2\textsuperscript{42} D118A dominant lethality. The starting template was pGAL-cdc2\textsuperscript{42} D118A; the 5′- and 3′-primers utilized were the same as used in the PCR mutagenesis of cdc2\textsuperscript{42} D118A (see above). 30 pmol of each primer and 2 fmol of template were used in a reaction volume of 100 µl. The PCR cycling parameters were 30 cycles of 94 °C for 15 s, 50 °C for 20 s, and 72 °C for 2 min. The library of PCR products obtained was extracted with PCI (phenol:chloroform:isoamyl alcohol, 25:24:1), ethanol precipitated, and resuspended in 20 µl of sterile distilled H\textsubscript{2}O. The resulting library of fragments was digested with BamHI plus HindIII and ligated into BamHI plus HindIII-cleaved pGPK2. The resulting library of pGPK2 plasmids was transformed into E. coli SURE cells by electroporation; ~24,000 ampicillin-resistant transformants were pooled, and plasmid DNA was extracted and resuspended in 200 µl of sterile distilled H\textsubscript{2}O.

Expression of the cdc2\textsuperscript{42} D118A mutant gene on plasmids is lethal to wild-type cells and does not give rise to viable transformants (16). Therefore, intragenic cdc2\textsuperscript{42} D118A suppressors were identified by an increased transformation frequency of W303–1A cells with the pGPK2 plasmid library containing mutated cdc2\textsuperscript{42} D118A genes (see above). 5-µl aliquots of the plasmid library were transformed into W303–1A cells, and half of the transformants were incubated on SC-Leu media at 30 °C and half on SC-Leu at 35 °C. A total of 1400 Leu\textsuperscript{+} transformants were obtained. These were transferred to secondary temperature-dependent growth phenotypes were performed on SC-Leu media at 23 and 16 °C (see “Results”). Plasmid DNA from yeast transformants was recovered into E. coli SURE cells and subjected to dideoxy sequencing protocols to confirm the presence of the original G12V mutation and the appearance of new coding region mutations. To separate the single mutations identified as cdc2\textsuperscript{42} D118A suppressors from the G12V mutation, the Eagl-BamHI restriction fragment from the double mutant genes, which only contained the new single mutations, was substituted for the wild-type Eagl-BamHI fragment in pPGK2E-CDC42. The presence of only the single mutations was confirmed by automated DNA sequencing (Vermont Cancer Center DNA Sequencing Facility). To further analyze two of the single mutant genes identified in the screen (S86P and S89P), they were placed under the control of the wild-type CDC42 promoter and integrated into the genome. The single mutant genes were excised from plasmid pGPK2E by NdeI plus SacI digestion, which generates the mutant genes without a promoter, and then inserted into plasmid pBS315(CDC42) that had been digested with NdeI plus SacI, releasing the wild-type gene. The resulting single mutant genes were excised from the plasmids by NotI plus SacI digestion and inserted into the integrating plasmid pRS306, which had been digested with NotI plus SacI. These plasmids were then integrated into the genome of the diploid strain JD6–11 (cdc2–23:TRP1/CDC42 ura3–52/ura3–32) at the ura3–52 locus by cutting the pRS306 derivative plasmids with BamHI, which has a unique site in the UR43 gene, and transforming JD6–11
with the linearized plasmids, selecting for Ura- transformants at 23 °C. Stable Ura- transformants were subjected to tetrad analysis to follow the ura3-52::cdc2412V::URA3 or ura3-52::cdc24118A::URA3 marked loci. In more than 10 tetrads each, Ura- Trp- haploid cells could not be isolated (data not shown), indicating that the cdc24118A and cdc24118B mutant genes do not encode functional Cdc42 proteins.

**Cell Fractionation and Immunoblot Analyses**—Cell fractionation experiments were performed as described previously (38). Briefly, cells containing PGK promoter-driven cdc24 mutant genes on plasmids were grown in SC-Leu liquid media to midlog phase at 23 °C. 1 × 10^6 cells were collected, washed with water, resuspended in 200 µl of lysis buffer (0.5% sarkohe, 140 mM NaCl, 50 mM Tris, pH 8.0) with protease inhibitors (0.5 mM phenylmethylsulfonyl fluoride and 1:1000 dilutions of 1 mg/ml stock of aprotinin in water, 1 mg/ml stock of N-tosyl-L-phenylalanine chloromethyl ketone in 95% ethanol, 1 mg/ml stock of leupeptin in water, and 1 mg/ml stock of pepstatin in methanol), and lysed on ice by vortexing with 425–600 µm acid-washed glass beads. Greater than 90% cell lysis was verified by light microscopy. Cells lysates were spun at 5000 rpm for 4 min at 4 °C; the 5000 rpm 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. To assess the relative amount of Cdc42p in each fraction, equal volumes of each fraction were resuspended in the same volume of lysis buffer. For immunoblots, protein samples were diluted 1:1 in SDS-lysis buffer (57) containing 40% buffer (57) containing 40% Cdc42p have been recently isolated in D. melanogaster and S. cerevisiae as analogous to domains of human Cdc42p that have been implicated in the binding of guanine nucleotides (see Fig. 5). The S86P, T138A, and L165S mutations are in regions of Cdc42p indicated in the binding of guanine nucleotides (Ref. 58; see “Discussion”). Also, the conserved G-4 domain of GTPases, which has been implicated in nucleotide exchange activity (Ref. 58; see “Discussion”).

**RESULTS**

**Temperature-dependent Interaction Between Cdc24p and Cdc42p**

The cdc24118A mutant displays a dominant negative phenotype at 23 °C but not at 30 °C or higher temperatures, and overexpression of Cdc24p can suppress this phenotype (38). These data suggested that the cdc24118A, C188S mutant negative phenotype may be due to the nonfunctional binding of Cdc24p by mutant Cdc42p. Cdc24p could not interact with the endogenous wild-type Cdc42p. It also suggested that this interaction could occur at 23 but not 30 °C. To test this hypothesis, the interaction between these proteins in the yeast two-hybrid protein system was examined. In frame fusion proteins between Cdc24p and the GAL4 transcriptional activation domain in plasmid pGAD2F and between Cdc42p and the GAL4 DNA binding domain in pAS1-CYH2 were generated. The C188S mutation was found in a region in which dominant negative phenotype was due to binding of Cdc24p by mutant Cdc42p (16, 38). A two-hybrid protein interaction between Cdc24p and Cdc42p was observed at 23 but not 30 or 34 °C (Fig. 1). This interaction correlates with the temperature dependence of the cdc24118A mutant phenotype and further supports the hypothesis that the dominant negative phenotype was due to binding of Cdc24p by mutant Cdc42p. Our data are consistent with a model in which Cdc42p binds Cdc24p within the cell at 23 °C, but not allowing the endogenous wild-type Cdc42p to bind, and that interaction is lost at higher temperatures, but we cannot rule out the possibility that our data are a result of a unique behavior of mutant Cdc42p.

**Isolation of Mutations in cdc24118A,C188S That Inhibit Interactions with Cdc24p**

To define the domain(s) of Cdc42p that interact with Cdc24p, PCR-generated mutations that reduced the two-hybrid interactions with Cdc24p, using a blue-to-white colony color change at 23 °C, were introduced into the cdc42118A,C188S gene in pAS1-CYH2. From nitrocellulose filters of ~800 colonies, eight colonies of white or pale blue color were chosen for further characterization. The plasmid DNA from these eight colonies was recovered into E. coli, and three plasmids were found to have no CDC42 insert; these colonies were white in the assay, as would be expected. The remaining five plasmids displayed the characteristic cdc24p restriction enzyme banding pattern and contained single point mutations in the cdc42118A,C188S gene resulting in the following amino acid changes: V44A, S86P, I117S, T138A, and L165S. All of the new mutant proteins showed a reduced interaction with Cdc24p (Table II), and all were equally expressed in S. cerevisiae as shown by immunoblot analysis (data not shown).

The V44A mutation lies in the putative effector domain (see below), and the I117S is next to the starting D118A mutation in the conserved G-4 domain of GTPases, which has been implicated in the binding of guanine nucleotides (see Fig. 5). The S86P, T138A, and L165S mutations alter in regions of Cdc42p that are analogous to domains of human Cdc42p that have recently been implicated in responsiveness to Cdc24p-mediated nucleotide exchange activity (Ref. 58; see “Discussion”). Also, the S86P mutation lies in a region in which dominant negative alleles of D. melanogaster Cdc42p have been recently isolated (Ref. 59; see below).

**Effects of the T35A Effector Domain Mutation on the**
round unbudded cells seen in these mutant cell cultures at 37 °C (data not shown). Surprisingly, the T35A mutation can suppress the lethal growth defect associated with the cdc42<sup>D118A</sup> mutation (Fig. 2A), and neither the single nor double mutant gene could complement the cdc42<sup>-1A</sup> mutant at 37 °C. Interestingly, the V44A effector domain mutation (see above) could suppress both the cdc42<sup>D118A</sup> growth and morphological defects. Taken together, these data suggest that the Cdc24p guanine-nucleotide exchange factor interacts with Cdc42p through its effector domain (see “Discussion”).

**Suppression of cdc42<sup>G12V</sup> by the K183–187Q Mutation**—To examine the role of the C-terminal polylysine region in targeting of Cdc42p to the plasma membrane, the four Lys residues were altered to uncharged Glu residues in either the wild-type or cdc42<sup>G12V</sup> mutant gene. The intragenic K183–187Q mutation was able to suppress the cdc42<sup>G12V</sup> lethality, but the quintuple mutant gene was unable to complement the cdc42<sup>-1A</sup> mutant at 37 °C (Fig. 2A). This result suggested that this polylysine region plays an important role in the function of Cdc42p. Interestingly, the quadruple K183–187Q mutant gene can complement the cdc42<sup>-1A</sup> allele at 37 °C (Fig. 2A), suggesting that some of this mutant protein can be properly localized and, hence, functional.

To address the mechanism of K183–187Q suppression, the subcellular localization of the K183–187Q mutant protein was examined using cell fractionation protocols (Fig. 3B). Under these experimental conditions, the wild-type Cdc42 protein fractionated predominately into the particulate pool (Fig. 3B). The Cdc42<sup>K183–187Q</sup> protein fractionated into both soluble and particulate pools (Fig. 3B), indicating that the K183–187Q mutation leads to partial loss of membrane localization. The Cdc42<sup>G12V,K183–187Q</sup> protein has a similar fractionation pattern as the Cdc42<sup>D118A</sup> protein (data not shown). The probability that this fractionation pattern is due to the K183–187Q mutation is bolstered by the fractionation patterns of two other suppressors of the G12V mutation (see below), which show a predominantly particulate fractionation pattern (Fig. 3B). This result is consistent with the K183–187Q mutant phenotypes described above and suggests that the K183–187Q mutation is affecting Cdc42p function by altering its subcellular localization.

**Effects of Pak Kinase Deletions on cdc42<sup>G12V</sup> Lethality**—To test the hypothesis that cdc42<sup>G12V</sup> lethality was due to an improper interaction with a downstream effector(s) at the plasma membrane, the effects of Pak kinase deletions on cdc42<sup>G12V</sup> lethality were examined. The pGAL-cdc42<sup>G12V</sup> plasmid was transformed into strains that had individual single deletions in either of the three Pak kinases, CLA4, STE20, or SKM1, as well as the corresponding double deletion mutants, and growth and morphological phenotypes on galactose-containing media at 23 °C were assayed (Table II; Fig. 4). The cdc42<sup>G12V</sup> lethality was still observed in the cdc42 and ste20Δ strains

---

3 T. J. Richman and D. I. Johnson, manuscript in preparation.
FIG. 3. Analysis of the K183–187Q mutation. A, wild-type and mutant CDC42 genes under the control of the PGK promoter were transformed into DJTD2–16A cells (cdc42–16A), and transformants were selected on SC-Leu media at 23 °C. Individual transformants were streaked to SC-Leu plates and incubated at 23 and 36 °C. B, protein extracts of wild-type W303–1A cells transformed with plasmids containing CDC42 genes under the control of the PGK promoter were spun at 10,000 × g to produce pellet (P) and supernatant (S) fractions. Equal volumes of protein fractions were run on a 12.5% polyacrylamide gel. Cdc42 protein was detected using affinity-purified anti-Cdc42p antibodies (1:500 dilution) and horseradish peroxidase-conjugated goat anti-rabbit IgG secondary antibody (1:1000 dilution) with the ECL detection system.

| Protein kinase present* | Ste20p | Cla4p | Skm1p | Growth on 2% galactose | Morphological phenotypes† |
|-------------------------|--------|-------|-------|------------------------|---------------------------|
|                         | P      | P     | P     | P                      | P                         |
| +                       | +      | +     | +     | 4 30 0 5 61 (M)         |                           |
| ∆                       | +      | +     | +     | + 3 10 76 (EB)          | +                        |
| +                       | +      | +     | +     | + 3 10 76 (EB)          | +                        |
| +                       | +      | +     | +     | + 3 10 76 (EB)          | +                        |
| +                       | +      | +     | +     | + 3 10 76 (EB)          | +                        |

* Cells containing the indicated deletions (∆) were transformed with the pGAL-cdc42G12V plasmid, selecting for Leu+ transformants on SC-Leu media at 23 °C. Individual transformants were streaked on SC-Leu plates and incubated at 23 and 36 °C. Protein extracts of wild-type W303–1A cells transformed with plasmids containing CDC42 genes under the control of the PGK promoter were spun at 10,000 × g to produce pellet (P) and supernatant (S) fractions. Equal volumes of protein fractions were run on a 12.5% polyacrylamide gel. Cdc42 protein was detected using affinity-purified anti-Cdc42p antibodies (1:500 dilution) and horseradish peroxidase-conjugated goat anti-rabbit IgG secondary antibody (1:1000 dilution) with the ECL detection system.

† Abbreviations for morphological phenotypes are as follows: NB, normal budded cells; NU, normal un budded cells; LRU, large, round un budded cells; LRSB, large, round cells with ≥1 small bud; AB, abnormal budded cells, which were either the cdc42G12V multibudded phenotype (M) or the elongated bud phenotype seen in cla4δ strains (EB) (30). Numbers are reported as a percentage of 200 cells counted and are representative of at least two independent experiments.

single deletion mutants as well as the cla4Δskm1Δ and ste20Δskm1Δ double deletion mutants, but cdc42G12V expression was not lethal in the skm1Δ mutant (HD2–1-2B), with 68% of the cells appearing normal in morphology (Table III). In addition, cdc42G12V expression was still lethal in a rga1Δ mutant (data not shown), which has a deletion in one of the Cdc42p GTPase-activating proteins Rga1p. These results suggested that cdc42G12V lethality was due, in part, to an interaction with the Skm1p protein kinase. However, when the morphological phenotypes of the cla4Δ cells were examined, a dramatic change from the typical cdc42G12V morphological phenotype was observed (Fig. 4; Table III). Instead of the large, multibudded cell phenotype observed when overexpressing cdc42G12V in wild-type or ste20Δ cells (Fig. 4), a new phenotype of large, round cells with one or more small buds (76%) as well as large, round un budded cells (~10%) was observed. This cellular morphology was also observed in skm1Δ cells but at a lower frequency (26 versus 76%). The large, round unbudded phenotype is similar to the cdc24 loss of function or dominant negative phenotype; however, the presence of small buds on the large, round cells suggested that bud emergence had occurred in these cells, but growth was restricted to the mother cell and not directed into an enlarging bud. Taken together, these data suggest that the mechanism of cdc42G12V action is through interactions with Skm1p and Cla4p but not Ste20p or Rga1p (see “Discussion”).

Isolation of Temperature-dependent Intragenic Suppressors of cdc42G12V—A PCR-generated mutant library was screened for intragenic suppressors of the cdc42G12V mutant gene by increased transformation frequency of wild-type W303–1A cells (see “Experimental Procedures”). Based on the above-mentioned suppressor results, three types of mutations were envisioned arising from this screen: (i) mutations that affect the localization of Cdc42p, such as the C1885 and K183–187Q mutations, (ii) mutations in the effector domain, such as the T35A mutation, and (iii) loss of function or null mutations as well as true revertants of the G12V mutation. Leu+ transform-
methylene blue.

temperatures.
cdc42 genes were selected on SC-Leu plates at 23° C. Individual trans-

changes (Fig. 5). The fourth plasmid contained the original
transitions resulting in the S86P, S89P, and C157R amino acid

mutant (16).

within plasmids containing the
into DJTD2–16A cells), which is a result similar to that seen

mids containing the mutant genes could not be transformed

of mutations. A total of 1400 Leu

terature-dependent phenotypes, thereby eliminating the third type

mutant may therefore be due to its inability to interact with a

Cdc42 GTPase interacts with several pro-

G12V mutation along with two coding region mutations, result-

in the S89P and K128E amino acid changes. Given the similarity

in phenotype between the single S89P mutant and the double S89P,K128E mutant, it is likely that the K128E

mutation is silent. Interestingly, the S89P mutation is analog-
ous to the S89F and S89L dominant negative mutations identi-
fied in the C. elegans ras homolog let60 (62) and D. melano-
gaster Cdc42 (Ref. 59; see “Discussion”).

To further analyze these mutations, they were separated away
from the G12V mutation (see “Experimental Proce-
dures”). Wild-type W303–1A cells containing the cdc42S86P or
cdc42S89P single mutant gene in plasmid pPGK2E retained the

cold-sensitive phenotype displayed in the G12V double mu-
tants (data not shown) and had an increase in the percentage of

unbudded cells in the population (Table IV, top). Cells contain-
ing the cdc42C157R single mutant gene did not exhibit a strong
cold-sensitive phenotype, but they did have an increase in unbudded cells in the population. Similar growth phenotypes

were observed when the single mutant genes were expressed in the

cdc42–1ts mutant strain DJTD2–16A (Table IV, bottom). In

addition, the single mutant genes could not complement the
cdc42–1ts mutation at 37 °C (Table IV, bottom), indicating that

they did not encode functional proteins at that temperature. The cdc42S86P or cdc42S89P single mutant gene could not serve as the sole copy of CDC42 in a cdc42Δ background (see “Ex-

perimental Procedures”), further substantiating the theory that

these alleles encode nonfunctional proteins.

For the S86P and S89P mutations, the mechanism of sup-

pression of the cdc42G12V mutation was not due to loss of

membrane attachment, because both show a particulate frac-
tionation pattern that was similar to the wild-type Cdc42p

fractionation pattern (Fig. 3B). In addition, the 16 °C dominant

negative phenotype could not be suppressed by overexpression of Cdc24p or by overexpression of the Cdc42p effector Cla4p

(data not shown), suggesting that the mechanism of action of

these alleles is different from that of the dominant negative

cdc42D118A allele (see above) and not due to sequestration of the

Cdc42p downstream effector. Interestingly, the S86P mu-
tation was also identified in the screen for mutations that

reduced a two-hybrid protein interaction between Cdc24p and
Cdc42D118A,C188Sp at 23 °C (see above; Table II). Cdc42D118A,C188Sp,S86Sp

p does not interact with Cdc24p in the two-hybrid protein system at 23 °C (Table II) or at 16 °C (data not shown), indicating a different dominant negative mecha-
nism for this allele.

DISCUSSION

In genetic and biochemical experiments with numerous Ras-
related proteins, a region between residues 26 and 48 has been
identified as being required for interactions with downstream
effectors (8, 63, 64). The first indication that this so-called
"effector domain" may be an important region of Cdc42p came from a sequence comparison between functional homologs of Cdc42p and other closely related GTPases (14). Functional
homologs of Cdc42p from S. pombe, C. elegans, and humans can complement the S. cerevisiae cdc42–1ts mutation and are 80–
85% identical to Cdc42p, especially in the highly conserved region between residues 26 and 50 (Fig. 5). The human Rac1 protein is 74% identical to Cdc42p but cannot complement the
cdc42–1ts mutation, indicating that it is not a functional homolog. Interestingly, the only region of Rac1p that is significantly
different from functional Cdc42p homologs is residues 41–52, the region in Ras-like proteins that interacts with effector proteins. The inability of Rac1p to complement the cdc42–1ts mutant may therefore be due to its inability to interact with a Cdc42-specific effector.

The S. cerevisiae Cdc42 GTPase interacts with several pro-

TABLE IV

Charaterization of cdc42G12V intragenic suppressors

Top, W303–1A cells containing pPGK2 plasmids with the indicated cdc42 gene were analyzed. These results are representative of at least three independent determinations. Bottom, DJTD2–16A cells (cdc42–1ts) containing pPGK2E plasmids with the indicated single mutant cdc42 genes were selected on SC-Leu plates at 23° C. Individual transformants were streaked to SC-Leu plates and incubated at the indicated temperatures.

| Mutant gene | Unbudded cells | Dead unbounded cells |
|-------------|----------------|---------------------|
|             | 30° C | 16° C |                  |                  |
| Wild type   | 48    | 49    | 12                |
| G12V,S86P   | 63    | 70    | 44                |
| G12V,S89P   | 69    | 75    | 39                |
| G12V,C157R  | 75    | 86    | 58                |
| Wild type   | 50    | 48    |                   |
| S86P        | 49    | 80    |                   |
| S89P        | 42    | 59    |                   |
| C157R       | 40    | 57    |                   |

| Mutant gene | Growth of cdc42G12V intragenic suppressor cells |
|-------------|-----------------------------------------------|
|             | 16° C | 23° C | 30° C | 37° C |
| None (cdc42–1ts) | +     | +     | +     | +     |
| Wild type   | +     | +     | +     | +     |
| S86P        | –     | +     | –/–   | –     |
| S89P        | –     | +     | –/–   | –     |
| C157R       | +     | +     | +     | +     |

n

- Cells were grown at 30°C to midlog phase and sonicated prior to determining the budding index. n = 100 cells.
- Cells were grown at 30°C to midlog phase and then shifted to 16°C for 20 hr (for G12V double mutants) or 45 hr (for single mutants) prior to determining the budding index. n = 100 cells.
- Cells were grown at 30°C to midlog phase and then shifted to 16°C for 20 hr. The percentage of dead cells was determined by staining with methylene blue. n = 100 unbudded cells.
FIG. 5. Summary of new cdc42 mutations. Intragenic suppressors of cdc42\(^{G12V}\) are boxed, and mutations that interfere with Cdc24p-Cdc42D118A, C188Sp interactions are circled. The amino acid sequences of the known Cdc42 homologs are shown to accent the high degree of amino acid identity at the mutation sites. The overlines indicate the five domains (G-1 through G-5) of Ras-related GTPases that have been implicated in nucleotide binding/hydrolysis (8). The Gly\(^{12}\) and Asp\(^{118}\) sites are indicated by asterisks. See text for details on new mutations.
proteins, including the Ste20, Cla4, and Skm1 protein kinases (30, 32, 34, 61) that are predicted to function downstream in the cell polarity pathway. This prediction is based, in part, on the inability of these proteins to interact with the Cdc42 Thr35A effector domain mutant protein. The Thr35 residue lies within the G-2 domain of Ras-related GTPases (8), which is predicted to change conformation upon GTP binding. The results presented herein further define the cdc42 Thr35A mutation as an effector domain mutation, but the ability of the Thr35A mutation to suppress the dominant negative cdc42 D118A morphological phenotypes and to disrupt interactions with Cdc24p suggests that this region of Cdc42p may also interact with the Cdc24p exchange factor. Another mutation (V44A) in the effector domain was identified by its ability to disrupt the interaction between Cdc42 D118A.C188S and Cdc24p, providing further support for this role of the effector domain, and recently, the Thr35A mutation in the human Cdc42p was found to disrupt responsiveness to Cdc24p-mediated nucleotide exchange activity (58). Taken together, these data suggest that the so-called “effector domain” plays multiple roles in the interactions of Ras-related GTPases with their regulatory and effector proteins.

The ability of the K183–187Q mutation to suppress the cdc42 G12V dominant lethality is due, in part, to the partial delocalization of the mutant protein from the plasma membrane. As opposed to the nonfunctional cdc42 C188S mutant gene that cannot complement the cdc42-1ts mutant (16), the ability of the cdc42 K183–187Q mutant gene to complement the cdc42-1ts mutation suggests that this mutation has an intermediate effect on Cdc42p function. In addition, these results suggest that the polylysine domain of Cdc42p is necessary but not sufficient for complete plasma membrane localization. This is an important point, because Cdc42p is targeted to a specific location on the plasma membrane at sites of polarized growth (38) as opposed to general plasma membrane localization of Ras proteins. This C-terminal polylysine region is not found in most Ras-like GTPases, and its positive charges may be functioning in interactions with negatively charged components, either protein or phospholipid, at the membrane site. Whether these interactions play a role in the specific targeting of Cdc42p or in enhancing membrane association is unclear at this point. Interestingly, deletion of this region in the mammalian Cdc42p led to loss of interaction with phosphatidylinositol 4,5-bisphosphate-containing vesicles (65); phosphatidylinositol 4,5-bisphosphate has also been shown to enhance nucleotide exchange with Cdc42Hs (65). Further support for the importance of the polybasic region in Cdc42p function comes from the isolation of a new temperature-sensitive cdc42 mutation (K186R) within the polybasic region (15).

The ability of the skm1Δ and cla4Δ mutations to suppress the growth and/or morphological phenotypes of the cdc42 G12V mutation suggest that the Cdc42p G12V mutant protein is exerting its lethal effects through these two protein kinases but not through the Ste20p protein kinase. Interpretation of these phenotypes at the protein-protein interaction level is complicated, however, by the presence of endogenous wild-type Cdc42p in these cells. For instance, the lethality of the cdc42 G12V mutation could be due either to a direct effect of Cdc42p G12V on a cellular process or to an indirect effect of Cdc42p G12V on the interactions between wild-type Cdc42p and another protein in the cell. The reversal of cdc42 G12V lethality in a skm1Δ mutant can be explained by postulating a role for Skm1p in either mediating Cdc42p G12V lethality or in inhibiting the function of the endogenous wild-type Cdc42p in these cells. Skm1p may be functioning either through a direct interaction with Cdc42p or through an indirect interaction with other downstream effectors such as Cla4p. The mechanism by which cdc42 G12V lethality is restored in cla4Δskm1Δ and ste20Δskm1Δ double deletion mutants is unclear at this point, but it could reflect the inability of Cdc42p G12V p-expressing cells to grow in the presence of only a single Pak-like kinase; again this could be due to a nonfunctional interaction between either Cdc42p G12V p or endogenous wild-type Cdc42p with the remaining Pak-like kinase. It should be noted that in two-hybrid protein studies, Cdc42p G12V p interacts comparably with Cla4p and Ste20p (30, 61); two-hybrid interactions between Cdc42p G12V p and Skm1p have not been reported.

The presence of Cla4p, either as the sole Pak kinase in the cell or in combination with Ste20p and/or Skm1p, does seem necessary for the cdc42 G12V-dependent generation of multibudded cells. In addition, the absence of Cla4p, and to a lesser extent Skm1p, shifts the Cdc42p G12V mutant phenotype from a multibudded morphology to a large, round cell with one or more small buds, suggesting that Cla4p and Skm1p are not necessary for bud emergence but are necessary for restricting growth to the enlarging bud. This phenotype was reminiscent of the phenotype observed when cdc24 Δts cells were arrested with hydroxyurea and then released into 37 °C (restrictive temperature) media (66), the phenotype that first suggested Cdc24p also functioned later in the cell cycle beyond bud emergence. This phenotype was also reminiscent of Cdc24p overexpression phenotypes (35), of downstream mutants of the polarity pathway such as rho1 mutants or pck1 mutants (67–69), and of Skm1p overexpression phenotypes (32). These data suggesting a role for Cla4p in restricting growth to the enlarging bud are also consistent with those previously obtained for cla4 mutations in wild-type backgrounds (30).

To identify other domains of Cdc42p that are important for function, intragenic suppressors of the cdc42 G12V dominant lethality were isolated. Given the different mechanisms of suppression observed for the Thr35A and K183–187Q mutations, it was important to assay both the ability of these suppressors to complement the cdc42-1ts mutation and the subcellular fractionation of the mutant proteins to distinguish between loss of function mutations and loss of localization mutations. The C157R mutation lies in a domain implicated in the responsiveness of the human Cdc42p to Cdc24p-mediated nucleotide exchange activity (58) and within the G-5 domain of Ras-related GTPases, which functions in the binding of guanine nucleotides (8). The Cys157 residue is unique to Rho/Rac/Cdc42 proteins in this domain, but we have not pursued the C157R mutation further at this time because it does not exhibit a phenotype on its own. The fractionation patterns of the S86P and S89P mutant proteins, as well as their inability to complement the cdc42-1ts mutation or act as the sole copy of Cdc42p within the cell, suggested that these mutations suppressed the cdc42 G12V phenotype by generating a nonfunctional albeit properly localized protein. However, the dominant negative phenotype of these mutant genes at 16 °C suggested that these mutant proteins were able to negatively interact with some component of the pathway, possibly sequestering it away from the endogenous Cdc42p. It is unlikely that the sequestered component was Cla4p or Cdc24p, since overexpression of either was unable to suppress the dominant negative phenotype. In addition, the S86P mutation disrupted the interaction between Cdc42 D118A.C188S and Cdc24p in a two-hybrid protein assay at both 23 and 16 °C, suggesting that the sequestered component was not Cdc24p. Interestingly, the paradigmatic cdc42 T17N dominant negative allele also could not be suppressed by overexpression of Cdc24p.4 Taken together, these data suggest the mechanism of action of these dominant negative alleles is dif-

4 M. Ziman and D. I. Johnson, unpublished results.
ferent from that of the cdc42D118A allele.

The S86P and S89P mutations are within a domain of Cdc42p (residues 82–120) and other GTPases in which dominant negative mutations have been recently isolated (59, 62). In addition, mutations in this domain lead to diminished responses to Cdc24p-mediated nucleotide exchange activity (58), suggesting that this domain of Cdc42p plays an important role in its function. The analogous domain in S. cerevisiae Ras2p is involved in the interaction between Ras2p and its GTPase-activating protein, Ira2p (70, 71). This domain in the Ras crystal structure corresponds to the turn between loop 6 and the α3 helix (72), a region of the protein that is predicted to be in close proximity to bound nucleotide. Introduction of additional Pro residues into this region (Fig. 5) could have a profound effect on the conformation of the protein and/or its ability to bind nucleotide, thereby leading to loss of interactions with both guanine-nucleotide exchange factors and GTPase-activating proteins.

Overall, these studies have identified the effector domain of Cdc42p as being important for interactions with both downstream effectors and the upstream guanine-nucleotide exchange factor Cdc24p and have identified two new domains of Cdc42p as being important for function and/or membrane localization. Biochemical interaction and genetic suppressor studies in the future may further define the regions of the effector domain that are necessary for interactions with multiple Cdc42p effectors and regulators and may elucidate the mechanism of Cdc42p targeting to the plasma membrane.

Acknowledgments—We thank D. Beach, B. Benton, J. Kurjan, P. Miller, M. Snyder, and W. White for strains and reagents; T. Hunter for

REFERENCES

1. Druhin, D. G., and Nelson, W. J. (1996) Cell 84, 335–344
2. Chant, J. (1994) Trends Genet. 10, 328–333
3. Madden, K., Costigan, C., and Snyder, M. (1992) Trends Cell Biol. 2, 22–29
4. Herskovitz, I., Park, H.-O., Soder, S., Valta, N., and Peter, M. (1995) Cold Spring Harbor Symp. Quant. Biol. 60, 717–727
5. Pringle, J. R., Bi, E., Harkins, H. A., Zahnner, J. E., de Virgilio, C., Chant, J., Corradese, K., and Fares, H. (1995) Cold Spring Harbor Symp. Quant. Biol. 60, 729–734
6. Chant, J., and Pringle, J. R. (1991) Curr. Opin. Genet. Dev. 1, 342–350
7. Druhin, D. G. (1991) Cell 65, 1093–1096
8. Bourne, H. R., Sanders, D. A., and McCormick, F. (1991) Nature 349, 117–127
9. Boguski, M. S., and McCormick, F. (1993) Nature 366, 643–654
10. Bender, A., and Pringle, J. R. (1989) Proc. Natl. Acad. Sci. U. S. A. 86, 9786–9800
11. Chant, J., and Herskovitz, I. (1991) Cell 65, 1203–1212
12. Ruggieri, R., Bender, A., Matsui, Y., Powers, S., Takai, Y., Pringle, J. R., and Matsumoto, K. (1992) Mol. Cell. Biol. 12, 758–766
13. Johnson, D. I., and Pringle, J. R. (1990) J. Cell Biol. 111, 143–152
14. Johnson, D. I. (1993) The ras Superfamily of GTPases (Lacal, J. C., and collaborators, ed) pp. 297–312, CRC Press, Inc., Boca Raton, FL
15. Miller, P. J., and Johnson, D. I. (1991) J. Cell Biol. 112, 561–572
16. Mizen, B. M., Brian, J. M., Ouellette, L. A., Church, W. R., and Johnson, D. I. (1991) Mol. Cell. Biol. 11, 3537–3544
17. Yamochi, I., Tanaka, H., Nonaka, H., Maud, A., Misha, T., and Takai, Y. (1994) J. Biol. Chem. 269, 1077–1083
18. Matsu, Y., and Ohn, A. (1992) Mol. Cell. Biol. 12, 5690–5699
19. Imai, J., Tohee, A., and Matsu, Y. (1996) Genes and Development 12, 359–369
20. Nonaka, H., Tanaka, K., Hijano, H., Fujii, N., Kohn, H., Ukimasa, M., Mino, A., and Takai, Y. (1995) EMBIO J. 14, 5931–5938
21. Qadota, H., Pinto, C., Inoue, S. B., Arisawa, M., Anraku, Y., Zheng, Y., Watanabe, T., and Ohya, Y. (1996) J. Cell Biol. 135, 1277–1289
22. Marcus, S., Polverino, A., Chang, E., Roberta, D., Cobb, M. H., and Wiggler, M. H. (1995) Proc. Natl. Acad. Sci. U. S. A. 92, 6180–6184
23. Simon, M. N., De Virgilio, C., Souza, B., Pringle, J. R., Abo, A., and Reed, S. I. (1995) Nature 376, 702–705
24. Han, M., and Sternberg, P. W. (1991) Genes Dev. 5, 2188–2198
25. Marshall, S. M. (1993) Trends Biochem. Sci. 18, 250–254
26. Polakis, P., and McCormick, F. (1993) J. Biol. Chem. 268, 19157–19160
27. Zheng, Y., Glafein, J. A., and Cerione, R. A. (1996) J. Biol. Chem. 271, 23815–23819
28. Shat, B., Adams, A., and Pringle, J. R. (1991) J. Cell Biol. 110, 395–405
29. Madaule, P., Axel, R., and Myers, A. M. (1997) Proc. Natl. Acad. Sci. U. S. A. 94, 779–783
30. Qadota, H., Anraku, Y., Botstein, D., and Ohya, Y. (1994) Proc. Natl. Acad. Sci. U. S. A. 91, 9317–9321
31. Watanabe, M., Chen, C.-Y., and Levin, D. E. (1994) J. Biol. Chem. 269, 16829–16836
32. Pavan, M. C., Bernardi, A., and Parmeggiani, A. (1996) EMBO J. 15, 1107–1111
33. Wood, D. R., Poulet, P., Wilson, B. A., Khalil, M., Tanaka, K., Cannone, J. F., and Tamanoi, F. (1994) J. Biol. Chem. 269, 5322–5327
34. Milburn, M. V., Tong, L. J., Devoe, A. M., Brunger, A., Yamauch, Z., Nishimura, S., and Kim, S. H. (1990) Science 247, 939–945