Mutants in the S. cerevisiae PKCI Gene Display a Cell Cycle–Specific Osmotic Stability Defect

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Abstract. The PKCI gene of Saccharomyces cerevisiae encodes a homologue of the Ca²⁺-dependent isozymes of mammalian protein kinase C (Levin, D. E., F. O. Fields, R. Kunisawa, J. M. Bishop, and J. Thorner. 1990. Cell. 62:213–224). Cells depleted of the PKCI gene product display a uniform phenotype, a behavior indicating a defect in the cell division cycle (cdc). These cells arrest division after DNA replication, but before mitosis. Unlike most cdc mutants, which continue to grow in the absence of cell division, PKCI-depleted cells arrest growth with small buds. We created conditional alleles of PKCI to explore the nature of this unusual cdc defect. In contrast to PKCI-depleted cells, all of the conditional pkcl mutants isolated were suppressed by the addition of CaCl₂ to the medium, suggesting that the mutant enzymes could be activated by Ca²⁺. Arrest of growth and cell division in the conditional mutants was accompanied by cessation of protein synthesis, rapid loss of viability, and release of cellular material into the medium, suggesting cell lysis. This conclusion was supported by the observation that a pkcl deletion mutant was capable of proliferation in osmotically stabilized medium, but underwent rapid cell lysis when shifted to hypo-osmotic medium. We have incorporated these observations into a model to explain the cdc-specific arrest of pkcl mutants.

Materials and Methods

Strains, Growth Conditions, and Transformations

All yeast strains used in this study (Table I) were derivatives of EGI23, MATα leu2-3,112 ura3-52 trp1-1 his4 can12 (Siliciano and Tatchell, 1984),
except RY262, X3119-12A, and HMSFI. Yeast cultures were grown in YEP (1% yeast extract, 2% bactopeptone) supplemented either with 2% glucose, or 2% galactose plus 0.1% sucrose, as required. Synthetic minimal dextrose medium (SD) (Sherman et al., 1986) supplemented with the appropriate nutrients was used to select for plasmid maintenance. Yeast transformation was by the lithium acetate method (Ito et al., 1983). General genetic manipulation of yeast cells was carried out as described (Sherman et al., 1986). For random spore germination, ascii were treated with β glucuronidase (Sigma Chemical Co., St. Louis, MO) for 8 h to separate spores and ether for 2 min to kill remaining diploid cells (Dawes and Hardie, 1974).

Escherichia coli strains DH5α (Hanahan, 1983) HB101, (Boyer and Roulland-Dussoix, 1969), and TG1 (Sambrook et al., 1989) were used for the propagation of all plasmids and phage. E. coli strain AB1886/pGW249 was used for ultraviolet mutagenesis. Phage M13mp18 (Norrander et al., 1983) was used to generate single-stranded template DNA for sequence determination. E. coli cells were cultured in Luria broth or YT medium and transformed, or infected with M13 by standard methods (Maniatis et al., 1982).

**Ultraviolet Mutagenesis**

The method used for ultraviolet mutagenesis of the PKC1-bearing plasmid was modified from one developed by T. Davis (University of Washington, Seattle, WA, personal communication). Plasmid YCP50[PKC1] (10 μg/ml; Levin et al., 1990) was irradiated with 53 J/m² of 254-nm light using a GE 15 W GIST5 germicidal bulb. This level of irradiation resulted in ~90% plasmid inactivation, as judged by transformation efficiency with strain AB1886/pGW249 (gift of T. Davis). AB1886/pGW249 was grown to OD₆₀₀ = 0.6 in LB medium containing 30 μg/ml kanamycin. The culture (10 ml) was centrifuged at 6,000 g for 5 min, and the pellet was washed and resuspended in 5 ml of λ diluent (100 mM NaCl, 20 mM Tris, pH 7.5, 1 mM MgSO₄, 0.01% gelatin). The cells were irradiated with 1.5 J/m² of 254-nm light to induce the error-prone DNA repair system mediated by pGW249 (Langerg et al., 1981), diluted (1:10 ml) in LB, and allowed to recover for 1 h at 37°C. This level of irradiation resulted in a reduction in plating efficiency of ~50%. The culture was harvested by centrifugation, resuspended in ice-cold 50 mM CaCl₂, and incubated at 0°C for 20 min. Cells were harvested and resuspended in 1 ml of 50 mM CaCl₂, and incubated at 0°C for 20 min with 2.5 μg of irradiated plasmid DNA. The suspension was heat shocked at 37°C for 2 min, diluted to 1 ml with LB, incubated at 37°C for 1 h, and spread on LB plates containing 50 μg/ml carbenicillin (200 μl of culture/plate). Plasmid DNA was recovered from ~3,000 transformants.

**Nucleic Acid Manipulations**

DNA was prepared from yeast strains by the method of Winston et al. (1983), and plasmid DNA was prepared from E. coli using the alkalali lysis method (Maniatis et al., 1982). Plasmid-borne pkcl sequences were recovered from yeast DNA preparations using polymerase chain reactions (PCR; 100 ng of PsI-digested yeast DNA per reaction). PCR was carried out using a Gene-Amp kit (Perkin Elmer Cetus Instruments, Norwalk, CT) following the manufacturer’s procedure. 30 cycles of the following thermal cycling profile was performed using a DNA Thermal Cycle (Perkin Elmer Cetus Instruments): 1 min at 42°C (annealing), 2 min at 72°C (extension), and 1 min at 94°C (denaturation). One of the oligonucleotide primers (17-mer) in each reaction was designed to hybridize within the region of PKC1 that was deleted from the chromosomal copy. This resulted in selective amplification of the plasmid-borne copies of pkcl. The entire PKC1 locus was recovered on three PCR-generated fragments. PCR products were treated with Klenow fragment to create blunt ends, and the resulting fragments were isolated from agarose gels for ligation into the SmaI site of M13mpl8.

DNA sequence analysis was conducted by theideoxy chain-termination method (Sanger et al., 1977) after subcloning of PCR-generated fragments into M13mpl8. In all cases in which an alteration from the wild-type sequence was identified, DNA fragments generated from duplicate PCR were subjected to sequence analysis. In no case was a PCR-generated mutation found. Oligonucleotide primers were synthesized by Operon Technologies, Inc. (Alameda, CA) for use in PCR experiments and DNA sequence determination.

**Photomicroscopy and Flow Cytometry**

Fluorescence microscopy was conducted using the DNA-staining dye 4',6-diamidino-2-phenylindole (DAPI) at 1 μg/ml. Cells were photographed using a 40× objective on a photomicroscope (Zeiss Universal; Carl Zeiss, Inc., Thornwood, NY). A selective ultraviolet filter (Carl Zeiss, Inc.) was used for viewing DAPI stained nuclei. Flow cytometry was used to determine the DNA content of yeast cells as described by Hutter and Eipel (1979). After fixing in 70% ethanol, cells were treated exhaustively with pancreatic RNAses A, stained with propidium iodide and analyzed for fluorescence using a flow cytometer (model EPICS 752; Coulter Corp., Hialeah, FL).

**Protein Synthesis**

Protein synthesis was measured in 1-ml aliquots of cultures grown in SD

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**Table I. S. cerevisiae Strains**

| Strain     | Genotype                                | Source                  |
|------------|------------------------------------------|-------------------------|
| EG123      | MATα leu2-3,112 ura3-52 trpl-1 his4 can1  | I. Herskowitz*          |
| 1783       | MATα EG123                               | I. Herskowitz           |
| 1788       | MATα/MATα isogenic diploid of EG123     | I. Herskowitz           |
| FL100      | MATα/MATα 1788 pkcl1A::LEU2/PKCI        | Levin et al., 1990      |
| FL103      | MATα EG123 pkcl1A::LEU2 (pGALL::PKC1)   | Levin et al., 1990      |
| FL104      | MATα/MATα 1788 pkcl1A::LEU2/pkcl1A::LEU2 (pGALL::PKC1) | Levin et al., 1990 |
| FL106      | MATα EG123 pkcl1A::LEU2 (YCP50[PKC1])   | Levin et al., 1990      |
| DL106      | MATα EG123 pkcl1A::LEU2 (YCP50[PKC1])   | This study              |
| DL247      | MATα/MATα 1788 bck1A::URA3/BCK1         | Lee and Levin, 1991     |
| DL251      | MATα/MATα 1788 bck1A::URA3/bck1A::URA3  | Lee and Levin, 1991     |
| DL376      | MATα pkcl1A::LEU2                       | This study              |
| DL504      | MATα EG123 pkcl1A::LEU2 (YCP50[pkcl-3*]) | This study              |
| DL506      | MATα EG123 pkcl1A::LEU2 (YCP50[pkcl-2*]) | This study              |
| DL511      | MATα EG123 pkcl1A::LEU2 (YCP50[pkcl-1])  | This study              |
| DL519      | MATα/MATα 1788 pkcl1A::LEU2/pkcl2A::LEU2 (YCP50[pkcl-2*]) | This study |
| LR684-C    | MATα EG123 cdc35-10                      | K. Tatchell‡            |
| RY262      | MATα rpb1-1 ura3-52 his539*              | R. Young†               |
| X3119-12A  | MATα cly7-1 his6 ade2 trp                | Y. G. S. C.*            |
| HMSFI      | MATα sec1-1 SUC2 mal gal2 CUP1           | Y. G. S. C.             |

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and adjusted to 1 A600 U/ml. Cells were pulse labeled for 10 min with 50 nCi of l-(4,5-3H)leucine (0.5 Ci/mmol; Amersham Corp., Arlington Heights, IL). Incorporation was stopped after 10 min with 1 ml of 20% TCA. Samples were boiled for 5 min to hydrolyse RNA, and labeled material was allowed to precipitate for 1 h at 0°C. The insoluble material was collected by filtration through filters (GF/B, Whatman Inc., Clifton, NJ), which were washed four times with cold TCA and dried, and then the radioactivity was measured using a Beckman LS500TD Liquid Scintillation Counter (Beckman Instruments, Inc., Fullerton, CA).

**Cell Lysis**

Cells were labeled for 12 h at 25°C with 1 μCi/ml of [5,6-3H] uridine (47 Ci/mmol for Ura⁺ strains, 10 mCi/mmol for LR864-C; Amersham Corp.) in SD medium. Labeled cultures were washed four times with fresh SD by centrifugation at 3,000 g for 3 min. Cells were resuspended in SD with an excess (50 μg/ml) of uridine to quench uptake of labeled uridine after release. Cultures of conditional mutants (and controls) were shifted to 37°C and aliquots were removed at various times. Cells were removed by centrifugation and samples from the supernatant fractions were spotted onto Whatman 3MM paper for liquid scintillation counting. The fraction of labeled material released into the medium was determined by dividing the dpm in the supernatant by the dpm in the sample before centrifugal removal of the cells. For measurement of immediate cell lysis, a pkclΔ::LEU2 mutant (DL376) and a PKCI+ control strain (FL106) were labeled in SD supplemented with 1 M sorbitol, washed with SD sorbitol, and resuspended in SD. Cells were removed as above, and the radioactive material remaining in the supernatant fraction was measured.

**Results**

Mammalian isozymes of PKC are organized as two-domain proteins (Parker et al., 1986; Coussens et al., 1986). They possess a catalytic domain (~30 kD), highly conserved among all protein kinases, and a unique amino-terminal regulatory domain, which is responsible for binding activating cofactors. The S. cerevisiae PKCI gene encodes a homolog of the Ca²⁺-dependent isozymes of mammalian PKC that is essential for yeast cell growth and division (Levin et al., 1990). We isolated conditional pkcl mutants for use in two lines of experimentation. First, conditional alleles would allow further examination of the growth and division deficit associated with loss of PKCI function. Second, conditional regulatory mutants of PKCI, deficient in activation of the encoded protein kinase, would allow an examination of structure/function relationships within the regulatory domain. A screen for pkcl mutants was devised to include such activation-deficient alleles.

**Isolation of Conditional pkcl Alleles**

Conditional alleles of PKCI were isolated after ultraviolet mutagenesis of a centromere plasmid bearing the PKCI gene (YCp50[PCKI]). A mutagenized population of YCp50[PCKI] (~3,000 independent members) was used to transform a diploid strain that is heterozygous for the pkclΔ::LEU2 mutation (FL100). The resulting transformants (~12,000) were collected and induced to sporulate. After the remaining diploids were killed (see Materials and Methods), spores were allowed to germinate at 26°C on minimal medium supplemented with 100 mM CaCl₂ and 2 mM ZnCl₂, but lacking leucine. This selection allowed colonies to arise only from haploid spores bearing the lethal pkclΔ::LEU2 mutation complemented by a plasmid-borne allele of PKCI. The germination conditions were designed to be permissive for at least three types of pkcl mutants. Specifically, germination was induced at low temperature to allow temperature-sensitive mutants to arise; CaCl₂ was included to provide permissive conditions for mutants deficient in Ca²⁺ binding; and ZnCl₂ was included to support the growth of mutants with defective zinc-finger structures (Johnston, 1987; proposed to be required for DAG binding (Ono et al., 1989a). Colonies arising under permissive conditions were replicate plated onto rich medium under restrictive conditions (36°C; no CaCl₂ or ZnCl₂). Among ~3,000 colonies screened, three mutants failed to grow under restrictive conditions. These mutants were back crossed to an isogenic PKCI+ strain (EG123 or 1783) for meiotic segregation analysis. In each case the conditional defect always and exclusively cosegregated with the combination of the pkclΔ::LEU2 mutation and YCp50[PCKI], indicating the presence of recessive defects in the plasmid-borne PKCI gene.

The behavior of the three conditional pkcl mutants was examined under various growth conditions (Fig. 1). A strain carrying pkcl-1 (DL511) grew only in the presence of exogenous CaCl₂—MgCl₂ at the same concentration did not support growth of this mutant. Strains carrying pkcl-2 (DL506) or pkcl-3 (DL504) were temperature sensitive for growth, but their growth defects at the restrictive temperature were suppressed by CaCl₂. The lowest concentration of CaCl₂ required to suppress each of the three mutants was ~25 mM, but the efficacy of suppression increased with increasing CaCl₂ concentrations up to 100 mM. MgCl₂-containing medium poorly supported growth of the pkcl-2 mutant. Neither ZnCl₂ at 2 mM nor MnCl₂ at 10 mM (the maximum tolerated concentrations) suppressed any of the pkcl mutants (not shown). A strain whose only functional copy of PKCI is under the inducible control of the GALI promoter (FL103) fails to grow on glucose-containing medium in response to depletion of the PKCI gene product (Levin et al., 1990). This strain was not rescued by CaCl₂ (Fig. 1), indicating that Ca²⁺ remediation of the conditional pkcl mutants is dependent on expression of the mutant alleles.

Molecular clones of the mutant pkcl alleles were recovered from the plasmids and subjected to DNA sequence analysis. Each allele differed from the PKCI+ gene by a single nucleotide change, which in each case was located within the region predicted to encode the catalytic domain of the putative protein kinase (Fig. 2). The pkcl-1 allele carries an A to T transversion at nucleotide position 2501, which results in replacement of Tyr 834 with His. This change is within the predicted ATP-binding site of the PKCI-encoded protein kinase. The pkcl-2 allele carries a C to T transition at nucleotide position 2660, which results in replacement of Thr887 with Leu. This change is within the predicted ATP-binding site of the PKCI-encoded protein kinase. The pkcl-3 allele carries a G to A transversion at nucleotide position 2660, which results in replacement of Leu887 with Ser. Only hydrophobic residues occupy this position in known protein kinases (with the single exception of cdc2*, which possesses a cysteine).

Cells depleted of the PKCI gene product arrest growth and division with a uniform terminal phenotype (Levin et al., 1990), a behavior that indicates a defect in the cell division cycle. We compared the terminal phenotypes of the conditional pkcl mutants to that of PKCI-depleted cells (strain FL104). In contrast to PKCI depletion, which results in arrest after two or three rounds of cell division (Levin et al., 1990), the conditional pkcl mutants all displayed "first-cycle"
arrest (data not shown). Like PKCI-depleted cells, the three conditional pkcl mutants arrested growth with small buds and single nuclei (Fig. 3). The arrest phenotype was somewhat less uniform for the conditional mutants (70-75% small-budded cells) than for the PKCI-depleted cells (92% small-budded cells), the former arresting with a relatively high frequency (25-30%) of unbudded cells. Additionally, the bud sizes of cells arrested by conditional pkcl mutations appeared to be slightly smaller than those of PKCI-depleted cells. The reduced level of morphological uniformity among arrested pkcl cells was corroborated by flow cytometric analysis of the DNA content of these cells. Whereas PKCI-depleted cells arrested nuclear division uniformly after DNA replication (~90%; Levin et al., 1990), the conditional pkcl mutants ceased division with only 67-73% postreplicative nuclei (data not shown).

Loss of PKCI Function Results in Cessation of Protein Synthesis and Rapid Loss of Viability

Because the conditional pkcl mutants ceased growth and cell division under restrictive conditions, it was of interest to determine if protein synthesis continues upon arrest. Logarithmically growing cultures were pulse labeled with 3H-leucine at various times after shift to the restrictive temperature. A pkcl-2 mutant (DL506) ceased protein synthesis (<10% of the starting level of 3H-leucine incorporation) within 3 h of shift to the restrictive temperature (Fig. 4). A temperature-sensitive RNA polymerase II mutation (rpb1l in RY262) resulted in cessation of protein synthesis within 2 h after the shift. A secretion mutant (secl-1 in HMSFI) was used as a negative control. The temperature-sensitive secl-1 mutation results in rapid growth arrest, but protein synthesis continues unabated for several hours at the restrictive temperature (Novik and Schekman, 1979).

We also examined the viability of the conditional pkcl mutants after shift to the restrictive temperature. Cultures were shifted to 36°C for various times and plated for viability at the permissive temperature (26°C). The pkcl mutants displayed a rapid decline in viability upon shift to the restrictive temperature. After 3 h at 36°C, only 1-2% of the population was capable of forming colonies at 26°C (Fig. 5). This is in contrast to the slow decline in viability observed when mRNA synthesis was arrested with the rpb1l mutation (in RY262) - 39% of this population formed colonies after 8 h at 36°C. A similarly slow decline in viability was observed.

Figure 1. Suppression of the growth defects of conditional pkcl mutants with exogenous Ca2+. Cells were streaked onto YEP-glucose medium with the indicated supplement and incubated for 48 h at either the permissive temperature (26°C) or the restrictive temperature (36°C). CaCl2 or MgCl2 was present at 100 mM. Strains are (clockwise from top): FL103 (pkdAUEU2 [pGAL1::PKQ]); DL511 (pkd1); DL506 (pkd-2); DL504 (pkd-3); DLI06 (PKCI+).
when protein synthesis was inhibited in wild-type cells (EG123) using 20 μg/ml cycloheximide (data not shown). These results suggest that the pkcI-associated defect is more detrimental than would be accounted for simply by diminution of RNA or protein synthesis.

pkcI Mutants Exhibit a Cell Lysis Defect

Deletion of a gene corresponding to a dominant suppressor of pkcI mutations (designated BCKI) results in a temperature-sensitive cell lysis defect (Lee and Levin, 1992). Although cell lysis was not microscopically evident in growth arrested pkcI cells, a lysis defect could explain the cessation of protein synthesis and coincident loss of viability of the conditional mutants. To test the possibility that pkcI mutants lyse under restrictive conditions, we examined the release of RNA at the restrictive temperature from cells labeled with [3H]uridine. A pkcI-2 mutant (DL519) released 24% of its radioactive material into the medium by 3 h after shift to the restrictive temperature (Fig. 6). Lysis of a bckIΔ::URA3 mutant (DL251) was more rapid and extensive, reaching a plateau of 42% after 2 h. A known cell lysis mutant, cly7-1 (X3119-12A), released labeled material more slowly and for a longer period (6 h) than did the pkcI-2 mutant. A strain that carries a temperature-sensitive mutation in adenylate cyclase (cdc35-10) in strain LR684-C was used as a negative control. Mutants in CDC35 arrest growth and cell division at G1 (Pringle and Hartwell, 1981). The cdc35-10 mutant did not release more than 7% of its labeled material into the medium at any time up to 8 h after shift to the restrictive temperature.

The temperature-sensitive lysis defect associated with the bckIΔ::URA3 deletion is suppressed by the addition of osmotic stabilizers to the medium (Lee and Levin, 1992). Missense mutations in a variety of genes that are not involved in osmotic stability are suppressed by osmotic stabilizing agents, presumably through osmotic support of unstable mutant proteins (Hawthorne and Friis, 1964). Therefore, osmotic remediation of a mutant may be taken as evidence of a cellular osmotic stability defect only if the mutant does not produce a defective protein that might be subject to osmotic stabilization. We tested the ability of cells bearing the pkcIΔ::LEU2 mutation to form colonies on medium supplemented with osmotic stabilizers. A diploid that is heterozygous for the pkcIΔ::LEU2 mutation (FL100) was induced to sporulate and tetrads were dissected on rich medium supplemented with 1 M sorbitol. Haploid pkcIΔ::LEU2 segregants gave rise to colonies at 30°C on this medium (Fig. 7). Suppression of the pkcIΔ::LEU2 mutation (DL376) could also be achieved by substitution of sorbitol with a variety of monosaccharides at 1 M (e.g., glucose or galactose), disaccharides at 0.5 M (e.g., lactose or maltose), 10% polyethylene glycol, or saltsthat could be incorporated into the medium at a concentration of at least 0.5 M without causing toxicity (e.g., NaCl and KCl; Fig. 7).

Cells deleted at PKC1 underwent rapid cell lysis upon shift to medium lacking osmotic stabilizers. Strain DL376, grown in medium supplemented with 1 M sorbitol, was diluted into medium lacking sorbitol and plated for viability on sorbitol-containing medium at various times. Within the first three min, 80% of the population had lost viability as compared with cells diluted into sorbitol-containing medium. This loss of viability was accompanied by release of 52% of the radioactive material from cells labeled with [3H]uridine (as compared with 7% for the isogenic PKC1+ strain, 1783).
Figure 3. Arrest phenotype of conditional pkcl mutants. Photomicrographs of PKCl-depleted cells (FL104; top) and pkcl-2\(^e\) cells (DL519; bottom). Strain FL104 was shifted from logarithmic growth in YEP-galactose to YEP-glucose at 30°C for 8 h. Strain DL519 was shifted from growth in YEP-glucose at 26°C to 36°C for 4 h. DNA was stained with DAPI and cells were photographed under phase or ultraviolet fluorescence light sources using a 40× objective.

Figure 4. Rate of protein synthesis of a temperature-sensitive pkcl mutant as a function of time at the restrictive temperature. Cells were grown in minimal medium at 26°C and shifted to 36°C at time zero. Aliquots were taken at the times indicated, and labeled with [\(^{1}\)H]leucine for 10 min. Strains were DL106 (wild type; o), DL506 (pkcl-2; △), RY262 (rpbl-1; ▲); and HMS11 (sec1-1; ●).

Figure 5. Viability of temperature-sensitive pkcl mutants as a function of time at the restrictive temperature. Cells were grown in YEP-glucose medium at 26°C and shifted to 36°C for the indicated times. Aliquots were diluted and spread onto YEP-glucose plates at the permissive temperature and colonies were counted after 48 h. Strains were DL106 (wild type; o), DL504 (pkcl-3; △), DL506 (pkcl-2; ▲), and RY262 (rpbl-1; ●).
Figure 6. Cell lysis of a conditional pkcl mutant. Cells were labeled with [3H]uridine at 25°C, washed, and shifted to 37°C at time zero. Aliquots were taken at the times indicated and the amount of radioactive material released into the medium was measured. Strains were DL247 (wild type; ▲), DL519 (pkcl-2; ○), DL251 (bek1Δ:URA3; □), X3119-12A (cly7-1; ●), and LR684-C (cdc35-10; △).

remaining cells lost viability gradually over the next two hours (data not shown). Microscopic examination of pkclΔ::LEU2 cells after 3 min in medium lacking sorbitol revealed a high frequency of nonrefractile "ghosts" (~70%), a further indication of cell lysis. Nearly all of the lysed cells appeared to have been budded, with a variety of bud sizes represented.

Figure 7. Osmotic stabilizing agents suppress the growth defect of a pkcl deletion mutant. A PKCl+ strain (1783; left) and a pkclΔ::LEU2 strain (DL376; right) were streaked onto YEP-glucose plates with the indicated supplements and allowed to incubate at 30°C for 48 h. Sorbitol was present at 1 M; KCl or NaCl was at 0.5 M.

Discussion

Conditional Alleles of PKCI Are Suppressed by Ca2+

The S. cerevisiae PKCl gene encodes a homolog of the Ca2+-dependent isozymes of mammalian PKC that is essential for yeast cell growth and division. We used ultraviolet mutagenesis to generate conditional alleles of the PKCl gene. Three mutant alleles were isolated: one was dependent on exogenous CaCl2 for growth, and two were temperature sensitive for growth. The growth defects of the temperaturesensitive mutants were suppressed by exogenous CaCl2. Ca2+ was unique among divalent cations in its ability to rescue these conditional mutants. Because the regulatory domain of PKC isozymes binds activating cofactors (Parker et al., 1986; Coussens et al., 1986), we anticipated that regulatory domain mutants deficient in activation of the protein kinase, might be among the CaCl2 remedial alleles of PKCl isolated. However, all three conditional mutants carry their mutations at different sites within the catalytic domain. Most surprisingly, the CaCl2-dependent allele carries a base substitution mutation within the region predicted to encode the ATP-binding site of the catalytic domain.

Calcium remediation appears to be a universal feature of conditional PKCl mutations. Indeed, several independently isolated, temperature-sensitive pkcl mutants that are distinct from those described in this study, are all suppressed by exogenous CaCl2 (unpublished). Ca2+-remediation was dependent on expression of the mutant pkcl-encoded enzyme, indicating that Ca2+ augments the activity of the "crippled" enzyme, rather than bypassing the requirement for it. We propose that the mechanism of Ca2+-remediation of conditional pkcl mutants is through hyperactivation of the mutant enzyme to levels that allow an otherwise nonfunctional (but partially active) protein to carry out its assigned function. This activation may be direct, through Ca2+ binding to the regulatory domain; or it may be indirect, through (for example) stimulation of a Ca2+-activated phospholipase C (Chien and Cambier, 1990) to generate abnormally high concentrations of DAG.

Mutants in PKCI Display a Cell Cycle-Specific Osmotic Stability Defect

A yeast strain that conditionally expresses the PKCI gene, ceases growth and cell division with a uniform phenotype in response to depletion of the PKCI gene product, indicating a defect in the cell division cycle (Levin et al., 1990). Arrested cells have single, small buds and single nuclei in which the DNA has been replicated. The terminal phenotype of PKCI-depleted cells suggests a highly unusual cdc defect, because all previously described cdc mutants that arrest division after initiation of the cell cycle (i.e., blocked at any stage between Start and cytokinesis) continue cell growth after division has ceased (Johnston et al., 1977; Pringle and Hartwell, 1981).

Growth and division arrest of conditional pkcl mutants
resulted in a terminal phenotype that is very similar to that displayed by PKCI-depleted cells. This arrest was accompanied by cessation of protein synthesis, rapid loss of viability, and release of cellular material into the medium, suggesting cell lysis. We propose that conditional mutations in PKCI result in a cell cycle–specific osmotic stability defect. Because pkcl cells ceased growth and division with a uniform phenotype, the defect is apparently initiated at a specific point in the cell cycle. The small-budded arrest is proposed to result from manifestation of the defect at the time of bud emergence. Arrest of the nuclear cycle after DNA replication, but before mitosis, can be explained as follows: because initiation of DNA synthesis normally precedes bud emergence (Rivin and Fangman, 1980), and completion of replication (once initiated) does not require protein synthesis (Hereford and Hartwell, 1973; Burke and Church, 1991), loss of cellular integrity at the time of bud emergence might not interfere with DNA replication. However, since protein synthesis is required for the initiation of mitosis (Burke and Church, 1991), nuclear arrest would result after completion of DNA replication but before mitosis.

The pkcl-associated defect appears to be specifically manifested in budded cells. A pkcl deletion mutant was able to proliferate only in the presence of osmotic stabilizing agents. pkcl-deleted cells that possessed buds of any size underwent immediate lysis upon transfer to medium lacking osmotic stabilizers. This is in contrast to the small-budded arrest observed of PKCI-depleted cells or conditional pkcl mutants. Expression of the defect associated with PKCI depletion requires turnover of the PKCI-encoded protein. Likewise, expression of the defect in conditional pkcl mutants presumably requires turnover of phosphorylated substrates of the PKCI-encoded protein kinase. In contrast, expression of the defect associated with deletion of PKCI requires no such decay period. Therefore, cells deleted at PKCI should cease growth and division immediately upon reaching the point in the cell cycle at which PKCI is required (i.e., the execution point). Because cells bearing a pkcl deletion lysed immediately in the absence of osmotic stabilizing agents, regardless of the size of their buds, it seems likely that there is not a single PKCI execution point. Perhaps multiple execution points exist, one being more sensitive than the others (i.e., bud emergence) to reduced levels of PKCI activity or levels of phosphorylated substrate. This could explain the difference in terminal phenotypes between PKCI-depleted cells (or conditional pkcl mutants) and the pkcl deletion mutant.

Precedents exist for the uniform arrest of cells with conditional mutations affecting processes required at multiple points in the cell cycle. For example, CDC63 is allelic to PRT1 (Hanic-Joyce, 1985), a gene encoding a protein synthesis initiation factor (Keierleber et al., 1986). Although protein synthesis is required at several points in the cell division cycle (Burke and Church, 1991), mutations in PRT1/CDC63 result in a uniform arrest at Start (Bedard et al., 1981). It has been proposed that this is because execution of Start is particularly sensitive to disruption by diminution in the rate of biosynthesis of particular polypeptides (Hanic-Joyce et al., 1987). If the activity level of the mutant ptrl/cdc63-encoded initiation factor is either gradually reduced, or its elimination is incomplete (i.e., at a partially restrictive tempera-
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