Mutants in the *S. cerevisiae PKC1* Gene Display a Cell Cycle–Specific Osmotic Stability Defect

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Abstract. The *PKC1* gene of *Saccharomyces cerevisiae* encodes a homologue of the Ca^{2+}-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 *PKC1* 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, *PKC1*-depleted cells arrest growth with small buds. We created conditional alleles of *PKC1* to explore the nature of this unusual cdc defect. In contrast to *PKC1*-depleted cells, all of the conditional *pkc1* mutants isolated were suppressed by the addition of $CaCl_2$ to the medium, suggesting that the mutant enzymes could be activated by Ca^{2+}. 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 *pkc1* 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 *pkc1* mutants.

MEMBERS of the family of phospholipid-dependent, serine/threonine-specific protein kinases known collectively as protein kinase C (PKC)[1], respond to extracellular signals that act through receptor-mediated hydrolysis of phosphatidylinositol-4,5-bisphosphate to diacylglycerol (DAG) and inositol-1,4,5-trisphosphate (IP_3) (Hokin, 1985). DAG serves as a second messenger to activate PKC (Takai et al., 1979; Kishimoto et al., 1980; Nishizuka, 1986, 1988), and IP_3 functions to mobilize Ca^{2+} from intracellular stores (Berridge and Irvine, 1984). Seven distinct subtypes of mammalian PKC have been reported (Nishizuka, 1988; Kikkawa et al., 1989), several of which (α, βI, βII, and γ) require Ca^{2+} for activity (Kishimoto et al., 1980), others (δ, ϵ, and ζ) do not (Ohno et al., 1988; Ono et al., 1988, 1989*b*).

Mammalian PKC is thought to play a pivotal role in the regulation of a host of cellular functions through its activation by growth factors and other agonists. These functions include cell growth and proliferation (Rosengurt et al., 1984; Kaibuchi et al., 1985; Persons et al., 1988), release of various hormones (Negro-Vilar and Lapetina, 1985; Ohmura and Friesen, 1985), and control of ion conductance channels (Madison et al., 1986; Farley and Auerbach, 1986). Indirect evidence suggests that PKC induces the transcription of a wide array of genes, including the proto-oncogenes c-*myc*, c-*fos*, and c-*sis* (Kelly et al., 1983; Coughlin et al., 1985; Greenberg and Ziff, 1984; Kruijer et al., 1984; Colamonici et al., 1986), human collagenase (Angel et al., 1987), metallothionein II_A, and the SV-40 early genes (Imbra and Karin, 1986). Several transcription factors have been implicated in this response, including components of the AP-1 complex, AP-2, AP-3, and NF-κB (Lee et al., 1987; Imagawa et al., 1987; Chiu et al., 1987; Baeuerle and Baltimore, 1988). In at least one case (AP-2), it appears that activation of the transcription factor does not occur through direct phosphorylation by PKC (Luscher et al., 1989).

We reported previously the isolation of a gene from *Saccharomyces cerevisiae* that encodes a homolog of the α, β, and γ subtypes of mammalian PKC (*PKC1*; Levin et al., 1990). The *PKC1* gene is essential for cell growth and division. Here we report the isolation of conditional alleles of *PKC1* and the use of these alleles to characterize further the *pkc1*-associated defect. These studies revealed that loss of *PKC1* function results in a cell division cycle–specific osmotic stability defect.

Materials and Methods

Strains, Growth Conditions, and Transformations

All yeast strains used in this study (Table I) were derivatives of EG123, *MAT*α *leu2-3,112 ura3-52 trp1-1 his4 can1*^{r} (Siliciano and Tatchell, 1984),

1. *Abbreviations used in this paper*: DAG, diaglycerol; PCR, polymerase chain reactions; PKC, protein kinase C; YEP, 1% yeast, 2% bactopeptone.

except RY262, X3119-12A, and HMSF1. 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, asci 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 Roilland-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/m^2 of 254-nm light using a GE 15 W G15T8 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 OD_{600} = 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 $MgSO_4$, 0.01% gelatin). The cells were irradiated with 1.5 J/m^2 of 254-nm light to induce the error-prone DNA repair system mediated by pGW249 (Langer 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 $CaCl_2$, and incubated at 0°C for 20 min. Cells were harvested and resuspended in 1 ml of 50 mM $CaCl_2$, 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 10 ml with LB, incubated at 37°C for 1 h, and spread onto 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 alkali lysis method (Maniatis et al., 1982). Plasmid-borne *pkc1* sequences were recovered from yeast DNA preparations using polymerase chain reactions (PCR; 100 ng of *PstI*-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-mers) 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 *pkc1*. 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 M13mp18.

DNA sequence analysis was conducted by the dideoxy chain-termination method (Sanger et al., 1977) after subcloning of PCR-generated fragments into M13mp18. 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 RNAse 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

Table I. S. cerevisiae Strains

Strain	Genotype	Source
EG123	*MATα leu2-3,112 ura3-52 trp1-1 his4 can1*^{r}	I. Herskowitz‡
1783	*MAT***a** EG123	I. Herskowitz
1788	*MAT***a**/*MATα* isogenic diploid of EG123	I. Herskowitz
FL100	*MAT***a**/*MATα* 1788 *pkc1*Δ::*LEU2*/*PKC1*	Levin et al., 1990
FL103	*MATα* EG123 *pkc1*Δ::*LEU2* (p*GAL1*::*PKC1*)	Levin et al., 1990
FL104	*MAT***a**/*MATα* 1788 *pkc1*Δ::*LEU2*/*pkc1*Δ::*LEU2* (p*GAL1*::*PKC1*)	Levin et al., 1990
FL106	*MAT***a** EG123 *pkc1*Δ::*LEU2* (YCp50[*PKC1*])	Levin et al., 1990
DL106	*MATα* EG123 *pkc1*Δ::*LEU2* (YCp50[*PKC1*])	This study
DL247	*MAT***a**/*MATα* 1788 *bck1*Δ::*URA3*/*BCK1*	Lee and Levin, 1991
DL251	*MAT***a**/*MATα* 1788 *bck1*Δ::*URA3*/*bck1*Δ::*URA3*	Lee and Levin, 1991
DL376	*MAT***a** *pkc1*Δ::*LEU2*	This study
DL504	*MATα* EG123 *pkc1*Δ::*LEU2* (YCp50[*pkc1-3*^{ts}])	This study
DL506	*MATα* EG123 *pkc1*Δ::*LEU2* (YCp50[*pkc1-2*^{ts}])	This study
DL511	*MATα* EG123 *pkc1*Δ::*LEU2* (YCp50[*pkc1-1*])	This study
DL519	*MAT***a** /*MATα* 1788 *pkc1*Δ::*LEU2*/*pkc1*Δ::*LEU2* (YCp50[*pkc1-2*^{ts}])	This study
LR684-C	*MAT***a** EG123 *cdc35-10*	K. Tatchell§
RY262	*MATα rpb1-1 ura3-52 his539*^{am}	R. Young‖
X3119-12A	*MATα cly7-1 his6 ade2 trp*	Y. G. S. C.*
HMSF1	*MAT***a** *sec1-1 SUC2 mal gal2 CUP1*	Y. G. S. C.

* Y. G. S. C., Yeast Genetic Stock Center, Berkeley, CA.
‡ University of California, San Francisco, CA.
§ North Carolina State University, Raleigh, NC.
‖ Massachusetts Institute of Technology, Cambridge, MA.

and adjusted to 1 A_{600} U/ml. Cells were pulse labeled for 10 min with 50 nCi of L-(4,5-^{3}H)leucine (0.5 Ci/mol; Amersham Corp., Arlington Heights, IL). Incorporation was stopped after 10 min with 1 ml of 20% TCA. Samples were boiled for 5 min to hydrolyze tRNA, 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-^{3}H] uridine (47 Ci/mmol for Ura$^+$ strains, 10 mCi/mmol for LR684-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 *pkc1*Δ::*LEU2* mutant (DL376) and a *PKC1*$^+$ 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 PKC1* gene encodes a homolog of the Ca^{2+}-dependent isozymes of mammalian PKC that is essential for yeast cell growth and division (Levin et al., 1990). We isolated conditional *pkc1* mutants for use in two lines of experimentation. First, conditional alleles would allow further examination of the growth and division defect associated with loss of *PKC1* function. Second, conditional regulatory mutants of *PKC1*, deficient in activation of the encoded protein kinase, would allow an examination of structure/function relationships within the regulatory domain. A screen for *pkc1* mutants was devised to include such activation-deficient alleles.

Isolation of Conditional pkc1 Alleles

Conditional alleles of *PKC1* were isolated after ultraviolet mutagenesis of a centromere plasmid bearing the *PKC1* gene (YCp50[*PCK1*]). A mutagenized population of YCp50[*PKC1*] (~3,000 independent members) was used to transform a diploid strain that is heterozygous for the *pkc1*Δ::*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 $CaCl_2$ and 2 mM $ZnCl_2$, but lacking leucine. This selection allowed colonies to arise only from haploid spores bearing the lethal *pkc1*Δ::*LEU2* mutation complemented by a plasmid-borne allele of *PKC1*. The germination conditions were designed to be permissive for at least three types of *pkc1* mutants. Specifically, germination was induced at low temperature to allow temperature-sensitive mutants to arise; $CaCl_2$ was included to provide permissive conditions for mutants deficient in Ca^{2+} binding; and $ZnCl_2$ 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., 1989*a*). Colonies arising under permissive conditions were replicate plated onto rich medium under restrictive conditions (36°C; no $CaCl_2$ or $ZnCl_2$). Among ~3,000 colonies screened, three mutants failed to grow under restrictive conditions. These mutants were back crossed to an isogenic *PKC1*$^+$ strain (EG123 or 1783) for meiotic segregation analysis. In each case the conditional defect always and exclusively cosegregated with the combination of the *pkc1*Δ::*LEU2* mutation and YCp50[*PKC1*], indicating the presence of recessive defects in the plasmid-borne *PKC1* gene.

The behavior of the three conditional *pkc1* mutants was examined under various growth conditions (Fig. 1). A strain carrying *pkc1-1* (DL511) grew only in the presence of exogenous $CaCl_2-MgCl_2$ at the same concentration did not support growth of this mutant. Strains carrying *pkc1-2* (DL506) or *pkc1-3* (DL504) were temperature sensitive for growth, but their growth defects at the restrictive temperature were suppressed by $CaCl_2$. The lowest concentration of $CaCl_2$ required to suppress each of the three mutants was ~25 mM, but the efficacy of suppression increased with increasing $CaCl_2$ concentrations up to 100 mM. $MgCl_2$-containing medium poorly supported growth of the *pkc1-2* mutant. Neither $ZnCl_2$ at 2 mM nor $MnCl_2$ at 10 mM (the maximum tolerated concentrations) suppressed any of the *pkc1* mutants (not shown). A strain whose only functional copy of *PKC1* is under the inducible control of the *GAL1* promoter (FL103) fails to grow on glucose-containing medium in response to depletion of the *PKC1* gene product (Levin et al., 1990). This strain was not rescued by $CaCl_2$ (Fig. 1), indicating that Ca^{2+} remediation of the conditional *pkc1* mutants is dependent on expression of the mutant alleles.

Molecular clones of the mutant *pkc1* alleles were recovered from the plasmids and subjected to DNA sequence analysis. Each allele differed from the *PKC1*$^+$ 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 *pkc1-1* allele carries an A to T transversion at nucleotide position 2501, which results in replacement of Asn834 with Ile. This change is within the predicted ATP-binding site of the *PKC1*-encoded protein kinase. The *pkc1-2* allele carries a C to T transition at nucleotide position 3068, which results in replacement of Pro1023 with Leu. This mutation is at a position that is highly conserved as a proline residue among known protein kinases (Hanks et al., 1988). The *pkc1-3* allele carries a T to C transition 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 *PKC1* 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 *pkc1* mutants to that of *PKC1*-depleted cells (strain FL104). In contrast to *PKC1* depletion, which results in arrest after two or three rounds of cell division (Levin et al., 1990), the conditional *pkc1* mutants all displayed "first-cycle"

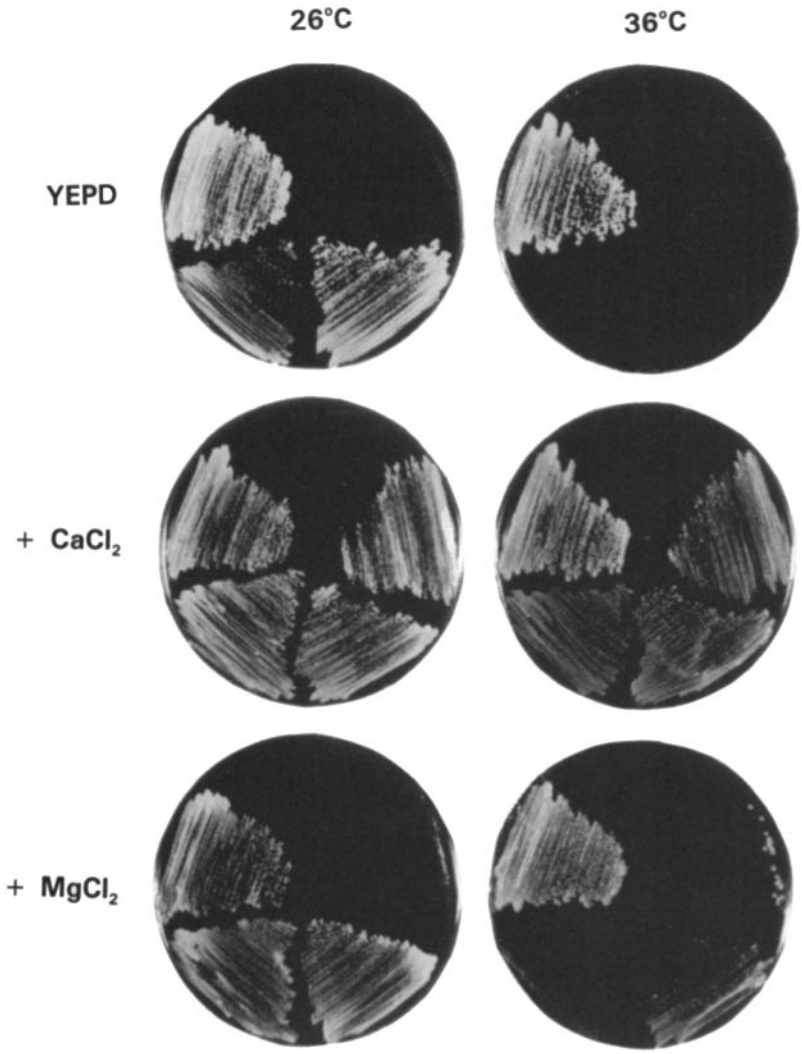

Figure 1. Suppression of the growth defects of conditional *pkcl* mutants with exogenous Ca^{2+}. Cells were streaked onto YEP-glucose medium with the indicated supplement and incubated for 48 h at either the permissive temperature (26°C) or the restrictive temperature (36°C). $CaCl_2$ or $MgCl_2$ was present at 100 mM. Strains are (clockwise from top): FL103 (*pkcl* Δ::*LEU2* [p*GAL1*::*PKCl*]); DL511 (*pkcl-1*); DL506 (*pkcl-2*); DL504 (*pkcl-3*); DL106 (*PKCl*$^+$).

arrest (data not shown). Like *PKCl*-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 *PKCl*-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 *PKCl*-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 *PKCl*-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 PKC1 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 ^{3}H-leucine at various times after shift to the restrictive temperature. A *pkcl-2* mutant (DL506) ceased protein synthesis (<10% of the starting level of ^{3}H-leucine incorporation) within 3 h of shift to the restrictive temperature (Fig. 4). A temperature-sensitive RNA polymerase II mutation (*rpb1-1* in RY262) resulted in cessation of protein synthesis within 2 h after the shift. A secretion mutant (*sec1-1* in HMSF1) was used as a negative control. The temperature-sensitive *sec1-1* 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 *rpb1-1* mutation (in RY262)—39% of this population formed colonies after 8 h at 36°C. A similarly slow decline in viability was observed

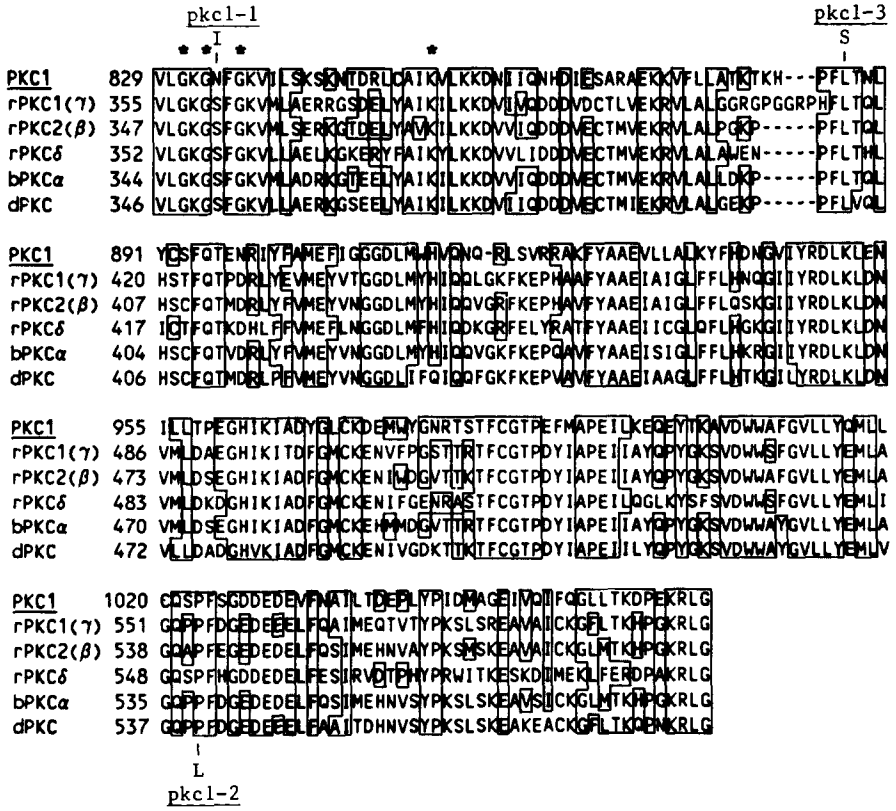

Figure 2. Conditional mutations within the catalytic domain of *PKC1*. Asterisks indicate conserved residues within the ATP-binding region. Sequences for the PKC isozymes can be found in the following citations: *PKC1* (Levin et al., 1990); rat isozymes, *rPKC1* (γ), *rPKC2* (β) (Knopf et al., 1986), and *rPKCδ* (Ono et al., 1988); bovine isozyme, *bPKCα* (Parker et al., 1986); and *D. melanogaster* isozyme, dPKC (Rosenthal et al., 1987).

when protein synthesis was inhibited in wild-type cells (EG123) using 20 μg/ml cycloheximide (data not shown). These results suggest that the *pkc1*-associated defect is more detrimental than would be accounted for simply by diminution of RNA or protein synthesis.

pkc1 Mutants Exhibit a Cell Lysis Defect

Deletion of a gene corresponding to a dominant suppressor of *pkc1* mutations (designated *BCK1*) results in a temperature-sensitive cell lysis defect (Lee and Levin, 1992). Although cell lysis was not microscopically evident in growth arrested *pkc1* 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 *pkc1* mutants lyse under restrictive conditions, we examined the release of RNA at the restrictive temperature from cells labeled with [^{3}H]uridine. A *pkc1-2* 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 *bck1*Δ::*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 *pkc1-2* 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 *bck1*Δ::*URA3* deletion is suppressed by the addition of osmotic stabilizers to the medium (Lee and Levin, 1992). Missence 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 *pkc1*Δ::*LEU2* mutation to form colonies on medium supplemented with osmotic stabilizers. A diploid that is heterozygous for the *pkc1*Δ::*LEU2* mutation (FL100) was induced to sporulate and tetrads were dissected on rich medium supplemented with 1 M sorbitol. Haploid *pkc1*Δ::*LEU2* segregants gave rise to colonies at 30°C on this medium (Fig. 7). Suppression of the *pkc1*Δ::*LEU2* mutation (in 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 salts that 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 [^{3}H]uridine (as compared with 7% for the isogenic *PKC1*$^+$ strain, 1783). The

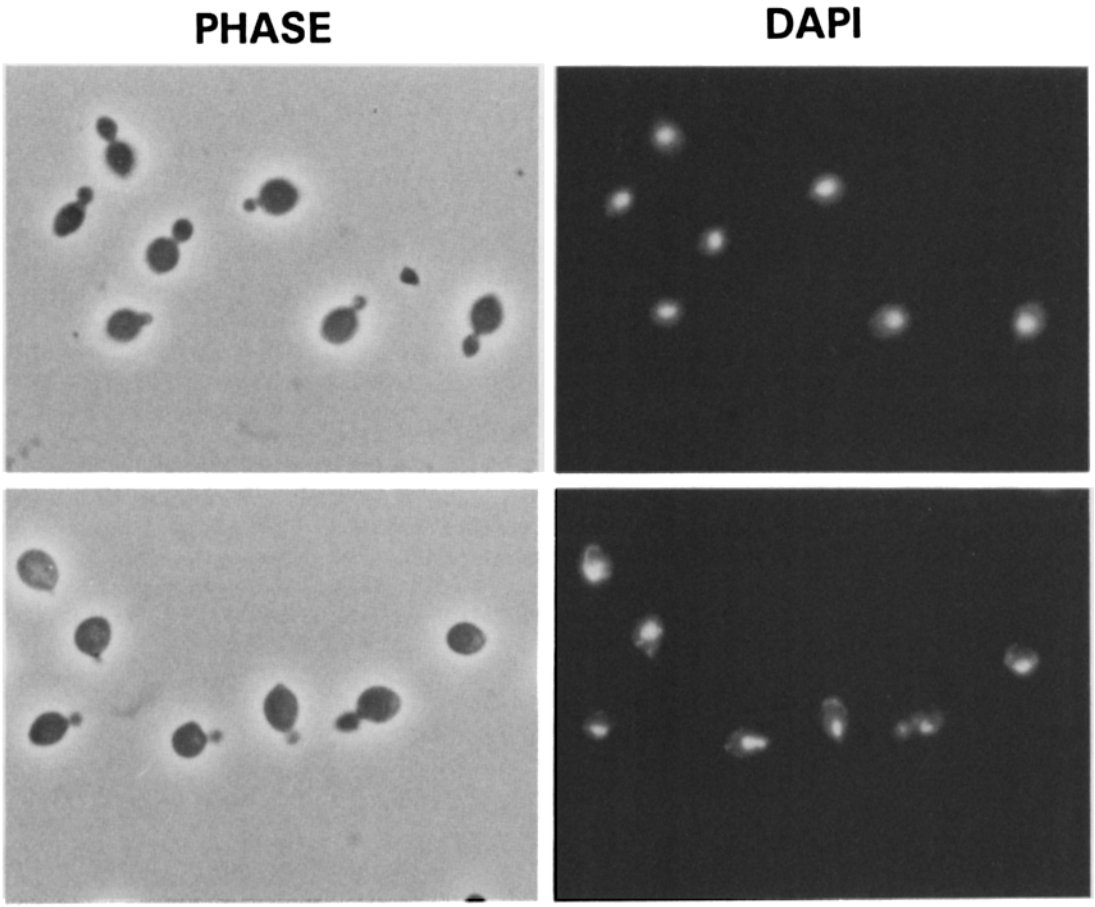

Figure 3. Arrest phenotype of conditional *pkcl* mutants. Photomicrographs of *PKCl*-depleted cells (FL104; *top*) and *pkcl-2*^{ts} cells (DL519; *bottom*). Strain FL104 was shifted from logarithmic growth in YEP-galactose to YEP-glucose at 30°C for 8 h. Strain DL519 was shifted from growth in YEP-glucose 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.

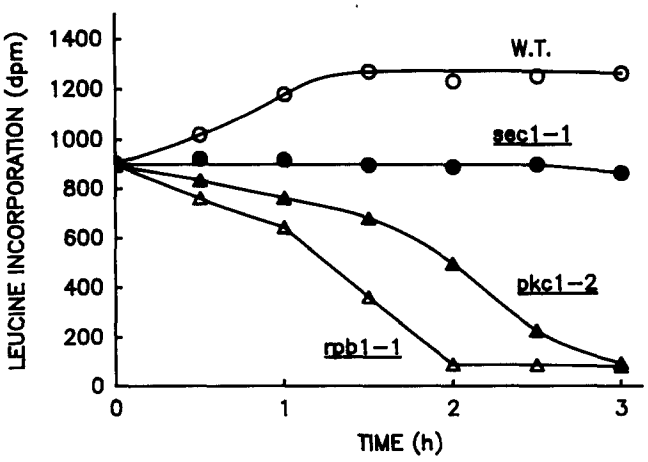

Figure 4. Rate of protein synthesis of a temperature-sensitive *pkcl* mutant as a function of time at the restrictive temperature. Cells were grown in minimal medium at 26°C and shifted to 36°C at time zero. Aliquots were taken at the times indicated, and labeled with [^{3}H]leucine for 10 min. Strains were DL106 (wild type; ○), DL506 (*pkcl-2*; ▲), RY262 (*rpbl-1*; △); and HMSF1 (*secl-1*; ●).

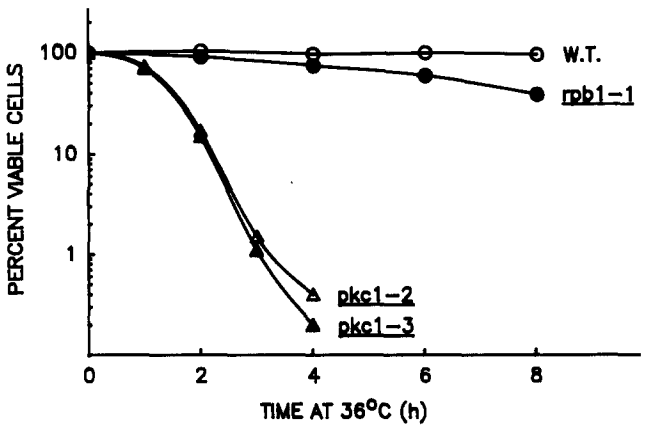

Figure 5. Viability of temperature-sensitive *pkcl* mutants as a function of time at the restrictive temperature. Cells were grown in YEP-glucose medium at 26°C and shifted to 36°C for the indicated times. Aliquots were diluted and spread onto YEP-glucose plates at the permissive temperature and colonies were counted after 48 h. Strains were DL106 (wild type; ○), DL504 (*pkcl-3*; ▲), DL506 (*pkcl-2*; △), and RY262 (*rpbl-1*; ●).

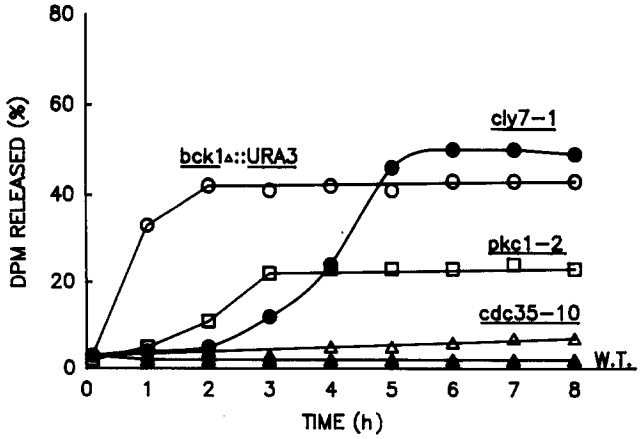

Figure 6. Cell lysis of a conditional *pkcl* mutant. Cells were labeled with [^{3}H]uridine at 25°C, washed, and shifted to 37°C at time zero. Aliquots were taken at the times indicated and the amount of radioactive material released into the medium was measured. Strains were DL247 (wild type; ▲), DL519 (*pkcl-2*; □), DL251 (*bck1Δ::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 *pkcl*Δ::*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.

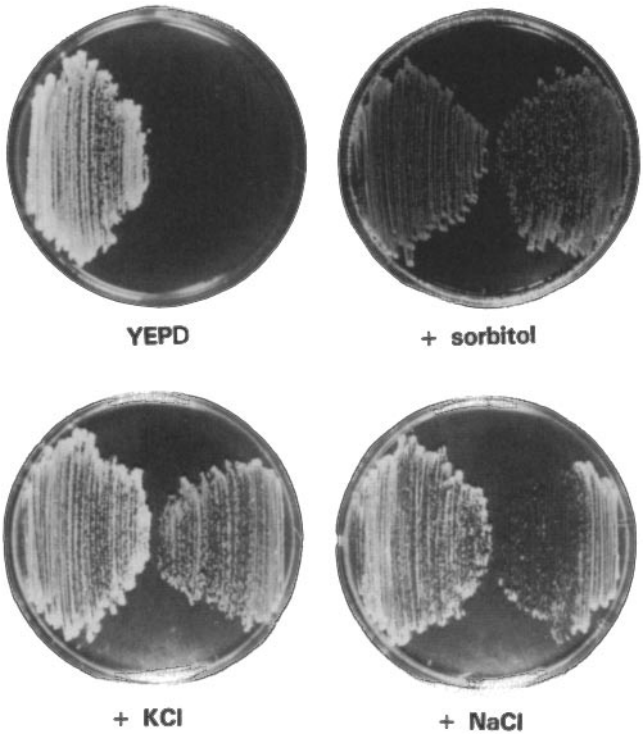

Figure 7. Osmotic stabilizing agents suppress the growth defect of a *pkcl* deletion mutant. A *PKC1*$^+$ strain (1783; *left*) and a *pkcl*Δ::*LEU2* strain (DL376; *right*) were streaked onto YEP-glucose plates with the indicated supplements and allowed to incubate at 30°C for 48 h. Sorbitol was present at 1 M; KCl or NaCl was at 0.5 M.

The cells that remained apparently intact after the first three minutes were almost exclusively unbudded (97%), suggesting that osmotic instability of the *pkcl*Δ::*LEU2* mutant manifests itself at the time of bud emergence and persists until cytokinesis.

Discussion

Conditional Alleles of PKC1 Are Suppressed by Ca^{2+}

The *S. cerevisiae PKC1* gene encodes a homolog of the Ca^{2+}-dependent isozymes of mammalian PKC that is essential for yeast cell growth and division. We used ultraviolet mutagenesis to generate conditional alleles of the *PKC1* gene. Three mutant alleles were isolated: one was dependent on exogenous $CaCl_2$ for growth, and two were temperature sensitive for growth. The growth defects of the temperature-sensitive mutants were suppressed by exogenous $CaCl_2$. Ca^{2+} 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 $CaCl_2$ remedial alleles of *PKC1* isolated. However, all three conditional mutants carry their mutations at different sites within the catalytic domain. Most surprisingly, the $CaCl_2$-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 *PKC1* mutations. Indeed, several independently isolated, temperature-sensitive *pkcl* mutants that are distinct from those described in this study, are all suppressed by exogenous $CaCl_2$ (unpublished). Ca^{2+}-remediation was dependent on expression of the mutant *pkcl*-encoded enzyme, indicating that Ca^{2+} augments the activity of the "crippled" enzyme, rather than bypassing the requirement for it. We propose that the mechanism of Ca^{2+}-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 Ca^{2+} binding to the regulatory domain; or it may be indirect, through (for example) stimulation of a Ca^{2+}-activated phospholipase C (Chien and Cambier, 1990) to generate abnormally high concentrations of DAG.

Mutants in PKC1 Display a Cell Cycle-Specific Osmotic Stability Defect

A yeast strain that conditionally expresses the *PKC1* gene, ceases growth and cell division with a uniform phenotype in response to depletion of the *PKC1* 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 *PKC1*-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 *PKC1*-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 *PKC1* result in a cell cycle–specific osmotic stability defect. Because *pkc1* 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 *pkc1*-associated defect appears to be specifically manifested in budded cells. A *pkc1* deletion mutant was able to proliferate only in the presence of osmotic stabilizing agents. *pkc1*-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 *PKC1*-depleted cells or conditional *pkc1* mutants. Expression of the defect associated with *PKC1* depletion requires turnover of the *PKC1*-encoded protein. Likewise, expression of the defect in conditional *pkc1* mutants presumably requires turnover of phosphorylated substrates of the *PKC1*-encoded protein kinase. In contrast, expression of the defect associated with deletion of *PKC1* requires no such decay period. Therefore, cells deleted at *PKC1* should cease growth and division immediately upon reaching the point in the cell cycle at which *PKC1* is required (i.e., the execution point). Because cells bearing a *pkc1* 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 *PKC1* execution point. Perhaps multiple execution points exist, one being more sensitive than the others (i.e., bud emergence) to reduced levels of *PKC1* activity or levels of phosphorylated substrate. This could explain the difference in terminal phenotypes between *PKC1*-depleted cells (or conditional *pkc1* mutants) and the *pkc1* 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 *prt1*/*cdc63*-encoded initiation factor is either gradually reduced, or its elimination is incomplete (i.e., at a partially restrictive temperature), cells may be able to traverse the cell cycle until they reach Start.

PKC1 Probably Functions in a Common Pathway with BCK1

The *S. cerevisiae BCK1* gene encodes a protein kinase that, when mutationally activated, bypasses the requirement for *PKC1* (Lee and Levin, 1992). Deletion of the *BCK1* gene results in a temperature-sensitive cell lysis defect that is specifically manifested in budded cells and is suppressible by osmotic stabilizing agents. The similarity of the defect associated with a *pkc1* deletion to that of a *bck1* deletion provides additional evidence that the protein kinases encoded by these genes function within a common pathway, perhaps playing a role in bud morphogenesis.

Previously identified cell lysis mutants may also function in the *PKC1*/*BCK1* pathway. Conditional mutations in the *CLY* genes result in temperature-sensitive cell lysis defects (Hartwell, 1967; Mortimer and Schild, 1985). Additionally, sorbitol-dependent fragile mutants undergo immediate cell lysis in the absence of osmotic stabilizers (Venkov et al., 1974). The systems perturbed by these mutations have not yet been identified. Perhaps they result in defects in cell wall metabolism. Mutations in the biosynthetic pathways for cell wall components could result in cell lysis defects; or perhaps more interestingly, mutations affecting the transport of cell wall components to their appropriate destinations could weaken the support structure.

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