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Dominant Mutations in a Gene Encoding a Putative Protein Kinase (*BCK1*) Bypass the Requirement for a *Saccharomyces cerevisiae* Protein Kinase C Homolog

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The *PKC1* gene of *Saccharomyces cerevisiae* encodes a homolog of mammalian protein kinase C that is required for yeast cell growth and division. To identify additional components of the pathway in which *PKC1* functions, we isolated extragenic suppressors of a *pkc1* deletion mutant. All of the suppressor mutations were dominant for suppressor function and defined a single locus, which was designated *BCK1* (for bypass of C kinase). A molecular clone of one suppressor allele, *BCK1-20*, was isolated on a centromere-containing plasmid through its ability to rescue a conditional *pkc1* mutant. The *BCK1* gene possesses a 4.4-kb uninterrupted open reading frame predicted to encode a 163-kDa protein kinase. The *BCK1* gene product is not closely related to any known protein kinase, sharing only 45% amino acid identity with its closest known relative (the *STE11*-encoded protein kinase) through a region restricted to its putative C-terminal catalytic domain. Deletion of *BCK1* resulted in a temperature-sensitive cell lysis defect, which was suppressed by osmotic stabilizing agents. Because *pkc1* mutants also display a cell lysis defect, we suggest that *PKC1* and *BCK1* may normally function within the same pathway. Suppressor alleles of *BCK1* differed from the wild-type gene in a region surrounding a potential PKC phosphorylation site immediately upstream of the predicted catalytic domain. This region may serve as a hinge between domains whose interaction is regulated by *PKC1*.

Members of the family of phospholipid-dependent, serine/threonine-specific protein kinases known collectively as protein kinase C (PKC) 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) (17). DAG serves as a second messenger to activate PKC (28, 40, 41, 65), and IP_3 functions to mobilize Ca^{2+} from intracellular stores (3). Seven distinct subtypes of mammalian PKC have been reported (27, 41), several of which (α, βI, βII, and γ) require Ca^{2+} for activity (28); others (δ, ε, and ζ) do not (44–46).

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 (25, 52, 56), release of various hormones (39, 43), and control of ion conductance channels (10, 36). 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* (8, 9, 12, 26, 29); human collagenase (1); metallothionein II_A; and the simian virus 40 early genes (21). Several transcription factors have been implicated in this response, including components of the AP-1 complex, AP-2, AP-3, and NF-κB (2, 7, 20, 31). In at least one case (AP-2), it appears that activation of the transcription factor does not occur through direct phosphorylation by PKC (35).

Although substantial progress has been made toward elucidating the pathways leading to PKC activation, the steps between this activation and subsequent nuclear events remain obscure. To dissect this signaling pathway further, it is useful to study the role(s) of PKC in systems that are amenable to rigorous genetic analysis. Deliberate mutation of a gene encoding an isozyme of PKC, followed by extragenic suppression of the resulting defect, is expected to reveal genes encoding other components of the pathway in which the PKC isozyme functions. 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* [33]). The *PKC1* gene is essential for cell growth and mitosis. Here we report the isolation of a class of dominant extragenic suppressor mutations that bypasses the requirement for *PKC1*. Molecular and genetic analysis of the suppressor locus, *BCK1*, revealed that it encodes a novel protein kinase required for osmotic stability at high temperature.

MATERIALS AND METHODS

Strains, growth conditions, and transformations. All yeast strains used in this study (Table 1) were derivatives of EG123, *MATα leu2-3,112 ura3-52 trp1-1 his4 can1*^{r} (62), except X3119-12A and those used for chromosomal mapping experiments. Yeast cultures were grown in YEP (1% yeast extract, 2% Bacto Peptone) supplemented either with 2% glucose or with 2% galactose plus 0.1% sucrose, as required. Synthetic minimal medium (SD [60]) supplemented with the appropriate nutrients was employed to select for plasmid maintenance and gene replacements. Yeast transformation was by the lithium acetate method (23). General genetic manipulation of yeast cells was carried out as described previously (60).

Bacterial strains DH5α (13), HB101 (5), and TG1 (58) were used for the propagation of all plasmids and phage. Phage M13mp18 and M13mp19 (42) were used to generate single-stranded template DNA for sequence determination. *Escherichia coli* cells were cultured in Luria broth or YT medium

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TABLE 1. *S. cerevisiae* strains

Strain	Genotype	Source or reference
EG123	*MATα leu2-3,112 ura3-52 trp1-1 his4 can1*^{r}	I. Herskowitz
1783	*MATa* EG123	I. Herskowitz
1788	*MATa/MATα* (isogenic diploid of EG123)	I. Herskowitz
FL100	*MATa/MATα* 1788 *pkc1Δ::LEU2/PKC1*	33
FL101	*MATa/MATα* 1788 *pkc1Δ::LEU2/PKC1* (p*GAL1::PKC1*)	33
FL102	*MATa* EG123 *pkc1Δ::LEU2* (p*GAL1::PKC1*)	33
FL105	*MATa/MATα* 1788 *pkc1Δ::LEU2/PKC1* (YCp50[*PKC1*])	33
FL106	*MATa* EG123 *pkc1Δ::LEU2* (YCp50[*PKC1*])	33
DL112	*MATa* EG123 *pkc1Δ::LEU2 BCK1-2*	This study
DL113	*MATa* EG123 *pkc1Δ::LEU2 BCK1-3*	This study
DL114	*MATa* EG123 *pkc1Δ::LEU2 BCK1-4*	This study
DL115	*MATa* EG123 *pkc1Δ::LEU2 BCK1-6*	This study
DL116	*MATa* EG123 *pkc1Δ::LEU2 BCK1-10*	This study
DL117	*MATa* EG123 *pkc1Δ::LEU2 BCK1-11*	This study
DL118	*MATa* EG123 *pkc1Δ::LEU2 BCK1-12*	This study
DL119	*MATa* EG123 *pkc1Δ::LEU2 BCK1-14*	This study
DL120	*MATa* EG123 *pkc1Δ::LEU2 BCK1-16*	This study
DL121	*MATa* EG123 *pkc1Δ::LEU2 BCK1-18*	This study
DL122	*MATa* EG123 *pkc1Δ::LEU2 BCK1-19*	This study
DL123	*MATa* EG123 *pkc1Δ::LEU2 BCK1-20*	This study
DL245	*MATa* EG123 *BCK1-20*	This study
DL247	*MATa/MATα* 1788 *bck1Δ::URA3/BCK1*	This study
DL248	*MATa/MATα* 1788 *pkc1Δ::LEU2/PKC1 BCK1*::pUC18[*URA3*]/*BCK1*	This study
DL250	*MATα* EG123 *BCK1*::pUC18[*URA3*]	This study
DL251	*MATa*/MATα 1788 *bck1Δ::URA3/bck1Δ::URA3*	This study
DL253	*MATα* EG123 *bck1Δ::URA3*	This study
DL283	*MATα* EG123 *pkc1Δ::LEU2 BCK1-20*	This study
DL327	*MATa/MATα* 1788 *bck1Δ::URA3/bck1Δ::URA3* (pRS314[*BCK1*])	This study
DL330	*MATa* EG123 *pkc1Δ::LEU2* (pRS314[*BCK1-20*])	This study
DL519	*MATa/MATα* 1788 *pkc1Δ::LEU2/pkc1Δ::LEU2* (YCp50[*pkc1-2ts*])	32
YPH149	*MATa ura3-52 lys2-801 his7 trp1-1* (Ade$^-$)	68
LR684-C	*MATa* EG123 *cdc35-10*	K. Tatchell
STX338-2C	*MATa cdc6-1 ura3 his7 leu1 ade1 trp1 tyr1 gal1*	Y.G.S.C.[a]
STX9-1A	*MATa arg3 ade2 gal2*	Y.G.S.C.
X3119-12A	*MATα cly7-1 his6 ade2* (Trp$^-$)	Y.G.S.C.

[a] Y.G.S.C., Yeast Genetic Stock Center, Berkeley, Calif.

(37). Transformation of *E. coli* with DNA and infection with M13 were done by standard methods (37).

Selection for plasmid loss. Selection for plasmid loss was imposed by using the uracil biosynthesis antagonist 5-fluoroorotic acid (5-FOA [4]). p*GAL1::PKC1*, which contains the uracil biosynthetic gene *URA3*, can be selected against by using 5-FOA. Mutants of FL102 (which carries p*GAL1::PKC1*) that were capable of growth on glucose-containing medium were patched onto 5-FOA-containing medium. FL102, used as a negative control, failed to papillate 5-FOA-resistant colonies, indicating its dependence on the plasmid for growth. The rate of p*GAL1::PKC1* loss in a strain that carries a chromosomal *PKC1*$^+$ gene (FL101) was approximately 10^{-3}, resulting in frequent papillation of 5-FOA-resistant colonies. Mutants of FL102 that were capable of papillating 5-FOA-resistant colonies were subjected to further analysis.

Nucleic acid manipulations. DNA was prepared from yeast strains by the method of Winston et al. (69), subjected to electrophoresis through agarose gels after digestion with restriction endonucleases, and transferred to nitrocellulose filters by standard procedures (64). Plasmids were rescued from genomic yeast DNA by transformation into *E. coli* DH5α. Plasmid DNA was prepared from *E. coli* by the alkali lysis method (37). Whole-cell RNA was prepared from proliferating *MATα* cells, fractionated by electrophoresis, and transferred to filters by the techniques described by Van Arsdell et al. (67).

The genomic library was constructed by using DNA partially digested with *Sau*3AI, followed by size fractionation of DNA fragments in a sucrose gradient (37). Fragments (10 to 20 kb) were cloned into the *Bam*HI site of dephosphorylated, centromeric plasmid pRS314 (61). The library was used to transform the temperature-sensitive strain DL519 (*pkc1Δ::LEU2* YCp50[*pkc1-2*]) to tryptophan prototrophy, followed by replicate plating of transformants onto YEP-glucose medium at restrictive temperature (34°C).

DNA sequence analysis was conducted by the dideoxy chain-termination method (59) following subcloning of restriction endonuclease fragments into M13mp18 and M13mp19. Oligonucleotide primers were synthesized by Operon Technologies, Inc. (Alameda, Calif.) for use in DNA sequence determination. The sequence shown in Fig. 3 was determined for both strands.

Nick-translated probes were labeled with [α-^{32}P]dATP (3,000 Ci/mmol) by *E. coli* DNA polymerase I. High-stringency hybridizations were carried out for 18 to 24 h at 42°C in 50% formamide–0.45 M NaCl–45 mM sodium citrate–4× Denhardt's solution–0.1% sodium dodecyl sulfate (SDS)–50 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES, pH 7.0)–200 μg of salmon testes DNA per ml. Reduced-stringency hybridizations were carried out for 12 to 15 h at 55°C without formamide in 0.9 M NaCl–90 mM sodium citrate; other components were present at the same concentrations as for high-stringency hybridizations. After hybridization, filters were washed twice in 0.3 M NaCl–30

mM sodium citrate–0.1% SDS for 20 min at 25°C and then once for 30 min at 42°C.

Gap repair. The 1.5-kb *Bgl*II fragment was excised from pRS314[*BCK1-20*], and the resulting plasmid was recircularized and passaged through *E. coli* DH5α before secondary digestion with *Bgl*II. This step ensured that no intact pRS314[*BCK1-20*] was present during the gap repair experiment. Strain 1783 (*BCK1*$^+$) was transformed with this linear gapped plasmid by selecting for tryptophan prototrophy. Integration of the plasmid sequences into the chromosomal locus was selected against because the plasmid carries a centromere. Gap-repaired plasmids were recovered from transformants and tested by restriction analysis for restoration of the missing *Bgl*II fragment. These plasmids were tested for suppressor function in strain DL519. The *BCK1*$^+$ gene was subcloned from pRS314[*BCK1*$^+$] into the multicopy plasmid pRS304 (which carries *TRP1* [61]) by using the *Xho*I and *Sst*I polylinker sites of both plasmids.

Integrative mapping of *BCK1*. The 2.4-kb *Eco*RI fragment of pRS314[*BCK1-20*], which carries the 5′-end of the putative *BCK1-20* locus, was cloned into a derivative of pUC18 harboring the *URA3* gene (inserted at the *Sma*I site). This construction was integrated by homologous recombination into the genome of a diploid strain that is heterozygous for the *pkc1*Δ::*LEU2* mutation (FL100). Integration of the plasmid at the genomic locus corresponding to the *Eco*RI fragment was confirmed by restriction and hybridization analysis, and the integrant was designated DL248. The integration event did not create a functional suppressor, as judged by the meiotic segregation pattern of viable haploids from DL248 (2:2). A Ura$^+$ segregant (DL250) was mated with the 12 *pkc1*Δ::*LEU2 BCK1* strains (DL112 to DL123) to examine the segregation pattern of the *URA3* gene with the *BCK1* mutations. A minimum of eight Leu$^+$ meiotic progeny from these crosses were examined for cosegregation of the Ura$^+$ marker.

***BCK1* gene replacement.** A deletion mutant allele of *BCK1* was constructed by the method of Rothstein (57). The C-terminal 1.5-kb *Bgl*II fragment of *BCK1* was cloned into the *Bam*HI site of a pUC18 derivative into which the *URA3* gene had been inserted at the *Sma*I site. The N-terminal 2.4-kb *Eco*RI fragment of *BCK1* was cloned into the *Eco*RI site of this construction in the same orientation (with respect to *BCK1*). The *BCK1* fragments in the resulting plasmid (pUC18[*bck1*Δ::*URA3*]) are separated by the *URA3* gene. A 4.9-kb *Sal*I fragment which carries the *bck1*Δ::*URA3* construction was isolated and used to transform a diploid strain (1788) by selecting for uracil prototrophy. Restriction and hybridization analysis of genomic DNA from the resulting transformants confirmed that transplacements had occurred at the *BCK1* locus. One such diploid transformant, heterozygous at the *BCK1* locus (*bck1*Δ::*URA3*/*BCK1*), was designated DL247.

Cell lysis. Cells were labeled for 12 h at 25°C with 1 μCi of [5,6-^{3}H]uridine per ml (47 Ci/mmol for Ura$^+$ strains and 10 mCi/mmol for LR684-C; Amersham) in SD. Labeled cultures were washed four times with fresh SD by centrifugation at 3,000 × *g* for 3 min. Cells were resuspended in SD containing an excess (50 μg/ml) of uridine to quench uptake of labeled uridine after release. Cultures were shifted to 37°C, and aliquots were removed at various times. The cells were removed by centrifugation, and samples from the supernatant fractions were spotted onto Whatman 3MM for counting. The amount of ^{3}H in the supernatant was measured with a Beckman LS500TD liquid scintillation counter. The fraction of labeled material released into the medium was determined by dividing the disintegrations per minute in the supernatant by the disintegrations per minute in the sample prior to centrifugal removal of the cells.

Chromosomal mapping of *BCK1*. *BCK1* was assigned to chromosome X by hybridization of a nick-translated probe, derived from *BCK1* (the 3.4-kb *Bam*HI fragment), to yeast chromosome-sized DNA. A strain designed for electrophoretic separation of chromosomal DNA (YPH149) by orthogonal field gel electrophoresis was used for this analysis (68); the nitrocellulose filter to which chromosomal DNAs had been transferred was the gift of Phil Hieter (The Johns Hopkins University).

Meiotic mapping allowed placement of the *BCK1* locus on chromosome X. Three markers were tested for linkage to the *URA3* gene integrated at the *BCK1* locus (DL250). For segregation of the *CDC6* and *CDC35* markers with respect to *URA3*, DL250 was crossed with STX338-2C and LR684-C, respectively. For segregation of *ARG3*, strain STX9-1A was crossed with EG123 for the purpose of constructing an *arg3-1 ura3-52* double mutant. An Arg$^-$ Ura$^-$ haploid segregant from this cross was mated with DL250 for meiotic mapping experiments.

Nucleotide sequence accession number. The EMBL accession number for *BCK1* is X60227.

RESULTS

Isolation of extragenic suppressors of a *pkc1* deletion mutant. To identify additional components of the pathway in which *PKC1* functions, we isolated extragenic suppressors of a *pkc1* deletion mutant. The rationale for this experiment was that mutations that bypass the requirement for *PKC1* may reside within genes that function in the same pathway as *PKC1*. We used a strain described previously (FL102 [33]) that carries a deletion at the chromosomal *PKC1* locus (*pkc1*Δ::*LEU2*) and a centromeric plasmid with *PKC1* fused to the inducible *GAL1* promoter (p*GAL1*::*PKC1*). Genes under the control of the *GAL1* promoter are induced by galactose but are repressed in the presence of glucose (24). Strain FL102 grows normally on galactose-containing medium, but it arrests growth and division on glucose-containing medium in response to the depletion of the *PKC1*-encoded protein. We selected spontaneous mutants of FL102 that could grow on glucose-containing medium. Three independent cultures, grown to saturation in YEP-galactose, were washed and spread on YEP-glucose plates (approximately 10^8 cells per plate). Approximately 300 colonies arose from this selection. We suspected that among these were mutants defective in the regulation of galactose metabolism (e.g., *GAL80* [48]), which inappropriately express *PKC1* from the *GAL1* promoter on glucose-containing medium. To eliminate such galactose regulatory mutants from the population, the ability of the glucose growers to survive loss of the plasmid bearing the *PKC1* gene was examined by selecting for resistance to 5-FOA (4) (see Materials and Methods). Twenty of the original 300 glucose-growing mutants of FL102 were not dependent on the *PKC1*-bearing plasmid for growth. Genomic DNA from each of these 20 mutants was subjected to hybridization analysis with a probe derived from *PKC1* to determine if they were truly growing in the absence of a functional *PKC1* gene. Twelve of these mutants possessed only the deleted allele of *PKC1* (Fig. 1), indicating the presence of extragenic suppressors of the *pkc1*Δ::*LEU2* mutation. These mutants represented a minimum of three independently isolated suppres-

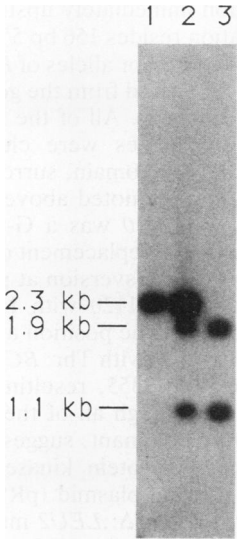

FIG. 1. Suppressors of a *pkc1* deletion bypass the requirement for *PKC1*. DNA (5 μg) was digested with *Eco*RI and prepared for hybridization with a nick-translated 2.3-kb *Eco*RI fragment from *PKC1*. Lanes: 1, DNA from the PKC1$^+$ diploid strain 1788; 2, DNA from the diploid strain FL100, which is heterozygous for the *pkc1* deletion (*pkc1*Δ::*LEU2*/*PKC1*$^+$); 3, DNA from the haploid strain DL112, 1 of 12 suppressor mutants of the *pkc1* deletion isolated. The hybridizing 1.9- and 1.1-kb *Eco*RI fragments result from the deleted allele and are due to cleavage at the single *Eco*RI site within the *LEU2* gene. The other 11 suppressor mutants (DL113 to DL123) displayed the same hybridization pattern as DL112.

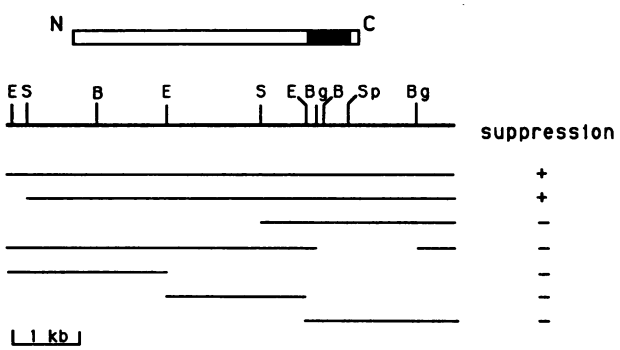

FIG. 2. Restriction and deletion maps of the *BCK1-20* suppressor locus. Deletions within the genomic DNA carried in pRS314[*BCK1-20*] were generated by restriction endonuclease digestion. In some cases, fragments were subcloned into pRS314. The ability (+) or inability (–) of the resulting plasmids to suppress a temperature-sensitive *pkc1* allele (DL519) is shown. The DNA fragments present in the plasmids are indicated. The box represents the predicted *BCK1*-encoded protein. The dark area indicates the putative catalytic domain. Abbreviations for selected restriction sites are as follows: B, *Bam*HI; Bg, *Bgl*II; E, *Eco*RI; S, *Sal*I; Sp, *Sph*I. N and C indicate the N terminus and C terminus of the protein, respectively.

sor mutations, which arose spontaneously at a frequency of between 10^{-8} and 10^{-7} per generation.

To test whether the ability of the suppressor mutations to rescue the *pkc1* deletion was dominant or recessive, we created diploid strains that were homozygous for the *pkc1*Δ::*LEU2* mutation and heterozygous for the suppressor mutations. Each of the 12 mutant strains described above was mated with a haploid strain (FL106) that carries the *pkc1*Δ::*LEU2* mutation and a centromeric plasmid-borne *PKC1* gene (YCp50[*PKC1*], which carries *URA3*). The resulting diploids were patched onto 5-FOA-containing medium to select for loss of the *PKC1*-bearing plasmid. All 12 diploids papillated 5-FOA-resistant colonies at a frequency comparable to that observed for a control strain that does not require the plasmid for growth (FL105). This result indicated that all of the suppressor mutant alleles were dominant to their wild-type alleles for suppressor function. An allelism test (described below) indicated that all 12 mutations were tightly linked to one another, suggesting that they all reside within the same locus. This locus was designated *BCK1* (for bypass of C kinase).

One representative mutant was selected for further analysis. Strain DL123, which carries the *pkc1*Δ::*LEU2* mutation and the *BCK1-20* suppressor, grew slowly on rich medium at 30°C (doubling time of 145 min compared with 90 min for the isogenic *PKC1*$^+$ *BCK1*$^+$ strain 1783). Upon shift to 37°C, DL123 ceased cell division in an apparently unsynchronized manner (not shown). To determine if this growth defect was the result of the dominant *BCK1-20* mutation or was a residual defect associated with the *pkc1*Δ::*LEU2* mutation, the effect of the *BCK1-20* mutation on growth was examined in a *PKC1*$^+$ background (DL245). Strain DL245 grew slowly at 30°C (110-min doubling time), but not as slowly as the double mutant. Additionally, DL245 was capable of growth at 37°C (180-min doubling time, compared with 100 min for strain 1783). These results indicate that the *BCK1-20* mutation and the *pkc1*Δ::*LEU2* mutation both contribute to the growth defect displayed by strain DL123.

Isolation of the *BCK1* locus. We isolated the *BCK1-20* locus for molecular and genetic characterization. A genomic library was constructed from a strain (DL123) that carries the *pkc1*Δ::*LEU2* mutation and the *BCK1-20* suppressor mutation in the *TRP1*, centromere-containing plasmid vector pRS314 (61). This library was used to transform a strain bearing a temperature-sensitive *pkc1* mutation (DL519 [32]) to tryptophan prototrophy, followed by a screen for growth at the restrictive temperature. Two transformants, from among 14,000 screened (approximately 10 genomic equivalents), were capable of growth at 34°C. The plasmids recovered from these yeast transformants contained identical 6.5-kb inserts (Fig. 2) and were both capable of suppressing the *pkc1*Δ::*LEU2* mutation. One of these plasmids, pRS314[*BCK1-20*], was subjected to further analysis. To localize the *BCK1* gene, a series of deletions was generated by restriction endonuclease digestion and tested for the ability to suppress the temperature-sensitive *pkc1* allele in DL519. Deletion analysis revealed that the region surrounding the 850-bp *Sal*I-*Bgl*II fragment (Fig. 2) was essential for suppressor function. The 850-bp *Sal*I-*Bgl*II restriction fragment was subcloned into M13mp18 and M13mp19 and subjected to DNA sequence analysis. This fragment possesses an open reading frame which spans the entire length of the subclone. Analysis of surrounding sequences revealed that this open reading frame extends for 4.4 kb (Fig. 2).

We showed by genetic analysis that the gene we had cloned truly corresponded to the *BCK1-20* locus. Using integrative transformation, we constructed a *BCK1*$^+$ strain whose only functional *URA3* gene was tightly linked to the wild-type (nonsuppressing) allele of the locus corresponding to the cloned DNA (DL250; see Materials and Methods). In crosses of DL250 to a *pkc1*Δ::*LEU2 BCK1-20* strain (DL123), the segregation pattern of *URA3* and *BCK1-20*

indicated complete linkage—among 35 surviving Leu$^+$ segregants (*pkc1*Δ::*LEU2 BCK1-20*; recovered from 35 tetrads), none were Ura$^+$. Strain DL250 was similarly used to test for allelism of *BCK1* to the other 11 suppressors. Results of these experiments indicated that all 12 suppressors are alleles of *BCK1* (data not shown).

BCK1 encodes a novel protein kinase. The uninterrupted open reading frame encoded by the *BCK1-20* locus corresponds to a polypeptide with a predicted length of 1,478 amino acids (calculated molecular size of 163 kDa; Fig. 3). This value assumes the use of the 5′-most methionine codon in the open reading frame. No consensus sequences for intron splicing (30) were identified within the open reading frame or in the sequence 5′ (89 bp) to the predicted translational initiation site. Neither of the consensus control sequences for transcription termination (22, 70) were found in the sequence (301 bp) following the translational termination site.

BCK1-20 was compared with sequences in the GenBank and National Biomedical Research Foundation data bases (50). The predicted *BCK1-20*-encoded protein possesses amino acid sequence similarity to members of the serine/threonine-specific protein kinase subfamily and includes hallmark sequences within its 30-kDa catalytic domain that are diagnostic of protein kinases (14). *BCK1-20* is not closely related to any known protein kinase, but the protein kinase with the greatest degree of similarity to *BCK1-20* is the *STE11*-encoded protein kinase from *S. cerevisiae* (54). This similarity is restricted to the predicted catalytic domain (45% identity; Fig. 4), which resides near the C terminus of the *BCK1-20*-encoded protein. The *BCK1-20* catalytic domain shares only 26% amino acid identity with that of *PKC1*, and no sequence similarity between these proteins is evident outside of this region. The extensive N-terminal sequence of the predicted *BCK1-20* protein is not similar to any sequence in the data bases. This sequence possesses a potential PKC phosphorylation site (at amino acid position Ser-1134 [18, 19]), suggesting that the *BCK1* protein might be a substrate for the *PKC1*-encoded protein kinase. Hydropathy analysis did not suggest the presence of a transmembrane region within the *BCK1*-encoded protein.

To identify the mutation responsible for creating the dominant *BCK1-20* suppressor, we isolated the wild-type allele of this gene by gap repair (47). A gapped version of pRS314[*BCK1-20*] which is missing the region corresponding to the 3′ end of *BCK1-20* (1.5-kb *Bgl*II fragment removed) was constructed. Upon transformation of the linearized plasmid DNA into strain 1783 (*BCK1*$^+$), the missing region was replaced with sequences derived from the genomic *BCK1*$^+$ locus by in vivo gap repair (see Materials and Methods). Repaired plasmids were recovered and tested for suppressor function after restoration of the missing fragment was confirmed by restriction analysis (data not shown). Among five independently repaired plasmids tested, two retained suppressor function and three did not. This result suggested that the suppressor mutation resides close to the removed sequence but not within it. During gap repair, short stretches of sequence on both sides of the missing fragment are also replaced by host sequence (16). The DNA sequence 5′ to the gapped region was determined for the repaired plasmids. The *BCK1* alleles that retained suppressor function were identical to the original *BCK1-20* allele in the 850 bp 5′ to the internal *Bgl*II site. The alleles that had lost suppressor function differed in this region by a single base change at position 3520 (Fig. 3). This G-to-C transversion results in replacement of Ala-1174 (in *BCK1*$^+$) with Pro (in *BCK1-20*) in a region immediately upstream of the catalytic domain. This mutation resides 156 bp 5′ of the gap endpoint.

Four additional suppressor alleles of *BCK1* (*BCK1-10*, *-11*, *-16*, and *-19*) were recovered from the genome and subjected to DNA sequence analysis. All of the mutations possessed by these suppressor alleles were clustered immediately upstream of the catalytic domain, surrounding the potential PKC phosphorylation site noted above (Fig. 3). The mutation possessed by *BCK1-10* was a G-to-T transversion at position 3437, resulting in replacement of Gly-1146 with Val; *BCK1-11* had a T-to-A transversion at position 3359, resulting in replacement of Ile-1120 with Lys; *BCK1-16* had a T-to-C transition at the same position as *BCK1-11*, resulting in replacement of Ile-1120 with Thr; *BCK1-19* had an A-to-C transversion at position 3355, resulting in replacement of Thr-1119 with Pro. Although all of the isolated suppressor alleles of *BCK1* were dominant, suggesting mutational activation of the encoded protein kinase, overexpression of *BCK1*$^+$ from a multicopy plasmid (pRS304[*BCK1*$^+$]) failed to suppress either the *pkc1*Δ::*LEU2* mutation or the conditional *pkc1-2* mutation in strain DL519.

Deletion of *BCK1* results in a temperature-sensitive cell lysis defect. To examine the phenotypic effect of loss of *BCK1* function, a deletion mutant of *BCK1* was constructed in vitro. A 2.3-kb fragment of *BCK1*, which includes part of the sequence encoding the catalytic domain, was replaced with the *S. cerevisiae URA3* gene (see Materials and Methods). This deletion allele (*bck1*Δ::*URA3*) was transplaced into a diploid strain (1788) with multiple auxotrophic markers by selecting for uracil prototrophy. Transformants were tested for possession of the deletion allele of *BCK1* by restriction and hybridization analysis (Fig. 5). Two independently derived diploids heterozygous at *BCK1* (*bck1*Δ::*URA3*/*BCK1*) were induced to sporulate, and tetrads were dissected. Four spores from each tetrad gave rise to colonies at 30°C, but two from each grew slowly. The slowly growing colonies carried the *bck1*Δ::*URA3* allele, as judged by segregation of the *URA3* marker and restriction and hybridization analysis (Fig. 5). The *bck1*Δ::*URA3* mutants failed to grow at 37°C. Microscopic examination of these nongrowing cells 4 h after the shift to the restrictive temperature revealed a high frequency (approximately 70%) of nonrefractile "ghosts" (not shown), suggesting that cell lysis was occurring at the restrictive temperature. Strains carrying the *bck1*Δ::*URA3* mutation could be rescued at the restrictive temperature with a centromeric plasmid bearing the *BCK1*$^+$ gene (pRS314[*BCK1*$^+$]). The temperature sensitivity of the *bck1*Δ::*URA3* mutation was suppressed by the addition of 100 mM $CaCl_2$, 100 mM $MgCl_2$, or 1 M sorbitol to the medium (Fig. 6). Other osmotic stabilizers, including 10% polyethylene glycol, 100 mM NaCl, and 1 M glucose, were also able to support growth of the *bck1*Δ::*URA3* mutant at 37°C (not shown), but overexpression of *PKC1*$^+$ from a multicopy plasmid (pRS304[*PKC1*$^+$]) failed to suppress the *bck1*Δ::*URA3*-associated defect. Microscopic examination of *bck1*Δ::*URA3* cells, growing in osmotically stabilized medium at the restrictive temperature, did not reveal any morphological defects.

To test directly the possibility that loss of *BCK1* function results in cell lysis at the restrictive temperature, the release of radioactive material from labeled cells was measured. Cells bearing the *bck1*Δ::*URA3* mutation (DL251), labeled with [^{3}H]uridine at 25°C, were washed and shifted to 37°C. Release of radioactive material from these cells into the medium reached a plateau of 42% after 2 h at the restrictive temperature (Fig. 7). This rapid release of material contrasts

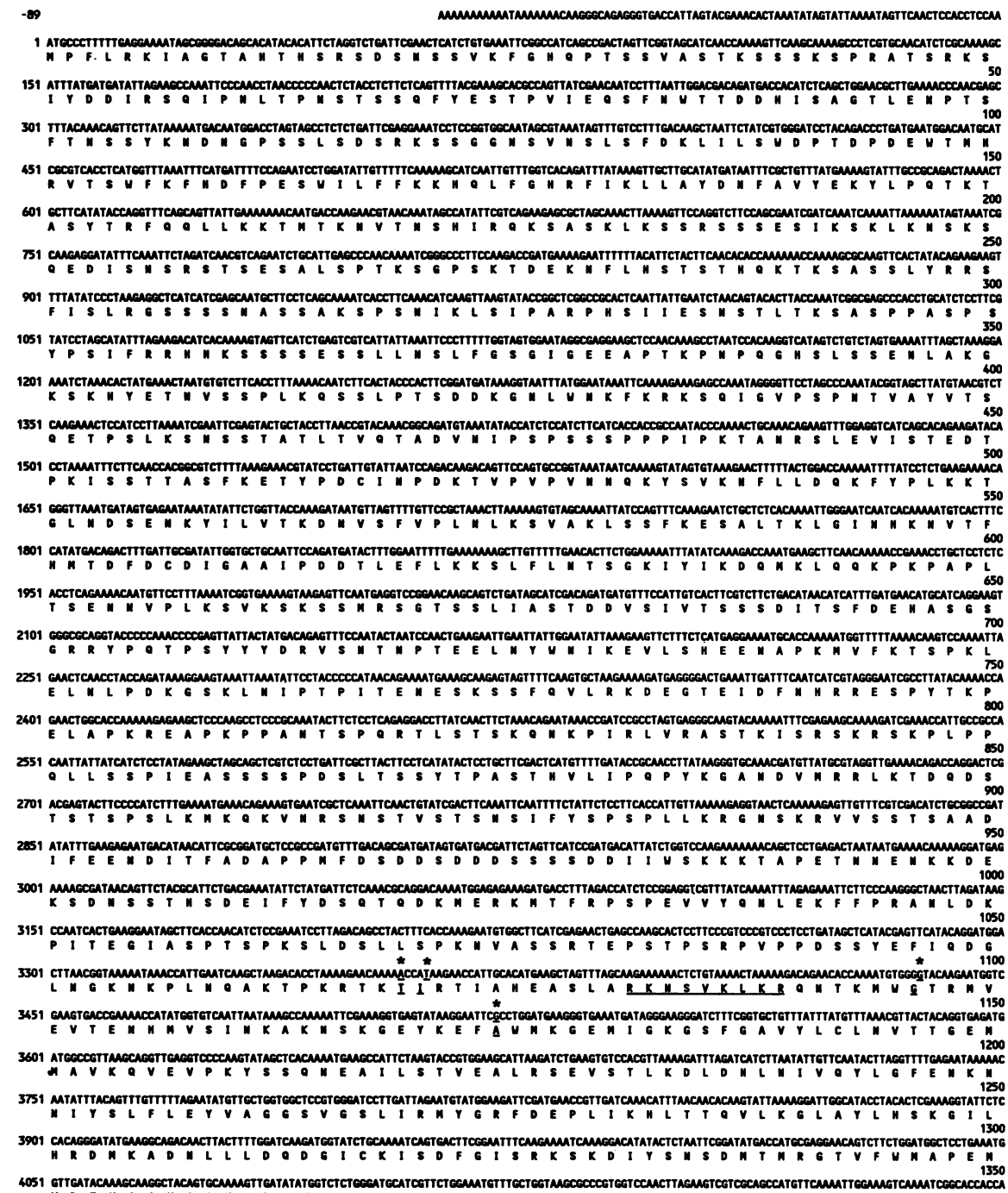

FIG. 3. Nucleotide sequence and predicted amino acid sequence of the *BCK1* gene. The predicted amino acid sequence starts with the first methionine codon in the open reading frame. The period indicates the termination codon. The asterisks indicate the mutation sites of *BCK1-19*, *-11/-16*, *-10*, and *-20*, from 5′ to 3′, respectively. Underlined amino acid sequence denotes a potential PKC phosphorylation site (at Ser-1134).

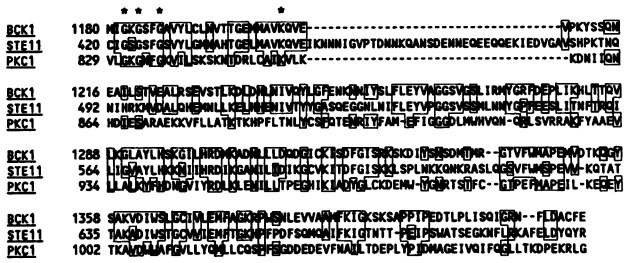

FIG. 4. Alignment of the predicted *BCK1* catalytic domain with those of *STE11* and *PKC1*. Residues that are identical in the *BCK1* protein and the other yeast protein kinases are boxed. The deduced amino acid sequences for the *STE11*-encoded protein kinase and the *PKC1*-encoded protein kinase C isozyme from *S. cerevisiae* have been published (33, 54). The sequences were aligned by eye, and gaps were introduced as indicated by dashes. Asterisks indicate conserved residues within the ATP-binding regions.

with results obtained with a known cell lysis mutant, *cly7*. The temperature-sensitive *cly7-1* strain (X3119-12A) lysed more slowly and continued to release labeled material for 6 h after the shift to the restrictive temperature. 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 (53). 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 the shift to the restrictive temperature.

The *bck1*-associated cell lysis defect was expressed preferentially in budded cells. A *bck1*Δ::*URA3* strain (DL251), growing logarithmically at 37°C in medium supplemented with 1 M sorbitol, was diluted into medium lacking sorbitol at the same temperature and plated for viability at various times (at 26°C on sorbitol-containing medium). Within the first 3 min, 67% of the population had lost viability compared

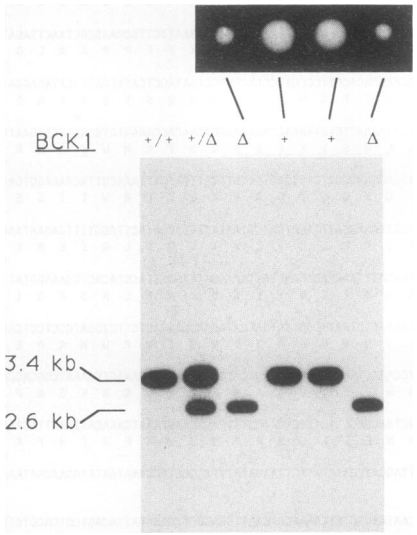

FIG. 5. Deletion of the *BCK1* gene. Genomic DNA was isolated from (left to right) a *BCK1*$^+$ diploid strain (1788), a diploid heterozygous for the *bck1* deletion (*bck1*Δ::*URA3*/*BCK1*$^+$; DL247), and the haploid segregants of a representative tetrad from DL247 (top). DNA (5 μg) was digested with *Bam*HI and prepared for hybridization with the nick-translated 3.4-kb *Bam*HI fragment from *BCK1-20*. The hybridizing 2.6-kb fragment is derived from the deleted allele.

with cells diluted into sorbitol-containing medium (Fig. 8). The remaining cells lost viability gradually over the next 3 h. Microscopic examination of these cells revealed that nearly all of the lysed cells appeared to have been budded. The cells that remained apparently intact after the first 3 min were almost exclusively unbudded (96%), suggesting that osmotic instability of the *bck1*Δ::*URA3* mutant manifests itself at the time of bud emergence and persists until cytokinesis.

Expression of *BCK1*. To examine the possibility that *BCK1-20*-mediated suppression is the result of increased levels of *BCK1* transcription, we measured the steady-state levels of *BCK1* RNA. A probe derived from the 3.4-kb *Bam*HI fragment of *BCK1* was hybridized with whole-cell *S. cerevisiae* RNA. Both *BCK1* and *BCK1-20* are transcribed into 5.5-kb mRNAs in proliferating *MAT*α cells (data not shown). The steady-state levels of *BCK1*- and *BCK1-20*-derived RNA were similar, indicating that the *BCK1-20* mutation does not result in transcriptional activation. No *BCK1*-derived RNA was detected from a *bck1*Δ::*URA3* mutant.

Mapping the chromosomal location of *BCK1*. To identify the chromosome on which the *BCK1* gene resides, the 3.4-kb *Bam*HI fragment of *BCK1* was hybridized with chromosomes from a strain of *S. cerevisiae* designed to allow electrophoretic resolution of all 16 chromosomal DNAs (YPH149 [68]). The probe hybridized exclusively to the band corresponding to chromosome X (data not shown). To determine the chromosomal location of *BCK1*, standard meiotic linkage analysis was conducted by using strains marked at the *CDC35*, *ARG3*, and *CDC6* loci. The latter two markers reside on the left arm of chromosome X, such that the order of these markers from left to right is *CDC6*, *ARG3*, *CEN10*, and *CDC35* (38). Tetrad analysis (Table 2) placed the *BCK1* gene 2.7 centimorgans centromere-distal to *ARG3*. This map position does not correspond to that of any previously described mutation (38).

To identify *S. cerevisiae* genes structurally related to *BCK1*, hybridization probes derived from the *BCK1* coding sequence were used to screen yeast genomic DNA under conditions of reduced stringency. A variety of probes, including the 750-bp *Eco*RI-*Sph*I fragment encoding the catalytic domain (Fig. 2), failed to detect any additional *BCK1*-related species (data not shown). This result suggests that the *BCK1* gene is not structurally redundant within the *S. cerevisiae* genome.

DISCUSSION

Isolation of the *BCK1* extragenic suppressor of a *pkc1* deletion. A yeast strain that conditionally expresses the *PKC1* gene, encoding a homolog of mammalian PKC, ceases growth and cell division uniformly at a specific point in the cell division cycle in response to depletion of the *PKC1* gene product (33). This strain was used to isolate extragenic suppressor mutations that support growth of mutants lacking a functional *PKC1* gene. All of the suppressor mutations isolated were shown by meiotic linkage analysis to be allelic to one another. Molecular analysis of the suppressor locus, designated *BCK1* (for bypass of C kinase), revealed a 4.4-kb open reading frame predicted to encode a previously unidentified protein kinase. The *BCK1* gene product, calculated to be 163 kDa, possesses a 30-kDa catalytic domain near its C terminus. This region is not closely related to any known protein kinase catalytic domain, sharing only 45% amino acid identity with its closest known relative, the protein kinase encoded by the *S. cerevisiae STE11* gene. The

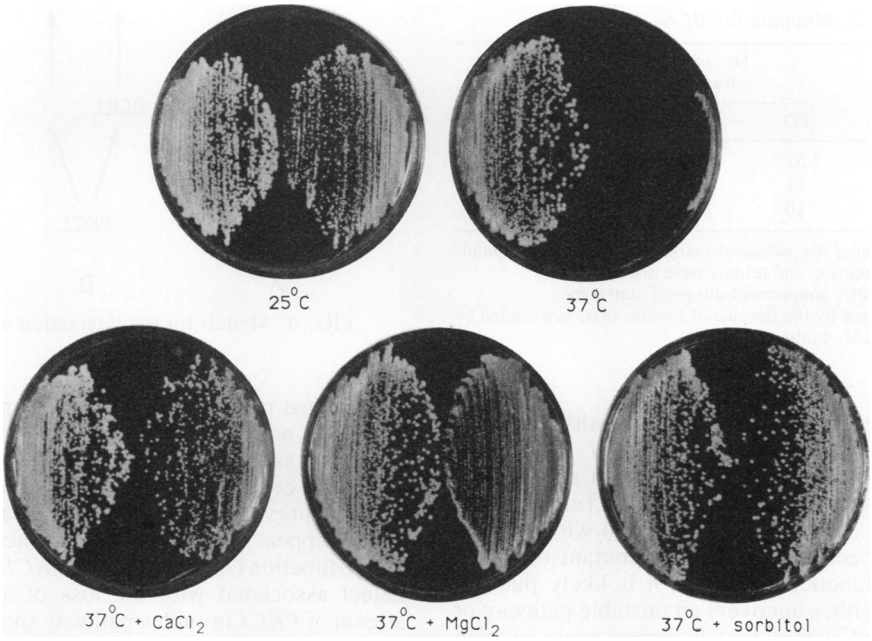

FIG. 6. Osmotic stabilizers suppress the temperature-sensitive growth defect of a *bck1* deletion. A *BCK1*$^+$ diploid strain (1788; left side of plates and a *bck*Δ::*URA3* diploid strain (DL251; right side of plates) were streaked onto YEP-glucose plates with the indicated supplements and allowed to incubate at either 25 or 37°C for 48 h. A diploid *bck1*Δ::*URA3* mutant was used because haploid mutants accumulated recessive extragenic suppressor mutations at a high frequency. $CaCl_2$ or $MgCl_2$ was present at 100 mM; sorbitol was present at 1 M.

N-terminal 130-kDa region of the *BCK1*-encoded protein bears no resemblance to any known protein. The fact that the predicted *BCK1*-encoded protein kinase is not closely related to the protein kinase encoded by *PKC1* diminishes the likelihood that *BCK1* has been mutationally recruited from another pathway to provide the *PKC1* function.

The suppressor alleles of *BCK1* were dominant to the wild-type gene for suppressor function, indicating that the mutant alleles had gained a function not possessed by the *BCK1*$^+$ gene. Suppressor alleles of *BCK1* carry mutations that result in single amino acid replacements clustered in a region of the protein immediately upstream of the catalytic domain. It is plausible that the N-terminal region of the *BCK1*-encoded protein serves as a regulatory domain which normally interacts with the catalytic domain. Mutations in the hinge region between these domains may interfer with such an interaction, resulting in an unregulated protein kinase. Deletion of *BCK1* did not result in suppression of *pkc1* mutations (unpublished data), a result that further supports the notion that *BCK1* suppressor mutations activate the encoded protein kinase. The observation that overexpression of *BCK1*$^+$ failed to suppress *pkc1* mutations may indicate that the wild-type Bck1 protein kinase is tightly regulated.

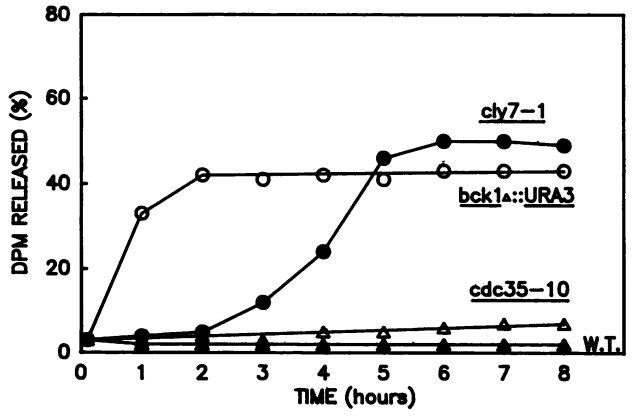

FIG. 7. Cell lysis of the *bck1* deletion mutant. Cells were labeled with [^{3}H]uridine at 25°C, washed, and shifted to 37°C at time zero. Aliquots were taken at the times indicated, and the amount of radioactive material released into the medium was measured. Strains were DL247 (wild-type; ▲), DL251 (*bck1*Δ::*URA3*; ○), X3119-12A (*cly7-1*; ●), and LR684-C (*cdc35-10*; △).

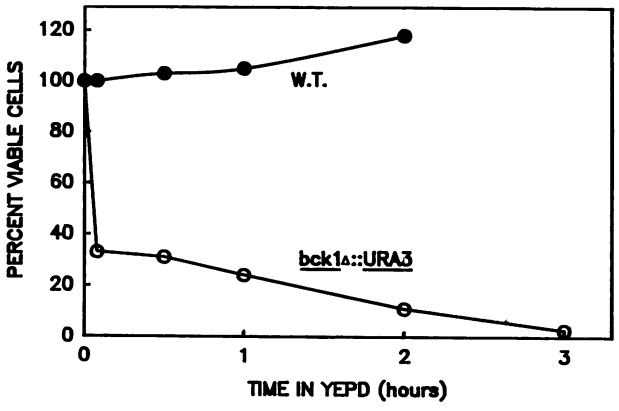

FIG. 8. Survival of the *bck1* deletion mutant after transfer to medium lacking osmotic stabilizing agents. Cells were grown logarithmically at 37°C in YEP-glucose medium supplemented with 1 M sorbitol and diluted at time zero into YEP-glucose. Aliquots were plated for viability at various times on sorbitol-containing medium at 25°C. Strains were DL247 (wild-type; ●) and DL251 (*bck1*Δ::*URA3*; ○).

TABLE 2. Mapping the *BCK1* gene

Cross (marker)[a]	No. of each tetrad type[b]:			Map distance (cM)[c]
	PD	NPD	T	
BCK1::*URA3* × *arg3*	53	0	3	2.7
BCK1::*URA3* × *cdc6*	11	2	50	53.8
BCK1::*URA3* × *cdc35*	10	5	33	81.5

[a] Haploid strains possessing the indicated markers were mated. Diploids were induced to undergo meiosis, and tetrads were dissected.
[b] PD, parental ditype; NPD, nonparental ditype; T, tetratype.
[c] Map units were calculated by the formula of Perkins (51), as modified by Mortimer and Schild (38). cM, centimorgans.

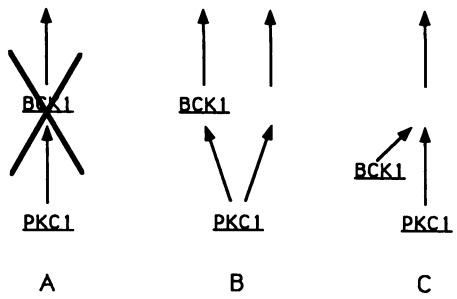

FIG. 9. Models for the interaction of *PKC1* and *BCK1*.

Deletion of *BCK1* results in a temperature-sensitive cell lysis defect. Cells bearing a deletion at the *BCK1* locus grew slowly at 30°C but displayed a cell lysis defect at 37°C that was suppressed by osmotic stabilizing agents. Temperature-sensitive phenotypes are normally associated with thermolabile proteins, but because the deletion mutant of *BCK1* does not express a functional protein, it is likely that the absence of this gene either uncovers an unstable pathway or results in a structural defect that becomes acute at high temperature. The observation that cells deleted at *BCK1* lysed rapidly after the shift to the restrictive temperature suggests that the latter possibility is more likely.

Evidence in support of a model in which *PKC1* and *BCK1* function in a common pathway comes from the similarity in the defects associated with loss of function of these genes. A strain that conditionally expresses *PKC1* ceases growth and division at a point in the cell division cycle shortly after bud emergence (33). Nuclear events arrest in these cells after DNA replication but before mitosis. We have shown recently that growth and division arrest of conditional *pkc1* mutants is accompanied by a cessation of protein synthesis and rapid loss of viability (32). Although cell lysis of conditional *pkc1* mutants is not microscopically evident, these cells release their contents into the medium after the shift to restrictive conditions. Moreover, a *pkc1* deletion mutant proliferates only in osmotically stabilized medium, and budded cells undergo rapid lysis after they are transferred to medium lacking osmotic stabilizers (32). A pattern of cell lysis similar to that displayed by the *pkc1* deletion mutant was observed of a *bck1* deletion mutant grown at the restrictive temperature after it was transferred to medium lacking osmotic stabilizers. We have proposed that conditional mutations in *PKC1* result in a cell division cycle-specific osmotic stability defect (32). Because *pkc1* cells cease growth and division with a uniform terminal phenotype, the defect is apparently initiated at a specific point in the cell cycle. The small-budded arrest phenotype is proposed to result from the manifestation of this defect at the time of bud emergence. Additionally, because initiation of DNA synthesis normally precedes bud emergence (55) and completion of replication does not require protein synthesis (6, 15), 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 (6), nuclear arrest would result after the completion of DNA replication but before mitosis. Indeed, this is the state of the nuclei in *pkc1*-arrested cells.

Interaction of *PKC1* and *BCK1*. Any model in which *PKC1* and *BCK1* are proposed to function in a common pathway must take into account two observations. (i) Because *BCK1* suppressor mutations bypass the requirement for *PKC1*, *BCK1* must not act at a point in the pathway before *PKC1*. This does not preclude the possibility that *PKC1* and *BCK1* act at the same point in the pathway through phosphorylation of a common substrate. (ii) *PKC1* is essential at all temperatures, but *BCK1* is only essential at 37°C. These results appear to rule out an unbranched pathway in which *BCK1* functions downstream of *PKC1* (Fig. 9A), because the defect associated with the loss of any component downstream of *PKC1* in such a pathway should be as severe as the defect associated with the loss of *PKC1*. Figure 9 outlines two models that are consistent with the observations described above. In the first model (Fig. 9B), *PKC1* regulates a bifurcated pathway in which *BCK1* functions on one branch. Loss of either branch of the pathway is not as deleterious as is loss of the entire pathway (by deletion of *PKC1*). In this model, the Pkc1 kinase either directly or indirectly activates the Bkc1 kinase. The dominant suppressor mutations of *BCK1*, which are clustered near a potential PKC phosphorylation site, may result in a conformational change approximating that induced through phosphorylation by Pkc1. In the second model (Fig. 9C), *BCK1* plays an auxiliary role in the pathway. In this case, loss of the presumptive regulatory function provided by *BCK1* weakens the pathway, but it does not have the devastating affect associated with complete loss of the pathway.

A mutation in the *BCK1* gene has been identified recently through its synthetic lethality with a *spa2* mutation (63). The *SPA2* gene encodes a protein that localizes to bud tips and "shmoo" (mating projection) tips and is proposed to play a role in the polarized cell growth required for bud formation and morphogenic differentiation prior to mating (11). Although *BCK1* and *SPA2* are not structurally related to each other, the synthetic lethality of mutations in these genes suggests that they may be functionally overlapping. By extension, *PKC1* may also participate in polarized cell growth and morphogenesis.

***BCK1* is structurally unique in the yeast genome.** Although it is possible that the *BCK1* function has been duplicated in *S. cerevisiae*, it is unlikely that the *BCK1* gene is structurally redundant. Probes derived from the *BCK1* gene failed to detect any related yeast loci under hybridization conditions of reduced stringency. These hybridization conditions have allowed the detection of duplicated (or triplicated) protein kinase genes previously in yeast by using probes derived from one member of a replicated gene family (34, 49, 66).

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ADDENDUM IN PROOF

Irie et al. (K. Irie, H. Araki, and Y. Oshima, Gene, in press) have isolated a dosage-dependent suppressor of a temperature-sensitive allele of *SMP3* (designated *SSP31*) that is identical to *BCK1*. The *SMP3* gene encodes a hydrophobic protein that influences the maintenance of heterologous plasmids in *S. cerevisiae*.

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