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Two Genes in *Saccharomyces cerevisiae* Encode a Membrane-Bound Form of Casein Kinase-1

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Two cDNAs encoding casein kinase-1 have been isolated from a yeast cDNA library and termed *CKI1* and *CKI2*. Each clone encodes a protein of ~62 000 Da containing a highly conserved protein kinase domain surrounded by variable amino- and carboxy-terminal domains. The proteins also contain two conserved carboxy-terminal cysteine residues that comprise a consensus sequence for prenylation. Consistent with this posttranslational modification, cell fractionation experiments demonstrate that intact CKI1 is found exclusively in yeast cell membranes. Gene disruption experiments reveal that, although neither of the two *CKI* genes is essential by itself, at least one *CKI* gene is required for yeast cell viability. Spores deficient in both *CKI1* and *CKI2* fail to grow and, therefore, either fail to germinate or arrest as small cells before bud emergence. These results suggest that casein kinase-1, which is distributed widely in nature, plays a pivotal role in eukaryotic cell regulation.

INTRODUCTION

Casein kinases are second messenger-independent protein kinases that are found in most eukaryotic cells (Pinna, 1990; Tuazon and Traugh, 1991). They are named for their unusual ability to phosphorylate acidic substrate recognition sites exemplified by the acidic heavily phosphorylated milk protein, casein. Two classes of enzyme are recognized in eukaryotic cells, termed casein kinase-1 (CK1) and casein kinase-2. Because a clear biological function has not been assigned to either enzyme, they are usually classified on the basis of their enzymological properties. CK1 is a monomer in solution with an unusually high isoelectric point and a strict requirement for ATP as cosubstrate. Because dephosphorylation of casein renders it a *less* efficient substrate for CK1 (Tuazon *et al.*, 1979), phosphorylated amino acid residues may comprise part of the substrate recognition site for the enzyme (Flotow and Roach, 1991; Meggio *et al.*, 1991). Casein kinase-2 is distinguished by its heterotetrameric structure ($\alpha\alpha'\beta\beta$), its extreme heparin sensitivity ($IC_{50} \leq 0.15$ μg/ml), its ability to use either ATP or GTP as cosubstrate, and its well-defined selectivity for acidic substrates (Pinna, 1990; Marshak and Carroll, 1991). When purified, both CK1 and casein kinase-2 are constitutively active.

We have turned to the yeast *Saccharomyces cerevisiae* as a biological system in which to study CK1 function and recently completed the isolation of CK1 from that organism (Kuret, unpublished data). Unlike previous studies, which identified a family of three CK1-like enzymes in yeast (Donella-Deana *et al.*, 1985; Szyszka *et al.*, 1985; Sternbach and Küntzel, 1987), we found that nearly all yeast CK1 activity purified as a single protein with a molecular mass of 54 kDa. This protein retained the enzymological characteristics described above for CK1. We now report the use of amino acid sequence data acquired from this preparation to clone two cDNAs encoding yeast CK1 and discuss the implications of the deduced primary structures for CK1 activity, localization, and function.

MATERIALS AND METHODS

Bacteria and Yeast Strains

Bacterial strain BB4 was used for all manipulations involving lambda phage (Short *et al.*, 1988). BB4 and BSJ72 were employed for growth of all single-stranded DNA templates (Kuret *et al.*, 1988) in conjunction with helper phage VCS-M13, (Stratagene, La Jolla, CA). MM294 and GM2136 were used for growth of all plasmids, which were prepared by the alkaline lysis method (Sambrook *et al.*, 1990). Lambda-ZAP (*Eco*RI digested, calf intestine alkaline phosphatase treated; Stratagene) was handled according to the manufacturer's instructions (Short *et al.*, 1988).

Yeast strains used in this study are summarized in Table 2. Strains were grown at 30°C in rich medium (YPD: 1% yeast extract, 2% Bacto-peptone, 2% glucose) or under selection in synthetic minimal

medium (SD) (Rose *et al.*, 1990) supplemented with appropriate nutrients. All yeast transformations were performed with lithium acetate (Ito *et al.*, 1983). Tetrad analysis and other genetic manipulations were performed by standard methods (Rose *et al.*, 1990).

Peptides and Amino Acid Sequence Determination

CK1 was purified from yeast strain (Kuret, unpublished data). CK1 (30 μg; 560 pmol) was dissolved in 50 mM tris(hydroxymethyl)aminomethane (Tris)-HCl, pH 8.0, and incubated with trypsin (0.2 μg) or chymotrypsin (1 μg) for 1 h at room temperature in a final volume of 80 μl for up to 1 h. Reactions were terminated by the addition of phenylmethylsulfonyl fluoride (PMSF) to a final concentration of 1.0 mM. The resulting peptides were separated in a 12% sodium dodecyl sulfate (SDS)-polyacrylamide gel (Kuret and Schulman, 1984), blotted onto polyvinylidene difluoride membranes (Matsudaira, 1987), and visualized by staining with 0.1% Coomassie Blue R-250 in 50% methanol (Matsudaira, 1987). Selected peptides were subjected to automated Edman degradation in an Applied Biosystems (Foster City, CA) model 475 gas phase sequenator operated according to the manufacturer's instructions.

Synthetic Oligonucleotide Probe

A portion of the amino acid sequence derived from overlapping peptides T2 and C1 (YKIGKKIG) was used to design a 16-fold degenerate pool of synthetic oligodeoxyribonucleotides having the 23-residue sequence:

TA(T/C)AA(A/G)ATIGGIAA(A/G)AA(A/G)ATIGG

The pool was synthesized, purified, and 5′-end labeled with T4 polynucleotide kinase and [γ-^{32}P]ATP as described previously (Kuret *et al.*, 1988; Sambrook *et al.*, 1990).

cDNA Library Construction and Cloning of CKI1

For mRNA isolation, 6×10^{10} log-phase SP1 cells were grown, harvested, lysed, and extracted as described previously (Rose *et al.*, 1990), except that the lysis buffer contained 0.5 mg/ml heparin. Poly(A)$^+$ RNA was purified twice by affinity chromatography over oligo(dT)-cellulose (Sambrook *et al.*, 1990). cDNA was prepared from 2 μg of Poly(A)$^+$ RNA by the method of Gubler and Hoffman (1983), methylated with *Eco*RI methylase, ligated to *Eco*RI linkers, digested with *Eco*RI, fractionated by size exclusion chromatography over BioGel (Bio-Rad, Richmond, CA) A-50M (Huynh *et al.*, 1985), and ligated into the *Eco*RI site of Lambda-ZAP (Short *et al.*, 1988). The resulting library consisted of 2.1×10^6 members, over 99% of which contained inserts on the basis of α-complementation (Sambrook *et al.*, 1990). The library was amplified (10^7-fold) in BB4 cells and stored at 4°C until used. The minimum insert size was 0.6 kb; the average insert size was ~1.5 kb.

An aliquot of this library (containing 25 000 recombinants) was plated at a density of 5000 plaques/100-mm plate. Phages were transferred from each plate to nitrocellulose filters in duplicate, denatured, baked, and prewashed by standard procedures (Sambrook *et al.*, 1990). Filters were then hybridized to 0.1 nM ^{32}P-labeled oligonucleotide in 6× SSC, 100 μg/ml denatured calf thymus DNA, 0.02% NaN_3, and 0.25% (wt/vol) instant nonfat milk for 16 h at 40°C. Hybridized filters were washed in 1× SSC, 0.1% SDS for 1 h at 37°C and subjected to autoradiography. Positive phage clones were plaque purified and excised from the Lambda-ZAP vector as described by Short *et al.* (1988).

Genomic Southern Analysis

Total yeast nucleic acids were isolated from 50 ml stationary-phase cultures of *S. cerevisiae* as described previously (Brill and Sternglanz, 1988). After ethanol precipitation, nucleic acids were resuspended in 10 ml of 50 mM MOPS, pH 8.0, 750 mM NaCl, 0.15% Triton X-100, 15% ethanol and loaded onto a 1.5-ml cation exchange column (Qiagen, Chatsworth, CA). After washing the column with 10 ml 50 mM MOPS, pH 7.0, 1.0 M NaCl, 15% ethanol, genomic DNA was eluted with 5 ml of 50 mM MOPS, pH 8.2, 1.25 M NaCl, 15% ethanol, precipitated with isopropanol, and resuspended in 10 mM Tris HCl, 0.1 mM EDTA, pH 8.0. Purified yeast genomic DNA (5 μg) was digested with the appropriate restriction enzyme for 6 h at 37°C, size fractionated in a 0.7% agarose gel, depurinated (20 min in 0.25 M HCl), denatured (20 min in 0.5 M NaOH, 1.5 M NaCl), neutralized (20 min in 1 M Tris HCl, pH 7.0, 2 M NaCl), and transferred to nylon membranes (Genescreen II; Dupont, Wilmington, DE) under vacuum (1 h at 50 mbar in 20× SSC).

Hybridizations were conducted for 12–15 h at 55°C (low stringency; Tm ≈ −40°C) or at 65°C (high stringency; Tm ≈ −30°C) in 6× SSC, 100 μg/ml denatured calf thymus DNA, 0.02% NaN_3, and 0.25% (wt/vol) instant nonfat milk. Radiolabeled probes (described below) were hybridized at concentrations of 2–10 ng/ml. After hybridization, filters were washed at room temperature 3 × 5 min in 2× SSC, 0.1% SDS, followed by 3 × 5 min in 0.1× SSC, 0.5% SDS. Finally, membranes were washed 30 min in 0.1× SSC, 0.5% SDS at 42°C (low stringency), or at 65°C (high stringency) and subjected to autoradiography. To prepare Southern blots for reuse, hybridized probes were stripped from nylon membranes by treatment with 0.4 M NaOH at 42°C for 30 min, followed by neutralization in 0.1× SSC, 0.1% SDS, 0.2 M Tris HCl, pH 7.5, at 42°C for 30 min.

The 942-bp *Hin*fI-*Bst*EII fragment of CKI1 served as a probe for the catalytic domain of CK1. Other probes included the full-length cDNAs of CKI1 (1930 bp) and CKI2 (1826 bp), which were released from pBluescript after digestion with *Eco*RI. After digestion with the relevant restriction enzyme, probes were separated in 0.8% agarose gels, isolated by adsorption to glass beads (Vogelstein and Gillespie, 1979), extracted with phenyl:chloroform:isopentanol (25:24:1) followed by chloroform:isopentanol (24:1), ethanol precipitated, and resuspended in 10 mM Tris HCl, pH 8.0, 0.1 mM EDTA. Purified probes were radiolabeled with [α-^{32}P]dATP to >10^8 cpm/μg by nick translation (Sambrook *et al.*, 1990).

Genomic Library Construction and Cloning of CKI2

Purified yeast genomic DNA (25 μg) was digested with *Hin*dIII and size fractionated in a 0.7% agarose gel. Fragments migrating between 2.3 and 2.7 kb were excised, isolated by centrifugal filtration (Costar), and ligated into calf intestine alkaline phosphatase-treated *Hin*dIII-digested pBluescript (pSK-; 200 ng). Colony filters containing the library were prepared, replica plated on nitrocellulose filters, and prewashed as described by Woods (1984). To clone CKI2, filters were probed at low stringency with the 942-bp *Hin*fI-*Bst*EII fragment of CKI1 (described above). The resultant CKI2 clone consisted of a 2.5-kb *Hin*dIII fragment that contained 75% of the CKI2 coding sequence. To obtain the full-length cDNA for CKI2, the 2.5-kb genomic CKI2 clone was nick translated and used to screen the Lambda-ZAP cDNA library (12 000 plaques) at high stringency as described above. Positive phage clones were plaque purified and excised from the Lambda-ZAP vector as described above.

DNA Sequencing

*Eco*RI fragments containing full-length cDNAs for CKI1 and CKI2 were cloned into pSK(−)ΔH in both orientations. This vector is identical to pSK(−) except that it contains a *Hin*cII-*Eco*RV deletion in the polylinker, making it useful for the gene disruptions described below. Single-stranded DNA templates were prepared from the resulting clones and used for DNA sequence analysis as described previously (Sanger *et al.*, 1977; Biggin *et al.*, 1983). The 5′-terminal non-coding sequences of CKI2 were determined from the 2.5-kb genomic clone by the dideoxy chain termination method applied to denatured double-

stranded DNA (Sambrook *et al.*, 1990). Thus, DNA sequences for CKI1 and CKI2 were determined for both strands.

Gene Disruptions

Gene replacements were performed as described by Rothstein (1983). The null allele of *CKI1* was engineered as follows: pSK(−)ΔH-*CKI1* was digested with *Bcl* I and *Hin*dIII, blunt-ended with Klenow polymerase, and ligated to a double-stranded *Hin*dIII linker (5′-CAAGCTTG). The resultant construction was digested with *Hin*dIII and ligated (in the reverse transcriptional orientation) to the 1107-bp *Hin*dIII fragment of *URA3* from YEp24 (Botstein *et al.*, 1979). Digestion of the resultant plasmid with *Eco*RI released a 1.9-kb DNA fragment containing *cki1::URA3* from pSK(−)ΔH that was used to transform W303 (see Table 2) cells to uracil prototrophy.

The deletion allele of *CKI2* was engineered as follows: pSK(−)ΔH-*CKI2* was digested with *Bcl* I and *Hin*dIII, blunt-ended with Klenow polymerase, and resealed with T4 DNA ligase. The resultant construction deleted 1189 bp of the CKI2 catalytic domain and recreated the *Bcl* I site. After digestion of the construction with *Bcl* I, it was ligated (in the same transcriptional orientation) to the 1764-bp *Bam*HI fragment of *HIS3*. Digestion of the resultant construction with *Eco*RI released a 2.4-kb DNA fragment containing *cki2::HIS3* from pSK(−)ΔH that was used to transform W303 cells to histidine prototrophy. Homologous recombination of both null alleles was confirmed by genomic Southern analysis performed at high stringency as described above.

Physical Mapping

CKI1 and *CKI2* were localized to their respective chromosomes by hybridizing nick-translated full-length cDNAs to yeast chromosomes at high stringency. Chromosome blots were prepared as described by Gerring *et al.* (1991) and were the gift of Naama Kessler.

Mapping was extended to high resolution by hybridizing the same probes to filters containing ≈1000 overlapping members of the yeast genomic library described by Olson *et al.* (1986).

Expression

Expression of CKI1 was monitored using the epitope addition method described by Field *et al.* (1988). The expression plasmid was built by first adding useful restriction sites (*Eco*RI, *Nde* I, and *Sal* I) to *CKI1* using polymerase chain reaction (PCR). Reactions (100 μl) contained PCR buffer (10 mM Tris HCl, pH 8.4, 50 mM KCl, 1.5 mM $MgCl_2$, 0.01% gelatin), 20 ng template DNA (20 ng pSK(−)*CKI1*), 200 μM deoxyribonucleotide triphosphates, 5 U Taq DNA polymerase (Perkin Elmer-Cetus, Norwalk, CT), and 100 pmol each of the oligonucleotide primers:

5′-CTCG AAT TCG TCG ACC CAT ATG

TCT CAA GTG CAA AGT CC^{20}

5′-AAA GAA TTC GGA TCC TCA CTG GAC GGT GGT GGG^{1675}.

The superscript numbers indicate the position of the 3′ nucleotide with respect to the nucleotide sequence shown in Figure 2. The resulting 1.7-kb PCR fragment was treated with Klenow polymerase, digested with *Eco*RI, and ligated into the *Eco*RI site of pSK(−) to create pSK(−)PCRA. A 1.7-kb *Sac* I/*Sal* I fragment containing *CKI1* was prepared from pSK(−)PCRA and ligated into *Sac* I/*Sal* I-digested pAD5 (a yeast expression plasmid containing the ADHI promoter, *LEU2* selectable marker, and the epitope coding sequences) (Field *et al.*, 1988). The final construction, pAD5-*CKI1*, was transformed into W303 cells to create strain JK5 (Table 2). The sequence of CKI1 in this strain was therefore changed from MSQV . . . to MYPYDVPDYASLGGPMSTHMSQV . . . and raises the molecular mass of CKI1 to 64.2 kDa.

Expression of epitope-tagged CKI1 was visualized on Western Blots. W303 and JK5 cells were grown under selection (100 ml cultures), harvested, resuspended in 2 ml of ice cold Buffer A (20 mM Tris-HCl, pH 7.4, 2 mM ethylene glycol-bis(β-aminoethyl ether)-*N,N,N′,N′*-tetraacetic acid, 2 mM PMSF, 0.1% 2-mercaptoethanol, 10% glycerol, 10 μg/ml leupeptin, 10 μg/ml pepstatin, 10 μg/ml aprotinin), and ruptured by vortexing 6 × 30 s with 2 g of glass beads (0.45 μm diameter). The homogenate was then centrifuged 10 min at 6000 × *g* to remove cell debris. The cloudy supernatant (S1) was removed and the pellet (P1) was washed three times with 1 ml of Buffer A. The washes and S1 were pooled and recentrifuged for 1 h at 170 000 × *g*. The supernatant (S2; 5 ml) was decanted, and the particulate fraction (P2) was resuspended in 0.5 ml of Buffer A. Aliquots of the S2 (20 μl) and P2 (5 μl) fractions were prepared for electrophoresis by boiling them in the presence of SDS-sample buffer as described previously (Kuret and Schulman, 1984). To assess proteolysis, JK31 and W303 cells were lysed in the presence of Buffer A containing 2% SDS, and the resulting lysate boiled immediately in the presence of SDS-sample buffer. Samples and prestained markers (Bio-Rad, Richmond, CA) were electrophoresed through a 9% SDS-polyacrylamide and transferred to nitrocellulose membranes in a Milliblot (Millipore, Bedford, MA) semidry transfer apparatus as described by Carter *et al.* (1990). Immunoreactivity was detected with monoclonal antibody 12CA5 (Field *et al.*, 1988; crude ascites fluid was used at 1:500 dilution) and goat anti-mouse IgG conjugated to alkaline phosphatase (Sigma, St. Louis, MO; 0.17 μg/ml).

RESULTS

Protein Sequence and Isolation of the CKI1 cDNA Clone

Digestion of yeast CK1 with low concentrations of trypsin or chymotrypsin produced discrete peptide products that were easily separated and visualized on SDS-polyacrylamide gels. Within 30 min of incubation with trypsin (1:150 wt/wt), nearly all CK1 protein could be isolated as a trypsin-resistant 41-kDa peptide (T1). Digestions that were continued for an additional 30 min released ~30% of the preparation as a 38-kDa peptide (T2). Incubation of CK1 with chymotrypsin (1:30 wt/wt) produced a complex pattern of peptides (many of which electrophoresed as doublets) that was dominated by a 42-kDa species (C1). When peptides T1, T2, and C1 were isolated and subjected to automated Edman degradation, we found that their amino termini were not blocked and that they provided sequence information with initial yields (i.e., the recovery of the amino-terminal residue in the sequenator relative to the amount of starting sample) averaging 10%. This low initial yield reflects losses incurred during proteolysis, electrophoresis, blotting on membranes, and coupling in the sequenator. Attempts to increase yields by substituting a neutral pH electrophoresis system (described by Moos *et al.*, 1988) for our standard electrophoresis conditions failed.

The amino acid sequences of the three peptides are shown in Figure 2. Because peptides T2 and C1 overlapped, we were provided with an unambiguous 27 residue amino acid sequence that was suitable for the design of a degenerate oligonucleotide probe. A cDNA

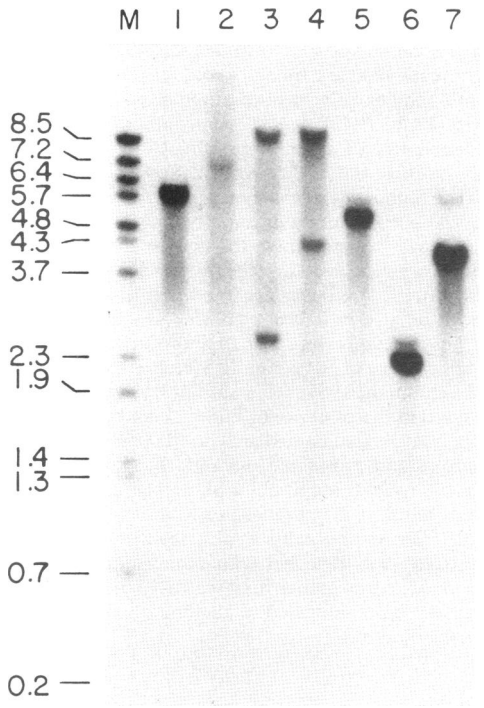

Figure 1. Genomic Southern hybridization with *CKI1* probe. Southern blots were prepared and probed at low stringency with the 0.94-kb fragment of *CKI1* as described in MATERIALS AND METHODS. Genomic DNA was digested with *Bam*HI (lane 1), *Eco*RI (lane 2), *Hin*dIII (lane 3), *Hpa* I (lane 4), *Nde* I (lane 5), *Hin*dIII + *Eco*RI (lane 6), and *Bam*HI + *Eco*RI (lane 7). Markers (lane M) consisted of end-labeled *Bst*EII-digested Lambda DNA.

library prepared in bacteriophage lambda was screened with this probe and yielded 10 hybridizing clones. Three of these positive clones were analyzed further and found to contain an open reading frame that encoded a protein homologous to the eukaryotic family of protein kinases (Hanks and Quinn, 1991). The gene encoding this kinase was designated *CKI1*. The nucleotide sequence of the longest of these cDNA clones (1.9 kb) is discussed below.

Isolation of a Gene Homologous to CKI1

To determine whether CK1 was encoded by multiple genes, genomic Southern analysis was carried out at low stringency using the 0.94-kb *Hin*fI-*Bst*EII fragment of *CKI1* as probe. This fragment corresponds to the protein kinase domain of *CKI1* (nucleotides 203 to 1145 in Figure 2). As shown in Figure 1, the probe hybridized strongly to *CKI1* and weakly to one other restriction fragment under a variety of conditions. This pattern was particularly clear after digestion with *Hin*dIII (Figure 1, lane 3), where the probe hybridized to 9- (authentic *CKI1*) and 2.5-kb fragments. These results suggested that a gene homologous to *CKI1* existed in *S. cerevisiae* and that it resided in part on a 2.5-kb *Hin*dIII fragment.

The 2.5-kb *Hin*dIII fragment was cloned, partially sequenced, and found to encode a protein kinase having extensive sequence similarity with *CKI1*. Thus, the gene encoding this kinase was designated *CKI2*. Because the 2.5-kb *Hin*dIII fragment did not contain the 3′ end of the *CKI2* gene, this fragment was used to reprobe the cDNA library at high stringency. The resultant cDNA was sequenced as described in MATERIALS AND METHODS and, together with sequence information obtained from a portion of the 2.5-kb *Hin*dIII fragment, yielded the complete nucleotide sequence of *CKI2*.

Nucleotide Sequence Analysis of CKI1 and CKI2

The nucleotide sequences of *CKI1* and *CKI2* are shown along with their predicted amino acid sequences in Figures 2 and 3, respectively. The longest open reading frame of *CKI1* (initiated by ATG) encodes a protein of 546 amino acids (calculated molecular mass and isoelectric point of 62 083 Da and 9.5, respectively). An in-frame stop codon is found 39 bp upstream from the predicted start codon. The predicted amino acid sequence contains all three peptides that were derived from purified CK1 (T1, T2, and C1). *CKI2* contains a large open reading frame that begins 330 bp upstream of the first in-frame ATG codon. A potential transcriptional control sequence (TATAAA) exists 268 bp upstream of this ATG codon, and the *CKI2* cDNA clone (which marks the 5′-end of one *CKI2* mRNA molecule) begins at position −105. Within this putative 105 bp untranslated region exists a start codon associated with a four amino acid open reading frame (position −80). Although the presence of upstream ATG codons that are not translation start sites is unusual, several examples have been identified in yeast (Cigan and Donahue, 1987). Assuming translation begins with the first in-frame methionine, *CKI2* encodes a protein of 538 amino acids (calculated molecular mass and isoelectric point of 61 736 Da and 9.3, respectively). The predicted amino acid sequence contains peptide T2 but not T1 or C1. Codon usage for both genes follows the trend observed in *S. cerevisiae* for proteins expressed at low levels (Sharp *et al.*, 1988).

Comparison of the deduced primary structures of CKI1 and CKI2 reveals a conserved structural organization (Figure 4). Both sequences begin Met-Ser- followed by ~60 hydrophilic amino acid residues that are weakly conserved (<30% sequence identity). Therefore, both proteins are likely substrates for Nα-acetylation on the penultimate serine residue (Arfin and Bradshaw, 1988). This region is followed by a typical 300-residue eukaryotic protein kinase domain that is nearly identical in the two proteins (>90% sequence identity). Sequence similarity ends abruptly with an ~150-residue glutamine-rich sequence that is predicted by the algorithm of Karplus and Schulz (1985) to comprise the most flex-

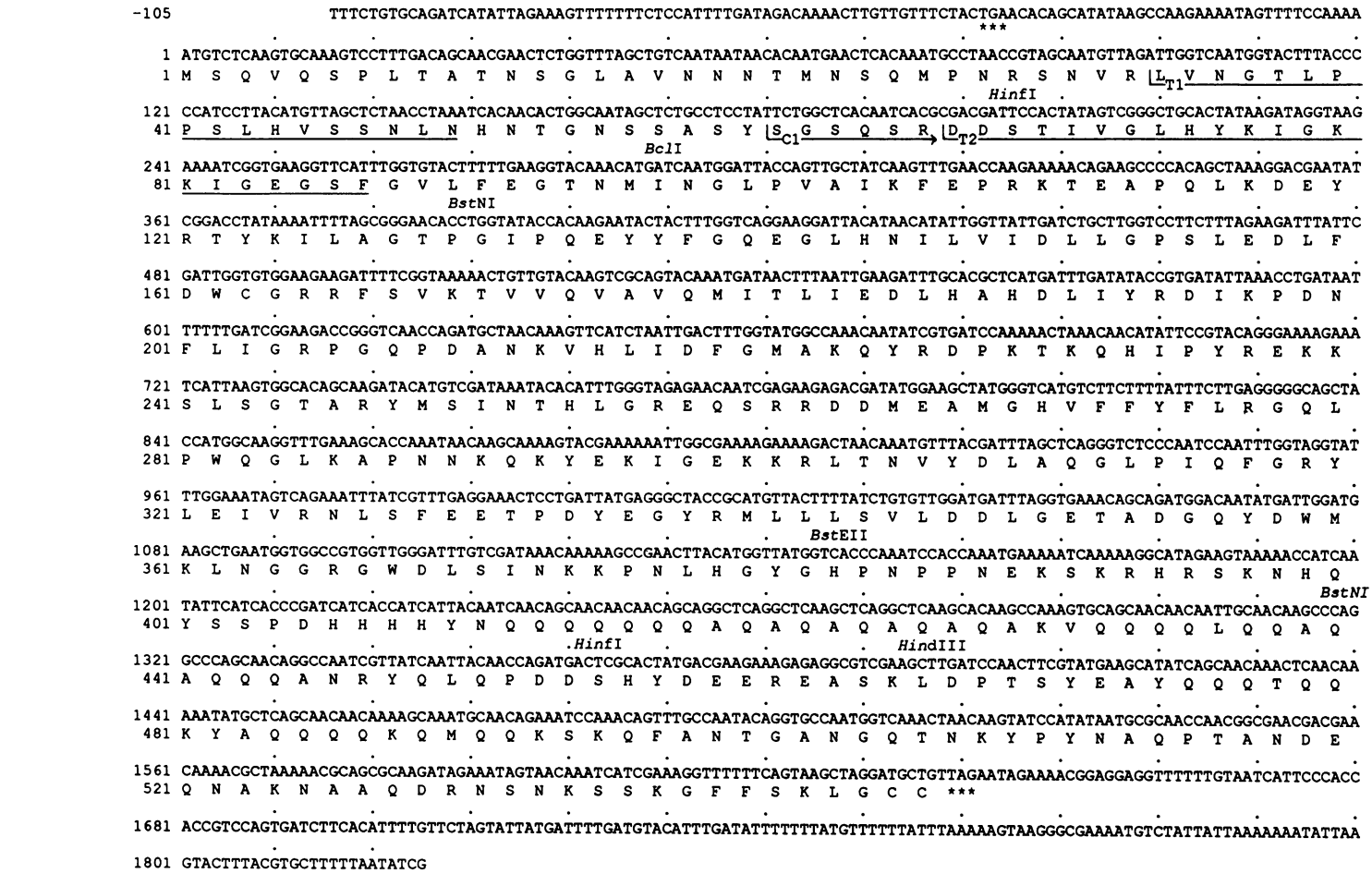

Figure 2. Nucleotide and deduced amino acid sequences of CKI1. In-frame stop codons are marked with three asterisks. Underlined amino acid residues correspond to the amino-terminal portion of peptides derived from trypsin (T1 and T2) or chymotrypsin (C1) digestion of CK1. These amino acid sequences were determined by automated Edman degradation. Restriction sites are indicated for *Bcl* I (position 291), *Bst*EII (position 1317), *Bst*NI (positions 391 and 1320), *Hin*dIII (position 1397), and *Hin*fI (positions 203 and 1361).

ible region of these proteins. Sequence similarity between the glutamine-rich regions of CKI1 and CKI2 is limited to a ≈30-residue sequence of unknown function. The final segment of both proteins consists of a 12-residue sequence (83% sequence identity) that ends in a pair of carboxy-terminal cysteine residues. This region is similar to the carboxy-terminal sequences shared by members of the GTP binding protein family, including *YPT1*, *SEC4*, *YPT3*, and *SAS1/SAS2* (Table 1). In those proteins, carboxy-terminal cysteine residues are thought to be linked to prenyl groups through thioether bonds and are necessary for membrane localization (Glomset *et al.*, 1990; Hall, 1990). In addition to cysteine residues, the carboxy-terminal regions of most of these proteins contain lysine residues that vary in number and distance from the cysteines. As in the case of cellular Ki-ras, these positively charged amino acids may assist in targeting proteins to membranes (Hancock *et al.*, 1989).

Although a comparison of CKI1's catalytic core (residues 76–363) with all sequences in the GenBank database (as of November 1, 1991) confirmed its similarity to eukaryotic protein kinases, few alignments produced identities better than the 20–25% observed among random members of the kinase family. The closest matches were with three mammalian CK1 isozymes (51–56% identity) (Rowles *et al.*, 1991) and with HRR25, a protein kinase associated with repair of damaged DNA in *S. cerevisiae* (50–52% identity) (Hoekstra *et al.*, 1991). Despite the similarity in nomenclature, CK1 is structurally unrelated to the catalytic (α) subunit of casein kinase-2 (Padmanabha *et al.*, 1990).

Alignment of CKI1 and CKI2 with the cAMP-dependent protein kinase (23% identity) is presented in Figure 4. Although the greatest similarity among these sequences is observed in the subdomains involved in nucleotide binding and catalysis (I, II, VI, VII, IX, and X) (Hanks and Quinn, 1991; Knighton *et al.*, 1991), most of the

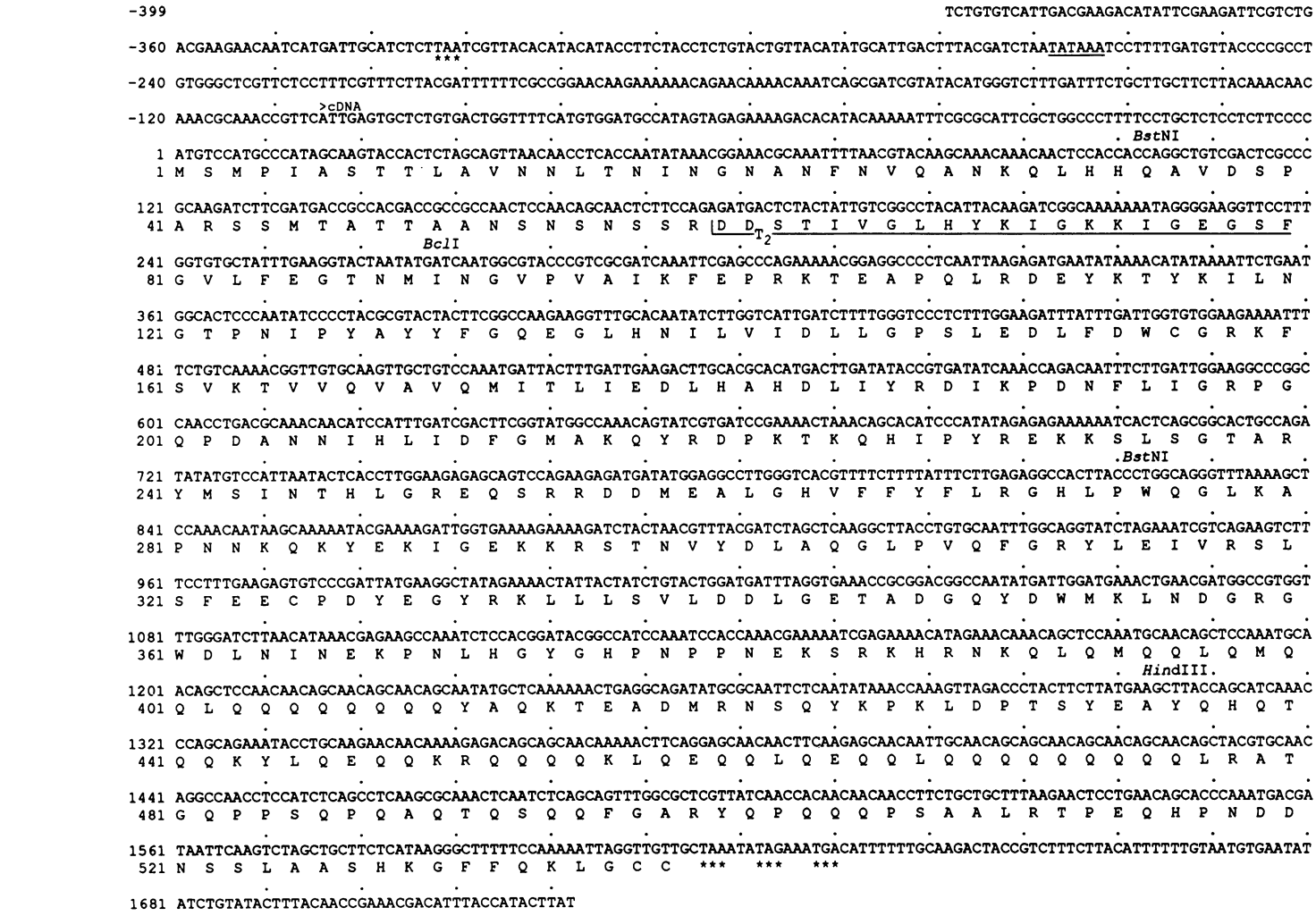

Figure 3. Nucleotide and deduced amino acid sequences of CKI2. In-frame stop codons are marked with three asterisks. Underlined amino acid residues correspond to the amino-terminal portion of tryptic peptide T2. A potential TATA box at position −268 is underlined. The 5′ end of the *CKI2* cDNA is indicated at nucleotide position −105. Restriction sites are indicated for *Bcl* I (position 267), *Bst*NI (positions 102 and 721), and *Hin*dIII (position 1303).

other invariant residues associated with protein kinases are identifiable as well. The principle exception occurs in subdomain VIII, where the common peptide triplet Ala-Pro-Glu is replaced by Ser-Ile-Asn. In the cyclic AMP-dependent protein kinase, the third residue (Glu) of this triplet forms a salt bridge with a conserved arginine residue located in subdomain XI (Knighton *et al.*, 1991). Although the Asn substitution can no longer bond in this manner, it may still make contact with domain XI through hydrogen bonds. Not surprisingly, the invariant arginine residue normally found 11 residues carboxy-terminal to a conserved hydrophobic amino acid in domain XI is not apparent in CKI1 or CKI2.

Chromosomal Location of CKI1 and CKI2

After hybridizing their full-length cDNAs to chromosome blots, CKI1 and CKI2 were localized to chromosomes XIV and VIII, respectively. The precise location of the genes within these chromosomes was determined by hybridizing the same probes to filters containing the yeast genomic library of Olson *et al.* (1986). Hybridization to clone 6572 placed *CKI1* on the long arm of chromosome 14, ~300 kb from the telomere on the physical map, and between *KEX2* and *RAS2* on the linkage map. Hybridization to adjacent clones 5283 and 7054 placed *CKI2* on the long arm of chromosome 8, ~280 kb from the centromere on the physical map, and distal to *CDC12* on the linkage map.

Casein Kinase-1 is Essential

To determine the phenotype resulting from the disruption of one or both *CKI* genes, deletion alleles were constructed in vitro. *CKI1* was inactivated by replacing its

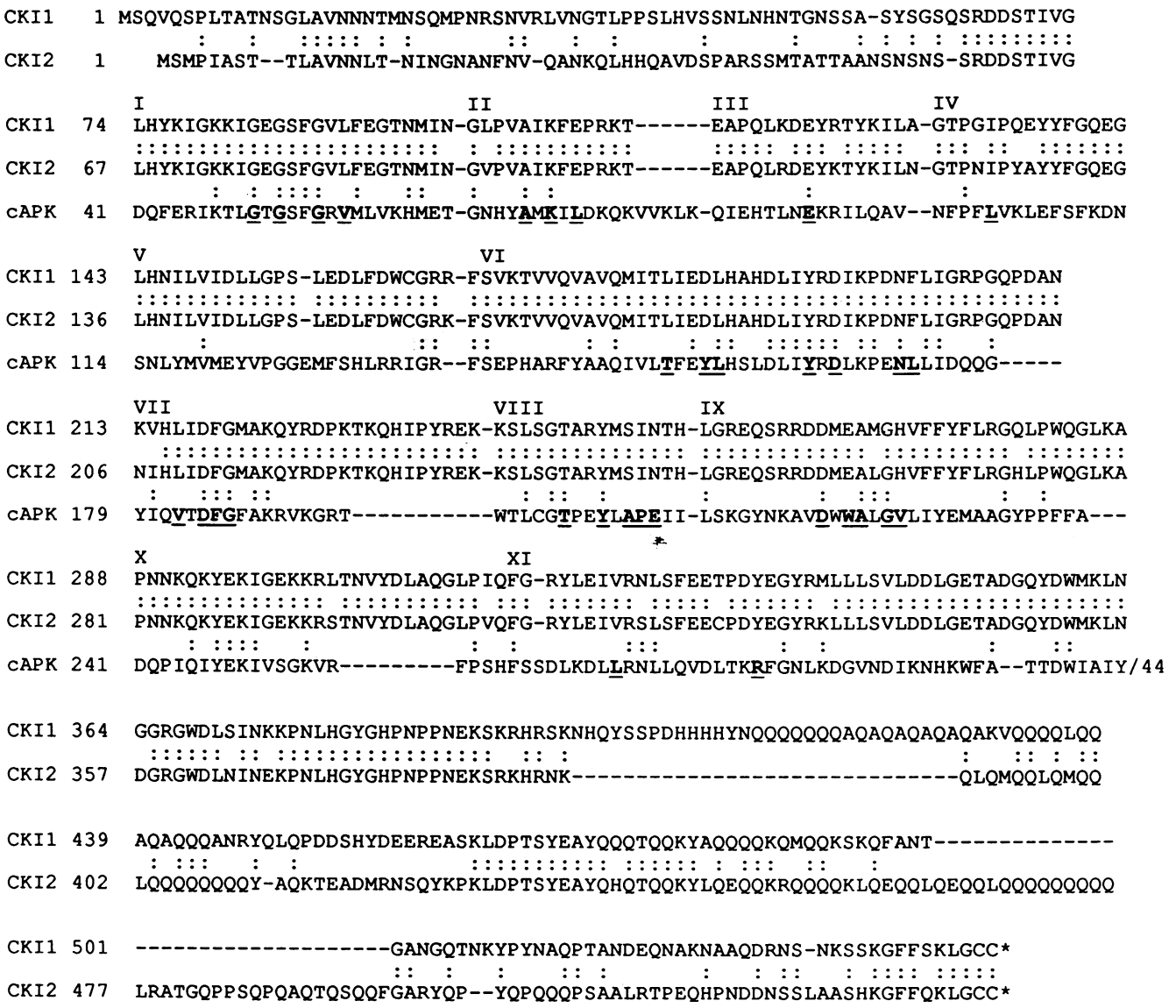

Figure 4. Sequence alignment of CKI1, CKI2, and the cAMP-dependent protein kinase (bovine C_α). Sequences were first aligned using the FASTDB program (Brutlag *et al.*, 1990), then adjusted by eye and divided into 11 subdomains as described by Hanks and Quinn (1991).

catalytic region (amino acid residues 96–465) with a 1.2-kb DNA fragment containing the *URA3* gene, whereas *CKI2* was inactivated by replacing its catalytic region (amino acids 91–434) with a 1.8-kb fragment containing the *HIS3* gene. Using these deletion alleles, heterozygous diploid strains JK 11 and JK21 (Table 2) were constructed.

JK11 and JK21 sporulated normally and, on tetrad dissection, yielded four viable colonies of haploid cells that were indistinguishable from wild-type cells (W303-1a) in growth rate (rich medium, 30°C). Genomic Southern analysis confirmed that URA^+ segregants contained the deletion allele of *CKI1* and the wild-type allele of *CKI2* and that HIS^+ segregants contained the deletion allele of *CKI2* and the wild-type allele of *CKI1* (Figure 5). Thus, cells that have only one *CKI* gene grow as well as wild-type cells that have two intact *CKI* genes.

Disruption of *CKI2* was also carried out in a homozygous diploid $cki1^-$ strain (JK31; created by mating strains JK11-1a and JK11-1b). The resultant strain, JK101, sporulated normally and, on tetrad dissection, consistently yielded only two viable colonies (Figure 5). None of the haploid colonies obtained in this way were histidine prototrophs, indicating that *CKI2* disruption is lethal in a $cki1^-$ background. Microscopic examination of spores that failed to grow into colonies after incu-

Table 1. Comparison among carboxy-terminal regions of yeast casein kinase-1 and low molecular weight GTP binding proteins

Locus	Species[a]	Sequence[b]
CKI1	Sc	534/ SSKGFFSKLGCC
CKI2	Sc	526/ SHKGFFSNLGCC
YPT1	Sc	194/ GQSLTNTGGGCC
SEC4 (YPT2)	Sc	203/ SGSGNSSKSNCC
ypt3	Sp	202/ DLNKKKSSSQCC
SAS1	Dd	196/ LGANNNKKKACC
SAS2	Dd	191/ CITPNNKKNTCC

[a] Sc, *Saccharomyces cerevisiae;* Sp, *Schizosaccharomyces Pombe;* Dd, *Dictyostelium discoideum.*

[b] Sequence data are the following: *YPT1,* Gallwitz *et al.,* 1983; *SEC4,* Salminen and Novick, 1987; *ypt3,* Miyake and Yamamoto, 1990; *SAS1* and *SAS2,* Saxe and Kimmel, 1990.

bation for 50 h at 30°C revealed single unbudded cells that remained the size of ungerminated spores. Thus, in a W303 background, haploid spores that are cki$^-$ either fail to germinate or germinate and arrest as small cells before bud formation. These results, taken together with the results described above, suggest that although neither of the two *CKI* genes is essential by itself, at least one *CKI* gene is required for yeast cell viability.

CKI is Associated With Membranes

Subcellular fractionation in conjunction with Western blotting was used to determine whether CK1 is membrane associated or soluble. To facilitate detection of the enzyme, CKI1 was tagged with a 19-amino acid peptide and expressed on a high-copy plasmid as described in MATERIALS AND METHODS. The peptide tag, which contained the influenza hemaglutinin epitope recognized by monoclonal antibody 12CA5 (Field *et al.,* 1988), was placed at the amino terminus of CKI1 to avoid interference with the putative site of prenylation found at its carboxyl terminus. The growth kinetics of the final overexpression strain (JK5) in rich and selective media were indistinguishable from its parent wild-type strain (W303).

When JK5 cells were lysed, boiled immediately in 2% SDS, and the whole-cell homogenate subjected to Western analysis with antibody 12CA5 as described in MATERIALS AND METHODS, epitope-tagged CKI1 was detected as a 66-kDa protein (Figure 6, lane 3). This mass approximated the 64.2-kDa mass predicted for tagged CKI1 and was not detected in strains that did not express the epitope-tagged CKI1 (W303; Figure 6, lane 4). When JK5 cells were lysed in the absence of SDS, epitope-tagged CKI1 underwent rapid proteolysis to smaller immunoreactive forms. The inclusion of protease inhibitors PMSF, leupeptin, pepstatin, and aprotinin during cell lysis helped retard but could not prevent proteolysis. Because the epitope tag was fused to the amino terminus of CKI1 and was necessary for immunodetection, only proteolysis occurring at the carboxyl terminus was detected. Nonetheless, membranes prepared from JK5 cells in the presence of protease inhibitors clearly contained intact epitope-tagged CKI1 along with a major proteolysis product of 52 kDa (Figure 6, lane 2). The cytosolic fraction from JK5 cells, however, contained only the 52-kDa fragment and no intact epitope-tagged CKI1 (Figure 6, lane 1). We conclude that epitope-tagged CKI1 associates exclusively with yeast cell membranes and that proteolysis occurring at the carboxyl terminus releases truncated forms of it into the cytosol.

DISCUSSION

In yeast, CK1 consists of two closely related isozymes that are encoded by the *CKI1* and *CKI2* genes and are characterized by a mass of ~62 kDa, an extremely alkaline isoelectric point (>9) and a conserved structural organization. In addition, yeast CK1 joins a growing list of proteins that contain two cysteine residues at their carboxyl termini. In *S. cerevisiae,* this "CC motif" functions as a substrate recognition sequence for a protein: geranylgeranyl transferase of which the *BET2* gene product is a subunit (Rossi *et al.,* 1991). Proven substrates for this protein prenyl transferase include the membrane-associated, CC motif-containing, small GTP binding proteins SEC4 and YPT1, both of which require prenylation for membrane attachment (Rossi *et al.,* 1991). Although it is likely that the association of CKI1 with membranes results from a similar mechanism, we cannot rule out the participation of other factors. Indeed, mammalian uroporphyrinogen III synthase contains the CC motif yet is classically described as a soluble enzyme (Tsai *et al.,* 1988).

Our model for yeast CK1 structure is presented in Figure 7. We predict that at least one geranylgeranyl group anchors the CK1 catalytic domain to cellular membranes via a flexible glutamine-rich sequence that functions as a "tether." Although CKI1 and CKI2 differ substantially in amino acid sequence outside their catalytic domains and CC motifs, this organization of domains is conserved and probably facilitates access to physiological substrates. Because CK1 is always isolated as a constitutively active enzyme (Tuazon and Traugh, 1991) and because its substrate selectivity requirements are still under investigation, it is unclear whether CK1 is regulated by a "pseudosubstrate" or "autoinhibitory" domain (Soderling, 1990) or where such a domain would exist in the primary structure we report here. With its short length and divergent amino acid sequence, the amino-terminal portion of CKI1 and CKI2 is unlikely to serve a regulatory function analogous to the amino-terminal regions of the cGMP-dependent protein kinase

Table 2. *S. cerevisiae* strains used in this study

Strain	Genotype	Source
SP1	*MATa ade8 his3 leu2 trp1 ura3 can1*	Kataoka *et al.*, 1985
W303	*MATa/MATα ade2-1/ade2-1 his3-11,15/his3-11,15 leu2-3,112/leu2-3,112 trp1-1/trp1-1 ssd 1-d2/ssd1-d2 ura3-1/ura3-1 can1-100/can1-100*	Sutton *et al.*, 1991
W303-1a	*MATa ade2-1 his3-11,15 leu2-3,112 trp1-1 ura3-1 ssd1-d2 can1-100*	Sutton *et al.*, 1991
W303-1b	*MATα ade2-1 his3-11,15 leu2-3,112 trp1-1 ura3-1 ssd1-d2 can1-100*	Sutton *et al.*, 1991
JK5	W303 [pAD5-*CK11*]	This study
JK11	W303 *CKI1/cki1::URA3*	This study
JK11-1a	W303-1a *cki1::URA3*	This study
JK11-1b	W303-1b *cki1::URA3*	This study
JK21	W303 *CKI2/cki2::HIS3*	This study
JK21-1a	W303-1a *cki2::HIS3*	This study
JK21-1b	W303-1b *cki2::HIS3*	This study
JK31	W303 *cki1::URA3/cki1::URA3*	This study
JK101	W303 *cki1::URA3/cki1::URA3 cki2::HIS3/+*	This study

(Takio *et al.*, 1984) or protein kinase C (Levin *et al.*, 1990). Alternatively, if regulation of CK1 activity occurs, it may be through posttranslational modification, through association with other polypeptides (e.g., a regulatory subunit), or through limitation of its access to physiological substrates by changes in its subcellular distribution. Regarding this last possibility, it has been proposed that the membrane association of other CC motif-containing proteins (YPT1 and SEC4) is reversible (Walworth *et al.*, 1989).

The predicted mass of yeast CK1 (≈62 kDa) is larger than the 54-kDa protein we purified at the outset (Kuret, unpublished data). As shown in Figure 6, the likely cause of this discrepancy is proteolysis that occurs in the glutamine-rich tether region, ~80 residues from the carboxyl terminus. Consistent with this hypothesis, the amino terminus of our purified CK1 is blocked and contains a peptide (T1) that is just 33 amino acids from the predicted amino terminus of CKI1. Apparently, detergent extraction coupled with partial proteolysis during purification can produce a soluble 54-kDa fragment with protein kinase activity (because digestion of this protein with trypsin generated peptide T2 from peptide T1 and because T1 is present only in CKI1, the 54-kDa prep-

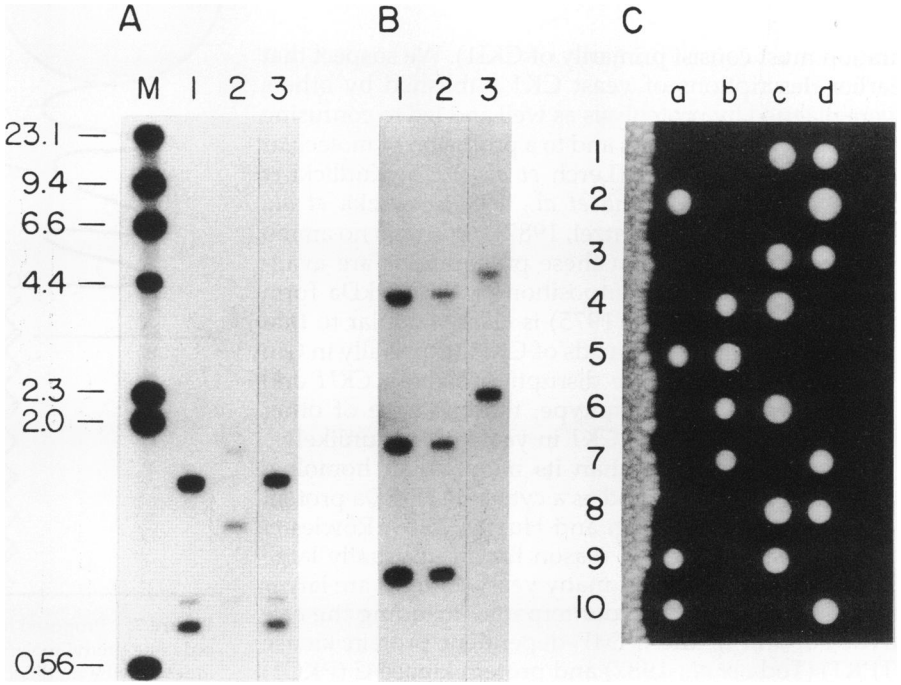

Figure 5. Phenotype of cki-cells. DNA from haploid strains W303-1a (lane 1), JK21-1a (lane 2), and JK11-1a (lane 3) was digested with *Bst*NI and prepared for genomic Southern analysis as described in MATERIALS AND METHODS. The resultant filter was probed at high stringency with the full-length cDNAs of A, *CKI2*, and B, *CKI1*. Markers (lane M) consisted of end-labeled *Hin*dIII-digested Lambda DNA. The positions of the *Bst*NI sites used for Southern analysis relative to the *Bcl* I and *Hin*dIII sites used for creating deletion alleles for *CKI1* and *CKI2* are shown in Figures 2 and 3, respectively. (C) Tetrad analysis of strain JK101. Haploid segregants from 10 tetrads were dissected and grown on rich medium at 30°C for 5 d. Each row represents a tetrad (a–d).

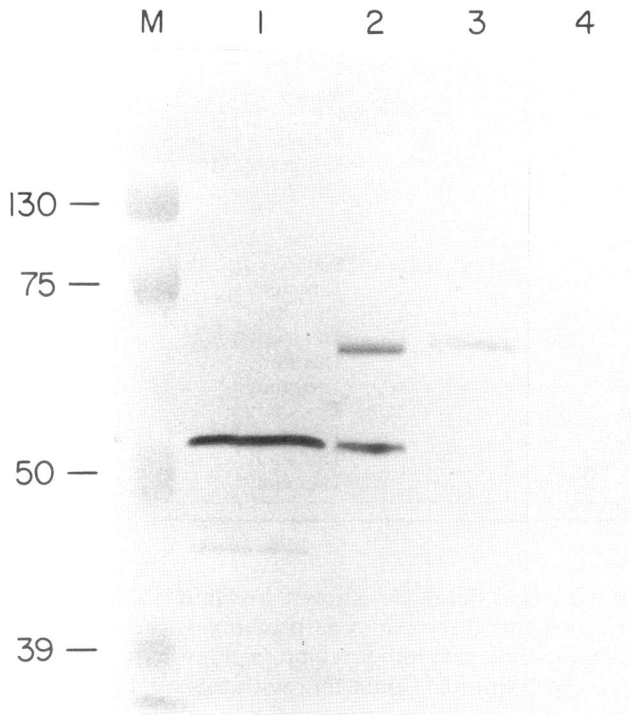

Figure 6. CKI1 is a particulate enzyme. Cytosol, membranes, and whole-cell lysates were subjected to Western analysis using monoclonal antibody 12CA5 as described in MATERIALS AND METHODS. Lane 1, cytosol from JK5 cells (S2); lane 2, membranes from JK5 cells (P2); lane 3, whole-cell lysate from JK5 cells prepared in 2% SDS with immediate boiling; lane 4, whole-cell lysate from W303 cells prepared in 2% SDS with immediate boiling. Prestained markers (lane M) included phosphorylase b (130 kDa), bovine serum albumin (75 kDa), Ovalbumin (50 kDa), and carbonic anhydrase (39 kDa).

aration must consist primarily of CKI1). We suspect that earlier descriptions of yeast CK1 published by others were plagued by proteolysis as well and led to confusing claims of molecular mass and to a profusion of molecular forms in the literature (Lerch *et al.*, 1975; Kudlicki *et al.*, 1980; Donella-Deana *et al.*, 1985; Szyszka *et al.*, 1985; Sternbach and Küntzel, 1987). Although no amino acid sequence data from these preparations are available, the amino acid composition of the 43-kDa form isolated by Lerch *et al.* (1975) is clearly similar to that of the first ≈380 amino acids of CKI1 (especially in Glu + Gln content). Because disruption of both *CKI1* and *CKI2* yields a clear phenotype, the existence of other functional homologs of CK1 in yeast cells is unlikely.

Yeast CK1 is larger than its mammalian homolog, which is typically isolated as a cytosolic 37-kDa protein (Kuret *et al.*, 1985; Singh and Huang, 1985; Rowles *et al.*, 1991). Although the reason for an unusually large CK1 in yeast is not clear, many yeast proteins are larger than their mammalian counterparts, including the catalytic subunit of the cAMP-dependent protein kinase (TPK1) (Toda *et al.*, 1987) and protein kinase C (PKC1) (Levin *et al.*, 1990). The deduced primary structure of the 37-kDa form of mammalian CK1 lacks carboxy-terminal cysteine residues and is probably not associated with membranes (Rowles *et al.*, 1991). Yet larger membrane-associated forms of mammalian CK1 may exist. Indeed, there is evidence for a 50-kDa protease-sensitive form of CK1 in mammalian nuclei (Delpech *et al.*, 1986).

Together, HRR25, CKI1, CKI2, and mammalian CK1 form a distinct branch of the protein kinase family. These enzymes contain neither a conserved Glu residue in subdomain VIII nor a conserved Arg residue in subdomain XI; two residues that are present in nearly all protein kinases identified to date and that are thought to interact with each other by forming a salt bridge (Knighton *et al.*, 1991). Thus far, in the CK1 family of kinases, these residues are most frequently substituted with Asn (in subdomain VIII) and Tyr (in subdomain XI). It is unclear whether these substitutions have a role in shaping the substrate selectivity of CK1. It would be of interest to determine if HRR25 is an efficient casein kinase in vitro.

Although CK1 activity has been identified in many eukaryotic systems, its purpose has been a mystery (Tuazon and Traugh, 1991). The current work dem-

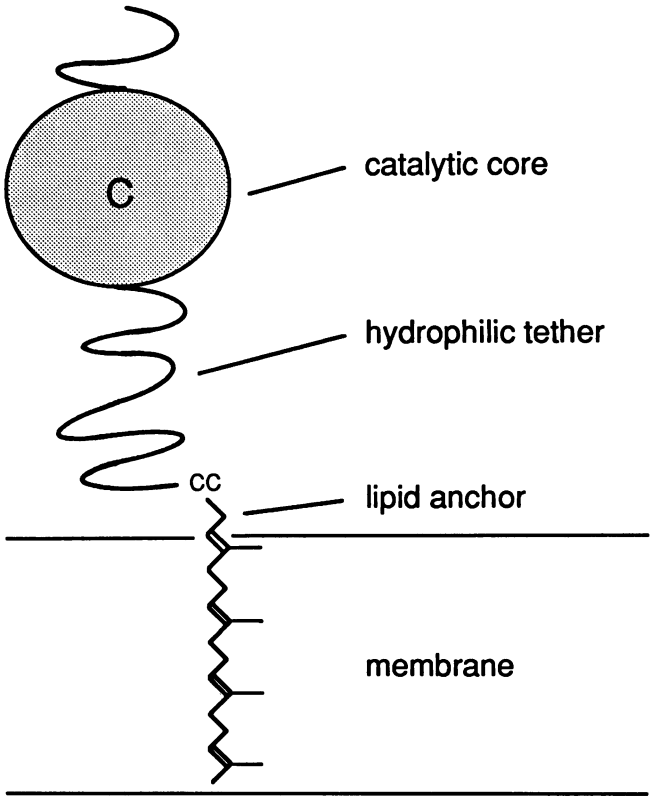

Figure 7. Proposed model for CK1 quarternary structure. Although only one prenyl moiety (geranylgeranyl) is shown, both cysteine residues may be modified with this lipid.

onstrates that CK1 plays a vital role in cellular function. Its surprising membrane localization suggests it has a more specialized function than thought previously and that it may regulate processes such as cross-membrane signaling or membrane trafficking. Interestingly, two other members of the CC motif-containing family, SEC4 and YPT1, associate with intracellular membranes and are thought to regulate intracellular transport of vesicles (Goud *et al.*, 1988; Segev *et al.*, 1988). Clearly, it will be of interest to determine the subcellular location of CK1. We now have the tools necessary to begin such a study and to continue the genetic and biochemical dissection of CK1 function in yeast.

We note that the sequences of CKI1 and CKI2 have been confirmed independently and have been deposited in Nucleotide Sequence Databases as YCK2 and YCK1, respectively (accession numbers M74552 and M74453).

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