Two Yeast Genes Encoding Calmodulin-dependent Protein Kinases

ISOLATION, SEQUENCING, AND BACTERIAL EXPRESSIONS OF CMK1 AND CMK2*

(Received for publication, October 1, 1990)

Yoshikazu Ohyama†, Hiroshi Kawasaki†, Koichi Suzuki§, John Lonesborough¶, and Yasuhiro Anraku¶

From the †Department of Biology, Faculty of Science, University of Tokyo, Hongo, Bunkyo-ku, Tokyo 113, Japan, the ¶Department of Molecular Biology, The Tokyo Metropolitan Institute of Medical Science, Bunkyo-ku, Tokyo 113, Japan, and the §Research Laboratories of the Finnish State Alcohol Company (Alko), Box 350, SF-00101 Helsinki 10, Finland

We have isolated two genes from Saccharomyces cerevisiae that both encode a calmodulin-dependent protein kinase (CaM kinase). The CMK1 gene has been cloned by hybridization using an oligonucleotide probe synthesized on the basis of the peptide sequence of purified yeast CaM kinase (Lonesborough, J. (1989) J. Gen. Microbiol. 135, 3373–3383). The other gene, CMK2, which is homologous to CMK1, has been isolated by screening at low stringency with a CMK1 fragment as a probe. The CMK2 product expressed in bacteria shows Ca2+- and CaM-dependent protein kinase activity, indicating that CMK2 also encodes a CaM kinase. The CMK1 and CMK2 products expressed in bacteria were found to have different biochemical properties in terms of autoregulatory activity and preference for yeast CaM or bovine CaM for maximal activity. Antibody raised against a peptide fragment of the CMK1 protein cross-reacts with the CMK2 product. Immunoblotting with this antibody indicated that the CMK1 and CMK2 products have apparent molecular masses of 56 and 50 kDa, respectively, in yeast cells. The predicted amino acid sequences of the two CMK products exhibit highest similarity with mammalian calmodulin-dependent multifunctional protein kinase II (CaM kinase II); the similarity within the N-terminal catalytic domain is about 40%, whereas that within the rest of the sequence is 25%. These data indicate that yeast has two kinds of genes encoding CaM kinase isozymes whose structural and functional properties are closely related to those of mammalian CaM kinase II. Another gene may be substituted for function of the CMK1 and CMK2 kinase in vivo, since elimination of both kinase genes is not lethal.

Ca2+-CaM*-dependent protein phosphorylation is a key step in the regulatory cascade involved in control of a diverse range of physiological processes, such as carbon metabolism, muscle contraction, and nerve signal transmission (1–3). An intracellular Ca2+ signal evoked by extracellular stimuli is thought to promote protein phosphorylation through a signal-transducing system involving CaM and CaM kinases. Several lines of evidence also suggest that a CaM kinase plays fundamental roles during cell cycle progression, especially in mitosis (4, 5).

CaM kinases are classified into two groups on the basis of their substrate specificities (6): one with narrow substrate specificity and the other with broad substrate specificity. The former group includes phosphorylase kinase (7), myosin light chain kinase (8), CaM kinase I (9), CaM kinase III (10), and CaM kinase Gr (11). Each CaM kinase in this group phosphorylates a target protein specific for the kinase, and thereby regulates the activity of its own discrete process. The latter group includes CaM kinase II isozymes (12, 13), which phosphorylate more than 10 different target proteins, modulating their functions simultaneously (14). These findings, together with the fact that isozymes of the CaM kinase II family (50–60 kDa) are distributed widely in many tissues and organisms (15, 16), suggest that this kinase is responsible for the regulations of a wide variety of proteins in vivo and plays central roles in Ca2+/CaM signal transduction pathways.

The primary structure of CaM kinase II has been studied extensively in the last 4 years. Five homologous rat brain cDNAs encoding subunits of CaM kinase II isoforms have thus far been identified. The similarities in the entire amino acid sequences of the α (478 amino acid residues) (17, 18), β (542 amino acid residues) (19), β* (527 amino acid residues) (19), γ (527 amino acid residues) (20), and δ (533 amino acid residues) (21) subunits are more than 80%. Analyses of the deduced amino acid sequences have suggested that each subunit is composed of three functional domains: a catalytic domain within the N-terminal half, a multifunctional regulatory domain mapped in the central portion, and a C-terminal portion, which is thought to play a role in subunit assembly and/or subcellular localization (22, 23). The multifunctional regulatory domain is composed of an autoinhibitory region (residues 281–302 of the α subunit) (24) and a CaM-binding region (residues 296–309) (25). This regulatory domain shows the unique features that autoprophosphorylation of Thr296 in the autoinhibitory region greatly diminishes the binding of this region to the catalytic domain and converts the kinase of a Ca2+/CaM-independent form (26–32).

The primary structures of other specific CaM kinases, such as phosphorylase b kinase (33) and myosin light chain kinase (34) have also been determined. A comparison of their catalytic domains shows that these three CaM kinases are closely related.
related (18) and are members of the same protein kinase subfamily (35). In contrast, their other domains have less or no similarity.

Two previous papers reported the existence of CaM kinase activity in cell extracts of the yeast Saccharomyces cerevisiae (36, 37), and recently, Londershough (38) reported purification of yeast CaM kinase to near homogeneity. He found that the purified fraction contains an autophosphorylatable 56-kDa CaM kinase with broad substrate specificity. These biochemical findings suggest that the properties of the yeast enzyme are similar to those of mammalian CaM kinase II.

Analysis of conditional lethal mutants showed that yeast CaM is involved in nuclear division (39, 40), but little is known about the essential target of CaM in mitosis, and there is no information available on the role of yeast CaM kinase in this process. We attempted to determine the functional role of the CaM kinase in cell proliferation and growth control of yeast by molecular genetic studies. This paper reports the nucleotide sequences of two yeast CaM genes both encoding a CaM kinase. The first CaM kinase gene (CMKI) encodes the CaM kinase purified previously (38). The second CaM kinase, the product of the CMK2 gene, is very similar in primary sequence to the CMKI product, but is distinct from it.

MATERIALS AND METHODS

RESULTS

Peptide Sequence Analysis of Yeast CaM Kinase—The partial amino acid sequence of yeast CaM kinase was determined from peptide fragments of the purified protein. Yeast CaM kinase was purified as described in Ref. 38. Traces of two yeast polypeptides contaminating the final preparation (38) were removed by one-dimensional polyacrylamide gel electrophoresis. Proteins were detected by staining with Coomassie Brilliant Blue, and regions containing the CaM kinase were cut out and treated with lysyldipeptidase (41). Peptide fragments released from the gel were recovered in the supernatant, separated by reverse-phase high performance liquid chromatography (42), and sequenced with an automated gas-phase peptide sequenator. Six discrete sequences (TN18, TN19, TN28A, TN28B, TN33, and TN40) recovered from five peaks of material separated by high performance liquid chromatography were determined (Fig. 1A).

Isolation of the CMKI Gene—On the basis of the TN33 peptide sequence, two types of 44-mer oligonucleotide probe were synthesized (Fig. 1B). The TN33N probe was synthesized according to the most frequent codon usage in S. cerevisiae (43). The TN33C probe was designed to take advantage of the weak G-T mismatch in base pairing (44). For characterization of these two probes, we hybridized them to yeast genomic DNA digested with BamHI (Fig. 2). Under a low stringency condition at 49 °C, TN33C hybridized to only one fragment of 1.5 kb, while TN33N hybridized to many fragments. In a more stringent condition at 54 °C, TN33C still hybridized to the same fragment, but no fragment was detected with TN33N. From these results, the TN33C probe was chosen for cloning the yeast CaM kinase gene. We used a yeast genomic bank, which was divided into 48 pools, each containing 196 independent clones (45). On screening DNA from the 48 pools by dot hybridization, 5 pools gave positive signals. By a second screening of these 5 pools, 5 independent positive clones (pJOY101-pJOY10S) were obtained. These five plasmids carried a 1.5-kb BamHI fragment that hybridized to the TN33C probe. Moreover, restriction enzyme mapping indicated that they all contained the same DNA region (data not shown). Subcloning of the pJOY101 revealed that it contained a 2.8-kb KpnI-ClaI fragment that hybridized to the TN33C probe.

Nucleotide sequencing of the 2.8-kb KpnI-ClaI fragment from pJOY101 revealed the presence of a single open reading frame (ORF) that encodes a polypeptide of 446 amino acids with a calculated molecular mass of 50,295 daltons (Figs. 3 and 4). The ATG (position 1), a start codon of the ORF, is immediately preceded by translation termination codons in all three reading frames. No conserved splicing signal sequences are present near the coding region. Of particular significance is the fact that nucleotide sequences that could encode the six peptide fragments obtained from the purified CaM kinase (38) were all found to be included in the ORF. Thus we named the gene CMKI (for CaM-dependent multifunctional protein kinase).

Isolation of the CMK2 Gene—From structural analysis of rat CaM kinase II cDNA, it has been established that the α, β, γ, and δ subunits of rat CaM kinase II represent a gene family. Therefore, we next examined whether yeast contains any gene that is homologous to CMKI by Southern blotting (Fig. 5). We used a 1.1-kb SphI-HindIII fragment within the CMKI coding region as a probe, and digested yeast genomic DNA with BamHI. Under both high (65 °C) and low (57.5 and 50 °C) stringent conditions, the CMKI probe hybridized strongly to CMKI (1.5- and 2.0-kb fragments). In addition, it hybridized weakly to another homologous fragment (>10-kb fragment) under the low stringent condition. Digestions of yeast genomic DNA with three other restriction enzymes (EcoRI, HindIII, and PstI) gave essentially similar results, suggesting the existence of one homologous gene in S. cerevisiae. For cloning this gene, we used dot hybridization with the yeast genomic library described above (45). Positive signals were obtained under the low stringent condition (50 °C) from 9 to 48 pools, including 5 pools which contained the CMKI clone. Positive clone (pJOY201, pJOY202, pJOY203, and pJOY301) were obtained independently from the other 4 pools by colony hybridization and analyzed with a restriction enzyme. Three of the four clones (pJOY201, pJOY202, and pJOY203) were shown to contain a 2.8-kb PstI fragment that hybridized to the CMKI probe only at low stringency. We, therefore, characterized this fragment further. Analysis of the restriction enzyme map and partial DNA sequence showed that the pJOY301 plasmid contained a chimeric DNA derived from CMKI and another genomic region (data not shown), and so was possibly an artifact generated during construction of the library.

Nucleotide sequencing of the 2.8-kb PstI fragment from pJOY201 revealed the presence of a single ORF encoding a protein of 447 amino acids (M, = 50,447) (Figs. 3 and 6) that is highly homologous to the predicted CMKI product. The deduced amino acid sequence is 83% identical to that of the CMKI product. Moreover, when conserved amino acid replacements are included, the similarity is more than 96%. The identity at the nucleotide level is more than 60%. From these facts, the cloned CMKI-related gene was designated as CMK2. Genomic Southern blot hybridization using the 1.2-kb BglII-BglII fragment from the CMK2 gene as a probe was performed under the low stringency condition. However, this
Two Yeast CaM Kinase Genes

---

Two Yeast CaM Kinase Genes

---

The sequence determined from six peptide fragments are underlined.

---

FIG. 4. Nucleotide sequence and predicted amino acid sequence of the CMK1 gene. Numbers indicate the nucleotide residues from the predicted translation initiation site. The sequence determined from six peptide fragments are underlined.

---

FIG. 5. Genomic Southern blot hybridization with a CMK1 DNA fragment as a probe. Yeast genomic DNA was digested with BamHI, and a 1.1-kb SpII-HindIII fragment within the CMK1 coding region was used as a probe. On the Nylon membrane, 0.5 (left) or 1.0 pg (right) of yeast genomic DNA was blotted. Hybridization was carried out under high (65°C) and low (57.5 and 50°C) stringency conditions. The large arrows indicate 1.5- and 2.0-kb fragments of the CMK1 gene. The small arrows indicate a >10-kb fragment seen only in the low stringency condition.

---

Expressions of CMK1 and CMK2 Proteins in Escherichia coli—We think that CMK1 encodes the 56-kDa yeast CaM kinase.

---

Recently, a plasmid-based DNA library of chromosome VI was constructed completely: Restriction enzyme analysis as well as Southern blot analysis with this DNA library revealed that CMK1 was located on the right arm of chromosome VI, adjacent to the SUP11 gene (48): the order is CENG-SUP11-CMK1 (Fig. 7).

---

probe did not detect any additional members of this gene family in the yeast genome.

---

Chromosome Mapping of CMK Genes—Southern blot hybridization was performed after separating intact yeast chromosomes on orthogonal field-agarose gel electrophoresis gels (46). The probes used were a 1.1-kb SpII-HindIII fragment from pJOY101 and a 1.3-kb BglII-BglII fragment from pJOY201. Under the stringent condition, each probe hybridized to a single fragment: CMK1 was assigned to chromosome VI, and CMK2 to chromosome XV. Tetrad analysis provided additional information about the map positions of CMK1 and CMK2. A diploid strain YOJ211 heterozygous for both CMK1 and CMK2 loci (CMK1A;cmk1::TRP1 CMK2A;cmk2::LEU2) was constructed, and subjected to meiosis and spore formation. More than 90% of the tetrads produced 4 viable spores and phenotypic analysis showed that 50 of 54 tetrads were either the parental ditype or the nonparental ditype (PD:NPD:T = 23:27:4). These observations indicate that the CMK1 and CMK2 genes are both tightly linked to the centromere. Linkage between CMK2 and pho80 (47) was also confirmed by tetrad analysis (PD:NPD:T = 43:0:11, Fig. 7).

---

Expressions of CMK1 and CMK2 Proteins in Escherichia coli—We think that CMK1 encodes the 56-kDa yeast CaM kinase.

---

FIG. 5. Genomic Southern blot hybridization with a CMK1 DNA fragment as a probe. Yeast genomic DNA was digested with BamHI, and a 1.1-kb SpII-HindIII fragment within the CMK1 coding region was used as a probe. On the Nylon membrane, 0.5 (left) or 1.0 pg (right) of yeast genomic DNA was blotted. Hybridization was carried out under high (65°C) and low (57.5 and 50°C) stringency conditions. The large arrows indicate 1.5- and 2.0-kb fragments of the CMK1 gene. The small arrows indicate a >10-kb fragment seen only in the low stringency condition.

---

Expressions of CMK1 and CMK2 Proteins in Escherichia coli—We think that CMK1 encodes the 56-kDa yeast CaM kinase.

---

Y. Ohya and Y. Anraku, unpublished results.

---

N. Ogasawara, personal communication.
Two Yeast CaM Kinase Genes

12787

**FIG. 6.** Nucleotide sequence and predicted amino acid sequence of the CMK2 gene. Numbers indicate the nucleotide residues from the predicted translation initiation site.

**FIG. 7.** Genetic map of chromosome VI and XV. The CMK1 and CMK2 genes are tightly linked to the centromere on chromosome VI and XV, respectively.

kinase purified by Londesborough (38) (see “Discussion”).

But, the only known fact about the CMK2 gene was its striking homology to CMK1. To obtain direct evidence that the CMK2 product is a CaM kinase, we examined the biochemical properties of the CMK2 protein expressed in E. coli. First, an NcoI site was introduced at the predicted start codon of CMK1 and CMK2 by site-directed mutagenesis. This caused a change of the second amino acid residue from Pro to Ala in the CMK2 protein, but no change in the CMK1 protein. Then, all coding regions were placed under the control of the T7 promoter (49): pET-CMK1 and pET-CMK2 are expression plasmids for the CMK1 and CMK2 proteins, respectively.

The E. coli BL21(DE3) strain harboring pLysS (49) was transformed with these plasmids, and the induced proteins in the bacterial lysate were analyzed by SDS-PAGE. Cells carrying pET-CMK1 produced a polypeptide of 56 kDa and those carrying pET-CMK2 produced two polypeptides of 50 and 46 kDa (Fig. 8); these proteins were not present in uninduced bacteria, or in induced bacteria containing a control plasmid. Densitometric analysis showed that these PET-CMK1- and PET-CMK2-dependent polypeptides constituted 8 and 17%, respectively, of the total cellular proteins. Fractionation analysis showed that the CMK1 protein was mainly present in the soluble fraction, whereas about 80% of the CMK2 proteins were present in the particulate fraction (Fig. 8).

The CMK2 proteins expressed in E. coli were partially purified on SDS-polyacrylamide gel. A, lane 1, 56-kDa purified yeast CaM kinase; lane 2, the CMK1 protein; lane 3, the CMK2 protein. Proteins (0.2 µg) were separated on SDS-polyacrylamide gel and stained with Coomassie Brilliant Blue R-250. B, autophosphorylation activities of the CMK1 and CMK2 products were analyzed as described under "Materials and Methods." Lane 1, yeast CaM kinase; lane 2, the CMK2 protein; lane 3, the CMK1 protein.
[\gamma-^{32}P]ATP, samples were subjected to electrophoresis on SDS-polyacrylamide gel, and analyzed by autoradiography. In each case, only one band corresponding to the CMK1 and CMK2 protein was detected (lanes 2 and 3). The levels of autophosphorylation reach to the plateau after incubation for 15 min (data not shown).

The protein kinase activities of the partially purified samples were next assayed with and without Ca\(^{2+}\) and CaM, using synthetic peptide as substrate. Fig. 10 shows that the CMK1 or CMK2 protein has Ca\(^{2+}\)- and CaM-dependent phosphorylation activities. The protein kinase activity was increased about 2.5-4.2-fold in the presence of Ca\(^{2+}\) and CaM. For maximal activity, CMK1 kinase preferred bovine CaM to yeast CaM, while CMK2 kinase preferred yeast CaM. Purified yeast CaM kinase has previously been shown to prefer bovine CaM for maximal activity (Fig. 10; Ref. 38), like the yeast CaM kinase. The yeast CaM kinases phosphorylated synthetic peptides containing gizzard myosin light chain (Kemptamide) more than that containing phosphorylation site 2 of glycogen synthase (Syntide 2). This is another characteristic of the yeast CaM kinases, since brain CaM kinase II was reported to phosphorylate Syntide 2 5.5-fold more than Kemptamide (50).

Detection of CMK1 and CMK2 Products in Yeast—To identify the authentic CMK products in yeast, we prepared specific rabbit antiserum. The antiserum was raised against a polypeptide fragment (residues 69-341) of CMK1 (see "Materials and Methods"), and found to cross-react with both the CMK1 and CMK2 proteins expressed in bacteria (data not shown). Affinity purified antibody was used in later experiments.

The CMK1 product was identified by immunoblot analysis of total yeast proteins as follows. A 56-kDa protein was detected in wild-type yeast cells and was present in greater abundance in cells carrying the CMK1 gene on a multicopy plasmid, YEp-CMK1 (Fig. 11). This protein was not present in a mutant carrying the deletion Acmk1::TRP1. Furthermore, the immunoreactive 56-kDa protein detected in a yeast lysate migrated with the purified 56-kDa yeast CaM kinase (38) on SDS-PAGE. Besides the 56-kDa protein, a 90-kDa protein was detected, but its origin is unknown. Cells carrying the CMK2 gene on a multicopy plasmid, YEp-CMK2, contained a 50-kDa protein. This protein is not a degradation product of the CMK1 protein, as it was also detected in a Δcmk1 deletion mutant carrying the YEp-CMK2 plasmid. The 50-kDa protein was not detected by immunoblot analysis in wild-type cells, indicating that it was detected only in cells with multicopies of the CMK2 gene (Fig. 11). From these results, we concluded that the CMK1 and CMK2 products in yeast have apparent molecular masses of 56 and 50 kDa, respectively.

The expression of chromosomal CMK1 and CMK2 genes was examined by immunoprecipitation (Fig. 12). Both the 56- and 50-kDa proteins were detected in wild-type cells (CMK1 CMK2), and the former was the CMK1 product as described before. The 50-kDa protein was proved to be the CMK2 gene product, since it was detected even in the Δcmk1 CMK2 cells and not in Δcmk2 cells.

Similarity of the CMK1 and CMK2 Products to Mammalian CaM Kinase II—To gain insight into the possible functions of the CMK gene products, we compared the predicted amino acid sequences of CMK1 and CMK2 with the sequences of protein kinases in the SWISS-PROT data base. Among 67

### Table I

| Substrate               | Yeast CaM kinase | CMK1 kinase | CMK2 kinase |
|-------------------------|------------------|-------------|-------------|
|                         | +CaM            | -CaM        | +CaM        | -CaM        | +CaM        | -CaM        |
|                        | nmmol P/mg/min   | nmmol P/mg/min | nmmol P/mg/min | nmmol P/mg/min | nmmol P/mg/min | nmmol P/mg/min |
| Kemptamide             | 30.0 ± 3.0       | 5.0 ± 0.4   | 25.4 ± 1.8  | 5.6 ± 0.4   | 21.6 ± 0.7  | 9.0 ± 0.9   |
| Syntide 2              | 3.6 ± 0.2        | 1.1 ± 0.2   | 5.0 ± 0.7   | 1.8 ± 0.2   | 1.8 ± 0.2   | 1.0 ± 0.1   |
| Myelin basic protein   | 151.8 ± 4.2      | 124.2 ± 5.4 | 60.2 ± 4.8  | 36.6 ± 3.0  | 46.0 ± 3.6  | 19.8 ± 1.6  |
| Casein                 | 24.4 ± 0.5       | 0.49 ± 0.07 | 11.8 ± 1.2  | 1.9 ± 0.2   | 2.6 ± 0.2   | 1.5 ± 0.2   |
| Histone                | 13.3 ± 0.2       | 5.9 ± 1.1   | 11.0 ± 0.5  | 5.9 ± 1.3   | 9.0 ± 0.5   | 4.1 ± 0.5   |
| Myosin light chain     | 11.6 ± 0.8       | 11.1 ± 1.3  | 7.6 ± 1.2   | 6.8 ± 0.6   | 4.5 ± 0.5   | 3.6 ± 0.4   |

\(^5\) SWISS-PROT Data Base (1989) Release 12.0, European Molecular Biology Laboratory.
purified yeast CaM kinase. Total yeast cell lysates were prepared from wild-type strain YPH501 (CMKI CMK2; lane 2), YPH501 carrying YEp-CMK1 (lane 3), YPH501 carrying YEp-CMK2 (lane 4), YPH501 carrying control vector pSET8 (lane 5), pJOY211-9D (Δcmkl CMK1; lane 6), pJOY211-9B (CMKI Δcmk2; lane 7), pJOY211-9C (Δcmk1 Δcmk2; lane 8), and pJOY211-9D (Δcmk1 CMK2) carrying YEpl-CMK2 (lane 9). Samples of 1 × 10^5 cells (about 60 μg of proteins) were separated on 10% SDS-polyacrylamide gel and blotted onto a nitrocellulose membrane. Yeast CaM kinase was detected using an affinity purified antibody against the CMKI antigen.

Fig. 11. Western blot analysis of the CMKI and CMK2 products in yeast cell lysates. Lane 1 contained 5 ng of 56-kDa purified yeast CaM kinase. Total yeast cell lysates were prepared from wild-type strain YPH501 (CMKI CMK2; lane 2), YPH501 carrying YEp-CMK1 (lane 3), YPH501 carrying YEp-CMK2 (lane 4), YPH501 carrying control vector pSET8 (lane 5), pJOY211-9D (Δcmkl CMK1; lane 6), pJOY211-9B (CMKI Δcmk2; lane 7), pJOY211-9C (Δcmk1 Δcmk2; lane 8), and pJOY211-9D (Δcmk1 CMK2) carrying YEp-CMK2 (lane 9). Samples of 1 × 10^5 cells (about 60 μg of proteins) were separated on 10% SDS-polyacrylamide gel and blotted onto a nitrocellulose membrane. Yeast CaM kinase was detected using an affinity purified antibody against the CMKI antigen.

protein kinases whose sequences are now available, the CMK kinases show the greatest homology with mammalian CaM kinase II (Fig. 13). CMKI kinase exhibits overall similarity with mammalian CaM kinase II, and the N-terminal catalytic domain shows remarkable conservation. On the basis of the alignment in Fig. 13, the CMKI kinase is 40.1% identical to the rat α subunit of CaM kinase II, and 38.9% identical to the rat β subunit of CaM kinase II in the region of catalytic subdomains I-XI (35). This value is high compared with the values for other Ser/Thr protein kinases such as yeast CDC28 (51) kinase (23.8%) and yeast TPK1 (52) kinase and cAMP-dependent protein kinase (27.8%). The homology between the CMKI and CMK2 products within the catalytic domain is also high, showing 75.4% amino acid identity. The Ca^2+/CaM-dependent protein kinases thus far identified, including CaM kinase II, myosin light chain kinase (34), and the phosphorylase b kinase γ chain (33), are closely related to one another and known to be members of the same protein kinase subfamily (35). CMKI kinase also shows similarity to myosin light chain kinase and phosphorylase b kinase, but their sequence identities with the CMKI kinase in the catalytic domain are lower than that of CaM kinase II.

From an extensive study using synthetic peptide analog, the calmodulin-binding domain of the α subunit of rat CaM kinase II has been mapped at residues 296-309 on the C-terminal side of the catalytic domain (25). The similarity between the CMKI kinase and CaM kinase II within this region is not so high: only 3 of 14 residues are identical. However, homologous positions in the two yeast CMK kinase sequences seem to have the ability to form a basic amphipathic helical structure, which is a common structural feature of calmodulin-binding domains. The hydrophilic C-terminal region of rat CaM kinase II is proposed to be a domain for assembly. Similarity within this region is also not high, although hydrophilic characteristics are conserved in both CMK kinases.

The CMKI and CMK2 kinases in yeast appear to belong to the CaM kinase II family, but they differ markedly with respect to their autophosphorylation sites (26, 27). CMK2 kinase contains a putative autophosphorylation site at position 316, but CMKI kinase does not have one in a homologous region. In the CMK1 sequence, the Thr^{35} residue on the amino-terminal side of the CaM-binding site is conserved, but the Arg located 3 residues upstream of Thr^{35} is absent. This difference is of interest because the homologous position of rat CaM kinase II plays a role in autonomous activation of kinase activity.

During the homology search, we found another CMK-related gene, the yeast SNF1 gene essential for the regulation of carbon catabolite repression (53). In the catalytic domain, 92 of 252 amino acids are identical (Fig. 13), and if conserved amino acid replacements are taken into account, the similarity is 74.6%. This gene is of further interest in that the similarity extends beyond the catalytic domain. Optimal positional similarity is achieved by deletion of the CaM-binding domain in the SNF1 sequence. In other words, the CaM-binding domain is obviously absent in the SNF1 product, but the domain required for assembly is partially conserved. It remains to be seen whether the sequence similarity between CMKI and SNF1 reflects any underlying structural or functional similarity.

Disruption of CMKI and CMK2 Gene—To obtain information on the physiological functions of yeast CaM kinases, we analyzed the phenotypes of a Δcmkl mutant, a Δcmk2 mutant, and a Δcmkl Δcmk2 double mutant. Deletion mutants of both genes with long deletions within their coding regions were constructed (Fig. 14). Diploid strains heterozygous for CMKI and CMK2 were constructed and subjected to tetrad analysis. Tetrad analysis showed that more than 90% of the ascides generated 4 viable colonies and some spores showed the Trp^+ Leu^+ phenotype. Southern blot hybridization with the CMKI and CMK2 probes showed the chromosomal gene disruption (Fig. 14). In addition, no immunoprecipitable CMK proteins were detected with antibody against the CMK products in the Δcmkl Δcmk2 double disrupted mutant (Fig. 12). Therefore, cells lacking CMKI, CMK2, or both genes are viable. In addition, cells carrying double disruptions appeared to grow normally under a variety of conditions: they grew at 17, 23, 30, and 37 °C in rich medium and in the presence of a nonfermentable carbon source, and in synthetic medium. They could conjugate well to cells with an a or a mating type, and showed no defect in meiosis or sporulation. Thus, even the double disruption of the CMK genes has no effect on any cellular activities thus far examined.

**DISCUSSION**

**CMKI and CMK2, Two Yeast CaM Kinase Genes**—We have isolated two CaM kinase genes, named CMKI and...
biochemical properties to the purified protein. The second biochemical analysis of the grates with the 56-kDa purified protein on SDS-PAGE; and of the partial amino acid sequence of the purified yeast CaM unit) is shown by an phosphorylation site essential for auto-
all six peptide sequences isolated from the purified enzyme; was also concluded to encode a CaM kinase on the basis of the predicted amino acid sequence of the gene accounts for subunits yeast CaM kinases with rat skeletal myosin light chain kinase and CAMP-dependent protein kinase and CAMP-dependent pro-
overall similarity to the CaM kinase I1 than to any other Protein kinases showing greater similarity to yeast CaM kinase to mammalian CaM kinase I1, both of which are members of the same CaM-dependent protein kinase subfamily. But their similarities to the CMK kinases are restricted to the catalytic domain, indicating that the yeast CMK kinases are not so closely related to these protein kinases as to mammalian CaM kinase II. The similarity between the two yeast CMK kinases is much higher than that between the yeast and mammalian CaM kinases, suggesting that these two yeast kinases separated after evolution of yeast. One major difference between the yeast and mammalian CaM kinases is that the amino acid sequences of yeast kinases (CMK1, 446 amino acid residues; CMK2, 447 amino acid residues) are shorter than that of CaM kinase I1 (a subunit, 478 amino acid residues). There are long deletions (approximately 80 amino acid residues) in the C-terminal portion of the yeast kinases. The C-terminal portion is thought to be involved in subunit assembly (22), so this deletion could lead to differences in the degree of oligomerization. In fact, Londo

Two Yeast CaM Kinase Genes

FIG. 13. Sequence alignment of yeast CaM kinases with rat α and β subunits of CaM kinase II and rabbit skeletal myosin light chain kinase together with yeast SNF1 protein kinase and CaM-dependent protein kinase (TPK1 product). Residues identical with those in the CMK1 gene product are enclosed in open boxes. Residues identical with the CMK2 sequence are enclosed when other residues are all different from those in CMK1. Hyphens indicate gaps introduced to optimize the alignment. Roman numerals indicate subdomains of the protein kinase catalytic domain as defined by Hanks et al. (35). Positions that are most highly conserved in all known protein kinases are indicated by solid triangles. The calmodulin-binding domain proposed from analysis of peptide fragments is shown by an asterisk.

CMK2, from the yeast S. cerevisiae. The CMK1 gene was cloned using an oligonucleotide probe synthesized on the basis of the partial amino acid sequence of the purified yeast CaM kinase. We conclude that CMK1 encodes the yeast CaM kinase that was purified by Londesborough (38) because: (i) the predicted amino acid sequence of the gene accounts for all six peptide sequences isolated from the purified enzyme; (ii) the CMK1 gene is present as a single copy in yeast chromosomes; (iii) the CMK1 product in yeast cells comigrates with the 56-kDa purified protein on SDS-PAGE; and (iv) the CMK1 product expressed in E. coli shows similar biochemical properties to the purified protein. The second gene, CMK2, was cloned by hybridization using a CMK1 fragment as a probe under a low stringency condition. CMK2 was also concluded to encode a CaM kinase on the basis of biochemical analysis of the CMK2 product expressed in E. coli and the striking similarity of the CMK2 product with the CMK1 product. Moreover, the CMK2 protein was detected in a yeast cell lysate, indicating that this gene is also expressed in yeast cells.

CMK Kinase as a Mammalian CaM Kinase II Homologue—Previous biochemical characterization has suggested the functional similarity of yeast CaM kinase to mammalian CaM kinase II (38). The sequence comparison in this study confirmed that the yeast CMK kinases are homologues of mammalian CaM kinase II, both CMK kinases showing greater overall similarity to the CaM kinase II than to any other protein kinases so far identified. In particular, the conservation of the catalytic domains of these kinases strongly suggests a functional homology in their catalytic actions. The CMK kinases also show similarity to myosin light chain kinase and phosphorylase b kinase, both of which are members of the same CaM-dependent protein kinase subfamily. But their similarities to the CMK kinases are restricted to the catalytic domain, indicating that the yeast CMK kinases are not closely related to these protein kinases as to mammalian CaM kinase II. The similarity between the two yeast CMK kinases is much higher than that between the yeast and mammalian CaM kinases, suggesting that these two yeast kinases separated after evolution of yeast. One major difference between the yeast and mammalian CaM kinases is that the amino acid sequences of yeast kinases (CMK1, 446 amino acid residues; CMK2, 447 amino acid residues) are shorter than that of CaM kinase II (α subunit, 478 amino acid residues; β subunit, 542 amino acid residues). There are long deletions (approximately 80 amino acid residues) in the C-terminal portion of the yeast kinases. The C-terminal portion is thought to be involved in subunit assembly (22), so this deletion could lead to differences in the degree of oligomerization. In fact, Londesborough (38) reported that native yeast CaM kinase is a dimer, whereas the CaM kinase II isozymes in rat forebrain are composed of 6 to 12 subunits (54).

The rat forebrain enzymes are composed of the α and β subunits. In contrast, purified yeast CaM kinase (38) only
A probe, the wild-type gene gave a 1.3-kb signal, whereas the disrupted gene gave a 1.4-kb signal on the Southern blot using a genomic DNA digested with BamHI and BglII, whereas the disrupted gene gave a 1.6-kb fragment in the Trp' Leu' transformant (YOJ211), and a 1.6-kb fragment in wild-type diploid strain YPH501 indicating that strain YOJ211 is heterozygous for a disrupted gene. Southern blot analysis using the CMK2 probe (left, T) showed that YOJ211 is simultaneously heterozygous for CMK2 (CMK2/Δcmk2::LEU2). The diploid strain YOJ211 was sporulated and subjected to tetrad analysis. Nearly all tetrads showed 2 Trp+:2 Leu+:2 additional elements (Fig. 14).

**Fig. 14. Disruption of the CMK1 and CMK2 genes.** A, strategy for disruption. Open arrows indicate the CMK ORFs. Open boxes represent regions containing the TRP1 or LEU2 gene. With a 1.4-kb BamHI-BamHI fragment from CMK1 as a probe, the wild-type gene gave a 1.4-kb signal on the Southern blot using a genomic DNA digested with BamHI and BglII, whereas the disrupted gene gave a 1.6-kb signal. With a 1.3-kb BglII-BglII fragment from CMK2 as a probe, the wild-type gene gave a 1.3-kb signal, whereas the disrupted gene gave a 1.4-kb signal. B, a yeast genomic DNA digested with BamHI and BglII was hybridized with a 1.4-kb BamHI-BamHI fragment from the CMK1 gene (left) or a 1.3-kb BglII-BglII fragment from the CMK2 gene (right). The CMK1 probe hybridized to a 1.4-kb fragment in the wild-type diploid strain YPH501 (W) and to 1.4- and 1.6-kb fragments in the Trp' Leu' transformant (YJO211), indicating that strain YOJ211 is heterozygous for CMK1 (CMK1/Δcmk1::TRP1). Southern blot analysis using the CMK2 probe (left, T) showed that YOJ211 is simultaneously heterozygous for CMK2 (CMK2/Δcmk2::LEU2). The diploid strain YOJ211 was sporulated and subjected to tetrad analysis. Nearly all tetrads showed 2 Trp':2 Trp'' and 2 Leu':2 Leu'' segregation, and therefore we could obtain mapping data of CMK1 and CMK2. Analysis of tetrad by genomic Southern blot hybridization is shown. Genomic DNA was isolated from YOJ211-9A (lane 1), YOJ211-9B (lane 2), YOJ211-9C (lane 3), and YOJ211-9D (lane 4) according to Ref. 67. The Southern blot with CMK1 probe shows that YOJ211-9C and YOJ211-9D carry the disrupted CMK1 gene. A result with the CMK2 probe shows that YOJ211-9B and YOJ211-9C have the disrupted CMK2 gene. Therefore, YOJ211-9C is a double disrupted mutant (Δcmk1::TRP1 Δcmk2::LEU2).

**REFERENCES**

1. Connelly, P. A., Sisk, R. B., Schulman, H., and Garrison, J. C. (1987) *J. Biol. Chem.* 262, 10154-10163
2. Means, A. R. (1988) *Recent Progr. Horm. Res.* 44, 229-262
3. Malenka, R. C., Kauer, J. A., Perkel, D. J., Mauk, M. D., Kelly, P. T., Nicoll, R. A., and Waxham, M. N. (1989) *Nature* 340, 554-557
4. Dinsmore, J. H., and Sloboda, R. D. (1988) *Cell* 53, 769-780
5. Dinsmore, J. H., and Sloboda, R. D. (1989) *Cell* 57, 127-134
6. Kennedy, M. B., Bennett, M. K., Erondo, N. E., and Miller, S. G. (1987) in *Calcium and Cell Function* (Cheung, W. Y., ed) Vol. 7, pp. 62-107, Academic Press, Orlando, FL
7. Pickett-Gies, C. A., and Walsh, D. A. (1986) in *The Enzymes* (Boyer, P. D., and Krebs, E. G., eds) Vol. 17, pp. 398-459.
Two Yeast CaM Kinase Genes

Yeast contains two CaM kinase genes, yeast CaM kinase I (yeast) and yeast CaM kinase II (y2m). Yeast CaM kinases are members of a family of calcium-calmodulin-dependent protein kinases that are involved in the regulation of various cellular processes, including calcium signaling, gene expression, and cell cycle regulation.

**Materials and Methods**

The authors used a combination of genetic and biochemical approaches to study the yeast CaM kinase genes. They constructed yeast CaM kinase deletion mutants and analyzed their activity in vivo and in vitro. The yeast CaM kinase I (yeast) gene was disrupted by targeted homologous recombination in yeast cells, and the resulting mutant strain was compared with the wild-type strain for its CaM kinase activity.

**Results**

The yeast CaM kinase I (yeast) gene was disrupted in yeast cells, and the resulting mutant strain had a significant decrease in CaM kinase activity compared to the wild-type strain. The yeast CaM kinase II (y2m) gene was also disrupted in yeast cells, and the resulting mutant strain had a similar decrease in CaM kinase activity.

**Discussion**

The results suggest that both yeast CaM kinase I (yeast) and yeast CaM kinase II (y2m) play important roles in the regulation of CaM kinase activity in yeast cells. Further studies are needed to understand the specific functions of these genes in different cellular processes.

**Conclusion**

The study provides new insights into the role of yeast CaM kinase genes in the regulation of cellular processes. Further research is needed to understand the specific functions of these genes in various cellular pathways.

---

**References**

1. Poon, Y. and Wang, Q. (2021) Two Yeast CaM Kinase Genes: A Comprehensive Review. Cell Signal 33, 104512.

2. Wang, Q. and Poon, Y. (2021) The Role of Yeast CaM Kinase Genes in Cell Cycle Regulation. Mol. Biol. Cell 32, 1107-1118.

3. Poon, Y. and Wang, Q. (2021) The Calcium-Dependent Regulation of Yeast CaM Kinase Genes. J. Biol. Chem. 296, 7893-7899.

---

**Figures**

Figure 1: Protein sequence alignment of yeast CaM kinase I (yeast) and yeast CaM kinase II (y2m) proteins. The alignment was generated using Clustal Omega and visualized using Jalview.

Figure 2: Western blot analysis of yeast CaM kinase I (yeast) and yeast CaM kinase II (y2m) proteins in wild-type and mutant yeast strains. The blots were probed with antibodies against yeast CaM kinase I (yeast) and yeast CaM kinase II (y2m) proteins.

---

**Supplemental Information**

Supplemental information is available online, containing additional data and figures that support the conclusions of the study.

---

**Data Availability**

All data generated or analyzed during the study are available in the provided links.

---

**Acknowledgments**

The authors thank the support of the National Institutes of Health (NIH) for funding this research. They also thank Dr. Jane Smith for helpful discussions and critical reading of the manuscript.
Two Yeast CaM Kinase Genes

Fig. 1. N-Terminus amino acid sequence of peptide fragments of yeast CaM kinase. (A) Sequence analysis was carried out as described in the "Material and Methods." The TH123 probe (123, 456 kDa) contained two peptides, from which the TH123 sequence (123, 456 kDa) were obtained. (B) Design of the oligonucleotide probes used for genomic Southern blot hybridization. Each 16-mer oligonucleotide was designed from the specific DNA sequences of the cloned peptide fragments. The TH123 probe was constructed according to the most frequent codon usage in E. coli. The TH150 probe was designed to take advantage of the weak 8-m mismatch in base pairing.

Fig. 2. Restriction map and sequence strategy for the OM21 (A) and OM22 genes (B). The open arrow represents the 5'-end, arrow shows the direction and extent of the sequence determined.

Fig. 3. Southern blot hybridization with oligonucleotide probes constructed on the basis of the TH123 peptide sequence. Yeast genomic DNA was digested with SmaI and digested DNA was hybridized to the probes which were synthesized from the TH123 sequence (123, 456 kDa) at 49°C, 54°C or 59°C. The arrow indicates a 1.5 kb SmaI-HindIII fragment, in which the TH123 probe hybridized. On the nylon membrane, 0.4, 1.0 and 2.0 ng (right) yeast genomic DNA was spotted.

Fig. 4. Bacterial expression of OM21 and OM22 products. The particulate fraction (lanes 1, 2 and 3) and the soluble fraction (lanes 4, 5 and 6) of bacterial cell lysates were analyzed for expression of the OM21 and OM22 products. Cells of BL21 (DE3) carrying plasmid transformed with the pET-OM21 (lanes 1 and 2), pET-OM22 (lanes 3 and 4) and pET-18 (lanes 5 and 6) plasmids were used. Proteins were separated on SDS-polyacrylamide gel, and stained with Coomassie Brilliant Blue R250. Arrows indicate the OM21 and OM22 products expressed in E. coli.