Cloning of a Calmodulin Kinase I Homologue from Schizosaccharomyces pombe

By using 35S-labeled calmodulin (CaM), we have isolated a full-length cDNA clone expressing the Schizosaccharomyces pombe homologue of calmodulin kinase I (CaMK-I), a gene we have named cmk1. It has been previously shown in mammals that CaMK-I is a member of a CaM-dependent protein kinase cascade that ultimately regulates transcription factors such as ATF and cAMP-response element-binding protein. The cmk1 cDNA encodes a 335-amino acid protein with significant homology to mammalian CaMK-I, including a conserved sequence for phosphorylation by CaM kinase kinase. We have expressed the cmk1 cDNA in bacteria and yeast, and we have shown that it is a CaM-dependent protein kinase. A truncation mutant of cmk1 (d320) failed to bind CaM, indicating that the CaM-binding domain is at the extreme C terminus of the protein. The mRNA for cmk1 is expressed in a cell cycle-dependent manner, peaking at or near the G1/S boundary. Overexpression of wild-type cmk1 in S. pombe caused no apparent effects on growth and division. However, mutation of a predicted regulatory site (Thr-192) to aspartic acid resulted in hyperactivation of CMK1 activity in the presence of CaM and causes cell cycle arrest in vivo. Arrest is also accompanied by morphological defects. These results suggest the presence of a CaM-dependent protein kinase cascade in yeast and indicate that cmk1 may be important in cell cycle progression, a process known to be dependent on CaM in eukaryotic cells.

The calcium receptor calmodulin (CaM) has been implicated in the regulation of a number of cellular processes. Through the regulation of literally dozens of CaM-dependent enzymes, CaM can potentially affect a broad spectrum of intracellular mechanisms in response to the second messenger, calcium (1, 2). Work from our laboratory and others (3–6) has shown that CaM plays an important role in controlling cell proliferation in eukaryotic cells. Calmodulin is essential for viability and cell proliferation in every organism where it has been possible to test this requirement experimentally (4, 7, 8).

Reversible protein phosphorylation plays an important role in the control of cell proliferation. Therefore, it would be reasonable to hypothesize that the function of CaM in cell cycle control might be mediated either through regulation of CaM-dependent kinases or phosphatases. There are several CaM-dependent protein kinases (9–14), and at least one CaM-dependent phosphatase (PP2B or calcineurin) (15). Recent evidence suggests that CaM-dependent protein kinases play a role in cell cycle regulation. Studies in Aspergillus nidulans (16) and Schizosaccharomyces pombe (17) suggest negative regulation of G1/S progression by CaMK-I. In addition, CaMK-II seems to regulate positively G1/S progression in both A. nidulans (6) and mammalian cells (5). CaMK-I and CaMK-IV are of interest recently due to the discovery of a distinct activating kinase, CaM kinase kinase, which phosphorylates and enhances the protein kinase activity of these enzymes (18).

Because few genes for CaM-dependent enzymes have been cloned and studied from genetically tractable model systems, we undertook to isolate cDNAs encoding CaM-binding proteins from the yeast S. pombe. This study reports the cloning and characterization of a cDNA encoding the S. pombe homologue of CaMK-I. The gene, which we have named cmk1, encodes a 38-kDa protein that is most homologous to CaMK-I from rat. It binds CaM in a Ca2+-dependent manner and is activated by Ca2+/CaM. Mutation of a putative regulatory Thr residue to Asp results in hyperactivation of the CMK1 kinase in the presence of Ca2+/CaM. We have been able to determine that the CaM-binding domain is at the extreme C terminus of the protein. In addition, the levels of the cmk1 mRNA are cell cycle-regulated, being maximal coincident with S-phase. Overexpression of the normal kinase had no effect on the vegetative growth of cells, which expression of the T192D variant caused cell cycle arrest, and morphological defects. Our studies suggest that the CaM kinase cascade, previously described in mammalian cells, may be conserved in yeast, providing a genetic model system in which to study CaM-dependent signal transduction mechanisms in eukaryotic cells. In addition, the data also suggest that CaMK-I may act to regulate proliferation in eukaryotic cells.

MATERIALS AND METHODS

Synthesis of 35S-CaM and Expression Library Screening—To produce labeled CaM, log phase cells containing the expression plasmid pKK233-SpCaM (which contains the full-length S. pombe CaM cDNA) were grown to mid-log phase at 37 °C, washed in sterile water, and resuspended in SO4-free M9 medium (19). Cells were incubated 15 min before the addition of 100 μCi per ml of TransLabel (ICN Radiochemicals) and incubated further for 4 h at 37 °C. Calmodulin was purified by phenyl-Sepharose chromatography (20) and assayed by SDS-PAGE and CaM overlay to ensure the protein was functional. An S. pombe Uni-ZAP cDNA expression library (obtained from Alison Pidoux and Zac Cande) was screened using 35S-labeled S. pombe CaM by described methods (17). Plugs containing positive plaques were selected and re-screened in secondary and tertiary screens. Positives were plaque-
Calmodulin Kinase Homologue in Fission Yeast

RESULTS

Isolation of Fission Yeast cmk1 by Expression Screening with 35S-SpCaM—In order to isolate cDNAs encoding CaM-binding proteins, we screened a UniZAP S. pombe cDNA expression library with S. pombe CaM radiolabeled with [35S]methionine as described under "Materials and Methods." Plaques that bound 35S-SpCaM were selected and processed through secondary and tertiary screenings to obtain pure plaques. Plasmid DNA was recovered by co-infection of XL-1 Blue cells with the helper phage R408, which led to excision of a Bluescript plasmid containing the cDNA insert. Plasmids were first analyzed by restriction digestion with EcoRI and XhoI and confirmed by nucleic acid hybridization. For nucleic acid hybridization, the full-length cmk1 cDNA was labeled by the oligo-labeling procedure using the Prime-a-Gene kit (Promega). All restriction enzymes used were from Promega. Total RNA was isolated by the Trizol method (Life Technologies, Inc.). All PCR reactions were done using Pfu polymerase and standard conditions (Stratagene). PCR products were sequenced to confirm their accuracy. For Northern blots and dot blots, the full-length cmk1 cDNA was radiolabeled with [35S]methionine. This gene has been previously cloned by M. Yanagida's lab and us (23, 24). Initial screening of the S. pombe cDNA expression library with S. pombe CaM, and secondary screenings to obtain pure plaques. Plasmid DNA was rescued by co-infection of XL-1 Blue cells with the helper phage. Preparation of cDNA for the complete cDNA was determined using P81 phosphocellulose paper as described previously (5). For Western blots and CaM overlays, was performed as described previously (17).

Yeast Methods—All yeast culture techniques were as described (21). The strains used are all based on SP130 (17). Cells containing expression constructs were grown either in the presence of thiamine (5 mM) to repress expression or in the absence of thiamine to permit expression. Protein extracts were made by vortexing cells in extraction buffer containing glass beads (21).

Expression of the GST tag or GST-tagged CMK1 (and mutant variants) was assayed by Western blot using a goat, anti-GST antisera (Sigma). Detection of the primary antibody was performed either using a horseradish peroxidase-conjugated anti-goat secondary antibody and the ECL detection kit (Amersham Pharmacia Biotech). For immunofluorescence detection, a fluorescein isothiocyanate-labeled anti-goat secondary antibody (Sigma) was used according to standard methods (22).

For cell synchronization, cdc25-22 cells were grown at 25 °C to a density of 1 × 10^7 cells per ml, shifted to 36 °C for 4.25 h, and then returned to 25 °C by addition of an equal volume of chilled medium. Samples were taken at 20-min intervals, and septation index was calculated to determine the peak of septation. For cell synchronization, cdc25-22 cells were grown at 25 °C to a density of 1 × 10^7 cells per ml, shifted to 36 °C for 4.25 h, and then returned to 25 °C by addition of an equal volume of chilled medium. Samples were taken at 20-min intervals, and septation index was calculated to determine the peak of septation.
sequencing of the 5' and 3' ends of the CBP2 did not reveal homology to any known proteins as determined from searching of the GenBank™. However, sequencing of an internal 0.6-kilobase pair EcoRI fragment revealed that CBP2 encodes a homologue of calmodulin-dependent protein kinase I (CaMK-I). The complete sequence and conceptual translation of CBP2 is shown in Fig. 1A. The cDNA encodes a putative protein of 335 amino acids, with a predicted molecular mass of 38 kDa. Comparison of the predicted CBP2 protein to protein sequences in GenBank™ showed that it has the highest homology to rat CaMK-I (40% identity), followed by human CaMK-I (Fig. 1B). Based on this comparison, it can be concluded that CBP2 encodes the S. pombe homologue of CaMK-I, representing the first CaMK-I isofrom to be isolated from yeast. We have named the gene cmk1 in conformance with S. pombe nomenclature.

By using oligonucleotide primers derived from the 5'- and 3'-untranslated regions of the cmk1 gene, we next amplified genomic DNA in order to obtain the genomic sequence corresponding to the cmk1 open reading frame. At the time we obtained the cDNA, we also isolated a genomic clone by PCR. The genomic clone was significantly larger than the cDNA as estimated by agarose gel electrophoresis. This indicated that the cmk1 gene contained introns. Since then, cmk1 has been sequenced at the Sanger Center Pombe Genome Project. Examination of their sequence both confirmed our cDNA sequence and indicated the presence of four introns. Fig. 2 is a diagrammatic representation of the intron locations in the cmk1 gene.

Expression of cmk1 mRNA Is Cell Cycle-regulated—As part of our standard characterization of the cmk1 gene, we examined mRNA levels during the course of a synchronous cell cycle. Preliminary Northern blot analysis showed that a single mRNA species hybridized to a cmk1 cDNA probe, so dot-blot analysis was employed. We first produced synchronized cell cultures using a temperature-sensitive cdc25-22 strain as described previously (23), and the septation index was used to gauge synchrony. Samples were taken at 20-min intervals, total RNA prepared, equal amounts dot-blotted on Nytran filters, and the filter probed with a radiolabeled full-length cmk1 cDNA probe. As shown in Fig. 3, the levels of cmk1 mRNA increased rapidly (6-fold as estimated by PhosphorImager analysis) at a time coincident with entry into S-phase, followed by an equally rapid decline at or near the end of S-phase. In contrast, probing the same blot with a radiolabeled cDNA probe for S. pombe EF1α revealed no change in mRNA levels over the same period. These results indicate that cmk1 mRNA levels are regulated in a cell cycle-dependent manner and increase coincident with DNA replication in S. pombe.

Characterization of the CaM-binding Domain—Calmodulin-binding domains are typically amphipathic helical regions, often located in the C-terminal portion of the enzyme (25). In the case of CaMK-I homologues, the CaM-binding domain is juxtaposed to an autoinhibitory region (26, 27) and is near the C terminus of the protein. Inspection of the CMK1 protein suggested the presence of a homologous sequence. To delineate the CaM-binding domain in the CMK1 protein, PCR-based mutagenesis was used to insert a stop codon after amino acid position 320 of the cmk1 open reading frame (named d320). Both the normal and mutant (d320) cDNAs were subcloned into pGEX-2T, expressed in bacteria, and purified as GST fusion proteins. Western blotting confirmed that the GST tag, full-length CMK1, and the CMK1-d320 version were expressed in E. coli. Calmodulin overlays were then used to compare CaM binding of the wild-type and truncated proteins (Fig. 4). The results of the CaM overlay show that full-length CMK1 protein was capable of binding CaM, whereas the deletion mutant lacking the C-terminal 15 amino acid residues was unable to bind CaM. Therefore, as predicted from examination of mammalian CaMK-I sequences, the CaM-binding domain is situated at the extreme C terminus of the CMK1 protein.

*cmk1 Encodes a CaM-dependent Protein Kinase*—By using bacterially synthesized GST-CMK1, we next determined if the GST-CMK1 had CaM-dependent kinase activity in a phosphorylation assay using a peptide substrate modeled on the CaMK-I phosphorylation site in synapsin I (11). As shown, the GST-CMK1 was able to phosphorylate the site 1 peptide in a Ca2+/CaM-dependent manner, with the addition of CaM stimulating activity approximately 10-fold (Fig. 5). This indicates that S. pombe cmk1 encodes a CaM-activated protein kinase.

**Mutation of the Putative CaM-KK Phosphorylation Site Results in Hyperactivation**—Previous studies have shown that mammalian CaMK-I activity is increased by addition of CaM. In addition, hyperactivation occurs after phosphorylation of CaMK-I by an activating kinase known as CaM kinase kinase (CaM-KK) (18, 28, 29). This hyperactivation occurs via phosphorylation of a Thr residue within a region termed the T-loop (27). Since this region and the corresponding Thr residue is conserved in the S. pombe CMK1 protein (see Fig. 2), we tested
whether or not mutation of Thr-192 would affect cmk1 kinase activity.

In previous studies, mutation of Thr to Asp in CaM kinase II mimicked phosphorylation and created an enzyme that was constitutively active (17). We would predict based on those studies that mutation of Thr-192 to Asp-192 would result in an enzyme that would behave as if it had been phosphorylated by CaM-KK. The result would be a CMK1 enzyme that displays hyperactivated levels of kinase activity but with only the addition of Ca\(^{2+}\)/CaM.

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\begin{align*}
\text{Calcium} & \quad \text{EGTA} \\
66 \text{ kDa} & \quad 27 \text{ kDa}
\end{align*}
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Fig. 4. Analysis of bacterially expressed GST-CMK1. A, GST tag alone (GST), GST-CMK1 (CMK1), or GST-CMK1/d320 (d320) were purified as described under “Materials and Methods.” Equal amounts of protein were resolved by SDS-PAGE, transferred to Immobilon-P filters, and then probed with \(^35\)S-CaM buffer containing either 1 mM Ca\(^{2+}\) (Calcium) or 2 mM EGTA (EGTA). B, CaM-dependent kinase activity of bacterially synthesized GST-CMK1 was assayed as described. Reactions were carried out either with (+CaM) or without (−CaM) the addition of Ca\(^{2+}\)/CaM.

as a control for a simple change in sequence. Alanine was expected to have no effect on basal CaM-stimulated activity.

The mutated cDNAs were then cloned into the \textit{S. pombe} expression vector pESP-1, and proteins were expressed as GST fusions in yeast. pESP-1 contains the \textit{S. pombe} nmt1 promoter and a selectable LEU2 marker. In the absence of thiamine, nmt1 is expressed at high levels, whereas in the presence of thiamine expression is repressed (30). Cells were transformed with each of the three expression plasmids, and transformants were selected on medium lacking leucine but containing thiamine. This allowed for selection of cells carrying the expression plasmid and prevented expression in case the normal or mutant proteins were deleterious to the cell. The presence of GST-tagged proteins was assayed in cells grown in the presence or absence of thiamine by Western blot. Samples prepared from equal numbers of cells were resolved by SDS-PAGE, transferred to Immobilon-P filters, and then probed with anti-GST antisera (Sigma), which was in turn localized by chemiluminescent detection procedure (ECL, Amersham Pharmacia Biotech). The results showed that similar amounts of GST tag, CMK1, CMK1-T192A, and CMK1-T192D were produced in cells (not shown).

Samples containing equal amounts of the normal and mutant kinases were then tested for kinase activity in the presence or absence of Ca\(^{2+}\)/CaM. Normal, T192A, and T192D proteins were expressed in yeast and purified by glutathione-Sepharose affinity chromatography. Yields were assessed with a protein quantification kit (Bio-Rad). Equal amounts of protein were then used in kinase assays to test for CaMK-I activity. The results show that both the normal and Thr-192 versions of CMK1 showed a modest increase in activity in the presence of Ca\(^{2+}\)/CaM (Fig. 5). However, in the presence of CaM, the T192D mutant kinase showed 15-fold greater activity than did the wild-type protein under identical conditions (Fig. 5). These results indicate that mimicking phosphorylation of Thr-192 does lead to a hyperactivation of CMK1 kinase activity and suggests that CMK1 like mammalian CaMK-I is normally regulated by an activating kinase in vivo.

Effects of Expressing CMK1 in Vivo—During the course of the experiment in which CMK1 and the mutant kinases were synthesized and purified from yeast, we noticed that cells expressing the T192D version grew much more slowly (Fig. 6). Therefore, we decided to examine growth more carefully to determine whether expression of the normal or mutant kinases had an effect on cell proliferation and cell morphology. Previous studies in which we expressed a constitutively active CaMK-II enzyme showed an arrest of cell proliferation (17). To determine whether increased levels of CMK1 had any obvious phenotypic effects, cells containing the wild-type or mutant pESP1-CMK1 plasmids were grown either in the presence or
absence of thiamine, and cultures were compared for changes in rates of cell proliferation and cellular morphology (Fig. 7). Microscopic examination of cells revealed that increased levels of both normal and CMK1-T192A proteins caused no changes in cell size or shape or rates of proliferation and density at saturation (Figs. 7 and 8). However, expression of the T192D mutant caused cell cycle arrest and marked changes in cell morphology (Figs. 7 and 8). The onset of these defects was coincident with the known kinetics of nmt1 promoter-dependent expression (30). It should be noted that although only a few cells are shown in Fig. 8, a broader visual examination of cells expressing the T192D mutant kinase indicated that the cells shown in Fig. 8 are representative of the terminal phenotype.

**DISCUSSION**

In this study, we have used the binding of $^35$S-CaM to screen a cDNA expression library for clones that express CaM-binding proteins. From the expression library screen, we obtained a number of clones, of which one was found to encode the S. pombe homologue of CaM-K-I, the gene that we have named cmk1. The gene encodes a protein kinase with a predicted molecular mass of 39 kDa and that is 40% identical at the primary amino acid sequence level to rat and human CaMK-I (11, 31). In addition, it was possible to obtain a partial genomic clone through PCR amplification of cmk1 sequences from S. pombe genomic DNA. The results of the sequence analysis comparison between the cDNA clone we isolated and the genomic sequences in the Sanger Center Pombe Genome Data base indicated the presence of four introns within the open reading frame (8). The same organization is conserved in both the A. nidulans and mammalian CaM genes (32, 33). Determination of whether or not the intron locations are conserved will have to await the isolation of genomic clones for CaMK-I from mammalian sources.

Like mammalian CaMK-I, the present study has shown the yeast enzyme to be a CaM-dependent protein kinase. CMK1 also contains a putative phosphorylation site for CaM-KK (Thr-192). The observation that the T192D mutation produces a hyperactive enzyme is consistent with the conclusion that CMK1 activity is regulated in a manner homologous to mammalian CaMK-I (18, 27–29, 31, 34–36). The purification of yeast CaMK-KK is the subject of ongoing work.

Our results also indicate that the levels of cmk1 mRNA are cell cycle-regulated and appear to increase coincident with the S-phase of the cell cycle. Several other genes display this same pattern of expression in S. pombe. Recent studies have identified a cis-acting element, the MCB box, which is the binding site for a transcription factor complex (37–39). Whether or not the expression of the cmk1 gene is under the control of this element will have to await the isolation of complete genomic clone containing the regulatory region. At present it is not known whether or not CMK1 activity is required for cell cycle progression. We do know from the overexpression experiments that increased levels of CMK1 appear to have no effect, either positive or negative, on the proliferation rate of S. pombe cells. This result was not surprising since previous studies in which a normal mouse CaMK-II cDNA was overexpressed gave the same results (17). It is noteworthy that we have not been successful so far in attempts to express truncation mutants of CMK1 that lack the CaM binding domain, either in bacteria or yeast. One possible reason is that these versions, which we would predict to be constitutively active, are fatal in vivo.

The fact that overexpression of CMK1 has no obvious effects on cells has another implication. Previous studies in which other CaM-binding proteins have been expressed in S. pombe have resulted in a variety of effects on cell proliferation and morphology. Overexpression of either calcineurin (24) or EF1α$^2$ has been found to cause reduced rates of proliferation and defects in cell shape and septation. Given the results of the present study, it must be concluded that these effects are

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2 C. D. Rasmussen, unpublished observations.
specifically due to calcineurin and EF1a and not simply due to titration of intracellular CaM by increased levels of a CaM binding. Finally, attempts at subcellular localization using immunofluorescence indicated that the CMK1 protein is distributed evenly throughout the cell, indicative of a cytoplasmic localization. Previous studies in rat have suggested a similar intracellular distribution (40).

The immediate question now is whether CMK1 acts in a pathway homologous to the CaM-dependent kinase cascade previously described in mammalian cells. Recent studies have indicated the presence of homologues of the transcription factors ATF1, CREB (41–44). Whether or not these are substrates for CMK1, its role in eukaryotic signaling is yet to be determined. Ultimately, the question will be whether CMK1 acts in vivo or in vitro as a CaMK I homologue in Fission Yeast.

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