Identification and Characterization of the CLK1 Gene Product, a Novel CaM Kinase-like Protein Kinase from the Yeast Saccharomyces cerevisiae

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The CLK1 gene of Saccharomyces cerevisiae encodes a 610-residue protein kinase that resembles known type II Ca\(^{2+}\)/calmodulin-dependent protein kinases (CaM kinases), including the CMK1 and CMK2 gene products from the same yeast. The Clk1 kinase domain is preceded by a 162-residue N-terminal extension, followed by a 132-residue C-terminal extension (which contains a basic segment resembling known calmodulin-binding sites) and is as similar to mammalian CaM kinase (38% identity to rat CaM kinase a) as it is to yeast CaM kinase (37% identity to Cmk2). However, Clk1 shares 52% identity with Rck1, another putative protein kinase encoded in the S. cerevisiae genome. Clk1 tagged with a c-myc epitope (expressed in yeast) and a GST-Clk1 fusion (expressed in bacteria) underwent autophosphorylation and phosphorylated an exogenous substrate (yeast protein synthesis elongation factor 2), primarily on Ser. Neither Clk1 activity was stimulated by purified yeast protein synthesis elongation factor 2, primarily on Ser. and phosphorylated an exogenous substrate (yeast pro-

Phosphorylation of specific proteins is an important mechanism for regulating many cellular processes (1). Protein kinases that catalyze the transfer of the \(\gamma\)-phosphate of ATP to the side chains of serine, threonine, and/or tyrosine residues comprise a highly conserved enzyme superfamily (2). In eukaryotic cells, protein kinases control cellular function by altering the catalytic activity, localization, state of assembly, and/or stability of their target substrates. Protein kinases themselves are highly regulated by mechanisms as diverse as binding of small molecule effectors (such as cyclic 3',5'-AMP or diacylglycerol), association with regulatory proteins (such as cyclins or small GTP-binding proteins), and direct phosphorylation by other protein kinases.

Calcium ion is thought to be a critical second messenger in many cell types (3). In the unicellular eukaryote, Saccharomyces cerevisiae (budding yeast), Ca\(^{2+}\) is required for cell growth and is essential for cell cycle progression (4, 5). Many of the effects of Ca\(^{2+}\) are mediated via binding of the ion to Ca\(^{2+}\)-binding proteins. One such protein found ubiquitously in all eukaryotes is calmodulin (CaM) (6). Upon binding Ca\(^{2+}\), CaM undergoes a conformational change (7) that enables the Ca\(^{2+}\)/CaM complex to bind to its target proteins (8). The S. cerevisiae gene (CM1) encoding yeast CaM has been cloned and shown to be essential for cell viability (9). Because Cmk1 is indispensable and its role is to modulate the function of other proteins, it follows that one or more of its specific targets is also necessary for cell survival. One Cmk1-binding protein that is critical for mitotic progression is Nuf1, a component of the spindle pole body (10, 11). Another Cmk1-binding protein that is essential for properly localized cell surface growth is Myo2, a so-called class V myosin required for the translocation of secretory vesicles along actin cables (12, 13).

Another CaM target is the class of type II Ca\(^{2+}\)/CaM-activated protein kinases, which display a broad substrate specificity, designated “CaM kinases” (14). In mammalian cells, there are at least 17 distinct CaM kinase isotypes encoded by at least four discrete genes (15, 16). These enzymes are thought to regulate many cellular functions in response to changes in internal Ca\(^{2+}\) concentration. Even S. cerevisiae, a unicellular eukaryote, possesses two genes (CMK1 and CMK2) that encode Ca\(^{2+}\)/CaM-dependent protein kinases with sequence homology, structural organization, and in vitro biochemical properties similar to their mammalian counterparts (17, 18). Despite the presumed importance of this class of enzyme in animal cells, yeast strains lacking either Cmk1 or Cmk2 (or both) are viable and display no detectably deleterious phenotype under standard growth conditions. However, critical function(s) of Cmk1 and Cmk2 might not be manifested, because additional protein kinases may be present that can substitute for Cmk1 and

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Cmk2. Ampale precedent for such a situation in S. cerevisiae exists. For example, the yeast genome contains three different genes (TPK1, TPK2, and TPK3) that encode isoforms of the catalytic subunit of cyclic 3',5'-AMP-dependent protein kinase (19). Cells lacking any one or any pair of these genes are viable, but cells lacking all three are not.

It was initially brought to our attention that the nucleotide sequence upstream of the YEF3 gene on chromosome 12 that was originally deposited in GenBank<sup>(20)</sup> contained a partial leu2-<sup>was originally deposited in GenBank</sup> gene. This region was cloned and sequenced, and it encodes a protein kinase with characteristics similar to those of the CaM kinase like protein kinase, which we designate CLK1. To determine if this genomic segment actually contained the CaM kinase-related gene, we cloned and sequenced this region, delineated the open reading frame that encodes this CaM kinase-like protein kinase, which we designate CLK1, and examined in detail the biochemical properties and cellular localization of this enzyme.

**EXPERIMENTAL PROCEDURES**

**Organisms and Growth Conditions—**S. cerevisiae strain YPH501 (MATαMATα ade2-101 ade2-101 his3-12α his3-200 leu2-<sup>2</sup> lys2-801<sup>lan</sup> lys2-801<sup>trp</sup>1-163/spc1-163 ura3<sup>-52</sup> ura3-52) was used as the parental strain for the construction of all mutant strains, unless noted otherwise. A probe-defensive strain, B72168 (MATαMATα ade2-101 prb1<sup>-</sup>-1122 prc1<sup>-</sup>-407 gal2<sup>−</sup>), was used for experiments involving enzyme expression and immunoprecipitation. Strain AS306 (MATα GAL<sup>−</sup> ade2-101 his3-11, 15 leu2-3, 113 trp-1-1 ura3-1 con-100), originally designated CRY1 and obtained from R. S. Fuller (Dept. of Biological Chemistry, University of Michigan, Ann Arbor, MI), was used for experiments involving gene induction by galactose. Yeast cells were grown either in a rich medium containing 2% glucose (YPD), or 2% galactose (YPGal) or in a synthetic medium containing 2% glucose (YPGlc) or 2% galactose (YPGal) that was supplemented with 2% glucose (YPGlc) or 2% galactose (YPGal) to yield the desired growth characteristics and metabolic activities. Yeast cell growth was monitored by measuring absorbance at 660 nm.

**DNA Manipulations**—Bacterial and yeast strains were grown on agar plates containing galactose or glucose as carbon sources. Yeast DNA was isolated as described<sup>(19)</sup>. Cell lysates containing any one or any pair of these genes are viable, but cells lacking all three are not.

**Isolation of Genomic DNA Containing the CLK1 Gene—Oligonucleotides** were synthesized in the DNA Synthesis Facility (University of California, Berkeley, CA) by IDT, Inc. (Corvallis, IA). To screen an S. cerevisiae genomic DNA library for segments derived from the portion of chromosome 12 upstream of the YEF3 gene (20), a probe based on the published sequence of this region (27) (5'-CAATGGTATGTGTTCTCCGCGATT-3') was prepared, end-labeled with polynucleotide kinase (Boehrer Mannheim) and [γ-<sup>32</sup>P]ATP, and separated from unincorporated nucleotide using a NICK<sup>®</sup> column (Pharmacia Biotech Inc.) (28). The radioabeled probe was hybridized to five nitrocellulose filter replicas (15-cm diameter) of plates (~8,000 colonies/plate) containing a yeast genomic DNA library in the plasmid vector, pBS32 (gift of F. Spencer and P. Hieter, Dept. of Molecular Biology and Genetics, Johns Hopkins University School of Medicine, Baltimore, MD). The bacterial cells were grown, transferred to filters, and screened by hybridization using standard procedures (26). Two independently isolated clones (YCpMM1.4 and YCpMM1.6), which reproducibly hybridized to the probe in subsequent secondary and tertiary tests, were recovered.

**Plasmid Constructions—**Restriction endonucleases and other DNA-modifying enzymes were purchased from Boehringer Mannheim, New England Biolabs, or Stratagene and were used as recommended by the manufacturer. Conventional methods were used for the construction, propagation, and purification of all plasmids (26). DNA fragments separated on agarose gels were recovered from the gels via adsorption to powdered glass (GeneClean<sup>™</sup>, Bio 101, Inc.). For certain purposes, it was necessary to eliminate the EcoRI site in the vector Bluescript SK (Stratagene), which was accomplished by cleaving with EcoRI, filling in the resulting 5'-overhangs by treatment with T4 DNA polymerase in the presence of all four deoxyribonucleoside 5'-triphosphates (26), and incubating with calf intestinal DNA ligation and ATP. A 3-kb Apo fragment was excised from YcpMM1.6 and inserted into the Apo site of this modified version of Bluescript SK, yielding plasmid pMM12. The same fragment was ligated in the reverse orientation in the standard Bluescript SK, producing pMMA3.

B. Baum, personal communication.

To generate a version of Clk1 containing a 16-residue epitope from the bacterial (Escherichia coli) bacteria-encoded lambda enterotoxin (–LEEQKLISEEDLLRKR–COOH) in frame to the C-terminal end of Clk1, the polymerase chain reaction and a three-primer scheme (28) were used. For this purpose, the template DNA was YcpMM1.6, the 5'-primer was 5’-AAAGAGCAGCCTAGGCCC-3’, the join oligonucleotide was 5’-TTTCTGCTTCTTCTGTTTCCTTTGATAGCC-3’, and the 3’-primer encoding the tag was 5’-TTATCTCTTCCAGGAAAGCTCCTGCTGATTAATCTGCTGAG-3’. The amplification was achieved using the following program: four cycles of denaturation (94 °C, 30 s), annealing (40 °C, 30 s), and synthesis (72 °C, 30 s) followed by 16 cycles under the same conditions, except that annealing was carried out at 45 °C. The resulting product was ligated into the vector, pCR<sup>™</sup>1000 (Promega), according to the manufacturer’s instructions, to generate plasmid pCR-MM1. A NorI-KpnI fragment of pCR-MM1 was used to replace the corresponding NorI-KpnI segment in plasmid pMMR2, thereby creating plasmid pMM15. To express full-length c-Myc-tagged CLK1 from its own promoter, a PstI-KpnI fragment of pMM15 was ligated into YEp352 (29) that had been previously digested with PstI and KpnI, yielding pMM16. Polymerase chain reaction was also used to construct a derivative of Clk1 containing a C-terminal truncation. For this purpose, the template DNA was pMM15-<sup>2</sup>, the 5’-primer was 5’-GGGGATTCCCTCATGTTAAAATAAGGGCC-3’, which included a BamHI site (boldface type) 7 base pairs upstream of the sequences corresponding to the ATG (underlined) at the start of the CLK1 open reading frame, and the 3’-primer was 5’-CCCGTGCATCTAATTGGAAACATCGGAA-3’, which included two stop codons (underlined) after codon 486 of the Cln1 protein sequence immediately followed by a methionine (boldface type). Amplification was achieved using the following program: 22 cycles of denaturation (94 °C, 30 s), annealing (37 °C, 30 s), and synthesis (72 °C, 30 s). To remove any over-hanging 3’-extensions, the resulting product was incubated with T4 DNA polymerase (26) and then ligated into Bluescript SK that had been cleaved with EcoRI, yielding plasmid pBS-ΔC. A BamHI-SalI fragment was excised from pBS-ΔC and inserted immediately downstream of the GAL1 promoter in the vector, YEp351GAL (30), that had been cleaved with BamHI and SalI, yielding plasmid pMM25. To place expression of full-length CLK1 under GAL1 promoter control, the HindIII fragment in pMM25 was replaced by the HindIII fragment from pMM3, generating plasmid pMM5. To express a catalytically inactive mutant version of full-length CLK1 from the GAL1 promoter, the HindIII fragment in pMM25 was replaced with the HindIII fragment from plasmid pMM45 (described below), which contains the clk1(K201R) allele that was generated by in vitro site-directed mutagenesis (see “In Vitro Site-directed Mutagenesis”), yielding plasmid pMM52. To express a catalytically inactive mutant version of the C-terminally truncated derivative of Clk1 under the control of the GAL1 promoter, several steps were required. First, the BamHI-SalI fragment of pMM42 (described below) was inserted into pBluescript SK that had been digested with BamHI and SalI. Second, a Clal-EcoRI fragment of the resulting plasmid was then replaced by the Clal-EcoRI fragment of pMM45, generating pMM55. Finally, the BamHI-SalI fragment of pMM25 was replaced with the BamHI-SalI fragment of pMM55, to yield the desired plasmid pMM56.

To facilitate purification, chimeras containing various portions of Clk1 fused to the C terminus of Schistosoma japonicum gluthionine S-transferase (GST) were constructed. A chimera containing codons 135–610 of CLK1 joined in frame to the GST coding sequence was generated by ligating the EcoRI-KpnI fragment of pMM3 into the E. coli expression vector, pGEX-3x (Pharmacia), that had been digested with SmaI and KpnI, yielding pMM23. A chimera containing all 610 codons of the CLK1 gene joined in frame to the GST coding sequence was generated in two steps. First, the BamHI-EcoRI fragment of pMM25 was ligated into pGEX-3x that had been cleaved with BamHI and EcoRI, yielding plasmid pMM36. Second, the EcoRI fragment of pMM36 was ligated into the EcoRI site of pMM3, yielding plasmid pMM37, which expresses the desired fusion. A chimera containing codons 1–486 of the CLK1 gene joined in frame to the GST coding sequence was generated by replacing the HindIII fragment in pMM36 with the HindIII fragment from pMM25, yielding plasmid pMM42, which expresses the GST-Clk1(487–610) fusion. A chimera containing the wild-type (K201R) allele fused in frame to the GST coding sequence was constructed in two steps. First, the HindIII fragment in pMM37 was replaced with the HindIII fragment of pMM21 (see “In Vitro Site-directed Mutagenesis”), yielding plasmid pMM44. Second, the EcoRI fragment of pMM44 was replaced with the EcoRI fragment of pMM3, to yield pMM45.

In Vitro Site-directed Mutagenesis—To generate a template for con-
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structing the Lys-to-Arg mutation at codon 201 of the Clk1 coding sequence into a C-terminal fragment of the plasmid-derived 9E10 antibody was used to coat the double-stranded replicative form of the phage vector, M13mp19 (New England Biolabs), and single-stranded phage DNA was prepared from the resulting construct using standard procedures (26). Using a commercial in vitro mutagenesis kit (Amersham Corp.) and the mutagenic primer, 5'-GCTTTTTAATAATATGATGTCGACGTT-3' (mis- matched base underlined), mutant DNA was generated and purified, according to the manufacturer's recommendations. Introduction of the desired mutation was confirmed by direct nucleotide sequence analysis (31). The corresponding fragment was excised from the replicative form of the mutant phage by digestion with EcoRI and PstI and ligated into pMM15 to generate plasmid pMM21. The PstI-KpnI fragment of pMM21 was inserted into the corresponding sites in YEp352 to yield plasmid YMM32.

DNA Sequence Determination, Physical Mapping, and Transcriptional Analysis of the CLK1 Locus—Plasmid pMM22 was purified using a Magic Mini-Prep<sup>TM</sup> kit (Promega), and the nucleotide sequence of most of the insert was determined on both strands by the dideoxynucleotide method and a double-stranded DNA sequencing procedure (32) using the Sequenase® (U.S. Biochemical Corp.) form of T7 DNA polymerase, [α-<sup>32</sup>P]dATP (Amersham) (32). The corresponding fragment was excised from the replicative form of the mutant phage by digestion with EcoRI and PstI and ligated into pMM15 to generate plasmid pMM21. The PstI-KpnI fragment of pMM21 was inserted into the corresponding sites in YEp352 to yield plasmid YMM32.

To confirm the map location of the CLK1 locus, an internal (EcoRI-NotI) fragment of the CLK1 coding sequence was excised from plasmid pMM22, purified by gel electrophoresis, radiolabeled by a random primer method (24), and hybridized to nitrocellulose filters containing yeast DNA fragments (gift of G. Anderson, Dept. of Plant Biology, University of California, Berkeley) separated by orthogonal field gel electrophoresis (35). The same probe was also hybridized to a set of filters containing an ordered array of fragments of the yeast genome in a bacteriophage λ vector (36), generously provided by L. Riles and M. Olson (Dept. of Genetics, Washington University School of Medicine, St. Louis, MO).

Total RNA and poly(A)<sup>+</sup>- RNA species were isolated as described (23). Poly(A)<sup>+</sup> RNA was fractionated in a formaldehyde-containing agarose gel, transferred to nitrocellulose filter, and hybridized against the same EcoRI-NotI probe derived from pMM22 as described (26).

Construction of ckl1 Null Mutations—The EcoRI-NotI fragment in pMM22 was replaced with an EcoRI-NotI fragment from pJ2217 (37), which contains the entire HIS3 gene, to yield pMM11. The ApoI fragment of pMM11 was excised and used for DNA-mediated transformation of diploid strain YPH501. His<sup>+</sup> colonies were analyzed by restriction endonuclease digestion and DNA hybridization (38) using a 2.5-kb EcoRI-Sall fragment excised from Tc1pMM1.6 as the probe to identify transformants in which the CLK1 locus on one homolog had been replaced by the HIS3/λHSV alleles. Such a ckl1-1::HIS3/CLK1 heterozygous diploid (YMM33) was sporulated to yield ascospores (gift of M. Pausch, this laboratory). Reactions were terminated by digestion with EcoRI and PstI and ligated into pMM15 to generate plasmid pMM21. The PstI-KpnI fragment of pMM21 was inserted into the corresponding sites in YEp352 to yield plasmid YMM32.

Production of Rabbit Polyclonal Anti-Clk1 Antibodies—As determined by immunoblotting (39) prior to immunization, two rabbits (1188 and 1189) displayed negligible cross-reaction against a cell-free extract of yeast strain YMM32 containing an appropriate plasmid was grown in a selective medium (SGlc or SGal) to late exponential phase (a value of 100 on a Klett-Summerson photoelectric colorimeter equipped with a number 66 red filter). The cells were harvested by centrifugation, washed by resuspension and recentrifugation in sterile distilled deionized water, resuspended in 400 µl of lysis buffer (25 mM Tris-HCl, pH 7.5, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride), and lysed by five 1-min bursts of vigorous vortex mixing with an equal volume of glass beads (0.45–0.5-mm diameter) alternating with 1-min periods of cooling on ice. The resulting crude extract was separated from the beads by placing the bead-lasylate slurry in a conical Eppendorf tube punctured at the bottom with 25-gauge syringe needle and recovered into 50 µl of the same lysis buffer. Immobilization of the antibody was achieved by adsorption to 20 µg of Protein A-Sepharose beads (Pharmacia), washed three times by resuspension and recentrifugation in lysis buffer, and resuspended in kinase assay buffer (50 mM Tris-HCl, pH 7.5, 0.1 mM EDTA, 1 mM DTT, 5% glycerol). For each reaction, a sample of the resuspended immune complexes (typically 5 µl, representing ½% of the total volume) was incubated for 10–20 min at either 25 or 30 °C in a final volume of 40 µl in kinase assay buffer containing 10 µCi of [γ-<sup>32</sup>P]ATP (3 Ci/mmol, Amersham) in the presence and absence of various divalent cations (Mg<sup>2+</sup>, Mn<sup>2+</sup>, and/or Ca<sup>2+</sup>), with or without purified yeast Cdc1 protein (0.5 µg) (gift of M. Pausch, this laboratory). Reactions were terminated by the addition of 40 µl of SDS-PAGE sample-loading buffer (41) followed immediately by boiling for 2 min. After removal of the beads by centrifugation in a microcentrifuge, the quenched reaction mixtures were subjected to SDS-PAGE (41). To fix the gel and remove any unincorporated label, the gel was soaked for at least 2 h in 10% acetic acid, 40% methanol and then in ΔH<sub>2</sub>O for at least 2 h to further reduce the residual radioactivity. After drying the gel onto a filter paper backing, the radioactive species present were visualized by autoradiography using x-ray film (XAR, Kodak). To normalize for the amount of protein present and to determine unambiguously the migration position of the Clk1 polypeptide, another portion of the same immune complexes was blotted to nitrocellulose and probed with the 9E10 antibody, as described under “Immunoblot Analysis of Proteins”.

Assays of Clk1 Protein Kinase Activity—Autophosphorylation was measured as the incorporation of radioactivity from [γ-<sup>32</sup>P]ATP into a species that co-migrated with authentic Clk1 protein and was immunoprecipitable with anti-Clk1 antibodies. To measure the phosphotransferase activity of Clk1, immune complexes generated as described immediately below were incubated under the same conditions but in the presence of various exogenously added protein substrates. For these preliminary assays of yeast strain YMM32 containing an appropriate plasmid was grown in a selective medium (SGlc or SGal) to late exponential phase (a value of 100 on a Klett-Summerson photoelectric colorimeter equipped with a number 66 red filter). The cells were harvested by centrifugation, washed by resuspension and recentrifugation in sterile distilled deionized water, resuspended in 400 µl of lysis buffer (25 mM Tris-HCl, pH 7.5, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride), and lysed by five 1-min bursts of vigorous vortex mixing with an equal volume of glass beads (0.45–0.5-mm diameter) alternating with 1-min periods of cooling on ice. The resulting crude extract was separated from the beads by placing the bead-lasylate slurry in a conical Eppendorf tube punctured at the bottom with 25-gauge syringe needle and recovered into 50 µl of the same lysis buffer. Immobilization of the antibody was achieved by adsorption to 20 µg of Protein A-Sepharose beads (Pharmacia), washed three times by resuspension and recentrifugation in lysis buffer, and resuspended in kinase assay buffer (50 mM Tris-HCl, pH 7.5, 0.1 mM EDTA, 1 mM DTT, 5% glycerol). For each reaction, a sample of the resuspended immune complexes (typically 5 µl, representing ½% of the total volume) was incubated for 10–20 min at either 25 or 30 °C in a final volume of 40 µl in kinase assay buffer containing 10 µCi of [γ-<sup>32</sup>P]ATP (3 Ci/mmol, Amersham) in the presence and absence of various divalent cations (Mg<sup>2+</sup>, Mn<sup>2+</sup>, and/or Ca<sup>2+</sup>), with or without purified yeast Cdc1 protein (0.5 µg) (gift of M. Pausch, this laboratory). Reactions were terminated by the addition of 40 µl of SDS-PAGE sample-loading buffer (41) followed immediately by boiling for 2 min. After removal of the beads by centrifugation in a microcentrifuge, the quenched reaction mixtures were subjected to SDS-PAGE (41). To fix the gel and remove any unincorporated label, the gel was soaked for at least 2 h in 10% acetic acid, 40% methanol and then in ΔH<sub>2</sub>O for at least 2 h to further reduce the residual radioactivity. After drying the gel onto a filter paper backing, the radioactive species present were visualized by autoradiography using x-ray film (XAR, Kodak). To normalize for the amount of protein present and to determine unambiguously the migration position of the Clk1 polypeptide, another portion of the same immune complexes was blotted to nitrocellulose and probed with the 9E10 antibody, as described under “Immunoblot Analysis of Proteins”.

Phosphoamino acid analysis of the radioactivity incorporated into Clk1 derivatives, or other phosphoacceptor substrates, either after incubation with [γ-<sup>32</sup>P]ATP in vitro or after labeling of yeast cells in vivo by growth of cultures (5 ml) in LPSM medium (42) with 0.2 mCi of [γ-<sup>32</sup>P]PO<sub>4</sub>/ml for 2 h was performed using standard methods for protein hydrolysis and two-dimensional electrophoresis (43).

Assays of CuM Binding—A culture (200 ml) of E. coli strain BL21(DE3) (44) carrying plasmid pMM23 was grown at 37 °C to an A<sub>600</sub>
of 0.5, and then expression of the GST-Clk1(356–610) fusion protein encoded in the plasmid was induced by treatment of the cells with isopropyl 1-thio-β-D-galactopyranoside at a final concentration of 0.5 mM for 2 h. The cells were collected by centrifugation, resuspended in TBS containing 0.1% Triton X-100 and 1 mM phenylmethylsulfonyl fluoride, and lysed by four 3-s bursts of sonic irradiation at the optimum setting of a probe sonicator (model W1850, Heat Systems-Ultrasonics, Inc.). The crude lysate was clarified by centrifugation for 20 min at 15,000 rpm in the SS-34 rotor of a Sorvall refrigerated centrifuge. The resulting supernatant solution contained nearly all of the fusion protein and was loaded onto a bed (0.5 ml) of glutathione-Sepharose 4B (Phar- 
macia) in a minicolumn. After loading, the column was washed with 2 ml of TBS containing 0.1% Triton X-100 and then with 2 ml of TBS containing 2 mM CaCl₂. A solution (10 µl) containing 2 mM CaCl₂ and 10 µg of purified CmCl₁ protein (gift of M. Pausch, this laboratory) was applied to the column. The flow-through fraction and six subsequent washes (0.3 ml each) with TBS containing 2 mM CaCl₂ were all col- 
lected. The GST-Clk1(356–610) fusion was then eluted with four washes (0.3 ml each) of TBS containing 5 mM glutathione. All of the fractions were subjected to SDS-PAGE and transferred to nitrocel- 
lose. The content of CmCl₁ in the various fractions was determined by immuno blotting of the nitrocellulose using rabbit polyclonal anti-Cmk₁ antibodies (45). The content of fusion protein in each fraction was determined by resolving samples on an identical gel and staining with Coomassie Brilliant Blue dye.

**Immunofluorescence Detection of Clk₁—Subcellular distribution of Clk₁ was assessed using minor modifications of standard procedures for fixation and immunolabeling, and labeling of yeast cells by indirect immuno- 
fluorescence (46). Permeabilized cells were incubated with a 1:300 dilution of primary antibody for 1.5 h at room temperature. Prior to use, 
rabbit polyclonal anti-Clk1 antiserum 1188 was depleted for nonspecific 
immunoreactivity by incubation with nitrocellulose filter strips on 
which a large excess of proteins from an extract of a clkΔ strain had 
been immobilized. To remove excess primary antibody, wells were washed 10 times (20 µl each) with the sorbitol-containing phosphate 
buffer. The slides were then incubated for 1.5 h at room temperature 
with either goat anti-rabbit immunoglobulin antibodies (when the pri- 
mary antibody was serum 1188) or sheep anti-mouse immunoglobulin 
antibodies (when the primary antibody was 9E10 mAb), which were 
conjugated to fluorescein isothiocyanate (Boehringer Mannheim). To 
remove excess secondary antibody, wells were washed again 10 times 
in the same way. Before viewing, slides were dipped briefly in 1 mg/ml 
4,6-diamino-2-phenylindole; then a drop of Citifluor (Citifluor Ltd.) was 
placed in each well, and a coverslip was secured on top using nail polish. 
Slides were examined using the ×100 objective of a Nikon Optiphot 
epifluorescence microscope equipped with an ultraviolet filter and pho- 
tographed using Kodak Ektachrome Elite 400 slide film.

**Isolation of the RCK1 Gene and Construction of rck1 Null Mutations—Based on the published sequence of RCK1 (47), an oligonucleo- 
teotide (5′-GGTGTCGCCCGCGGCGGTATTGGGT-3′) was synthesized, 
end-labeled, purified, and used to probe bacterial colonies containing a 
yeast genomic DNA library as described above. In this way, a plasmid 
(pMM46) was isolated that contained the entire RCK1 gene, as judged 
by restriction enzyme digestion and Southern blot hybridization analysis 
of the insert. A ClaI fragment containing the RCK1 gene was excised from pMM46 and inserted into the corresponding site of Blue- 
script SK, yielding plasmid pMM48. pMM48 was cleaved within the 
RCK1 coding sequence by digestion with NdeI, and the resulting 5′- 
overhangs were converted to flush ends by incubation with the Klenow 
fragment of E. coli DNA polymerase I and all four deoxyribonucleoside 
5′-triphosphates (200 µM). Linearized plasmid was then ligated to a 
SalI fragment containing the entire LEU2 gene, which had been excised from plasmid pJ283 (37) and converted to blunt ends by the same 
procedure, yielding plasmid pMM49. A XbaI-SalI fragment of pMM49 
containing the rck1-1::LEU2 disruption allele was introduced by DNA- 
mediated transformation into haploid strains YPH499, YMM3.1a 
(clk1Δ LEU2), YMM15, YMM18, YMM20, and YMM21.

RESULTS

The Clk1::myc fusion protein encoded an open reading frame homologi- 
uous to known CaM kinase-like protein kinases. To determine if the genomic region upstream of the deposited se- 
quence of the YEF3 gene (27), which encodes a yeast translation elongation factor, contains a bona fide open reading frame, we used a synthetic oligonucleotide probe based on the deposited sequence to isolate chromosomal fragments from a yeast genomic DNA library. We then determined the complete nucleotide sequence of this entire region, which revealed a continuous open reading frame with the capacity to encode a 610-residue polypeptide (Fig. 1). Using the BLAST algorithm (47) to compare the deduced protein against all available data 
bases, the greatest degree of amino sequence identity was shared with known and biochemically well characterized CaM 
kinases from both mammals and yeast (Fig. 2). Hence, the gene was designated CLK1, for CaM kinase-like protein kinase. 
Hybridization of a probe internal to the CLK1 coding region to a yeast chromosome blot and to a yeast contig set confirmed that 
CLK1 resides on the right arm of chromosome 12 (data not shown), as reported for the immediately juxtaposed YEF3 gene 
(27), just centromere-distal to the CDC42 gene.

While our work was in progress, another laboratory isolated a putative protein kinase gene, RCK1, from S. cerevisiae and 
also noted that a portion of the data base entry for YEF3 had homology to it (48). This group also reisolated and resequenced the 
DNA upstream of the YEF3 gene and reported a larger and continuous open reading frame, which they designated RCK2 (48). RCK2 is the same locus as CLK1; however, the open reading frame reported for RCK2 (48) is prematurely termin- 
ated. Likewise, sequence determined for this same region of chromosome 12 and deposited in GenBankTM (accession num- er U20865) as part of the international effort to determine the complete nucleotide sequence of the entire S. cerevisiae gene- 
ome, also contains errors that alter the CLK1 open reading frame. The corrected and full-length CLK1 gene product shares 
significantly greater identity to the predicted RCK1 gene product 
than it does to CaM kinases (Fig. 2); yet, Rck1 is 98 codons shorter than Clk1, primarily because it lacks the prominent 
C-terminal extension present in Clk1. To ensure that the re- 
ported RCK1 sequence was not incomplete at its 3′-end, we reisolated and resequenced this gene. Our sequence agrees 
completely with that previously reported (48).

**CLK1 Is an Expressed Gene and Encodes the Predicted Polypeptide—Poly(A)+ RNA was isolated from MATα cells and from 
MATα cells treated with α-factor, separated by agarose gel electrophoresis, and hybridized to an internal portion 
(1.1-kb EcoRI-Apal fragment) of the CLK1 gene. Two transcrip- 
tions were detected (data not shown): a major one (≈2,600 nucleotides) and a more minor one (≈2,100 nucleotides). Nei- 
theter transcript appeared to be induced after a 1-h exposure to 
mating pheromone. Both of these mRNAs are of more than 
sufficient length to encode the entire CLK1 open reading frame 
(1830 nucleotides) as well as 5′- and 3′- untranslated regions and a poly(A) tail.

To confirm that the CLK1 sequence represents one continu- 
ous open reading frame and that the predicted protein is actu- 
ally produced in cells, an epitope tag was placed immediately 
downstream of and in frame with the last amino acid (residue 
610) at the C terminus of the deduced CLK1 coding sequence. As judged by immunoblotting with the 9E10 mAb directed 
against the epitope tag, a single polypeptide was readily detec- 
ted, even when expressed from the CLK1 promoter (Fig. 3A). The Clk1::myc protein also cross-reacted with a rabbit polyclonal 
antibody raised against a GST-Clk1Δ487–160 fusion protein that lacks the deduced C-terminal extension (data not shown). The apparent molecular mass of the epitope-tagged
polypeptide (as estimated from its mobility upon SDS-PAGE relative to standards of known molecular mass) was 89 kDa. Even if the 2 kDa contributed by the C-terminal epitope tag is subtracted, this value is significantly higher than the molecular mass calculated for the Clk1 polypeptide (68.7 kDa) based on its predicted amino acid sequence. However, in extracts of normal yeast cells, the rabbit polyclonal anti-Clk1 antibodies recognized a single species with an apparent molecular mass of 87 kDa (see Fig. 5A), confirming that the authentic protein also migrates with an anomalously slow mobility. Both the N- and C-terminal portions of Clk1 are rich in charged amino acids, and both segments contain several uninterrupted tracts of acidic residues. Such highly negatively charged regions in other proteins confer a markedly slower electrophoretic mobility upon SDS-PAGE than that predicted by the amino acid sequence (29, 49).

The CLK1 Gene Product Possesses Ser/Thr-specific Protein Kinase Activity—

FIG. 1. Nucleotide sequence and deduced amino acid sequence of the CLK1 gene. Numbers above the line indicate the position of the bases in the nucleotide sequence (where +172 represents the A of the ATG initiator codon of the coding sequence). Numbers to the left and right of each line represent the position of the amino acids (in one-letter code) in the deduced open reading frame. The GenBank™ accession number for this sequence is U23464.
polypeptide contains all of the most highly conserved residues and hallmark motifs found in known Ser/Thr-specific protein kinases (2, 50). Except for Rck1, Clk1 is significantly more similar to the CaM kinase superfamily than it is to other classes of protein kinases, such as the yeast cyclic 3',5'-AMP-dependent protein kinase catalytic subunit (TPK1 gene product) (51) (Fig. 2). In fact, Clk1 is as similar to mammalian CaM kinases as it is to yeast Cmk1 and Cmk2 (Fig. 2). When compared with mammalian CaM kinases, however, Clk1 has a significantly longer N-terminal extension upstream of the kinase domain and a 31-residue Gly-rich insert situated between conserved domains VI (HRDXKPPNLL) and VII (LADFGQL). Most strikingly, residues 493–513 of Clk1 (but not the corresponding region of Rck1) possess the highly basic character found in other CaM-binding sites (8). Furthermore, when a 17-residue segment of this same region (residues 504–520) is displayed as an α-helix, positively charged residues are arrayed on the side of the helix opposite from hydrophobic residues, reminiscent of the amphipathic nature of other known CaM-binding sequences (52).

For these reasons, it was of interest to determine whether Clk1 is an active protein kinase and whether its catalytic activity is affected by yeast CaM (CMD1 gene product). The Clk1 derivative tagged with the C-terminal c-Myc epitope allowed the use of an immune complex kinase assay to assess, first, the autophosphorylation activity of Clk1. The Clk1::myc polypeptide was collected from extracts of yeast cells expressing the Clk1::myc protein was present at a level identical to that of Clk1::myc (Fig. 3B). These results demonstrated that Clk1 is capable of autophosphorylation.

The effect of other divalent metal ions and purified yeast Cmd1 was then examined using the same assay method (Fig. 3C). Either Mg$^{2+}$ or Mn$^{2+}$ (but not Ca$^{2+}$) supported autophosphorylation, but Mg$^{2+}$ was significantly more efficacious. With Mg$^{2+}$ present, the addition of Ca$^{2+}$ and/or Mn$^{2+}$ had no marked effect (either stimulatory or inhibitory). Under all conditions tested, the addition of Cmd1 produced no significant enhancement of incorporation (Fig. 3C).

To determine the nature of the residues autophosphorylated in vitro, GST-Clk1 was incubated with [$\gamma$-32P]ATP and 2 mM Mg$^{2+}$, resolved by SDS-PAGE, transferred electrophoretically to a polyvinylidene difluoride filter (Immobilon-P, Millipore), and located by autoradiography. The corresponding region of the filter was excised, and bound protein was subjected to acid hydrolysis (43). Hydrolysis products were mixed with nonradioactive phosphoserine and phosphothreonine (as carriers and internal standards), resolved by two-dimensional electrophoresis, and visualized by both ninhydrin staining and autoradiography. Radioactivity present in each spot was quantitated using a PhosphorImager (Molecular Dynamic, Inc.). Of the total radioactivity, 90% was present as phosphoserine and only 10% as phosphothreonine (data not shown). There was no detectable phosphotyrosine.

We were concerned that binding of the 9E10 mAb to the epitope tag on the C terminus of Clk1::myc might interfere with binding of yeast Cmd1, might mimic the effect of Cmd1 binding, or might otherwise disrupt proper regulation. Therefore, we also expressed Clk1 and various derivatives in bacterial cells as GST-Clk1 fusions. The GST fusions were purified by adsorption to and elution from glutathione beads. In solution, both GST-Clk1 and a C-terminally truncated derivative, GST-Clk1(Δ487–610), displayed autophosphorylation activity (Fig. 4), in contrast, a catalytically inactive mutant derivative, GST-Clk1(K201R), failed to incorporate any detectable radioactivity. During production and purification, some of the GST-Clk1 suffered proteolytic degradation and was converted to a size
Phosphotransferase Activity of Clk1—Several exogenously added proteins were tested as potential phosphoacceptor substrates for Clk1 both in the immune complex kinase assay and using the soluble GST-Clk enzyme. Neither β-casein nor autophosphorylation site sequence in mammalian CaM kinases was detectably phosphorylated by either bead-bound or soluble Clk1. In contrast, a modest degree of incorporation into myelin basic protein was observed under both assay conditions (data not shown).

During the course of these studies, our attention was drawn to the possibility that yeast translation elongation factor 2 (EF-2), product of the EFT1 and EFT2 genes (54), might serve as an effective substrate for Clk1 for several reasons. First, a novel type of Ca$^{2+}$/CaM-dependent protein kinase activity (dubbed Ca$^{2+}$/CaM-dependent protein kinase III) has been implicated in the phosphorylation and regulation of mammalian EF-2 by in vitro biochemical studies (55). Second, the purified mammalian enzyme is able to phosphorylate yeast EF-2 and has a reported molecular mass (~95 kDa) that is in the same size range as the apparent molecular mass of Clk1 (56). Third, a similar activity has been detected and partially purified from yeast (57); however, the gene encoding this Eft1/Eft2 kinase has not yet been identified. Finally, as dosage suppressors of the growth-inhibitory effect of overproduced C-terminally truncated Clk1 (see “Discussion”), we isolated genes implicated in translation and RNA binding (58).

Partially purified yeast EF-2 (generous gift of A. C. Nairn, Rockefeller University, New York) was readily phosphorylated by both GST-Clk1 and GST-Clk1(Δ487–610), but not at all by the catalytically inactive GST-Clk1(K201R) mutant (Fig. 4). The presence of Ca$^{2+}$ and purified Cmd1 did not significantly stimulate the activity of GST-Clk1 to phosphorylate yeast EF-2. In contrast, the autophosphorylation activity of purified yeast Clk1, a CaM kinase with a typically broad substrate specificity (17, 18), was greatly stimulated by the presence of Ca$^{2+}$ and Cmd1; yet, this enzyme was unable to phosphorylate yeast EF-2 under any conditions. Thus, the ability of GST-Clk1 to phosphorylate yeast EF-2 was remarkably specific.

To determine the nature of the residues in yeast EF-2 phosphorylated by Clk1 in vitro, the yeast EF-2 preparation was incubated in solution with purified GST-Clk1, [γ-32P]ATP, and Mg$^{2+}$ and then subjected to SDS-PAGE. The resulting gel was dried onto a filter paper backing (Whatman, 3MM), and the blot was incubated with the 9E10 antibody and visualized using enzyme-linked chemiluminescence (B). Reactions were conducted, as in A, with the immune complexes prepared from cells expressing Clk1::myc in the presence and absence of the various divalent cations indicated, with and without the addition of purified Cmd1 protein (yeast CaM) (C).

Fig. 3. Immune complex assay of Clk1 kinase activity isolated from yeast. Extracts were prepared, as described under “Experimental Procedures,” from a clk1Δ yeast strain (YMM5.1a) carrying vector alone (YEp352), the same plasmid overexpressing Clk1::myc (pMM16), or the same plasmid overexpressing the catalytically inactive Clk1::K201R::myc mutant (pMM22). Immune complexes were captured on Protein A-Sepharose beads after incubation of the extracts with an anti-c-Myc mAb (9E10), washed with TBS, and incubated with 2 mM Mg$^{2+}$ and [γ-32P]ATP, as described in detail under “Experimental Procedures.” The products of the reaction were subjected to SDS-PAGE in a 10% gel. The gel was dried onto filter paper and analyzed by autoradiography (A). Another gel containing portions of the same immune complexes was transferred to a nitrocellulose filter, and the blot was incubated with the 9E10 antibody and visualized using enzyme-linked chemiluminescence (B). Reactions were conducted, as in A, with the immune complexes prepared from cells expressing Clk1::myc in the presence and absence of the various divalent cations indicated, with and without the addition of purified Cmd1 protein (yeast CaM) (C).

Fig. 4. Solution assay of Clk1 kinase activity using bacterially expressed enzyme. Extracts were prepared, as described under “Experimental Procedures,” from an E. coli strain (DH5α) expressing either GST-Clk1 (from pMM37), GST-Clk1(K201R) (from pMM45), and pGST-Clk1(Δ487–610) (from pMM42). These fusion proteins (~0.5 μg each; purified by adsorption and elution from glutathione beads, as described under “Experimental Procedures”) or a sample of highly purified Cmk1 enzyme (~500 μg) (17) was incubated with [γ-32P]ATP and 2 mM Mg$^{2+}$ in the presence of 2 mM Ca$^{2+}$ and 0.5 μg of Cmd1 (+) or in the presence of 1 mM EGTA (−), either alone (−) or in the presence (+) of a partially purified preparation (~0.5 μg of total protein) of yeast translation elongation factor 2 (YEF-2) (generous gift of A. Nairn). The products of the reaction were resolved using SDS-PAGE. The gel was dried onto filter paper and analyzed by autoradiography. The migration positions of the proteins indicated were confirmed (data not shown) by immunoblotting a gel containing otherwise identical samples with rabbit polyclonal anti-Clk1 antibodies and anti-Eft1/Eft2 antibodies (provided by A. Nairn).
determine if Clk1 can associate with Cmd1. First, a GST-Clk1(135–610) fusion protein was produced and purified from E. coli cells. Glutathione-agarose beads were loaded with an excess of this fusion protein, washed exhaustively, and then loaded with purified yeast Cmd1 (10 μg) in the presence of 2 mM Ca²⁺. An essentially identical experiment was performed on another column in which the Cmd1 was loaded in buffer containing EGTA. The flow-through of each column was collected. The columns then were washed twice with the appropriate buffer, and the bound GST-Clk1(135–610) was eluted with glutathione. The resulting fractions were resolved by SDS-PAGE, and the distribution of Cmd1 was analyzed by immunoblotting with rabbit anti-Cmd1 antibodies. Either with or without Ca²⁺, all of the Cmd1 protein was in the flow-through and in the initial wash fraction; none was detected in the eluted fraction, which contained all of the GST-Clk1(135–610) (data not shown). Thus, Cmd1 was unable to bind tightly to the C-terminal segment of immobilized Clk1.

As a second means to attempt to detect association of Clk1 with Cmd1, both proteins were overproduced in yeast cells from multicopy plasmids. CLK1 and CMD1 were expressed from the GAL1 promoter, and CLK1::myc was expressed from its own promoter. Clk1 (or Clk1::myc) was then immunoprecipitated from extracts of such cells using either the rabbit polyclonal antibodies (or the 9E10 mAb). The immunoprecipitates were resolved by SDS-PAGE and examined by immunoblotting. No Cmd1 was detectable, although copious amounts of Clk1 (or Clk1::myc) were present (data not shown).

As a third means to assess the ability of Clk1 to bind Cmd1, we used a CaM overlay method, as described previously for Cmk1 and Cmk2 (17). Extracts of E. coli cells expressing GST-Clk1 fusions were resolved by SDS-PAGE, transferred to filters, and incubated in the presence and absence of Ca²⁺ with purified Cmd1 that had been freshly labeled by reaction with ¹²⁵I-labeled Bolton-Hunter reagent. Under no condition tested was a detectable amount of radioiodinated Cmd1 protein retained by the GST-Clk1 bound to the filter, whereas a readily detectable amount of label was retained by an equivalent amount of purified Cmk1 protein (data not shown).

Overexpression of CLK1 Inhibits Yeast Cell Growth—Removal of the C-terminal CaM-binding sequences downstream of the catalytic domain in CaM kinases generates a CaM-independent and constitutively active enzyme (15, 16, 59, 60). To examine the role of the C-terminal domain of Clk1, high copy plasmids were constructed that express from the galactose-inducible GAL1 promoter full-length Clk1 (pMM51), the catalytically inactive full-length Clk1(K201R) mutant (pMM52), the C-terminally truncated Clk1(Δ487–610) (pMM25), or a catalytically inactive version (K201R) of the C-terminally truncated Clk1(Δ487–610) (pMM56) were resolved by SDS-PAGE, transferred to a nitrocellulose filter, and incubated with rabbit polyclonal anti-Clk1 antibodies raised against GST-Clk1(Δ487–610) as the antigen, as described under "Experimental Procedures." B, overexpression of Clk1 and C-terminally truncated Clk1 is growth-inhibitory. The same five strains described in A were restreaked to single colonies from glucose-containing plates onto selective medium containing either 2% glucose (SGlGlc-Leu; Glucose) or 2% galactose (SGal-Leu; Galactose). The plates were incubated at 25 °C for 4 days and then photographed.

FIG. 5. The C-terminal sequence of Clk1 contains a negative regulatory domain. A, overproduction of Clk1 and Clk1 derivatives. Samples (~20 μg) of extracts of a yeast strain (AS306GAL) harboring vector alone (Yepl351GAL) (129), and the same plasmid expressing from the GAL1 promoter full-length Clk1 (pMM51), the catalytically inactive full-length Clk1(K201R) mutant (pMM52), the C-terminally truncated Clk1(Δ487–610) (pMM25), or a catalytically inactive version (K201R) of the C-terminally truncated Clk1(Δ487–610) (pMM56) were resolved by SDS-PAGE, transferred to a nitrocellulose filter, and incubated with rabbit polyclonal anti-Clk1 antibodies raised against GST-Clk1(Δ487–610) as the antigen, as described under "Experimental Procedures." B, overproduction of Clk1 and C-terminally truncated Clk1 is growth-inhibitory. The same five strains described in A were restreaked to single colonies from glucose-containing plates onto selective medium containing either 2% glucose (SGlGlc-Leu; Glucose) or 2% galactose (SGal-Leu; Galactose). The plates were incubated at 25 °C for 4 days and then photographed.
Fig. 6. Clk1 is located in the cytosol and excluded from the nucleus. Wild-type cells (YPH499) harboring a plasmid (pMM12), which expresses Clk1 from its own promoter on a multicopy plasmid (YEp531), and an otherwise isogenic strain (YMM3.1a), carrying the clk1-1Δ:HIS3 mutation and harboring the vector alone, were grown to mid-exponential phase, fixed, affixed to slides, permeabilized, and stained with 4,6-diamino-2-phenylindole (right side) and then with primary and secondary antibodies, as described in detail under “Experimental Procedures.” The primary antibody was a 1:300 dilution of a rabbit polyclonal anti-Clk1 antiserum (1188) that had been depleted of nonspecific cross-reacting activity by preadsorption to nitrocellulose filter replicas of extracts of clk1-1Δ:HIS3 cells separated by SDS-PAGE. The secondary antibodies were a 1:200 dilution of fluorescein isothiocyanate-conjugated goat anti-rabbit immunoglobulin antiserum (left side).

not shown), whereas wild-type cells examined with the same antibody showed no detectable staining, as has also been observed by others who have used this same anti-epitope antibody for examining yeast cells by indirect immunofluorescence (62).

CLK1 Is Not an Essential Gene—To determine if the CLK1 locus is an essential gene, a deletion-insertion allele was constructed. This mutation removed 40% of the CLK1 coding region (including more than a third of the kinase domain) and substituted the HIS3 gene (Fig. 7A). Diploid His⁺ transformants heterozygous for the mutation were readily obtained (Fig. 7B), and sporulation of such a diploid strain (YMM3) yielded many tetrads containing four viable spores and in which His⁻:His⁻ segregated 2:2 (Fig. 7B). This result indicated that CLK1 function is not required for normal vegetative growth on rich or minimal medium.

Because of the similarity of CLK1 in certain respects to the CMK1 and CMK2 loci, it was also of interest to determine the phenotype of cells lacking all three genes. For this purpose, a spore carrying the clk1-1Δ:HIS3 allele was mated with a haploid of opposite mating type that carried the cmk1-1Δ:HIS3 and cmk2-1Δ::TRP1 mutations (17). The resulting diploid (YMM6) was sporulated and dissected. In 3 tetrads out of the 40 analyzed, His⁺:His⁺ segregated 2:2 and both of the His⁺ spores were Trp⁺. Restriction endonuclease digestion and Southern blot hybridization analysis with appropriate DNA probes confirmed that the His⁺ Trp⁺ spores from such tetrads did indeed contain all three mutant alleles. All of these clk1Δ cmk1Δ cmk2Δ triple mutant haploids grew essentially like wild-type cells on rich medium containing glucose, galactose, or glycerol as the carbon source or on minimal selective medium (data not shown).

Because the predicted product of the RCK1 gene shares an even greater degree of amino acid sequence identity to Clk1 than do Cmk1 and Cmk2, it was of obvious interest to determine the phenotype of a clk1Δ rck1 double mutant. For this purpose, a rck1::LEU2 disruption allele was constructed and introduced into appropriate haploid and diploid strains, as described under “Experimental Procedures.” It was possible to obtain clk1Δ rck1 double mutant haploids at the expected frequency either from sporulation of diploids heterozygous at both loci and via direct transformation of clk1Δ haploids with rck1 mutant DNA. Thus, RCK1 and CLK1/RCK2 do not constitute an essential gene pair, as previously reported (48).

With the strains we generated, it was also possible by appropriate crosses to construct strains to attempt to recover clk1Δ rck1 cmk1Δ cmk2Δ quadruple mutant cells. Viable quadruple mutant spores were obtained at the expected frequency and were shown to possess all four mutant loci by restriction endonuclease digestion and Southern blot hybridization analysis using appropriate DNA probes. The growth of these clk1Δ rck1 cmk1Δ cmk2Δ quadruple mutant cells was essentially indistinguishable from wild-type cells on both rich medium and minimal selective medium (data not shown), suggesting that CLK1, RCK1, CMK1, and CMK2 do not constitute an extended and essential gene family.

DISCUSSION

CaM kinases are thought to mediate many important regulatory signals in mammalian cells (16, 60). Therefore, it was a somewhat surprising observation that yeast cells lacking both of the known CaM kinase homologs, Cmk1 and Cmk2, displayed no detectably deleterious phenotype (17, 18). Our initial motivation in examining the CLK1 locus in detail was the possibility that the protein kinase encoded by this gene might have properties similar to other Ca²⁺/CaM-dependent protein kinases and might overlap in function with Cmk1 and Cmk2. In this way, the existence of the CLK1 gene might account for the observed viability of cmk1Δ cmk2Δ double mutants. However, the biochemical findings presented here demonstrate that, although the CLK1 gene product shares certain sequence features with other known and well characterized Ca²⁺/CaM-dependent protein kinases, Clk1 is not a Ca²⁺/CaM-dependent protein kinase in vitro and does not appear to interact to any detectable extent with yeast CaM. Furthermore, the genetic results presented here indicate that neither CLK1 nor its homolog, RCK1, are essential genes and that CLK1, RCK1, CMK1, and CMK2 do not constitute a gene family with overlapping functions.

On the other hand, the properties of Clk1 are interesting and novel. Clk1 was able to phosphorylate the yeast translation elongation factor 2, which is thought to be the substrate of a
Ca\(^{2+}\)/CaM-dependent protein kinase in both mammalian cells and yeast (56, 57). The substrate selectivity of Clk1 is highlighted by the fact that Cmk1 was unable to phosphorylate yeast EF-2, even though Cmk1 has a rather broad substrate specificity in vitro (17, 18). Furthermore, overexpression of a C-terminally truncated, and presumably hyperactive, form of Clk1 led to severe growth inhibition, and this toxicity required the catalytic activity of the kinase. Thus, the C-terminal domain of Clk1 is a negative regulatory element, as has also been demonstrated for the mammalian CaM kinases (16, 59, 60). Indeed, overexpression of C-terminally truncated derivatives of Cmk1 and Cmk2 also markedly inhibit yeast cell growth (58).

Our data do not support the conclusion that Clk1 is a Ca\(^{2+}\)/CaM-responsive enzyme. We were unable to detect any effect of Cmd1 on Clk1 activity or to observe any stable association between Clk1 and Cmd1. On the other hand, it is possible that, in the immune complex assay, binding of the mAb to the C-terminal epitope altered the conformation of the C-terminal domain so as to alleviate its negative regulatory effect and, thereby, cause CaM independence. Although less likely, it could also be argued that, even in the GST-Clk1 fusion, the structure of the C-terminal domain is perturbed, leading to constitutive activation of the enzyme and precluding binding of Cmd1. Furthermore, even for mammalian CaM kinases, the degree of CaM-dependence varies for different phosphoacceptor substrates (16, 60). Thus, it is also possible that Clk1 might have displayed a greater responsiveness to CaM activation if the “correct” substrate had been used.

The molecular mass of Clk1 is larger than that of Cmk1 and Cmk2 (and mammalian CaM kinases) but in the same size range as that reported for partially purified activities from mammalian cells (56) and yeast (57) that phosphorylate yeast EF-2 in a Ca\(^{2+}\)/CaM-dependent manner. Despite its apparent lack of CaM responsiveness, Clk1 was able to phosphorylate EF-2. Studies by others have indicated that hyperphosphorylation of EF-2 inhibits translation (63, 64). Two other observations are at least consistent with a potential role for Clk1 in regulation of protein synthesis. First, Clk1 is confined to the cytosol and, thus, is most abundant in the same subcellular compartment where translation occurs. Second, as a genetic approach to examine the physiological function of the Clk1, dosage suppressors and chromosomal mutations were isolated that ameliorate the growth inhibitory effect of overproduction of C-terminally truncated Clk1. All of the dosage suppressors analyzed to date are genes that encode RNA-binding proteins and other factors known to be involved in translation (58).

The RCK1 locus is the gene most closely related to CLK1 in the S. cerevisiae genome. RCK1 was identified by another group via its ability, when expressed in a Schizosaccharomyces pombe rad1 mutant, to suppress the UV sensitivity of these cells (48). These workers isolated CLK1/RCK2 because of its homology to RCK1. Their subsequent work, which has continued to involve the expression of these S. cerevisiae genes in S. pombe, suggests that the presence of Clk1 and Rck1 extends the G2 phase of the cell cycle in S. pombe (65). In this regard, it has also been reported that a constitutively active form of mouse CaM kinase also inhibits the G2/M transition when expressed in S. pombe (66). However, we find that S. cerevisiae clk1A and rck1 single mutants and clk1A rck1 double mutants are no more UV-sensitive than otherwise isogenic wild-type cells (58), suggesting that the normal role of Clk1 (and Rck1) is not in repair of radiation damage (48) or in checkpoint control (65). At least as judged by budding pattern, cultures of cells growth-inhibited by overexpression of C-terminally truncated Clk1 do not accumulate cells with large buds, as would be diagnostic of a G2-specific arrest in S. cerevisiae (67). If Clk1 is a EF-2 kinase in vivo, one explanation for the ability of hyperactive Clk1 to inhibit growth in S. cerevisiae (and to retard the G2/M transition in S. pombe) is that the resulting hyperphosphorylation of EF-2 inhibits the synthesis of proteins, like cyclins, that are especially critical for cell cycle progression.

We attempted to determine if the state of EF-2 phosphorylation in S. cerevisiae is affected by the status of the CLK1 gene. However, anti-Eft1/Eft2 antibodies available to us (gift of A. C. Nairn) were not effective in immunoprecipitating detectable amounts of radioactive EF-2 protein from extracts of yeast cells labeled by growth in minimal medium containing \([^{32}P]P\)O\(_4\). In contrast, immunoprecipitation of Clk1 from cells labeled in this fashion demonstrated that Clk1 is a phosphoprotein in vivo, that the label is almost exclusively present in phosphoserine, and that neither the C-terminal truncation (487–610) nor the catalytically inactive mutant (K201R) shows any significant diminution in incorporation (58). Thus, major phosphorylation sites in vivo do not reside in the C-terminal domain and can arise from the action of other cellular protein kinases(n),

\(^{5}\) M. Melcher, unpublished observations.
and not obligatorily from autophosphorylation.

Despite the sequence relatedness of Ctk1 to Rck1, Cmk1, and Cmk2, these genes do not constitute a family with shared and essential functions, since a ctk1Δ rck1Δ cmk1Δ cmk2Δ quadruple mutant was viable. No other closely related genes are present in the S. cerevisiae genome. Thus, these protein kinase activities do not appear to serve any function that is critical for vegetative growth but rather may be necessary for the cell to adjust efficiently to changing growth conditions. In this regard, the type II Ca2+/Calmodulin-dependent protein kinases seem to contribute to the ability of yeast cells to adjust to heat stress (68). Thus, it is possible that Ctk1 and its related protein kinases help adjust cellular metabolism to cope with environmental stress.

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