Identification of a Calcium/Calmodulin-dependent Protein Kinase That Phosphorylates the Neurospora Circadian Clock Protein FREQUENCY*

Received for publication, July 20, 2001, and in revised form, August 29, 2001
Published, JBC Papers in Press, September 10, 2001, DOI 10.1074/jbc.M106905200

Yuhong Yang, Ping Cheng, Gang Zhi, and Yi Liu‡
From the Department of Physiology, the University of Texas Southwestern Medical Center, Dallas, Texas 75390-9040

Phosphorylation of circadian clock proteins represents a major regulatory step that controls circadian clocks. In Neurospora, the circadian clock protein FREQUENCY (FRQ) is progressively phosphorylated over time, and its level decreases when it is hyperphosphorylated. In this study, we showed that most of the kinase activity phosphorylating FRQ in vitro was calcium/calmodulin-dependent, and the endogenous FRQ in the Neurospora extracts was phosphorylated by a Ca/CaM-dependent kinase-like activity. From Neurospora cell extracts, an ~50-kDa Ca/CaM-dependent kinase (CAMK-1) that can specifically phosphorylate FRQ was purified. In vitro, this kinase accounts for near half of the FRQ kinase activity, and it can phosphorylate the FRQ region that contains the three known functionally important phosphorylation sites. To understand the function of camk-1 in vivo, it was disrupted in Neurospora by gene replacement. After germination from ascospores, the camk-1 null strains grew slowly, indicating that CAMK-1 plays an important role in growth and development of Neurospora. This phenotype was transient however, revealing redundancy in the system. Analysis of the camk-1 null strain revealed that the deletion of camk-1 affected phase, period, and light-induced phase shifting of the circadian conidiation rhythm. Taken together, our results suggest that multiple kinases may phosphorylate FRQ in vivo.

Circadian clocks are responsible for controlling a wide variety of physiological, behavioral, cellular, and biochemical activities in most eukaryotic and certain prokaryotic organisms. At the molecular level, circadian oscillators consist of autoregulatory negative feedback loops, in which the positive elements of the loop activate the transcription of the negative elements, whereas the negative elements feed back to block their own transcriptional activation by the positive elements (1–7).

In the Neurospora frq-wc-based circadian feedback loops, WHITE COLLAR-1 (WC-1) and WC-2, two (Per-Amt-Sim) domain-containing transcription factors, form heterodimeric complexes to activate the transcription of frequency (frq) (8–10), whereas two forms of FRQ (large and small FRQ) are present in homodimeric complexes that feed back to repress their own transcription, probably by interacting with the WC-1/WC-2 complex (3, 11–15). Besides its role in repressing frq transcription, FRQ proteins positively regulate protein levels of both WC-1 and WC-2, thereby forming positive feedback loops interlocked with the negative feedback loop (10, 15, 16). These positive feedback loops appear to be important for maintaining the robustness and stability of the clock.

Although transcriptional regulation seems to play a major role in rhythm generation, it is becoming more and more evident that various posttranscriptional mechanisms also have important influence on rhythm generation. Phosphorylation of clock proteins is one of the major posttranscriptional mechanisms that regulate circadian clock functions in all clock model systems, and the kinases responsible are important components of the circadian systems (11, 17–26). For example, in Drosophila, the DOUBLE-TIME protein, a casein kinase I, acts to phosphorylate the clock protein PERIOD. In a dbt null mutant, the steady-state level of PERIOD is high and hypophosphorylated, suggesting that the phosphorylation of PERIOD may lead to its degradation (21, 22). In Neurospora, FRQ protein is progressively phosphorylated over time after its synthesis, and extensive phosphorylation of FRQ results in its eventual degradation. Two lines of evidence support the conclusion that one of the functions of FRQ phosphorylation is to lead to its degradation (18). First, a kinase inhibitor that blocks FRQ phosphorylation in vivo reduces the degradation rate of FRQ and lengthens the period of the clock. Second, mutation of one phosphorylation site at Ser-513 of FRQ leads to a dramatic reduction of the rate of FRQ degradation and a long period (>30 h) of the clock. Thus, phosphorylation of FRQ is a major determining factor for FRQ stability and for the period length of the Neurospora circadian clock.

In this study, we have purified a Neurospora calcium (Ca)/calmodulin (CaM)-dependent kinase (CAMK-1) protein that phosphorylates FRQ. Our data showed that the majority of FRQ phosphorylation in vitro is Ca/CaM-dependent. In vitro kinase assays indicate that CAMK-1 can phosphorylate the FRQ region containing Ser-513. Disruption of the camk-1 gene suggests that camk-1 plays an important role in the normal growth and development of Neurospora. However, the camk-1 null phenotypes were transient, revealing redundancy in the...
Phosphorylation of FRQ by Ca/CaM-dependent kinase

system. The analysis of the conidiation rhythm revealed that the clock is affected in the \textit{camk-1} null strain.

**EXPERIMENTAL PROCEDURES**

**Strains and Culture Conditions**—The \textit{bd.a} strain was used as the wild-type strain in this study. Liquid culture conditions were the same as described previously (3). Media for \textit{Neurospora} slants were 1% Vogel's, 2% sucrose, and 1.5% agar. Measurement of circadian period was performed on race tubes containing glucose/arginine medium (1% Vogel's, 0.1% glucose, 0.17% arginine, 50 mg/ml biotin, and 1.5% agar). Densitometric analysis of race tubes and calculations of period length and phase were performed as described (12) using Chrono II version 11.1 (Dr. Till Roenneberg, Ludwig-Maximillian University, Munich, Germany).

Analysis of phase response to light was performed on race tubes containing acetate/casamino acid medium (1% Vogel's, 1.2% sodium acetate, 0.05% casamino acid hydrolysate, and 1.5% agar), which results in condensed conidia bands and a more precise measurement of phase (27). Race tubes were first grown in constant light for 24 or 36 h before being transferred into continuous darkness at the same time. Cultures were then grown in the dark for 26 h, and different individual cultures were given a 5-min light pulse (2500 lux) at different times (2–9 h after the start of the entire circadian cycle). The phase of the center of the conidiation bands of the light-treated culture (6 replicates for each time point) was compared with those of the control cultures (kept in constant darkness), and the amount of phase shifts was determined. The initial light to darkness transition was defined as circadian time (CT) 12. The phases of the cultures were calculated as the average phase for 4 consecutive days after the light treatment.

**Recombinant Proteins**—GST-FRQ and GST-PO4 fusion proteins (containing FRQ amino acids (aa) 425–683 or 487–630) were expressed in the \textit{Escherichia coli} strain BL21 cells (Invitrogen) by growing the bacteria at 37 °C to an \textit{A}_{600} of 0.5–0.7. The cultures were then transferred to 25 °C and induced with 1 mM isopropyl-1-thio-\textit{D}-galactopyranoside. After a 3-h induction, cultures were harvested, resuspended in 1/50 volume of phosphate-buffered saline, and purified on a glutathione-agarose column as recommended by the supplier (Sigma). The in-gel kinase assay was performed as described previously (28, 29). Total protein (20–40 μg) prepared from the wild-type strain was subjected to electrophoresis in an 8% SDS-PAGE gel that had been polymerized in the presence of 100 μg/ml of the purified GST or GST-FRQ protein. Following electrophoresis, the gel was washed twice with 50 mM Tris-HCl, pH 8.0, with 20% isopropyl alcohol for 30 min, twice with buffer B (50 mM Tris-HCl, pH 7.5, 5 mM \textit{β}-mercaptoethanol) for 30 min, and then denatured by incubating the gel twice for 30 min in buffer B containing 6 M guanidinium HCl. The proteins in the gel were then renatured overnight at 4 °C in buffer B containing 0.05% Tween 20 with at least four changes of the buffer. Then the gel was incubated in kinase buffer (25 mM HEPES-NaOH, pH 7.9, 1 mM MgCl2, 2 mM MnCl2, 25 μM ATP, and 10 μg/ml \textit{γ}-\textit{32P}ATP) containing 10 μg/ml \textit{γ}-\textit{32P}ATP at room temperature for 1 h. When used, 0.15 mM CaCl2 and 14 μg/ml CaM (Sigma) were added to the reaction buffer. Then the gel was washed 5–7 times in 5% trichloroacetic acid and 1% sodium pyrophosphate. The gel was then dried and subjected to autoradiography. Prestained protein standard markers (Life Technologies, Inc.) were used in all SDS-PAGE gels.

**In Vitro Kinase Assays**—To assay kinase activity, GST-FRQ or GST-PO4 fusion proteins (5 μg) were incubated with \textit{Neurospora} protein extracts (5–10 μg of total extracts or 40 μl of the chromatographic fractions) in a buffer containing 25 mM HEPES-NaOH, pH 7.9, 10 mM MgCl2, 2 mM MnCl2, 25 μM ATP, and 10 μg/ml \textit{γ}-\textit{32P}ATP (total reaction volume of 125 μl). When used, 0.15 mM CaCl2 and 14 μg/ml CaM were added to the reaction buffer. The reaction mixture was incubated at room temperature for 1 h before adding 0.5 ml of phosphate-buffered saline and 10 μl of glutathione-agarose beads. After a 30-min incubation at room temperature, the glutathione-agarose beads were washed twice in phosphate-buffered saline to remove free nucleotide before they were resuspended and boiled in 1× SDS-PAGE loading buffer. The samples were then subjected to SDS-PAGE. After electrophoresis, the gel was dried and subjected to autoradiography or stained with Coomassie Blue.

For detecting phosphorylation of the endogenous FRQ in the \textit{Neurospora} cell extracts, the \textit{Neurospora} cell extracts were prepared as described previously (11) in 50 mM HEPES-NaOH, pH 7.4, 150 mM NaCl, and 10% glycerol. The protein concentration of each extract was adjusted to 2 mg/ml with this buffer, and the phosphorylation reaction was initiated by adding 5 mM ATP and 5 mM MgCl2. The reaction mixture was incubated 2–4 h at room temperature before an equal volume of 2× SDS-PAGE loading buffer was added. The samples were then subjected to SDS-PAGE and Western blot analysis using FRQ antiseraum (11).

**Purification of CAMK**—All procedures were carried out at 4 °C, and both in-gel kinase and \textit{in vitro} kinase assays were used to monitor kinase activity. 100 mg of wild-type \textit{Neurospora} total extracts in 20 ml of 50 mM HEPES-NaOH, pH 7.4, 137 mM NaCl, and 10% glycerol were applied to a DEAE-Sepharose column (50-ml bed volume) that had been equilibrated with buffer A (50 mM Tris-HCl, pH 7.5, 1 mM dithiothreitol, 1 mM EDTA). The flow-through (containing CAMK-1) was collected and concentrated 4-fold. CM-Sepharose column (55-ml bed volume) was equilibrated with buffer A. The column was washed with a 2-column volume of buffer A before the proteins on the column were eluted by buffer A containing 100 mM NaCl. The eluted protein fraction was then loaded onto a CaM affinity column (1-ml bed volume, Amersham Pharmacia Biotech) that had been equilibrated with buffer A containing 5 mM MgCl2 and 1 mM CaCl2. The column was washed with buffer A containing 5 mM MgCl2, 1 mM CaCl2, and 100 mM NaCl. Proteins on the column were eluted first by buffer A containing 5 mM EGTA and then by buffer A containing 5 mM EGTA and 1 mM NaCl.

**Cloning and Disruption of camk-1**—Two \textit{Neurospora} EST sequences (SM1Hi23T and SM1c13T) were found to resemble other eukaryotic Ca/CaM-dependent kinases. To clone the genomic DNA of \textit{camk-1}, a potato virus X genome fragment was cloned into the pDONR vector (Invitrogen). Transformants containing homologous double recombination events were screened by polymerase chain reaction and Southern blot analysis. To make a homokaryotic \textit{camk-1} knock-out strain, the positive transformants were crossed with a wild-type strain. Individual sexual spores were picked and germinated on slants containing hygromycin (32).

**RESULTS**

A 50-kDa Ca/CaM-dependent Kinase, a Potential FRQ Kinase.—To identify the potential FRQ kinase(s), we first used the in-gel kinase assay, which allows us to estimate the size and the number of the potential kinases in this assay. GST or GST-FRQ (FRQ aa 425–683, containing the three known FRQ phosphorylation sites) expressed in \textit{E. coli} was purified and polymerized in SDS-PAGE gel. Total extracts of \textit{Neurospora} protein were separated on the gel by electrophoresis. Afterward, the gel was treated to remove SDS and allow proteins in the gel to renature. The gel was then incubated in kinase buffer containing \textit{γ}-\textit{32P}ATP, washed, dried, and exposed to x-ray film. If a kinase in the gel phosphorylates FRQ, it produces a band on the film. The approximate size of the kinase polypeptide can be estimated by comparison to the molecular weight markers on the gel.

Several kinase reaction conditions were tested, including the addition of Ca/CaM in the reaction buffer. No kinase was found to significantly phosphorylate FRQ, except when Ca/CaM was added in the reaction mixture. Fig. 1A shows that an ~50-kDa kinase specifically phosphorylated GST-FRQ in the presence of Ca2+ and CaM. The weak phosphorylation signals in the \textit{left panel} of Fig. 1A and in experiments with no kinase substrate (data not shown) indicate that the kinase was phosphorylated, a characteristic of all Ca/CaM-dependent kinases. Although a few other phosphorylation bands were also found by the assay, the similar intensities of signals between assays using GST or no substrate and assays using GST-FRQ suggest that these bands were most likely due to autophosphorylation of the kinases and not to phosphorylation of GST-FRQ. Fig. 1B demon-
strates that the phosphorylation of GST-FRQ by this kinase required both Ca\(^{2+}\) and CaM, because when both were omitted (data not shown) or when Ca\(^{2+}\) alone was added in the reaction buffer, very little autophosphorylation and GST-FRQ phosphorylation were detected.

To confirm these in-gel kinase assay results and to show that the Ca/CaM-dependent kinase activity was the major FRQ-phosphorylating kinase activity, we performed the standard solution in vitro kinase assay (see “Experimental Procedures”). As shown in Fig. 2A, the presence of Ca\(^{2+}\) and CaM in the reaction mixture significantly increased the kinase activity phosphorylating GST-FRQ (compare lane 3 to lane 6), whereas GST was not phosphorylated under the same conditions (data not shown and Fig. 8B). The majority of the kinase activity in lane 3 (−Ca/CaM) of Fig. 2A was likely due to the activation of the Ca/CaM-dependent kinase(s) by the low levels of Ca\(^{2+}\) and CaM present in the Neurospora extracts, because such activity can be significantly inhibited by either BAPTA (a specific Ca\(^{2+}\) chelator, exhibiting a 10-fold greater affinity for Ca\(^{2+}\) than for Mg\(^{2+}\)) or W-7 (a calmodulin inhibitor) (Fig. 2B). The residual kinase activity in the presence of BAPTA or W-7 probably reflects the fact that 1) some of the Ca/CaM-dependent kinase had already been activated by Ca\(^{2+}\) and CaM in the cell so that it was Ca/CaM-independent, and 2) other Ca/CaM-independent kinases could also phosphorylate GST-FRQ.

Together, these results suggest that the 50-kDa Ca/CaM-dependent kinase was the only kinase that significantly phosphorylated FRQ in the in-gel kinase assay, and the Ca/CaM-dependent kinase activity was responsible for most of the kinase activity phosphorylating FRQ in vitro.

Biochemical Purification of the Neurospora CAMK-1, the 50-kDa Ca/CaM-dependent Kinase—If the potential FRQ kinase is Ca/CaM-dependent, it should bind CaM in the presence of Ca\(^{2+}\), and its activity should be enriched by passing the Neurospora extracts through a CaM affinity column. After binding the Neurospora cell extracts onto a CaM affinity column, proteins were eluted by buffers containing EGTA. The results of both the in-gel kinase assay and the in vitro kinase assay showed that the 50-kDa kinase activity was indeed greatly enriched (data not shown and Fig. 3). The ability for this kinase to bind to CaM affinity column further confirmed that it was Ca/CaM-dependent. In addition, the CaM affinity column provided a powerful step for the biochemical purification of this protein.

By using both in-gel and in vitro kinase assays to monitor the

kinase activity and a combination of ion exchange columns and CaM affinity column purification (see “Experimental Procedures”), we purified this kinase to near-homogeneity from Neurospora cell extracts. Silver staining of the SDS-PAGE of the protein fractions from the final CaM column showed that only two closely separated protein bands could be seen at around 50 kDa (Fig. 3, upper panel). The protein of the lower band (−48 kDa) appeared to bind the CaM column more strongly than the protein in the upper band (−50 kDa), because the majority of the 48-kDa protein only eluted from the column by 1 M NaCl, 5 mM EGTA. Because the kinase activity profile of different CaM column fractions correlated with the protein profiles of both proteins (Fig. 3) and their molecular weights were very close to what we expected, both proteins were potential kinase candidates.

To reveal the identities of both proteins, protein fractions
were concentrated, separated by SDS-PAGE gel, and Coomassie-stained. Both bands were cut out and subjected to tryptic digestion, and the resulting peptides were subjected to mass spectral analysis. The resulting mass spectral results were used to search the existing protein data bases for matching proteins. Twenty four peptides from the tryptic digestion of the 50-kDa protein were found to match the *Neurospora* elongation factor 1- (45% coverage) (33). Whereas a few peptides from the tryptic digestion of the 48-kDa protein also matched the *Neurospora* elongation factor 1- (probably due to the contamination of the 50-kDa protein), the majority of the resolved peptides of the 48-kDa protein did not match any protein in the data bases. The predicted molecular mass and pI of the *Neurospora* elongation factor 1- were 49.7 kDa and 9.27, respectively, in close agreement with the results of the ion exchange column purification and the protein size on SDS-PAGE. In addition, elongation factor 1- from other organisms has been found to bind to CaM (34, 35). However, no known Ca/CaM-dependent protein kinase activity has ever been found to be associated with elongation factor 1-.

Because no known *Neurospora* Ca/CaM-dependent kinase was present in the protein data bases, to identify the 48-kDa protein, we searched the existing *Neurospora* EST data base for a putative Ca/CaM-dependent kinase. Two EST sequences of the same gene (SM1H12T3 and SM1C1T3) (36) were found to resemble other eukaryotic Ca/CaM-dependent protein kinases. By using the DNA sequence information of the ESTs, the full-length genomic DNA for the gene (*camk-1*) was cloned and sequenced.

**A**

*Neurospora camk-1* (415 aa)

```
ATG
```

**B**

```
Neu (1)  N--------------------------------------------------------SFANML-NRLHKGFPS-YDK------KSYESFGRT
Asper (1) N--------------------------------------------------------SFAMNF-KNSLGGFP-EYE------KSLYFTGR
Yeast (1) N--------------------------------------------------------FYSEVINSLLQDVQDPRILHAGYAF-
Human (1) N--------------------------------------------------------AGRESTSSWKK-FAEDIKITFEKL
Rat (1) N----------------------------------------------------------PGAVGPKWR-QAERIIRYDIFPQV
Dros (1) N----------------------------------------------------------PLPGKDSGKR-ARAKDLKEL-NK-Q-VSIEKNYLHG
At (1) N-------------------------------------------------------------SKNGGTA68SLQDVQDPRILHAGYAF-

Neu (30) CTGTVGREA-SGPT-GRV-KRIRKLKVNGE-YQVMLELQLR
Asper (30) CTGTVGREA-DCSS-GRV-AKISKKNVREQ-YRVEYELDILQG
Yeast (34) GEGPVGRWQAKLST-NEVADAILIKLQKNNQGQMYPYLIEDILQG
Human (30) GEGPEVGLAEZKAT-GKLAVTPKKNLKLKQNGE-SSIESINAVLJKR
Rat (27) GEGPESVIAZDRT-KQILVKAIDKILKQNGE-GSEMIEINAVLJKR
Dros (38) GEGPSVIAZDRT-KQILVKAIDKILKQNGE-ESEVILRJAFsnfMsgk
At (61) GEGPSVIAZDRT-KQILVKAIDKILKQNGE-ESEVILRJAFsnfMsgk

Neu (74) LKPIPLKFLVWDPKSKYTVKTVHLTVATGSCELQFRK-XXXKTKQLKTQWLG
Asper (74) LKPIPLKFLVWDPKSKYTVKTVHLTVATGSCELQFRK-XXXKTKQLKTQWLG
Yeast (74) LKPIPLKFLVWDPKSKYTVKTVHLTVATGSCELQFRK-XXXKTKQLKTQWLG
Human (76) LKPIPLKFLVWDPKSKYTVKTVHLTVATGSCELQFRK-XXXKTKQLKTQWLG
Rat (73) LKPIPLKFLVWDPKSKYTVKTVHLTVATGSCELQFRK-XXXKTKQLKTQWLG
Dros (95) LKPIPLKFLVWDPKSKYTVKTVHLTVATGSCELQFRK-XXXKTKQLKTQWLG
At (109) LKPIPLKFLVWDPKSKYTVKTVHLTVATGSCELQFRK-XXXKTKQLKTQWLG

Neu (129) AVYIKJNRENYLRLDPKPMHYIDRADAISDLVYLADFQAIKMLNKDEVEVTTMADPSGFH
Asper (129) AVYIKJNRENYLRLDPKPMHYIDRADAISDLVYLADFQAIKMLNKDEVEVTTMADPSGFH
Yeast (129) AVYIKJNRENYLRLDPKPMHYIDRADAISDLVYLADFQAIKMLNKDEVEVTTMADPSGFH
Human (131) AVYIKJNRENYLRLDPKPMHYIDRADAISDLVYLADFQAIKMLNKDEVEVTTMADPSGFH
Rat (128) AVYIKJNRENYLRLDPKPMHYIDRADAISDLVYLADFQAIKMLNKDEVEVTTMADPSGFH
Dros (155) AVYIKJNRENYLRLDPKPMHYIDRADAISDLVYLADFQAIKMLNKDEVEVTTMADPSGFH
At (165) AVYIKJNRENYLRLDPKPMHYIDRADAISDLVYLADFQAIKMLNKDEVEVTTMADPSGFH

Neu (189) PEKNLQGQKRPVWNSMVYTVLLGCVPSFLSNNQLCDSCSSGV-VCHRML
Asper (189) PEKNLQGQKRPVWNSMVYTVLLGCVPSFLSNNQLCDSCSSGV-VCHRML
Yeast (189) PEKNLQGQKRPVWNSMVYTVLLGCVPSFLSNNQLCDSCSSGV-VCHRML
Human (193) PEKNLQGQKRPVWNSMVYTVLLGCVPSFLSNNQLCDSCSSGV-VCHRML
Rat (192) PEKNLQGQKRPVWNSMVYTVLLGCVPSFLSNNQLCDSCSSGV-VCHRML
Dros (213) PEKNLQGQKRPVWNSMVYTVLLGCVPSFLSNNQLCDSCSSGV-VCHRML
At (224) PEKNLQGQKRPVWNSMVYTVLLGCVPSFLSNNQLCDSCSSGV-VCHRML

Neu (247) VSDNQKIIKLKCQKPEKMTQVSYTVLTHGLRQDSATNHLLPPEKAI
Asper (247) VSDNQKIIKLKCQKPEKMTQVSYTVLTHGLRQDSATNHLLPPEKAI
Yeast (278) DSIKQKIIKLKCQKPEKMTQVSYTVLTHGLRQDSATNHLLPPEKAI
Human (248) DSIKQKIIKLKCQKPEKMTQVSYTVLTHGLRQDSATNHLLPPEKAI
Rat (278) DSIKQKIIKLKCQKPEKMTQVSYTVLTHGLRQDSATNHLLPPEKAI
Dros (271) DSIKQKIIKLKCQKPEKMTQVSYTVLTHGLRQDSATNHLLPPEKAI
At (281) DSIKQKIIKLKCQKPEKMTQVSYTVLTHGLRQDSATNHLLPPEKAI

Neu (297) YLTG-------------------------------------------------ARLRGEMIKLARN--IEIA
Asper (295) YLTG-------------------------------------------------ARLRGEMIKLARN--IEIA
Yeast (329) YLTG-------------------------------------------------ARLRGEMIKLARN--IEIA
Human (297) YLTG-------------------------------------------------ARLRGEMIKLARN--IEIA
Rat (294) YLTG-------------------------------------------------ARLRGEMIKLARN--IEIA
Dros (320) YLTG-------------------------------------------------ARLRGEMIKLARN--IEIA
At (339) YLTG-------------------------------------------------ARLRGEMIKLARN--IEIA

**Fig. 4.** Gene structure of the *Neurospora camk-1* gene and protein sequence alignment of CAMK1 and other eukaryotic Ca/CaM-dependent protein kinases. *A,* gene structure of *camk-1.* Boxes and V-shaped lines represent exons and introns. Filled boxes indicate the open reading frame of the gene. *B,* amino acid sequence alignment of eukaryotic Ca/CaM-dependent protein kinases. The carboxyl ends of the proteins were not included in this alignment. The amino acids conserved in all proteins are boxed. The following abbreviations are used: Neu, *Neurospora* CAMK-1; Asper, A. nidulans (A822981); yeast, *S. cerevisiae* CMK2 (CA40281); human, CamKII-like protein kinase (NP_065130); rat, Rattus norvegicus Ca/CaM protein kinase I (Q68450); Dros, Drosophila melanogaster Ca/CaM-dependent protein kinase I (CA76937); At, Arabidopsis thaliana (A823555).
The predicted protein sequence of CAMK-1 was then used to compare with the mass spectral results of the 48-kDa protein, and we found that 27 peptides of the tryptic digestion of the 48-kDa protein matched CAMK-1, a coverage of 59.5%. The high coverage rate indicates that the purified 48-kDa protein is CAMK-1.

The Neurospora camk-1 Gene — Comparison of the genomic and cDNA sequences of the Neurospora camk-1 revealed that it contained 6 exons and 5 introns (Fig. 4A), encoding a protein of 415 aa with a predicted molecular mass and pi of 46.7 kDa and 7.7, respectively. The predicted molecular mass and pi of CAMK-1 were in close agreement with our purification profiles. Protein sequence alignment show that the Neurospora CAMK-1 is very similar to other eukaryotic Ca/CaM-dependent kinases (Fig. 4B), and its closest homologs are a Ca/CaM-dependent protein kinase of Aspergillus nidulans (71% identity and 82% similarity over the first 390 aa) and the CMK2 of Saccharomyces cerevisiae (53% identity and 71% similarity over the first 320 aa). Like other Ca/CaM-dependent kinases, the catalytic domain (the ~300-aa N-terminal region) of the Neurospora CAMK-1 is highly conserved, and the regulatory domain (C-terminal part of the protein) of the kinase is diverged.

Disruption of the Neurospora camk-1 Gene — To understand the function of the Neurospora camk-1 in vivo, the endogenous camk-1 gene was disrupted by gene replacement. The gene disruption vector was constructed by replacing the BamHI fragment (containing the region from exon 2 to part of exon 6) of the genomic DNA with a hygromycin-resistant gene (hph) cassette (Fig. 5A). The resulting vector was transformed into a wild-type Neurospora strain, and transformants were screened by Southern blot analysis to identify the ones that carried the homologous recombination events and have only one hph integration site. The positive transformants were then crossed with a wild-type strain, and the individual sexual spores (ascospores) were picked to germinate on hygromycin-containing slants to obtain homokaryon of the camk-1 knock-out (KO) strain. As the Southern blot analysis shown in Fig. 5B, the endogenous camk-1 was disrupted in a camk-1 KO homokaryon strain.

Unlike the heterokaryotic camk-1 KO strains, which showed no abnormal growth and clock phenotypes, the homokaryon of the KO strains showed a very different growth phenotype. Immediately after the sexual spores were germinated, the vegetative growth of the strains was slow, colonial, and restricted to the agar surface (Fig. 6A). In addition, the strains produced no abnormal growth and clock phenotypes, the homokaryon of the KO strains showed a very different growth phenotype. The KO strains showed a very different growth phenotype.
lating onto a fresh slant or into liquid culture, they grew much faster than before and became more like the wild-type strain. Usually, after 1–2 slant transfers or 1–2 days of growth in liquid culture, all KO strains completely phenocopied the wild-type strain, and the initial slow growth phenotype was no longer seen. In Fig. 6A, 7-day-old slants of a wild-type strain, a KO strain (newly germinated from a sexual spore), and a fast growing KO (“revertant”) strain are shown. As described above, low amounts of mycelia, aerial hyphae, and conidia were made by the KO strain, but the appearance of the KO revertant strain is indistinguishable from the wild-type strain.

When a fast growing KO strain was crossed with a wild-type strain, all progenies with hph-resistant gene showed the slow growth phenotype following ascospore germination, and they phenocopied the wild-type strain after 1–2 slant transfers. These data indicate that the slow growth phenotype of the KO strains is genetically heritable and can only be observed following ascospore germination.

Because of the dramatic phenotype reversal of the camk-1 KO strains, it is possible that the enzymatic activity of CAMK-1 also “reverted” back. To rule out this possibility, we examined the CAMK-1 kinase activity of the fast growing KO strains by the in-gel kinase assay. As shown in Fig. 6B, the 50-kDa phosphorylation band was absent in both KO strains, and the intensities of other phosphorylation bands were comparable between the wild-type and the camk-1 KO strains. This result indicates that the reversion of the KO strain was not due to the reversion of the CAMK-1 activity.

To examine whether CAMK-1 is an important kinase that phosphorylates FRQ in vitro, we compared the FRQ kinase activity between the wild-type and the fast growing camk-1 KO strains using the standard in vitro kinase assay. As shown in Fig. 6C, the deletion of camk-1 resulted in 40–50% reduction of the FRQ kinase activity in vitro. Together, these results suggest that CAMK-1 is an important FRQ kinase, but clearly it is not the only kinase that phosphorylates FRQ in vitro. The initial slow growth phenotype of the camk-1 KO strains suggests that it is important for the normal growth and development of Neurospora immediately after the germination of the sexual spores. However, it becomes dispensable after some period of growth. It is possible that its function is replaced by other kinases in the fast growing KO strains. Recently, most of the Neurospora genome sequence was determined. Searching through the Neurospora genome database (wolfgram.wi.mit.edu/annotation/fungi/neurospora/), we found that there are two additional Ca/CaM-dependent protein kinases in Neurospora. Therefore, the “reversion” of the phenotype is likely due to redundancy of the kinases.

Circadian Clock Is Affected in the camk-1 KO Strain—To examine whether the deletion of the camk-1 gene affected the function of the Neurospora circadian clock, the freshly germinated camk-1 KO strains (3–7-day-old) were inoculated onto the race tubes. The KO strains grew slowly at first (1–2 mm/day versus 3–4 mm/day of the wild-type strain), and then they either stopped growing completely or grew faster and eventually grew like the wild-type strain. Because of their initial slow growth rate, the running of the clock could not be judged. When their growth rate was faster, near wild-type circadian conidiation rhythms were observed. After they phenocopied the wild-type strain, their growth rate was very similar to that of the wild-type strain. Because of the dramatic phenotype reversal of the camk-1 KO strains, it is possible that the enzymatic activity of CAMK-1 also “reverted” back. To rule out this possibility, we examined the CAMK-1 kinase activity of the fast growing KO strains by the in-gel kinase assay. As shown in Fig. 6B, the 50-kDa phosphorylation band was absent in both KO strains, and the intensities of other phosphorylation bands were comparable between the wild-type and the camk-1 KO strains. This result indicates that the reversion of the KO strain was not due to the reversion of the CAMK-1 activity.

Phosphorylation of FRQ by Ca/CaM-dependent kinase

Phosphorylation of the Endogenous FRQ by Ca/CaM-dependent kinase—We next examined whether the phosphorylation profile and the circadian oscillation of constant darkness of the mutants (12.5 h) was about 2 h later than that of the wild type (t test: T = −9.24, p = 1.2E-07, n = 30), and the period of mutants (22.1 h) was about 20 min longer than that of the wild-type (t test: T = 4.9, p = 0.0001, n = 30). Thus, although CAMK-1 is not required for the function of the clock, the circadian clock is modestly affected in the fast growing camk-1 KO strains.

Previously, it has been shown that the inhibitors of CaM can inhibit light-induced phase shifting in Neurospora (37); therefore, we next examined the light-induced phase shifting in the camk-1 KO strain. Cultures grown in constant darkness were given 5 min of saturating light pulse (27) at different times to cover one entire circadian cycle and the phase shifts determined. Fig. 7B showed the light-induced phase response curves for the wild-type and the fast growing camk-1 KO strains. Although the general shapes of the phase response curves and the maximum phase shifts of both strains are similar, these data showed that the phase response to light is altered in the camk-1 mutant. First, during the middle of the day (CT8–16), there was a “dead zone” for the wild-type strain (relative unresponsive to light), but the same light pulses resulted in delays in the mutant, suggesting that the clock in the camk-1 mutant responds to light most of the day. Second, around CT22–24, when the light pulse resulted in the biggest phase shifts in both strains, the time when light resulted in largest phase shift was earlier in the mutant. At CT22, although the light pulse induced about 5 h of delay in the wild-type strain, the delay is twice as large in the mutant (~10 h). At CT24 (CT1), when light resulted in the largest phase shift for the wild-type (~10 h advance), the same light treatment caused 6 h of advance in the mutant. These results suggest that the disruption of camk-1 affected the light response of the clock.
FRQ were affected in the camk-1 KO strains. Freshly germinated KO strains (4–5-day-old) and fast growing strains were both used. Western blot analysis revealed that the phosphorylation profiles of FRQ in the KO strains were similar to wild type (data not shown). This result was expected because of the robust circadian rhythm and the small changes of phase and type (data not shown). This result was expected because of the apparent redundancy of the kinases and the quick reversal of the KO strains prohibited our efforts to determine the phosphorylation profiles of FRQ in the KO strains. Freshly germinated KO strains (4–5-day-old) and fast growing strains were both used. Western blot analysis revealed that the phosphorylation profiles of FRQ in the KO strains were similar to wild type. Because of the quick “reversal” of the KO strains, we suspect that even the newly germinated KO hyphae were partially reverted because 1) some aerial hyphae and conidia were seen on slants, and 2) KO strains grown in liquid culture also appeared to speed up the reversion process.

The apparent redundancy of the kinases and the quick reversal of the KO strains prohibited our efforts to determine the role of CAMK-1 to phosphorylate FRQ in vivo. To show that the endogenous FRQ was phosphorylated by a Ca/CaM-dependent kinase-like activity, we set up an in vitro assay to examine the phosphorylation of the endogenous FRQ in cell extracts. In this assay, the reaction was initiated by the addition of ATP and MgCl₂ into the Neurospora cell extracts, and the phosphorylation profile of FRQ was very similar to that of the in vivo phosphorylation profile. As shown in Fig. 8A, after the addition of ATP and MgCl₂, the hypophosphorylated (faster mobility in SDS-PAGE) endogenous FRQ species were progressively phosphorylated and became the slower mobility species in the gel (compare lane 0 to lane 1). ATP and MgCl₂ were added to the Neurospora cell extracts (2 mg/ml) to initiate the phosphorylation of the endogenous FRQ proteins. 1 or 3 min BAPTA was added to the extracts shown in the two right lanes. After 2 h, the reactions were stopped; the extracts were subjected to SDS-PAGE, and Western blot analysis was performed using FRQ antiserum. Various FRQ bands seen on the Western blot were the result of progressive FRQ phosphorylation and two alternatively translated FRQ forms (large FRQ and small FRQ) (11). The phosphorylation of FRQ was monitored by observing the mobility shifts of the lower molecular weight FRQ species compared with the higher molecular weight species. 0 indicates the Neurospora extracts at the beginning of the reaction. The arrow points to one of the large FRQ forms that was hypophosphorylated before the reaction. B, aliquots of the CaM column fractions (Fig. 3) were used in the in vitro kinase assay to examine the ability of the CAMK-1 to phosphorylate the small FRQ region containing the three known phosphorylation sites. GST-PO₄ was the substrate used in the assay. The reaction mixtures were subjected to 15% SDS-PAGE. The arrow indicates the position of the full-length GST-PO₄ protein. In lane 1, 10 μg of Neurospora extracts was used. Less than 0.1 μg of purified CAMK-1 was used in lanes 2–4.

Phosphorylation of FRQ by Ca/CaM-dependent kinase

![Fig. 8. Inhibition of the phosphorylation of the endogenous FRQ proteins by BAPTA and the ability of the purified CAMK-1 to phosphorylate the FRQ region containing the three known phosphorylation sites in vitro.](http://www.jbc.org/) A, ATP and MgCl₂ were added to the Neurospora cell extracts (2 mg/ml) to initiate the phosphorylation of the endogenous FRQ proteins. 1 or 3 min BAPTA was added to the extracts shown in the two right lanes. After 2 h, the reactions were stopped; the extracts were subjected to SDS-PAGE, and Western blot analysis was performed using FRQ antiserum. Various FRQ bands seen on the Western blot were the result of progressive FRQ phosphorylation and two alternatively translated FRQ forms (large FRQ and small FRQ) (11). The phosphorylation of FRQ was monitored by observing the mobility shifts of the lower molecular weight FRQ species compared with the higher molecular weight species. 0 indicates the Neurospora extracts at the beginning of the reaction. The arrow points to one of the large FRQ forms that was hypophosphorylated before the reaction. B, aliquots of the CaM column fractions (Fig. 3) were used in the in vitro kinase assay to examine the ability of the CAMK-1 to phosphorylate the small FRQ region containing the three known phosphorylation sites. GST-PO₄ was the substrate used in the assay. The reaction mixtures were subjected to 15% SDS-PAGE. The arrow indicates the position of the full-length GST-PO₄ protein. In lane 1, 10 μg of Neurospora extracts was used. Less than 0.1 μg of purified CAMK-1 was used in lanes 2–4.

**DISCUSSION**

Phosphorylation of FRQ is critical for the regulation of the Neurospora circadian clock. In this study, we purified a 50-kDa Ca/CaM-dependent kinase from Neurospora extracts to near-homogeneity and identified and cloned the kinase gene. The identified Neurospora Ca/CaM-dependent protein kinase-1 (camk-1) is very similar to other eukaryotic Ca/CaM-dependent kinases. However, our repeated failure to detect the peptides in this region by mass spectral analysis prevented us from mapping the exact phosphorylation sites. This is not uncommon because mass spectral analysis normally only resolves part of the peptide species of a protein. The ability for CAMK-1 to phosphorylate GST-PO₄ substrates containing single point mutations of the three sites suggests that it probably can phosphorylate more than one amino acid (data not shown).

The CAMK-1 Activity Is Not Rhythmic Under Circadian Conditions—In Drosophila, the level of the dbt mRNA and protein was essentially unchanged at different times of the day, but the nuclear localization of DOUBLE-TIME is clock-regulated (22, 38). To examine whether the activity of CAMK-1 was controlled by circadian clock in a wild-type strain, we performed the in-gel kinase assay to measure its kinase activity over two circadian cycles. Fig. 9 shows that despite the robust circadian oscillations of the level and phosphorylation patterns of FRQ proteins, the activity of CAMK-1 appeared to be relatively constant in vitro. Although this result suggests that the level of CAMK-1 protein and its activity are not clock-controlled, we cannot rule out the possibilities that the cellular free calcium concentration and the cellular localization of CAMK-1 are regulated by the clock.
kinases, with a highly conserved catalytic domain. To understand the function of \textit{camk-1}, it was disrupted in \textit{Neurospora} by gene replacement. The phenotypes of the \textit{camk-1} null strain suggest that it plays an important role in growth and development of a wild-type strain. However, the transient slow growth phenotype indicates redundancy in the system.

Several lines of evidence suggest that CAMK-1 phosphorylates FRQ. First, CAMK-1 is the only kinase that strongly phosphorylates FRQ in the in-gel kinase assay (Fig. 1). Second, the Ca/CaM-dependent kinase activity accounts for most of the kinase activities that phosphorylate FRQ in the \textit{Neurospora} extracts (Fig. 2), and the phosphorylation of the endogenous FRQ in \textit{Neurospora} extracts can also be inhibited by a Ca\textsuperscript{2+} chelator (Fig. 8). Third, \textit{in vitro}, CAMK-1 accounts for near half of the FRQ kinase activity (Fig. 6C), and it can phosphorylate the FRQ region aa 501–519, which contains the three known functionally important phosphorylation sites. Finally, in the \textit{camk-1} mutant strain, three parameters of the circadian conidiation rhythm were modestly affected, namely phase, period length, and light-induced phase shifting. However, the less severe than expected clock phenotype and the significant FRQ kinase activity in the \textit{camk-1} KO strains suggest that FRQ may be phosphorylated by multiple kinases \textit{in vivo}.

Despite the evidence to suggest the involvement of CAMK-1 in phosphorylating FRQ, the quick reversal of the \textit{camk-1} null strains and the redundancy of the kinases prevent us from making a firm conclusion on its \textit{in vivo} role. The redundancy of the kinases was indicated by the existence of two additional Ca/CaM-dependent protein kinases in the recently completed \textit{Neurospora} genome database. By using \textit{Neurospora} extract of a \textit{camk-1} KO strain, we found that the phosphorylation of the endogenous FRQ can still be inhibited by Ca\textsuperscript{2+} or CaM inhibitors (data not shown), suggesting that there is Ca/CaM-dependent kinase-like activity left in the \textit{camk-1} KO strain. Consistent with this notion, 50–60% of the \textit{in vitro} FRQ kinase activity could still be observed in the \textit{camk-1} KO strain. Therefore, it is likely that several kinases may phosphorylate FRQ at the same sites and regulate its function. If so, the deletion of \textit{camk-1} may not significantly affect the phosphorylation of FRQ. Thus, the effects on the circadian clock are less than expected due to the presence of a wild-type strain. However, the transient slow growth phenotype indicates redundancy in the system.

One drawback of the in-gel kinase assay method is that the kinase of interest must be able to refold correctly after the denaturation process, and it can function by itself. Therefore, our inability to detect other kinase activity by the in-gel kinase assay in the \textit{camk-1} KO strains (Fig. 6B) may be because those kinases fail to refold correctly during the renaturation process or need to form complexes with other proteins. By conventional \textit{in vitro} kinase assay, some weak but detectable Ca/CaM-independent kinase activities can be observed after the fractionation of the cell extracts of the \textit{camk-1} KO strains by ion exchange columns (data not shown), suggesting that other Ca/CaM-independent kinases may also phosphorylate FRQ.

Ca\textsuperscript{2+} and CaM-mediated regulations have long been implicated in the control of circadian clock systems of \textit{Neurospora} and other eukaryotic organisms (37, 39–45). In \textit{Neurospora}, Nakashima and colleagues (45) have shown that the circadian conidiation rhythm of \textit{Neurospora} can be phase-shifted by CaM antagonists; mutants with altered sensitivity to a CaM antagonist affect the circadian clock of \textit{Neurospora} (42); and CaM antagonists can inhibit light-induced phase shifting in \textit{Neurospora} (37). Although it is possible that the influences of those drugs on the \textit{Neurospora} clock are indirect or due to their side effects, our identification of CAMK-1 as a potential kinase that phosphorylates FRQ provides a possible molecular explanation for those studies. Our data showed that the elimination of CAMK-1, one major downstream substrate for CaM, led to changes in cellular free Ca\textsuperscript{2+}, period length, and light-induced phase shifting of the clock. Because the level and the phosphorylation profile of FRQ appear to be the determinants for phase and period of the clock (11, 18), the effects on the \textit{camk-1} mutant are probably due to small alteration of FRQ phosphorylation profile that we failed to detect. However, it is also possible that such effects on the clock are not related to FRQ phosphorylation and are due to some indirect effects.

Ca/CaM-dependent kinases have been shown to be involved in regulating many aspects of cellular functions of eukaryotic organisms, and they have broad substrate specificities (46, 47). Our current knowledge of Ca/CaM-dependent kinases comes mostly from studies of the mammalian enzymes (46, 47). The mammalian CaM kinase II is inactive when it is not phosphorylated. When the concentration of Ca\textsuperscript{2+} in the cytosol is raised by the opening of Ca\textsuperscript{2+} channels, the Ca\textsuperscript{2+}-binding protein CaM becomes activated. In the presence of activated CaM, CaM kinase II undergoes rapid autophosphorylation events within the regulatory domain of the protein, which then leads to the activation of the kinase. The activated kinase is deactivated upon dephosphorylation by phosphatases. Our in-gel kinase assay results showed that the autophosphorylation of CAMK-1 and its ability to phosphorylate GST-FRQ require both Ca\textsuperscript{2+} and CaM (Fig. 1 and data not shown). However, the actual CaM-binding domain and the autophosphorylation sites on CAMK-1 could be different from those of the mammalian CaM kinases because the regulatory domains of proteins (C-terminal portion of the proteins) are different from each other. In \textit{Neurospora}, the level of CaM is constant at different times of the day (45), but it is not known whether the concentration of cellular free Ca\textsuperscript{2+} oscillates daily. Therefore, although the in-gel kinase assay result (Fig. 9) suggests that the activity of CAMK-1 is not clock-regulated, we cannot rule out the possibility that its activity oscillates \textit{in vivo} due to changes in cellular concentration of free Ca\textsuperscript{2+} or circadian variation of CAMK-1 cellular localization. In conclusion, we have identified a \textit{Neurospora} Ca/CaM-dependent kinase that phosphorylates FRQ. Our future analyses of the two additional \textit{Neurospora} Ca/CaM-dependent kinases and biochemical purification of additional FRQ kinases from cell extracts of the \textit{camk-1} null strain should reveal the identity of additional FRQ kinases and their functions in regulating the \textit{Neurospora} circadian clock.

Acknowledgments—We thank Dr. Carl Johnson for seminal discussions of the results. We also thank Drs. James Stull and Steve Hammes for critical reading of the manuscript.

REFERENCES

Phosphorylation of FRQ by Ca/CaM-dependent kinase

Identification of a Calcium/Calmodulin-dependent Protein Kinase That Phosphorylates the *Neurospora* Circadian Clock Protein FREQUENCY

Yuhong Yang, Ping Cheng, Gang Zhi and Yi Liu

doi: 10.1074/jbc.M106905200 originally published online September 10, 2001

Access the most updated version of this article at doi: 10.1074/jbc.M106905200

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 46 references, 21 of which can be accessed free at http://www.jbc.org/content/276/44/41064.full.html#ref-list-1