Regulation of the *Neurospora* circadian clock by casein kinase II

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Phosphorylation of clock proteins represents an important mechanism regulating circadian clocks. In *Neurospora*, clock protein FREDQUENCY (FRQ) is progressively phosphorylated over time, and its level decreases when it is extensively phosphorylated. To identify the kinase phosphorylating FRQ and to understand the function of FRQ phosphorylation, a FRQ-phosphorylating kinase was purified and identified as casein kinase II (CKII). Disruption of the catalytic subunit gene of CKII in *Neurospora* resulted in hypophosphorylation and increased levels of FRQ protein. In addition, the circadian rhythms of *frq* RNA, FRQ protein, and clock-controlled genes are abolished in the CKII mutant. Our data suggest that the phosphorylation of FRQ by CKII may have at least three functions; it decreases the stability of FRQ, reduces the protein complex formation between FRQ and the WHITE COLLAR proteins, and is important for the closing of the *Neurospora* circadian negative feedback loop. Taken together, our results suggest that CKII is an important component of the *Neurospora* circadian clock.

[Key Words: Circadian; frequency; white collar-1; *Neurospora*; phosphorylation]

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degradation. A previous study suggested that one of the functions of FRQ phosphorylation is to trigger its degradation [Liu et al. 2000]. This conclusion was supported by two lines of evidence. 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 reduction of the rate of FRQ degradation and a very long period (>30 h) of the clock. Thus, phosphorylation of FRQ is an important determining factor for FRQ stability and for the period length of the clock. In addition, this study also suggested that FRQ phosphorylation may have other functions as well, because the mutation of another phosphorylation site at Ser 519 resulted in a slightly shorter period and low amplitude of the overt rhythm (Liu et al. 2000).

To identify the kinases that phosphorylate FRQ, we previously purified a Neurospora calcium/calmodulin (Ca/CaM)-dependent kinase (CAMK-1) protein from Neurospora extract [Yang et al. 2001]. 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 phenotype is transient and quickly reverts to the wild-type phenotype, revealing redundancy in the system. Although modest clock phenotypes were found in the camk-1 null strain, we failed to detect significant change of the FRQ phosphorylation profile in the mutant strains. Together, those data suggest that FRQ may be phosphorylated by multiple kinases in vivo. In this study, we purified another FRQ-phosphorylating kinase from the protein extracts of a camk-1 null strain. This kinase was identified as the Neurospora casein kinase II (CKII). The disruption of the gene encoding for the catalytic subunit of the kinase suggested that phosphorylation of FRQ by CKII is important for the normal function of the Neurospora circadian clock.

Results

CKII, a kinase that phosphorylates FRQ, was biochemically purified

During the purification of CAMK-1, we noted that there were several FRQ-phosphorylating Ca/CaM-independent kinase activities. One kinase activity peak eluted from the Q-Sepharose column at 0.45 M NaCl. To purify this kinase, we used the protein extracts from a camk-1 ko strain to eliminate the kinase activity of CAMK-1. With the use of an in vitro kinase assay to monitor its activity to phosphorylate GST–FRQ (Yang et al. 2001), this kinase was purified from the Neurospora cell extracts after a four-step purification protocol (Fig. 1A). The elution profile in Superdex 200 suggested that this kinase was either quite large or in a protein complex (~150–200 kD; data not shown). The results of the final step of the purification are shown in Figure 1, B and C. The peak of kinase activity was in fraction 18, eluting at 0.45 M NaCl. Silver staining of the SDS–polyacrylamide gel of the same protein fractions revealed five visible protein bands in fraction 18, with molecular weights ranging from 25 to 42 kD (Fig. 1C). Of the five protein bands, the levels of three correlated well with the activity profile.

To obtain the sequence identity of these three proteins (~40, 36, and 25 kD), these protein bands were excised

![Figure 1](https://genesdev.cshlp.org/content/genesdev/article/995/8/995/1/genesdev-995-fig1.png)

Figure 1. Biochemical purification of CKII, a FRQ phosphorylating kinase from Neurospora. (A) A diagram of the purification scheme for CKII. (B) Aliquots of the indicated final Mono Q column fractions were used for the in vitro kinase assay. B and F are the protein extracts loaded and the flow-through of the Mono Q column, respectively. The NaCl gradient is indicated at top. (C) Aliquots of the indicated Mono Q column fractions were subjected to 4%–15% SDS-PAGE, and the gel was subsequently silver stained. The three arrows denote the protein bands identified as containing the indicated CKII subunits. The two protein bands copurified with CKII were denoted by asterisks.
from the gel and subjected to tryptic digestion followed by mass spectral analysis. The resulting mass fingerprints of the digested peptides were used to search against the NCBI database. Two independent *Neurospora* ESTs, c3d10np and NCM3A11, were found to match the mass fingerprints of the digested peptides of the 40-kD protein, whereas no match was found for the mass fingerprints of the 36-kD and 25-kD proteins. BLAST searches revealed that these ESTs have strong homology to the eukaryotic CKII catalytic (α) or regulatory (β) subunits.

The complete genome of *Neurospora* was recently determined [http://wolfram.wi.mit.edu/annotation/fungi/neurospora/]. On the basis of the EST sequences and cDNA sequencing results, the corresponding full-length *Neurospora* CKII α and β subunits were identified and the entire ORF of the proteins determined. Eukaryotic CKII holoenzyme is a α2β2 heterotetramer, and most eukaryotic organisms have at least two distinct α subunits and two different regulatory β subunits. Only one α subunit and two β subunit genes were identified in the *Neurospora* genome, named *cka*, *ckb1*, and *ckb2*. When the entire ORFs of *CKA*, *CKB1*, and *CKB2* were used to compare with the mass fingerprints of the digested peptides of the three proteins, the 36-kD protein was found to match *CKB2* and more matches were found between the mass fingerprints of the 42-kD protein and *CKA* and *CKB1* (Fig. 1C). The 25-kD protein band was found to be the protein degradation products of the *CKA* and *CKB1*. The fact that these proteins were found to match all three CKII subunits indicated that CKII was the kinase we were after.

The predicted molecular weights for *CKA*, *CKB1*, and *CKB2* are 39.6 kD [336 amino acids], 37.1 kD [333 amino acids], and 32.6 kD [285 amino acids], respectively, similar to their sizes estimated by SDS-PAGE (Fig. 1C). Therefore, the size of the CKII heterotetramer should be ~150 kD, similar to the size estimated by the Superdex 200 elution profile. BLAST searches conducted with the amino acid sequences of the *Neurospora* CKII α and β subunits revealed that they were closely related to other eukaryotic CKII α or β subunits [Fig. 2]. Interestingly, the closest homolog of the *Neurospora* CKIA is a CKII α subunit from *Arabidopsis thaliana*, with 76% identity at the amino acid level spanning the entire ORF. The two *Neurospora* CKII β subunits are larger than most other CKII β subunits [23–30 kD], with an extended carboxyl terminus. *CKB1* and *CKB2* are most similar to two distinct CKII β subunits from fission yeast, with 45% and 59% amino acid sequence identity, respectively.

Disruption of the *cka* gene revealed its important role in growth and development of *Neurospora*

To investigate the function of the *Neurospora* CKII in phosphorylating FRQ in vivo, *cka*, the gene encoding for the catalytic subunit of the enzyme, was disrupted in *Neurospora* by repeat-induced point mutation (RIP) [Selker and Garrett 1988; Cambarerri et al. 1989]. To introduce random mutations to a target gene by RIP, the gene of interest is first duplicated in *Neurospora* by introducing an additional copy of this gene into a wild-type *Neurospora* strain. The resulting strain is then crossed with another wild-type strain. During sexual cycle, random but exclusively G-C to A-T point mutations will be introduced to the duplicated gene through an unknown gene-silencing mechanism. To mutate *cka*, a 2.5-kb genomic DNA containing the entire *cka* ORF and part of its promoter region was introduced into a wild-type strain. Afterward, a positive transformant was crossed with a wild-type strain, and individual sexual spores were picked and germinated. After the spores were germinated, we noticed that some of the progenies showed a slow growth rate. To determine whether mutations were introduced into *cka* in these strains, Southern blot analysis was performed. As shown in Figure 3A, the endogenous *cka* gene was disrupted in a RIP strain. Northern blot analysis further showed that *cka* expression was also mostly abolished in this mutant [Fig. 3B]. DNA sequencing of the mutant *cka* gene found many G-C to A-T point mutations in the gene, including multiple premature stop codons after amino acid 9 (data not shown). Finally, the in vitro kinase assay results showed that the CKII kinase activity was also abolished in this mutant, confirming that *CKA* is the only CKII α subunit in *Neurospora* [Fig. 3C]. Together, these results indicate that there are no transcription and translation of *cka* and no CKII kinase activity in the *ckaRIP* mutant.

The *ckaRIP* strains grew much more slowly than the wild-type strains, and they produced short aerial hyphae and little conidia. These phenotypes indicate that, although CKII is not essential for survival, it is important for the normal growth and development of *Neurospora*. To show the slow growth phenotype of the *ckaRIP* strains and to examine whether they exhibit circadian conidia production rhythm, they were analyzed by race tube assay in constant darkness. As shown in Figure 3D, the growth rate of the *ckaRIP* strain was <10% of the wild-type strain, 2–3 mm/day instead of 3–4 cm/day [note that each black line on the race tube represents one day’s growth]. Due to the slow growth and the meager production of conidia, the circadian phenotype of the *ckaRIP* strains could not be analyzed by the race tube assay. Our effort to identify partially functional *cka* allele was not successful, because all of the mutants obtained showed similar phenotype as described above and DNA sequencing revealed that they contained many mutations in the *cka* ORF, and some resulted in premature stop codons.

FRQ is hypophosphorylated and more stable in the *ckaRIP* strain

If CKII phosphorylates FRQ in vivo, we expected the phosphorylation profile of FRQ to be altered in the *ckaRIP* strains. In addition, if CKII phosphorylation of FRQ plays a role in triggering FRQ degradation, the steady-state level of FRQ should be high in the mutants. To test these hypotheses, Western blot analyses were performed to examine the FRQ phosphorylation profile for *Neurospora* cultures grown in constant light (LL) or
Figure 2. Amino acid sequence alignment of the Neurospora CKII and other eukaryotic CKII. (A) Sequence alignment of the catalytic (H9251) subunits of CKII proteins. (Neu) Neurospora CKA; (Arab) Arabidopsis thaliana (AAC17823); (Mus) Mus musculus (AAA96795); (Droso) Drosophila melanogaster (P08181); (Yeast) Saccharomyces cerevisiae (NP_014704). The amino acids conserved in all proteins are boxed. (B) Sequence alignment of the regulatory (H9252) subunits of CKII proteins. (Neub1) Neurospora CKB1; (Neub2) Neurospora CKB2; (Yeast) Saccharomyces cerevisiae (NP_011496); (Mus) Mus musculus (NP_034105); (Droso) Drosophila melanogaster (P08182); (Arab) Arabidopsis thaliana (P40229).
was mostly abolished in the ckaRIP strain. (B) Northern blot analysis showing that the expression of the cka gene was abolished in the ckaRIP strain. SacI was used to digest the genomic DNA. A 2.5-kb genomic DNA fragment containing the entire cka ORF was used as the probe. Because of the point mutations created by RIP, there is an additional SacI site in the mutated cka gene. The weaker signal in the mutant was due to less DNA loaded. (C) In vitro kinase assay showing that the CKII activity was abolished in the ckaRIP strain. Protein extracts [5 mg] were loaded onto a Q-Sepharose column and eluted by the NaCl gradient indicated above. A wild-type strain was used as the control. (D) Race tube assay showing the slow growth phenotype of the ckaRIP strain. The position of the cka mRNA is indicated by the arrow. The asterisk denotes the large ribosomal RNA bands nonspecifically recognized by the probe. (E) In vivo kinase assay showing that the CKII activity was abolished in the ckaRIP strain. Protein extracts [5 mg] were loaded onto a Q-Sepharose column and eluted by the NaCl gradient indicated above. A wild-type strain was used as the control. (F) Northern blot analysis showing that the expression of the cka gene was abolished in the ckaRIP strain. SacI was used to digest the genomic DNA. A 2.5-kb genomic DNA fragment containing the entire cka ORF was used as the probe. Because of the point mutations created by RIP, there is an additional SacI site in the mutated cka gene. The weaker signal in the mutant was due to less DNA loaded. (G) In vitro kinase assay showing that the CKII activity was abolished in the ckaRIP strain. Protein extracts [5 mg] were loaded onto a Q-Sepharose column and eluted by the NaCl gradient indicated above. A wild-type strain was used as the control. (H) Race tube assay showing the slow growth phenotype of the ckaRIP strain. The position of the cka mRNA is indicated by the arrow. The asterisk denotes the large ribosomal RNA bands nonspecifically recognized by the probe. (I) Disruption of the cka gene in Neurospora. (A) Southern blot analysis showing that the cka gene was disrupted in the ckaRIP strain. SacI was used to digest the genomic DNA. A 2.5-kb genomic DNA fragment containing the entire cka ORF was used as the probe. Because of the point mutations created by RIP, there is an additional SacI site in the mutated cka gene. The weaker signal in the mutant was due to less DNA loaded. (B) Northern blot analysis showing that the expression of the cka gene was abolished in the ckaRIP strain. The position of the cka mRNA is indicated by the arrow. The asterisk denotes the large ribosomal RNA bands nonspecifically recognized by the probe. (C) In vitro kinase assay showing that the CKII activity was abolished in the ckaRIP strain. Protein extracts [5 mg] were loaded onto a Q-Sepharose column and eluted by the NaCl gradient indicated above. A wild-type strain was used as the control. (D) Race tube assay showing the slow growth phenotype of the ckaRIP strain. The position of the cka mRNA is indicated by the arrow. The asterisk denotes the large ribosomal RNA bands nonspecifically recognized by the probe. (E) In vivo kinase assay showing that the CKII activity was abolished in the ckaRIP strain. Protein extracts [5 mg] were loaded onto a Q-Sepharose column and eluted by the NaCl gradient indicated above. A wild-type strain was used as the control. (F) Northern blot analysis showing that the expression of the cka gene was abolished in the ckaRIP strain. SacI was used to digest the genomic DNA. A 2.5-kb genomic DNA fragment containing the entire cka ORF was used as the probe. Because of the point mutations created by RIP, there is an additional SacI site in the mutated cka gene. The weaker signal in the mutant was due to less DNA loaded. (G) In vitro kinase assay showing that the CKII activity was abolished in the ckaRIP strain. Protein extracts [5 mg] were loaded onto a Q-Sepharose column and eluted by the NaCl gradient indicated above. A wild-type strain was used as the control. (H) Race tube assay showing the slow growth phenotype of the ckaRIP strain. The position of the cka mRNA is indicated by the arrow. The asterisk denotes the large ribosomal RNA bands nonspecifically recognized by the probe.

Circadian oscillation of FRQ is abolished in the ckaRIP strain

To examine whether the clock was still running in the ckaRIP mutant, liquid cultures of the mutant strain were grown in constant darkness and harvested at different times for two circadian cycles. Western blot analysis was performed to examine whether the level of FRQ showed circadian oscillation. In Figure 4, D and E show the results of one representative experiment. Similar to previously described results [Garceau et al. 1997], there was a robust circadian rhythm of the steady-state level of FRQ in the wild-type strain. In addition to the rhythm of FRQ amount, the phosphorylation states of FRQ also oscillated rhythmically in the wild-type strain, with FRQ being less phosphorylated at DD16–DD20 and extensively phosphorylated when its level decreased [Garceau et al. 1997]. For the ckaRIP strain, although the FRQ level decreased slightly after the LD transition and then increased at DD16, the level of FRQ fluctuated very little afterwards. These data indicate that the ckaRIP strain can
still respond to the LD transition, but there is no circadian oscillation of FRQ proteins. In addition, the level of FRQ protein is constantly high in the dark in the mutant, consistent with the notion that FRQ is more stable without phosphorylation by CKII. In contrast to the dramatic oscillations of FRQ phosphorylation states in the wild type, the FRQ phosphorylation pattern was the same at different time points after DD16 (Fig. 4D). These data indicate that the circadian oscillation of FRQ is abolished in the ckaRIP strain.

The levels of frq and clock-controlled genes do not change rhythmically and the amount of frq is high in constant darkness in the ckaRIP strain

Because the level of FRQ protein is constantly high in DD in the ckaRIP strain, we predicted that there should be no circadian oscillations of frq mRNA and clock-controlled genes. In addition, if the phosphorylation of FRQ by CKII affects FRQ’s role to feedback to repress the controlled genes. In addition, if the phosphorylation of FRQ

**Figure 4.** Western blot analyses showing that in the ckaRIP strain, the FRQ phosphorylation pattern was significantly altered, the FRQ level was higher and more stable, and FRQ protein oscillation was abolished. [A] Western blot analysis showing that in the ckaRIP strain, FRQ was mostly hypophosphorylated and its level was higher than that of the wild type. Cultures harvested in DD24 were used. [Left] The mobility range of the two alternatively translated FRQ forms is indicated at left. The top arrow indicates that the hyperphosphorylated LFRQ species found in the wild type was missing in the ckaRIP mutants. The bottom arrow indicates that the hypophosphorylated SFRQ species was found only in the ckaRIP strain. [Right] Densitometric analysis of the Western blot shown at left. [B,C] Western blot analysis showing that FRQ was more stable in the ckaRIP strain after a LD transition. Cultures were first grown in LL for 2 d before they were transferred into constant darkness and harvested at the indicated time. The top and bottom arrows at left of B indicate the hyperphosphorylated LFRQ bands in the wild-type strain and the hypophosphorylated SFRQ band in the ckaRIP strain, respectively. Densitometric analysis of the Western blot results of B is shown in C. [D] Wild type; [●] ckaRIP. [E] Western blot analysis showing that FRQ protein oscillation was abolished in the ckaRIP strain. Cultures were harvested in constant darkness at the indicated time. Densitometric analysis of the Western blot results in D is shown in E. Note that there was a circadian oscillation of the FRQ phosphorylation patterns in the wild type, whereas the FRQ phosphorylation pattern stayed unchanged in the ckaRIP strain at different times of the day after DD16. The representative results of three independent experiments are shown here. (○) Wild type; [●] ckaRIP.
even though they both are morning-specific genes. Together with the data in Figure 4, these data strongly suggest that FRQ phosphorylation by CKII is required for the function of the clock, and the hypophosphorylated FRQ proteins in the cka^RIP^ mutant are not fully functional. Therefore, the Neurospora CKII is an important component of the Neurospora circadian system.

To further show that the arrhythmic phenotype of the cka^RIP^ strain was not due to its inability to be entrained by light, we examined the light induction of frq and albino-3 [al-3] genes in the cka^RIP^ strain. As shown in Figure 5E, both frq and al-3 are rapidly induced by a 15-min light pulse in the mutant. This result suggests that CKII is not required for the light input of the clock.

Hypophosphorylated FRQ proteins in the cka^RIP^ mutant promote the complex formation between FRQ and the WC proteins

Although FRQ phosphorylation by CKII seems to destabilize FRQ, it is obvious that its phosphorylation also has additional roles in regulating the function of FRQ, because the hypophosphorylated FRQ in the cka^RIP^ mutant fails to close the circadian negative feedback loop, as reflected by the high levels of frq in constant darkness. It is possible that the lack of CKII-mediated phosphorylation of FRQ blocks the nuclear localization of FRQ [Ko-meili and O’Shea 1999], because it is an essential step for FRQ to function [Luo et al. 1998]. To test this possibility, nuclei preparations from both the wild-type strain and the cka^RIP^ strain were examined. As seen in Figure 6A, the mutant FRQ was found in the nucleus like the wild-type FRQ. Thus, CKII is not required for the nuclear localization of FRQ. Similar to previously described results [Talora et al. 1999; Schwerdtfeger and Linden 2000; Cheng et al. 2001a], WC-1 and WC-2 were greatly enriched in the nuclear fractions in both strains.

Another possible reason why FRQ fails to function in the cka^RIP^ mutant is that the phosphorylation of FRQ by CKII affects its interaction with the WC complex and therefore the negative feedback loop cannot be closed (Cheng et al. 2001a; Denault et al. 2001). To test this possibility, immunoprecipitation experiments using WC-1 or WC-2 antiserum were performed to compare the protein–protein interactions between FRQ and WC proteins in the wild-type and cka^RIP^ strains. The results of a representative experiment using WC-1 antiserum are shown in Figure 6B, C. Although FRQ was found to be associated with WC-1 and WC-2 in both strains, significantly more FRQ was coprecipitated with WC-1 in the mutant, suggesting that the hypophosphorylated FRQ in the cka^RIP^ mutant either stabilizes or promotes the formation of the FRQ–WC complex. Similar results were obtained in four independent experiments with WC-1 antiserum and with WC-2 antiserum to perform immunoprecipitation [Fig. 6C, data not shown]. In addition, we found that the wild-type FRQ that coprecipitated with the WCs lacked most of the hyperphosphorylated species [Fig. 6B, right; see also Fig. 6B in Cheng et al. 2001a]. These results suggest that, even in the wild-
Whether these positive feedback loops are still functional in the *cka* mutant, the levels of WC-1 and WC-2 in the *cka* mutant were compared with those of the wild-type strain. As shown in Figure 6D (cultures harvested in DD), the level of WC-1 in the mutant is slightly higher (~20%) than that of the wild type, whereas the increase of WC-2 is ~70% in the mutant. This data suggests that the hypophosphorylated FRQ is still able to positively regulate the levels of WC proteins and the high level of FRQ in the mutant is responsible for the increases of its WC levels. Because WC-1 is the limiting factor in the WC complex (Cheng et al. 2001b; Denault et al. 2001) and WC-1 is unstable without forming a complex with WC-2 (Cheng et al. 2002), the concentration of the WC complex in the *cka* mutant strain should be ~20% higher than that of wild type. Such an increase in WC complex is insufficient to explain the high levels of the *frq* mRNA, because there were five times more FRQ–WC complex found in the *cka* strain [Fig. 6C]. Therefore, the high levels of *frq* in the *cka* strain, is likely due to the following: (1) FRQ cannot repress the activity of WC complex efficiently; (2) there is a higher level of WC complex in the mutant.

Because WC-1 is known to be phosphorylated, and as it becomes hyperphosphorylated after a light pulse, (Talora et al. 1999; Schwerdtfeger and Linden 2000), we examined whether the phosphorylation pattern of WC-1 was altered in the *cka* mutant strain, WC-1 phosphorylation patterns of the *cka* strain in DD and after a light pulse were compared with those of the wild type. As shown in Figure 6E, the phosphorylation patterns of WC-1 were similar in both strains, and as in the wild type, the light treatment also resulted in the hyperphosphorylation of WC-1. Therefore, the phosphorylation of WC-1 did not appear to be altered in the *CKII* mutant. However, we do not know whether the activity of the WC complex is altered in the *CKII* mutant strain.

The RNA level of the catalytic subunit of CKII is constant under circadian conditions

Because the phosphorylation states of FRQ show a circadian rhythm in a wild-type strain, it is possible that the expression of CKII genes is controlled by a circadian clock. To test this hypothesis, Northern blot analysis was performed to examine whether the expression of *cka* is rhythmic in a wild-type strain over two circadian cycles. As shown in Figure 7, despite the robust circadian oscillation of *frq* mRNA, the level of *cka* mRNA appeared to be relatively constant at different times of the day. This result suggests that, like *dbt* in *Drosophila* (Kloss et al. 1998), the steady state level of *cka* is not clock controlled. However, we cannot rule out the possibility that the CKII activity and the cellular localization of CKII are regulated by the clock.

Discussion

In this study, we have biochemically purified the *Neurospora* CKII, a kinase that phosphorylates FRQ. Disrup-
The disruption of important for the normal function of the circadian clock. Phosphorylates FRQ in vivo and such phosphorylation is an important role in phosphorylating FRQ in vivo. Not make a firm conclusion on whether CAMK-1 plays

Neurospora (two additional Ca/CaM-dependent kinases were identified in the Neurospora genome database), we could not make a firm conclusion on whether CAMK-1 plays an important role in phosphorylating FRQ in vivo. In contrast, evidence herein strongly suggest that CKII phosphorylates FRQ in vivo and such phosphorylation is important for the normal function of the circadian clock. The disruption of cka, the only catalytic subunit of Neurospora CKII, resulted in dramatic changes in the FRQ phosphorylation pattern under different conditions. FRQ was mostly hypophosphorylated in the mutant strain, and some of the hypophosphorylated FRQ species were never found in the wild-type strains. Previously, it was shown that even the least-phosphorylated FRQ species in the wild type was phosphorylated [Garceau et al. 1997], suggesting that as soon as FRQ is synthesized, it is immediately phosphorylated. We have shown that the purified endogenous CKII can directly phosphorylate FRQ in vitro. In combination with the changes of the FRQ phosphorylation profile in the ckaRIP mutant, these data suggest that CKII can also directly phosphorylate FRQ in vivo. However, as we do not have the evidence to show the direct interaction of CKII and FRQ in vivo [the interactions between the kinase and its substrates are usually weak and transient], we cannot exclude the possibility that the changes in FRQ phosphorylation pattern in the ckaRIP mutant are due to indirect regulation of FRQ phosphorylation by CKII.

Using the Motif Scanner protein phosphorylation prediction software, we found that there are at least 29 potential primary CKII phosphorylation sites on FRQ. Although the purified CKII was able to phosphorylate the small region of FRQ containing Ser 501 to Ser 519 in vitro [data not shown], the comparison of the known CKII consensus sequence, [S/T]XX[D/E/Tp/Sp], with the sequences of these three known FRQ phosphorylation sites showed that they do not resemble the typical CKII phosphorylation sites. Thus, it is unclear whether the phosphorylation of this region by CKII in vitro is physiologically relevant in vivo. Our future identification of more FRQ phosphorylation sites and the analyses of their function should inform us about the function of the specific phosphorylation sites in regulating FRQ.

Despite the dramatic differences in the FRQ phosphorylation profile between the wild-type and the mutant strains, several FRQ phosphorylation species can still be identified in the ckaRIP mutant. These data indicate that at least one additional kinase can still phosphorylate FRQ in vivo. However, it is unclear whether the FRQ phosphorylation pattern in the ckaRIP mutant is due to phosphorylation of the CKII-unrelated sites or a slower FRQ phosphorylation rate in the mutant. It is possible that CAMK-1 is one of the kinases that phosphorylates FRQ in the ckaRIP mutant. Unfortunately, our efforts to obtain the double mutant (camk-1ko, ckaRIP) failed, most likely because the disruption of both genes is lethal.

A recent study by Gorl and colleagues has suggested that casein kinase I (CKI) is involved in regulating the stability of FRQ by phosphorylating one of the PEST domains of FRQ [Gorl et al. 2001]. The deletion of the PEST domain resulted in increased FRQ stability and long period rhythm (～28 h) of the clock. However, CKI failed to phosphorylate the previously identified FRQ phosphorylation sites. Interestingly, both CKI and CKII are Ser/Thr kinases that phosphorylate Ser/Thr surrounded by acidic residues. Therefore, it is possible that both kinases work together to phosphorylate FRQ.

FRQ phosphorylation has multiple roles

We have shown that in the ckaRIP mutant, the levels of FRQ protein, and frq mRNA are high and not rhythmic. In addition, there is no clock-controlled expression of clock-controlled genes. Interestingly, in constant darkness, the levels of ccg-2 was constantly low, whereas ccg-1 stayed at a high level in the ckaRIP mutant, suggesting that the clock regulation on these two genes are different despite the fact that they are both morning-

Figure 7. Northern blot analysis showing that the level of cka was constant at different times of the day. (A) Wild-type cultures were harvested in DD at the indicated time. Northern blot analyses were performed to examine the expression of frq and cka genes. The representative results from two independent experiments are shown. (B) Densitometric analysis of the Northern blot results of A. (○) frq; (●) cka.
specific genes. These data suggest that the clock function is disrupted in the mutant, and that CKII-mediated phosphorylation of FRQ is essential for the function of the FRQ in the clock.

A major conclusion of this study is that FRQ phosphorylation by CKII has several important roles in regulating the function of FRQ in the circadian clock (Fig. 8). First, consistent with previous results [Liu et al. 2000], phosphorylation of FRQ by CKII is important in regulating the stability of FRQ. We have shown that the FRQ level is higher and more stable in the cka\textsuperscript{RIP} mutant than in the wild type. Thus, like phosphorylation of PER by DBT in Drosophila [Price et al. 1998; Suri et al. 2000], the phosphorylation of FRQ by CKII may be important in determining the period length and the normal function of the clock. The constitutive high levels of FRQ in constant darkness are likely the result of increased stability of FRQ and high levels of frq mRNA.

Second, the phosphorylation of FRQ by CKII plays important roles in regulating the formation of the FRQ–WC complex and the closing the circadian negative feedback loop. We have shown that not only FRQ in the cka\textsuperscript{RIP} mutant can interact with the WC complex, but the amount of FRQ coprecipitated with the WC proteins is significantly greater than that of the wild-type FRQ. Even in a wild-type strain, the less phosphorylated FRQ forms are the major species found to be associated with the WC complex. In wild type, beside the oscillation of the level of FRQ, the phosphorylation pattern of FRQ also changes dramatically in a circadian fashion in constant darkness. Thus, the different ability for various FRQ-phosphorylated forms to interact with the WC complex is a mechanism that increases the amplitude of FRQ rhythm and results in robust oscillation of the clock, and the amplitude of the FRQ protein levels alone is not a complete reflection of the amplitude of the rhythm. Therefore, both the quantity and quality of different forms of FRQ should be taken into account when considering the amounts of FRQ protein.

Despite more complex formation between FRQ and the WCs in the cka\textsuperscript{RIP} mutant, the frq mRNA levels are high in constant darkness and are comparable with those in the frq\textsuperscript{0} strain. Although the concentration of the WC complex is 20% higher in cka\textsuperscript{RIP} mutant than wild type (Fig. 6D), such an increase is insufficient to explain the increase of the frq RNA level if the negative feedback is normal in the mutant, as there are about five times more of FRQ–WC complex found in the cka\textsuperscript{RIP} mutant [Fig. 6C]. In fact, these results suggested that the circadian negative feedback loop was impaired in the CKII mutant and FRQ failed to inhibit the transcriptional activation activity of the WC complex effectively in the CKII mutant. Although the interaction between FRQ and the WCs is necessary for the closing of the circadian negative feedback loop [Cheng et al. 2001a], these data suggest that the interaction alone is not sufficient for FRQ to act to repress the transcriptional activation of frq by the WC proteins. In addition, these data suggest that the interaction between FRQ and the WC proteins and the transcriptional repression are two separate events, and the lack of CKII phosphorylation of FRQ at certain sites in the cka\textsuperscript{RIP} mutant prohibits FRQ from carrying out its function. However, it is also possible that the these two aspects of the FRQ phosphorylation function are related to each other.

CKII (CK\textsubscript{II}) is also involved in the function of the Arabidopsis circadian clock [Sugano et al. 1998, 1999]. In vitro, CKII subunits of Arabidopsis have been shown to interact and phosphorylate the circadian clock-associated 1 (CCA1) protein. The phosphorylation of CCA1 by CK\textsubscript{II}-like activity in vitro affects the formation of a DNA–protein complex containing CCA1. In addition, overexpression of a regulatory subunit of CK\textsubscript{II} resulted in the shortening of the period of clock-controlled genes in Arabidopsis. Because the Neurospora WC complex binds to DNA and FRQ interacts with the WCs, it is possible that the CKII phosphorylation of FRQ influences the formation of the DNA–WC complex.

In Drosophila, the phosphorylation of TIM appears to have at least two different functions. The phosphorylation of TIM by SGG promotes the nuclear transfer of the PER/TIM complex [Martinck et al. 2001], whereas a tyrosine-linked phosphorylation of TIM by an unknown kinase is implicated in the proteasome-mediated degra-

Figure 8. Model for gene regulation within the Neurospora circadian oscillator. WC-1 and WC-2 form heterodimer to activate the transcription of frq. FRQ proteins interact with the WC-1/WC-2 complex to inhibit their transcriptional activation, forming the negative feedback loop. FRQ also positively regulate the levels of both WC-1 and WC-2, forming the positive feedback loops. The phosphorylation of FRQ have multiple roles in regulating the function of FRQ: promoting degradation of FRQ, reducing the interaction between FRQ and the WC complex, and important for the negative repression of frq transcription. WC-2 maintains the steady state of WC-1 by forming complex with WC-1 [Cheng et al. 2002].
dation of TIM [Naidoo et al. 1999]. DBT of Drosophila and the casein kinase 1e in mammals phosphorylate PER and may trigger its turnover [Price et al. 1998; Lowrey et al. 2000; Toh et al. 2001]. Although both CKI and CKII are Ser/Thr kinases, their structures and substrate specificities are significantly different, suggesting that they did not evolve from a common ancestral protein.

Clock effects versus growth and developmental effects

Unlike DBT and SGG in Drosophila [Price et al. 1998; Martinek et al. 2001] and CKII in Saccharomyces [Padmanabha et al. 1990], which are required for the survival of the organisms, CKII of Neurospora is not essential for the survival of Neurospora. However, the Neurospora CKII is important for the normal growth and development of the fungus. As a protein serine/threonine kinase that is ubiquitously found in eukaryotes, CKII is known to phosphorylate many substrates and regulate many processes in eukaryotic cells [Litchfield and Luscher 1993; Pinna and Meggio 1997]. However, in Neurospora, we think the clock defects in the ckaRIP mutant are probably not caused by its growth and developmental defects. First, in genetic screening for clock mutants by us and others, most of Neurospora growth and developmental mutants were found to have normal functional clocks [Brody and Martin 1973; Feldman and Atkinson 1978; Feldman 1983; Lakin-Thomas et al. 1990; Bell-Pedersen et al. 1992]. A partial list of the biochemical and morphological mutants screened for clock effects is reviewed by Lakin-Thomas et al. [1990]. From the 85 mutants examined, only 12 mutants were found to have clock phenotypes. Of the 12 mutants, 11 had small period shortening effects on the clock (<2–3 h) and only one mutant, ccl, which is deficient in fatty acid synthetase, showed fatty acid-dependent period-lengthening effects [Mattern et al. 1982]. Importantly, none of the mutant examined was found to exhibit arrhythmic phenotype, and all of the morphological mutants (the ckaRIP mutant should be categorized as one), in which some of them have severe growth and developmental defects, were found to have normal period length of the clock. The 73 mutations with normal period affect a wide range of metabolic re-

Materials and methods

Strains and culture conditions

The bd, a and bd, his-3, a [wild-type clock] strains were used as the wild-type strains in this study. Protein extracts from bd, camk-1ko were used for CKII purification. The 301-6 [bd, his-3, A] strain was the host strain for the his-3-targeting construct. Liquid culture conditions were the same as described previously [Aronson et al. 1994a]. Race tube assay medium contained 1× Vogel’s, 0.1% glucose, 0.17% arginine, 50 ng/mL biotin, and 1.5% agar. The genotype of the ckaRIP strain was bd, ckaRIP, his-3. Therefore, histidine (0.5 mg/mL) was added to the medium in all experiments [including the medium for the wild-type strain] when the ckaRIP strain was used. To obtain similar amounts of mycelium from liquid cultures of both the wild-type and the ckaRIP strains, the ckaRIP cultures were first grown for about 2 d before the wild-type cultures were inoculated.

In vitro kinase assay

To assay kinase activity, a GST–FRQ fusion protein [Yang et al. 2001] [5 µg] containing FRQ amino acids 425–683 was incubated with Neurospora protein extracts (5–10 µg) of total extracts or 40 µL of the chromatographic fractions in a buffer containing 25 mM HEPES (pH 7.9), 10 mM MgCl₂, 2 mM MnCl₂, 25 µM ATP, and 10 µCi/mL [γ-32P]ATP (total reaction volume of 125 µL). The reaction was incubated at room temperature for 1 h before adding 0.5 mL of PBS and 10 µL of glutathione–agarose beads. After a 30-min incubation at room temperature, the glutathione–agarose beads were washed twice in PBS before they were resus-

Purification of CKII from Neurospora extracts

All procedures were performed on an automatic fast protein liquid chromatography (FPLC) station [Amersham-Phamacia] at 4°C. Neurospora protein extracts (1.5 g) of the camk-1ko strain in 50 mM HEPES (pH 7.4), 137 mM NaCl, and 10% glycerol.
were applied on a Q-Sepharose column (80 mL bed volume) equilibrated with buffer A [50 mM Tris at pH 7.5, 1 mM DTT]. After washing with buffer A, the bound materials on the column were eluted with a 800-mL linear gradient from 20 mM NaCl to 500 mM NaCl buffer A. Fractions of 9 mL were collected and assayed for kinase activity. A total of 50 mL of active fractions (∼0.45 M NaCl) were pooled together and loaded on a hydroxyapatite column (7-mL bed volume) equilibrated with 10 mM potassium phosphate buffer (KPO4). The column was washed with 10 mM KPO4, followed by 10 mM KPO4 containing 1 M NaCl. The bound proteins were eluted with a 15-mL linear gradient from 50 mM KPO4 to 350 mM KPO4 (pH 7.5). A total of 1.5 mL of active fractions (0.24–0.34 M KPO4) were pooled together and concentrated to 0.5 mL. At this time, it was loaded on a Superdex 200 (10/30) gel filtration column equilibrated with buffer A containing 150 mM NaCl and eluted with a 10-mL linear gradient from 300 mM NaCl to 700 mM NaCl in buffer A. Fractions of 0.5 mL were collected and assayed for kinase activity. Active fractions were stored at −80°C.

Protein sequencing

Proteins in fraction 18 of the final Mono Q column were concentrated by Centricon spin column (Amicon) and loaded onto a Superdex 200 (10/30) gel filtration column equilibrated with buffer A containing 150 mM NaCl and eluted with the same buffer. A total of 2 ml of active fractions were pooled and loaded on a Mono Q (5/5) column and eluted with a 10-mL linear gradient from 300 mM NaCl to 700 mM NaCl in buffer A. Fractions of 0.5 mL were collected and assayed for kinase activity. Active fractions were stored at −80°C.

Disruption of cka in Neurospora

A 2.5-kb PCR fragment containing the entire CKA ORF and part of the promoter was cloned into pDE3dBH (Ebbole and Sachs 1990) and introduced into the his-3 locus of a wild-type strain [301-6: bd, his-3, A] by transformation. Southern blot analysis was performed to identify transformants that carried the additional copy of cka, and a positive transformant was crossed with a wild-type strain [bd, his-3, a]. Sexual spores of the cross were picked individually and germinated on slants containing histidine. Southern and Northern blot analyses were performed to identify strains in which cka was disrupted and cka was not expressed.

Protein and RNA analyses

Protein extraction, quantification, Western blot analysis, and immunoprecipitation assays were performed as described previously (Garceau et al. 1997; Cheng et al. 2001a). Neurospora nuclei were purified as described previously (Luo et al. 1998). Immunoprecipitates or equal amounts of total protein (40 µg) were loaded in each protein lane.

RNA extraction and Northern blot analysis were performed as described previously (Aronson et al. 1994a). Equal amounts of total RNA (20 µg) were loaded onto agarose gels for electrophoresis, the gels were blotted and probed with an RNA probe specific for frq, cka, and clock-controlled genes.

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