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Coiled-coil domain-mediated FRQ–FRQ interaction is essential for its circadian clock function in *Neurospora*

Ping Cheng, Yuhong Yang, Christian Heintzen[1] and Yi Liu[2]

Department of Physiology, University of Texas Southwestern Medical Center, 5323 Harry Hines Boulevard, Dallas, TX 75390-9040 and [1]Department of Genetics, Dartmouth Medical School, Hanover, NH 03755, USA

[2]Corresponding author
e-mail: Yi.Liu@UTSouthwestern.edu

The *frequency* (*frq*) gene, the central component of the *frq*-based circadian negative feedback loop, regulates various aspects of the circadian clock in *Neurospora*. However, the biochemical function of its protein products, FRQ, is poorly understood. In this study, we demonstrated that the most conserved region of FRQ forms a coiled-coil domain. FRQ interacts with itself *in vivo*, and the deletion of the coiled-coil region results in loss of the interaction. Point mutations, which are designed to disrupt the coiled-coil structure, weaken or completely abolish the FRQ self-association and lead to the arrhythmicity of the overt rhythm. Mutations of the FRQ coiled-coil that inhibit self-association also prevent its interaction with two other key components of the *Neurospora* circadian clock, namely WC-1 and WC-2, the two PAS domain-containing transcription factors. Taken together, these data strongly suggest that the formation of the FRQ–FRQ and FRQ–WC complexes is essential for the function of the *Neurospora* clock.

Keywords: circadian/clock/frequency/wc-1/wc-2

Introduction

Circadian clocks (period ~24 h) are found in almost all groups of organisms and they control a wide variety of daily endogenous (circadian) rhythms of molecular, physiological and behavioral activities in these organisms (Bünning, 1973; Dunlap, 1999). The identification of clock genes in several model systems and the understanding of how these genes are regulated have started to unveil how circadian clocks work at the molecular level (Hall, 1998; Dunlap, 1999; Johnson and Golden, 1999; Liu *et al*., 1999; Young, 1999; King and Takahashi, 2000; Somers *et al*., 2000). From these studies, a common mechanistic theme has emerged: clocks consist of a network of positive and negative interactors that form the core of the oscillators that establish the negative feedback loops generating the basic circadian rhythm (Aronson *et al*., 1994a; Crosthwaite *et al*., 1997; King *et al*., 1997; Allada *et al*., 1998; Darlington *et al*., 1998; Hardin, 1998; Ishiura *et al*., 1998; Rutila *et al*., 1998; Wang and Tobin, 1998; Glossop *et al*., 1999; Shearman *et al*., 2000).

In *Neurospora*, there is at least one circadian autoregulatory negative feedback loop (Dunlap, 1999; Merrow *et al*., 1999; Lakin-Thomas and Brody, 2000; Lee *et al*., 2000) with *frq* mRNA and FRQ proteins as the central components (Aronson *et al*., 1994a). The deletion of the *frq* locus leads to the loss of normal circadian rhythmicity (Aronson, 1994b). Mutations of *frq* result in long and short period length (from 16 to 35 h), arrhythmia, and loss of temperature and nutritional compensation of the clock (Aronson *et al*., 1994b; Liu *et al*., 2000). Levels of both *frq* mRNA and FRQ protein cycle with the same period as the overt rhythms, and FRQ protein acts negatively to repress its own transcription (Aronson *et al*., 1994a). Importantly, the rhythmic expression of *frq* is essential for the negative feedback loop because constitutive expression of *frq* results in the loss of the overt rhythm, and step changes in *frq* expression reset the phase of the clock (Aronson *et al*., 1994a; Garceau *et al*., 1997). Light and temperature, two of the most important environmental signals, reset the *Neurospora* clock by changing the levels of *frq* mRNA and FRQ protein (Crosthwaite *et al*., 1995; Liu *et al*., 1998).

Although transcriptional regulation of *frq* is important for rhythm generation, post-transcriptional regulation of *frq* also plays a major role in regulating the clock. First, temperature-regulated alternative initiation of FRQ protein translation produces two forms of FRQ: a long form of 989 amino acids (LFRQ) and a shorter form of 890 amino acids (SFRQ) (Garceau *et al*., 1997). This mechanism allow the *Neurospora* clock to function optimally over a wide range of temperatures (Liu *et al*., 1997). Phosphorylation of FRQ is another important step of regulation. FRQ protein is progressively phosphorylated over time and its level decreases after it is extensively phosphorylated (Garceau *et al*., 1997). Although the identity of the FRQ kinase(s) is not known, one important function of phosphorylation is to regulate the protein stability of FRQ and affect the period length of the clock (Liu *et al*., 2000).

WC-1 and WC-2, two PAS-domain-containing transcription factors, are the other two known components of the *Neurospora* clock. Not only are they two of the essential components of the light signal transduction pathway of the clock (Crosthwaite *et al*., 1997; Talora *et al*., 1999), but they are also required for normal circadian rhythmicity in constant darkness. In strains with lesions of either *wc-1* or *wc-2*, the levels of *frq* mRNA and FRQ are extremely low, and these low levels of *frq* and FRQ expression are unable to support the overt rhythmicity (Crosthwaite *et al*., 1997). Like the PAS-domain-containing transcription factors BMAL1 and CLOCK in mammals, and dCLOCK and CYC in *Drosophila*, WC-1 and WC-2 form heterodimers *in vivo* (Dunlap, 1999; Talora *et al*., 1999). Recently, it was reported that FRQ interacts with WC-1 and WC-2 *in vivo*, suggesting that

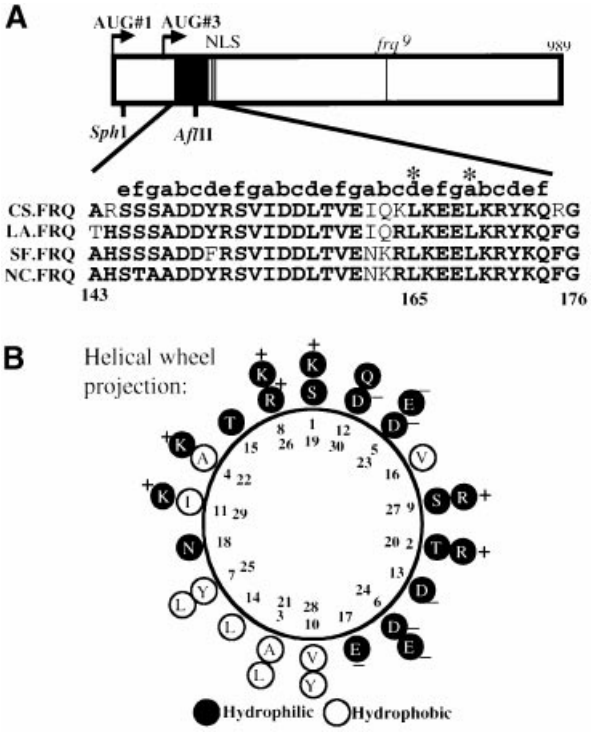

Fig. 1. The putative FRQ coiled-coil domain. (**A**) Schematic diagram of the FRQ ORF and sequencing alignments of the putative FRQ coiled-coil domain from four different fungal species. The open box represents the FRQ ORF. Arrows labeled AUG#1 and AUG#3 are the two alternative protein initiation sites that produce LFRQ and SFRQ. The filled black box represents the putative FRQ coiled-coil domain. NLS, nuclear localization signal. The line in the middle represents the location of the frq^9 mutation. In the sequence alignments, the bold letters are the conserved amino acids in different *frq* homologs. On the top of the alignment, letters *a–g* label the position of each amino acid within the helical heptad repeats. Asterisks mark the two amino acids (Leu165 and Leu169) that are mutated in this study. CS, *Chromocrea spinlosa*; LA, *Leptosphaeria australiensis*; SF, *Sordaria fimicola*; NC, *Neurospora crassa*. (**B**) Helical wheel projection of the FRQ coiled-coil domain. In the coiled-coils, seven residues allow the helix to make two turns to show a heptad repeat. The Ser145 denotes as position '1' in the helical wheel projection. The charge and the hydrophobicity of each residue are labeled.

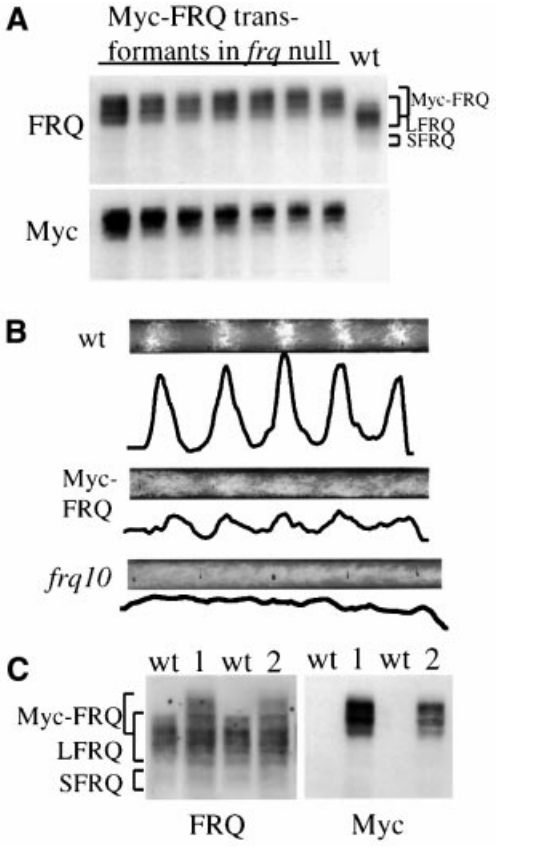

Fig. 2. The expression of Myc-FRQ in *Neurospora* and the rescue of the conidiation banding rhythm of the *frq* null strain by Myc-FRQ. (**A**) Western blot analysis showing the expression profile of Myc-FRQ in *Neurospora*. pKAJ120·Myc-FRQ was transformed into 93–4 (*frq* null strain) and seven independent transformants were selected for protein analysis. Cultures were grown and harvested in constant light (LL). The protein blot was first probed with FRQ antiserum (top) and then stripped and reprobed with a monoclonal c-Myc antibody (bottom). Note the slower mobility of Myc-FRQ bands relative to the wild-type FRQ. (**B**) The rescue of the circadian conidiation banding rhythm of *frq* null strain by Myc-FRQ. Both the original image of the race tubes and the densitometric analysis of the race tubes are shown. frq^{10} is the *frq* null strain. The race tubes shown are representative samples from six replicate tubes. (**C**) Expression of Myc-FRQ in an *frq*+ strain. pKAJ120·Myc-FRQ was transformed into 87–12 (frq^+) and two independent transformants (1 and 2) are analyzed along with the wild-type strain. The blot was probed with either FRQ (upper panel) or c-Myc antibody (lower panel).

FRQ might negatively regulate its own expression by inhibiting the transcriptional activation of the WC proteins (Denault *et al*., 2000). Together, these data suggest that the WC proteins might function as the transcription activators of *frq* and behave as the positive limb of the *Neurospora* circadian negative feedback loop (Dunlap, 1999).

Despite the importance of FRQ protein in the *Neurospora* clock, how it works in the *FRQ–WC* based circadian negative feedback loop is poorly understood. In this study, we identified the most conserved region of FRQ as a coiled-coil domain. We have shown that FRQ interacts with itself *in vivo*, and such interaction depends on the presence of the coiled-coil region. Point mutations in this region, designed to disrupt the coiled-coil structure, weaken or completely abolish the FRQ self-interaction, and lead to the arrhythmicity of the overt rhythm. In addition, mutant forms of FRQ that fail to self-associate, also fail to interact with WC-1 and WC-2, the two PAS-domain-containing transcription factors. Taken together, these data demonstrate that the formation of the FRQ–FRQ and FRQ–WC complexes are essential for the function of the *Neurospora* clock.

Results

FRQ interacts with itself

Analysis of the FRQ open reading frame (ORF) revealed that a 30 amino acid region (aa 145–174) near the N-terminus of the protein has potential to form a coiled-coil structure. MultiCoil and Paircoil scores indicate that this region has >90% probability of forming a dimer (Berger *et al*., 1995; Wolf *et al*., 1997). Previously, sequence comparison among different FRQ homologs has shown that this region is the most conserved region (Lewis *et al*., 1997) (Figure 1A), suggesting its important role for the function of FRQ. Coiled-coil domain is one of the common structural motifs mediating protein–protein inter-

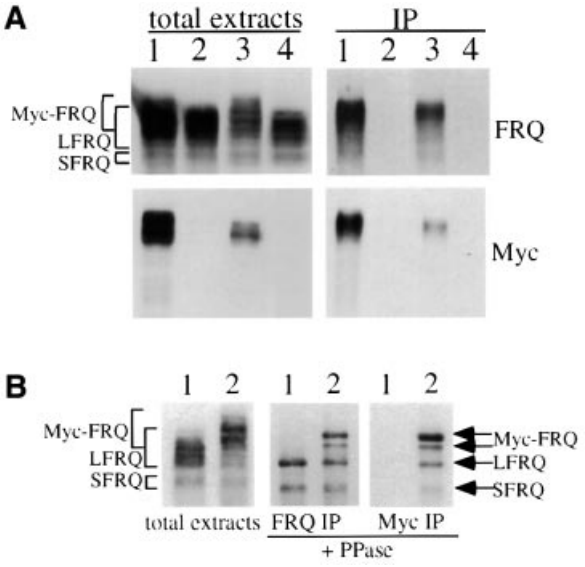

Fig. 3. FRQ interacts with itself *in vivo*. (**A**) Western blot analyses (probed with FRQ or c-Myc antibody) showing the expression of various FRQ forms in total extracts (left two panels) and in immunoprecipitates (right two panels). Immunoprecipitation was performed using a c-Myc monoclonal antibody. Note the lack of low molecular weight smear in the right bottom blot probed with c-Myc antibody. Lane 1, *frq*+, Myc-FRQ; LL; lane 2, wild type, LL; lane 3, *frq*+, Myc-FRQ; DD20; lane 4, wild type, DD20. (**B**) Phosphatase treatment after immunoprecipitation shows that Myc-FRQ interacts with the endogenous FRQ forms. Before phosphatase treatment, the protein extracts were immunoprecipitated with either FRQ (center panel) or c-Myc (right panel) antibodies. The cultures were grown and harvested in LL. Lane 1, wild type; lane 2, *frq*+, Myc-FRQ.

actions. It is a structure of heptad repeats (amino acid positions are labeled *a*–*g*), positions *a* and *d* are hydrophobic and form the helix interface that mediates protein–protein interactions, while other positions (*b*, *c*, *e*, *f* and *g*) are typically hydrophilic and form the solvent-exposed part of the structure (Lupas, 1996). The computer analysis reveals that this domain of FRQ consists of four heptad repeats. The helical wheel projection of this region of FRQ shows that the *a* and *d* positions are mostly hydrophobic and have the potential of forming the helix interface, while the rest of the positions are more hydrophilic (Figure 1B).

To demonstrate the function of this region in protein–protein interactions, we first examined its role in mediating FRQ self-association. We made a construct in which five c-Myc epitopes were inserted into the N-terminus (*Sph*I site; Figure 1A) of FRQ such that only the large FRQ form (LFRQ) is tagged. This Myc-tagged FRQ was expressed at a level comparable to that of the wild-type FRQ and it was also phosphorylated normally (Figure 2A, the various bands are the results of protein phosphorylation) (Garceau *et al*., 1997). The Myc-FRQ bands migrate more slowly than the wild-type FRQ in SDS–PAGE gel because of the addition of the five c-Myc epitopes (79 amino acids), and they are specifically recognized by a monoclonal c-Myc antibody (Figure 2A and C). However, this construct failed to express the small FRQ form (SFRQ, not tagged by c-Myc), suggesting that the region where the epitopes were inserted is important for the expression of SFRQ. The Myc-tagged FRQ is functional because it can rescue the circadian rhythm of conidiation banding of the *frq* null strain (frq^{10}) (Aronson *et al*., 1994b), albeit with a slightly shorter period (~21 h) than that of the wild-type strain (~22 h) (Figure 2B). The low amplitude and the short period rhythms of the Myc-FRQ transformants are likely to be due to the lack of expression of SFRQ, since both FRQ forms are required for optimal clock function (Liu *et al*., 1997).

To examine whether FRQ interacts with itself, the Myc-FRQ construct was transformed into a wild-type *Neurospora* strain. As can be seen from Figure 2C, all three forms of FRQ (endogenous LFRQ and SFRQ, and MYC-FRQ) are expressed in these transformants. Protein extracts from cultures grown in constant light (LL) or dark (DD20: 20 h after light/dark transition) were prepared. Immunoprecipitation was performed using the monoclonal c-Myc antibody. The smear of lower molecular weight FRQ signals in Figure 3A (upper right panel) suggests that the endogenous LFRQ and SFRQ were pulled down by Myc-FRQ. To obtain visually clearer results, the immunoprecipitates using either FRQ or Myc antibodies were treated with lambda phosphatase to dephosphorylate different FRQ forms. As shown in the center panel of Figure 3B, immunoprecipitation using FRQ antiserum pulled down both LFRQ and SFRQ in the wild-type strain, while in the Myc-FRQ strain, in addition to the two endogenous wild-type FRQ forms, two Myc-FRQ forms are also pulled down (Figure 3B, center panel). The expression of the smaller Myc-FRQ form is very likely to be the result of internal protein initiation from one of the six methionines within the five Myc epitope region. When c-Myc antibody was used to perform immunoprecipitation, as expected, the endogenous LFRQ and SFRQ are pulled down along with the Myc-FRQ forms, indicating the self-association of FRQ (Figure 3B). Comparison of the relative amounts of Myc-FRQ and the endogenous FRQ forms in the FRQ immunoprecipitation (IP) and the Myc IP samples, we estimate that there is ~50% of FRQ associated with each other to form complexes (Figures 3B and 4B). Since this comparison can only estimate the amount of FRQ that forms heterodimer and the immunoprecipitation procedures might also disrupt the complexes, the actual number *in vivo* should be >50%. In addition, yeast two-hybrid assays using FRQ regions containing this coiled-coil domain also showed that FRQ interacts with itself in yeast (data not shown). Together, these results demonstrate that both LFRQ and SFRQ interact with each other to form complexes *in vivo*.

Deletion of the coiled-coil domain eliminates the FRQ–FRQ interaction

To locate the region responsible for the FRQ–FRQ interaction, the Myc-FRQ construct was transformed into an frq^9 strain. In the frq^9 strain, a one nucleotide frame-shift deletion within the FRQ ORF results in the translation of a truncated FRQ protein of ~75 kDa (Aronson *et al*., 1994b). Although the truncated FRQ in frq^9 is only marginally stable, it can be detected by western blot analysis (Figure 4A, top panel). The various bands of frq^9 FRQ suggest that it is also phosphorylated. In the Myc-FRQ, frq^9 strain, both Myc-FRQ and frq^9 FRQ are expressed (Figure 4A, lane 3, left). When using Myc antibody to perform immunoprecipitation, the smaller frq^9

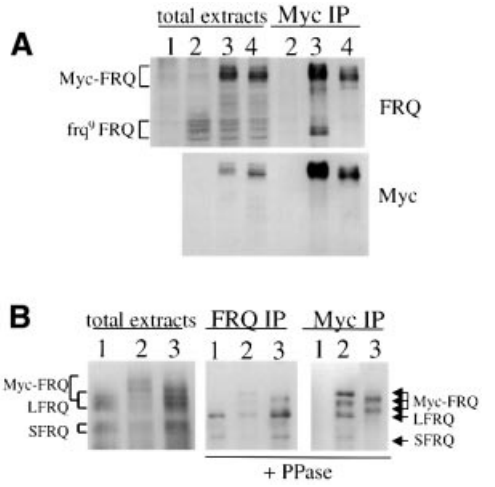

Fig. 4. Deletion of the putative coiled-coil domain abolishes FRQ–FRQ interaction. (**A**) The interaction between Myc-FRQ and the truncated *frq*^{9} FRQ. The cultures were grown in LL. Lane 1, *frq*^{10} (*frq* null); lane 2, *frq*^{9}; lane 3, *frq*^{9}, Myc-FRQ; lane 4, *frq*^{9}, Myc-FRQ1 (deletion of the coiled-coil region). The left four lanes are total protein extracts and the three lanes on the right are IP products with c-Myc antibody. The protein blots were probed with FRQ or c-Myc antibody. Note the disappearance of the *frq*^{9} FRQ bands in lane 4 of the IP samples. (**B**) Myc-FRQ with the deletion of the coiled-coil domain can no longer interact with the endogenous FRQ forms. Lane 1, wild type; lane 2, *frq*+, Myc-FRQ; lane 3, *frq*+, Myc-FRQ1. The protein extracts were immunoprecipitated with FRQ (center) or c-Myc (right) antibody and then treated with phosphatase to dephosphorylate various FRQ forms. The protein blots were probed with FRQ antiserum. Note the disappearance of the LFRQ and SFRQ bands in lane 3 of the Myc IP panel.

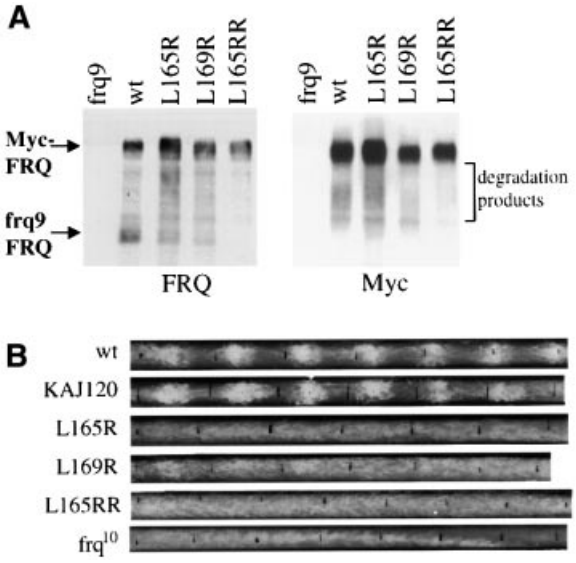

Fig. 5. Point mutations of the coiled-coil domain lead to the weakening or complete loss of the FRQ–FRQ interaction and loss of rescue of the conidiation banding rhythm in the *frq* null strain. (**A**) Leu165 and Leu169 are important residues for FRQ–FRQ interaction. Single (Leu165Arg and Leu169Arg) or double (Leu165ArgArg) mutations were introduced into pKAJ120·Myc-FRQ. Mutation constructs were transformed into 94–1 (*frq*^{9}, *his-3*), so that both Myc-FRQ and *frq*^{9} FRQ are expressed in these transformants. Various Myc-FRQ forms were immunoprecipitated down using c-Myc antibody. The protein blots were probed with FRQ or Myc antibody. Note the decrease of the *frq*^{9} FRQ signals in Leu165Arg and Leu169Arg samples, and its complete disappearance in 165ArgArg sample. The smearing signals between Myc-FRQ and *frq*^{9} FRQ are degradation products of Myc-FRQ. (**B**) Race tube results showing the arrhythmicity in constant darkness for the FRQ coiled-coil mutant strains. Leu165Arg, Leu169Arg and Leu165ArgArg mutations were introduced into pKAJ120. The mutations constructs were then transformed into the *frq* null strain (*frq*^{10}). The resulting transformants were first screened by western blot analysis for FRQ expression before being examined by race tube assays. At least 10 positive transformants were examined by race tube assays. The wild-type strain and the pKAJ120 transformants were used as controls. The race tubes shown are representative samples from six replicate tubes.

FRQ can be pulled down by the Myc-FRQ forms (Figure 4A, lane 3, right), but not in the negative control (*frq*^{9} strain; Figure 4A, lane 2). These data further indicate that FRQ self-associates and that the region responsible for the FRQ–FRQ interaction is in the region upstream of the *frq*^{9} mutation.

Since the putative coiled-coil domain is included in the *frq*^{9} FRQ, we made a construct (pMyc-FRQ1) in which the coiled-coil domain is deleted (FRQ aa 107–178) from Myc-FRQ to see whether the coiled-coil domain is necessary for the FRQ–FRQ interaction. This construct was transformed into either *frq*^{9} (Figure 4A) or the wild-type strain (Figure 4B). As predicted, Myc-FRQ1 did not co-precipitate with *frq*^{9} FRQ (Figure 4A, lane 4, right) or the endogenous LFRQ and SFRQ forms (Figure 4B, lane 3, right panel). The slightly faster mobility of the Myc-FRQ1 protein species is due to the deletion of the coiled-coil domain. These data demonstrate that this putative coiled-coil domain region is required for the FRQ–FRQ interaction. In addition, when this region is deleted from the wild-type FRQ, it no longer rescues the circadian banding rhythm of the *frq* null strain (data not shown), suggesting that this region and the FRQ–FRQ interaction mediated by this region are essential for the clock function of FRQ.

Mutations of the coiled-coil domain disrupt the FRQ–FRQ interaction and eliminate its clock function

To access the molecular surface involved in FRQ–FRQ interaction, we introduced point mutations at the critical *a* and *d* positions in the predicted coiled-coil structure (Figure 1B) that form the hydrophobic helix interface. Therefore, mutation of these amino acids to charged hydrophilic residues would be predicted to weaken or prevent the protein–protein interaction. Point mutations (to arginine) were introduced at one or both of the conserved leucines at position 165 and 169 (Figure 1), and the Myc-FRQ constructs with these mutations were transformed into the *frq*^{9} strain. Such mutations were predicted by the MultiCoil and Paircoil programs to reduce significantly the probability of forming coiled-coil structure (Berger *et al*., 1995; Wolf *et al*., 1997). As predicted, the single site mutations (Leu165Arg and Leu169Arg) significantly weakened the interaction between Myc-FRQ and *frq*^{9} FRQ, while in the double mutant (Leu165ArgArg: Leu165Arg and Leu169Arg), such interaction was completely abolished (Figure 5A). These data further support the role of the coiled-coil structure in the formation of the FRQ–FRQ complexes.

The ability of FRQ containing these mutations to rescue the circadian rhythm of conidiation of the *frq* null strain (*frq*^{10}) is a stringent test of the functional significance of the FRQ–FRQ interaction. These mutations were intro-

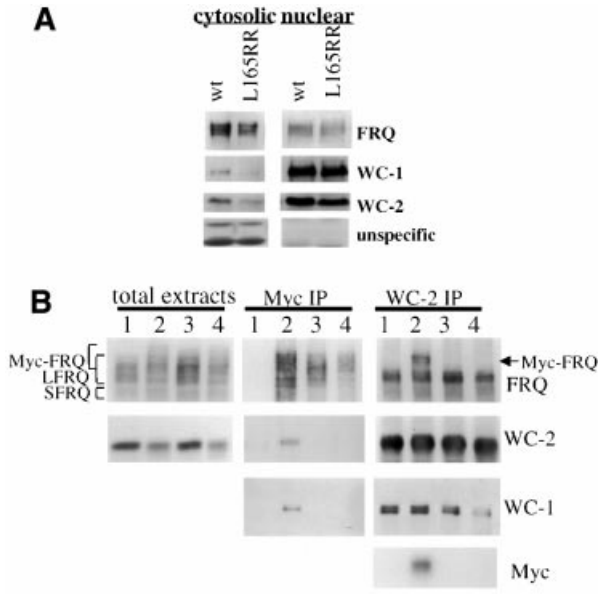

Fig. 6. The FRQ coiled-coil domain is not required for the nuclear localization of FRQ but it is necessary for the FRQ–WC interactions. (**A**) Western blot analysis of the nuclear and cytosolic preparations shows that the coiled-coil mutation does not affect the nuclear localization of FRQ. Wild-type strain and Leu165ArgArg mutant strain (*frq*^{10}, pKAJ120·L165RR) were grown and harvested in LL. The protein blots were probed with either FRQ, WC-1 or WC-2 antiserum. The two non-specific bands (bottom panels), recognized by the WC-2 antiserum, can only be found in the cytosolic fractions, suggesting that the nuclei preparations are free of cytosolic contamination. (**B**) The formation of the FRQ coiled-coil structure is required for the interaction between FRQ and the WC proteins. Total protein extracts from different strains grown in LL were immunoprecipitated by c-Myc or WC-2 antibody, and protein blots were probed with FRQ, WC-1, WC-2 or c-Myc antibody. Lane 1, *frq*$^{+}$; lane 2, *frq*$^{+}$, Myc-FRQ; lane 3, *frq*$^{+}$, Myc-FRQ1; lane 4, *frq*$^{+}$, FRQ-165RR.

duced into the wild-type *frq* construct (pKAJ120) and transformed into *frq*^{10}. None of the constructs, even the single mutation, was able to rescue to the normal circadian banding rhythm of *frq*^{10} (Figure 5B). The occasional very broad conidiation peaks in the Leu165Arg and Leu169Arg mutants suggest that the clock might be running with a very low amplitude in these mutant strains. The circadian phenotype of the Leu165ArgArg mutant strains is indistinguishable from that of the *frq* null strain. These data indicate that the coiled-coil domain-mediated FRQ–FRQ interaction is essential for the circadian function of FRQ in *Neurospora*, and even the weakening of the FRQ–FRQ interaction disrupts the circadian clock.

FRQ–FRQ interaction is required for its interactions with the WC proteins

Since the formation of the FRQ–FRQ complexes is required for the function of FRQ, we wondered what the biochemical role of such complexes is. Because the coiled-coil domain is proximal to the nuclear localization signal (NLS) of FRQ (Luo *et al*., 1998), it is possible that the formation of the FRQ–FRQ complex is important for the nuclear localization of FRQ. To examine this possibility, nuclei preparations from both the wild-type strain and the L165RR (pKAJ120·165RR transformed into *frq*^{10}) strain were made and the presence of FRQ and several other proteins was examined. As seen in Figure 6A, the mutant FRQ, like wild type, is found in the nucleus (Luo *et al*., 1998). In agreement with previous studies (Talora *et al*., 1999; Schwerdtfeger and Linden, 2000), both WC-1 and WC-2 are greatly enriched in the nuclear fractions. Thus, the formation of the FRQ–FRQ complex is not required for the nuclear localization of FRQ. The different nuclear and cytoplasmic distributions of FRQ and WCs suggest that FRQ nuclear localization is regulated by some unknown mechanisms.

Recently, it was reported that FRQ interacts with two PAS-domain-containing transcriptional factors, WC-1 and WC-2 (Denault *et al*., 2000), probably leading to the negative repression of *frq* transcription. To test the possibility that the formation of the FRQ–FRQ complex is important for the interaction between FRQ and the WC proteins, we examined the interactions between FRQ and the WC proteins. Wild-type strains containing Myc-FRQ with or without the coiled-coil mutations (FRQ1 or Leu165ArgArg) allowed the endogenous wild-type FRQ to be used as an internal control in the co-immunoprecipitation experiments. As expected, both WC-1 and WC-2 were specifically co-immunoprecipitated with the wild-type Myc-FRQ (Figure 6B, center panels, lane 2) when Myc antibody was used to perform immunoprecipitation. In contrast, Myc-FRQ containing the coiled-coil mutations (Figure 6B, center panels, lanes 3 and 4) failed to pull down either WC protein.

To confirm these results, the same extracts were immunoprecipitated with the WC-2 antiserum. As expected, the endogenous LFRQ and SFRQ co-immunoprecipitated with WC-2 in all strains. WC-2 also pulled down the wild-type Myc-FRQ (Figure 6B, right panels, lane 2), but it failed to pull down the Myc-FRQ containing the coiled-coil mutations (Figure 6B, right panels, lanes 3 and 4). In agreement with a previous report (Talora *et al*., 1999), WC-2 also interacts with WC-1 to form a heterodimer (Figure 6B, right panels). These data demonstrate that the formation of the FRQ coiled-coil structure is required for the interaction between FRQ and the WC proteins, and it is possible that this latter interaction is essential for FRQ to carry out its biochemical role in the circadian clock of *Neurospora*.

Discussion

Previous studies have shown that the *frq* gene is one of the central components of a *Neurospora* circadian negative feedback loop (Dunlap, 1999). In this study, we have investigated the role of FRQ protein–protein interactions. We have shown that FRQ interacts with itself *in vivo* through a coiled-coil domain. The data presented demonstrate that the coiled-coil-mediated FRQ–FRQ interaction is essential for its function in the *Neurospora* clock. We showed that point mutations of the coiled-coil structure weaken or prevent the FRQ self-association, and result in the arrhythmicity of the conidiation rhythm. Furthermore, we have also shown that the formation of the FRQ coiled-coil structure is required for the interaction between FRQ and the WC proteins. Taken together, these data indicate that the formation of the FRQ–FRQ and FRQ–WC complexes are essential for the function of the *Neurospora* clock.

Coiled-coils are one of the most commonly used protein structure motifs mediating protein–protein interactions (Lupas, 1996). The leucine zipper domain found in some transcription factors is the best known example (Landschulz *et al*., 1988; McKnight, 1991; Ellenberger *et al*., 1992). Previously, this region of FRQ was proposed to be a helix–turn–helix (HTH) DNA binding motif by computer analysis; however, the scores obtained for FRQ are low, and there is no experimental support for the hypothesis (Lewis *et al*., 1997). The evidence presented in this study strongly supports the formation of a coiled-coil structure by this region. First, we have shown that this region of FRQ mediates FRQ–FRQ interactions *in vivo*. Secondly, mutations that are designed to disrupt the coiled-coil hydrophobic interface weaken or completely abolish such protein–protein interaction. Thirdly, the interaction between FRQ and the WC complex and the loss of function for the mutant monomeric FRQ forms suggest that FRQ might function as a co-factor for the WC proteins. Although we do not know the orientation of the FRQ–FRQ coiled-coil complexes, the opposite charged residues flanking the hydrophobic core in the proposed coiled-coil structure (Figure 1B) suggest that FRQ might form anti-parallel coiled-coil complexes.

Previously, the sucrose gradient experiments suggested that FRQ existed mostly as a monomer although the results of the gel filtration experiments indicated that FRQ was in a large complex (Garceau *et al*., 1997). The failure to detect the FRQ–FRQ complexes in the sucrose gradient experiments might be due to limited resolution of the method, weak interactions among FRQ and the WCs, and the large size and various forms of FRQ. From the results of the co-immunoprecipitation experiments presented here (Figures 3 and 4), we estimate that most of the FRQ proteins form complexes with each other. We have formally shown the functional significance of the FRQ coiled-coil domain, and the simplest interpretation of our results is that FRQ proteins form dimers before interacting with the WC proteins. If this is the case, it suggests that another region of FRQ is responsible for the FRQ–WC interactions, and the formation of the FRQ–FRQ complex allows that to happen. However, it is also possible that the FRQ–FRQ and FRQ–WC complexes are mutually exclusive and FRQ interacts with the WCs through its coiled-coil domain. It is also not known which of the WC proteins FRQ interacts with and whether both WCs are required for such interactions. Examining these possibilities is the focus of our current research and it should provide us with better understanding on how these proteins work.

Is it possible that the monomeric FRQ is also functional? Several lines of evidence suggest that this is unlikely. First, the coiled-coil domain is the most conserved region in the FRQ ORF, suggesting that the protein–protein interaction mediated by this region is important for the function of FRQ. In this regard, the mutant strains with only the monomeric mutant FRQ is not functional (Figure 5B). In addition, we have shown that the mutant FRQ forms that failed self-association can no longer interact with the WC proteins, the positive elements in the circadian feedback loop.

Protein interactions of clock components are important regulatory steps for circadian negative feedback loops. In *Drosophila*, the formation of the PER–TIM complex is essential for the nuclear localization of both proteins (Vosshall *et al*., 1994; Saez and Young, 1996). Although *Drosophila* PER can interact to form a homodimer through the PAS domains, the functional significance of such interactions is unclear (Huang *et al*., 1993; Zeng *et al*., 1996). In mammals, three *mPer* genes (*mPer1*, *mPer2* and *mPer3*) are part of the circadian oscillator. The interactions among the three mPERs and mCRYs appear to be important for the nuclear localization of mPER proteins (Kume *et al*., 1999; Field *et al*., 2000; Yagita *et al*., 2000). Once in the nucleus, PER proteins, in both *Drosophila* and mammals, can then interact with the protein complex formed by two PAS-domain-containing transcription factors (dCLOCK–CYC in *Drosophila*, BMAL1–CLOCK in mammals) to inhibit their transcriptional activation of *per* and some clock-controlled genes (Darlington *et al*., 1998; Gekakis *et al*., 1998; Lee *et al*., 1999), thus closing the negative feedback loop in each system.

In contrast, the formation of the FRQ–FRQ complex does not affect the nuclear localization of FRQ (Figure 6A). Instead, we found that mutant forms of FRQ, which fail to interact with each other, also do not interact with WC-1 and WC-2 (Figures 5 and 6). Since WC-1 and WC-2 are thought to be the positive elements in the *Neurospora* negative feedback loop (Dunlap, 1999), the failure of FRQ to interact with the WC complex may break the feedback loop and cause the loss of rhythmicity in these mutants (Figure 5). If this is the case, the interactions between FRQ and the WC complex play an essential role in closing the negative feedback loop in *Neurospora*.

In conclusion, our studies demonstrated that FRQ associations with itself and the WC proteins are essential for the function of the *Neurospora* circadian clock. Our data are consistent with the negative feedback model in which FRQ is the negative element in the loop, and the WC proteins are the positive elements of the loop.

Materials and methods

Strains, culture conditions and race tube assay

Neurospora strains used in this study are bdA (clock wild type), 87–12 (*bd*; *A*; *his-3*), 93–4 (*bd*; *frq*^{10}; *his-3*), 94–1 (*bd*; *frq*^{9}; *his-3*) (Aronson *et al*., 1994a,b) and different *frq* mutant strains created in this study. Strains 87–12 (*frq* wild-type strain), 93–4 (*frq* null strain) and 94–1 (*frq*^{9}, making truncated FRQ) are the host strains for various *frq* mutation constructs described. Culture conditions were as described previously (Aronson *et al*., 1994a; Crosthwaite *et al*., 1995). Race tube assay media contains 1× Vogel's, 0.1% glucose, 0.17% arginine and 50 ng/ml biotin. Densitometric analyses of race tubes and calculations of period length were performed as described previously (Liu *et al*., 1997) using Chrono II version 11.1 (Dr Till Roenneberg, Ludwigs-Maximillian University, Munich, Germany).

Plasmid constructs and Neurospora transformation

pKAJ120 (containing the entire *frq* gene including its promoter and a *his-3* targeting sequence) is the parental plasmid for all *frq* constructs described in this study. To create the Myc-FRQ construct (pKAJ120·Myc-FRQ), a PCR fragment (using pCS2-Myc as the template, generously provided by Dr Hongtao Yu) containing five tandem repeats of c-Myc epitope (79 amino acids) was inserted into the *Sph*I site of pKAJ120 (Figure 1A). All the deletion and point mutations of the FRQ ORF were constructed using the Transformer Site-Directed Mutagenesis kit (Clontech Laboratories, Inc., Palo Alto, CA), and pUC19Mfrq was used as the *in vitro* mutagenic template (Liu *et al*., 2000). The mutagenic primers used were FRQ1 (to delete FRQ aa 107–178), 165Arg (to mutate Leu165 to Arg), 169Arg (to mutate Leu169 to Arg) and 165ArgArg (to

mutate both Leu165 and Leu169 to Arg). After mutagenesis, the region containing the mutation was subcloned into either pKAJ120 (for rescuing the circadian conidiation rhythm of *frq* null strain) or pKAJ120·Myc-FRQ (for immunoprecipitation assay). All constructs were confirmed by DNA sequencing, and they were targeted by transformation to the *his-3* locus of the host strains as previously described (Bell-Pedersen *et al*., 1996). For protein analyses, at least three or four independent transformants were examined. For race tube analysis, at least 10 independent positive transformants were examined.

Computer analysis of the FRQ coiled-coil structure

Web-based MultiCoil (http://nightingale.lcs.mit.edu/cgi-bin/multicoil) and Paircoil (http://nightingale.lcs.mit.edu/cgi-bin/score) programs were used to predicted the probability of coiled-coil formation (Berger *et al*., 1995; Wolf *et al*., 1997). They were also used to help design mutations to be introduced into the FRQ coiled-coil domain.

Generation of antisera against WC-1 and WC-2

Glutathione *S*-transferase (GST)–WC-1 (containing WC-1 aa 288–709) and GST–WC-2 (containing the entire WC-2 ORF) fusion proteins were expressed in BL21 cells and the inclusion bodies containing the recombinant proteins were purified. To purify the inclusion bodies, the pellets of 500 ml of *Escherichia coli* cultures (after 2 h of 1 mM isopropyl-β-D-thiogalactopyranoside induction) was resuspended in 40 ml of buffer A (50 mM Tris–HCl pH 8.3, 1 mM EDTA, 2 mM 2-mercaptoethanol) containing freshly added 0.1% lysozyme. The resuspensions were kept at room temperature for 40 min before the cells were broken by sonication (5″ × 30, Sonifier 450, VWR Scientific). The pellets were collected by centrifugation (9000 r.p.m. × 25′) and washed in 20 ml of buffer C (Tris–HCl pH 8.3, 2 mM EDTA, 2 mM 2-mercaptoethanol, 2 M urea, 0.5% Triton X-100). The resulting pellets were then resuspended in 8 M urea and the GST fusion proteins were then purified by electroelution after running on preparative 7.5% SDS–PAGE gels. WC-1 and WC-2 antisera were generated by immunizing New Zealand white rabbits with the purified recombinant proteins according to standard protocols. WC-2 antiserum was used for western blot analysis and immunoprecipitation assays without further purification. For WC-1 antiserum, it was affinity purified using nitrocellulose membranes blotted with purified GST–WC-1 protein.

Protein analyses

Protein extraction, quantification and western blot analysis are as described previously (Garceau *et al*., 1997). Equal amounts of total protein (~100 μg) were loaded in each protein lane and after the blots were developed by chemiluminescence (ECL, Amersham Pharmacia, Piscataway, NJ), they were stained by amido black to verify equal loading of protein (Liu *et al*., 1997). The purification of *Neurospora* nuclei was performed as described previously (Luo *et al*., 1998).

For IP, 0.5 mg of soluble protein extracts in extraction buffer (Garceau *et al*., 1997) were incubated at 4°C for 2 h with either the monoclonal c-Myc antibody (2 μg; Santa Cruz Biotechnology, Inc., Santa Cruz, CA) or WC-2 antiserum (1:250 dilution). Then protein G–agarose beads (10 μl) were added and incubated for 1–2 h at 4°C, washed four times with ice-cold extraction buffer before the IP products were resuspended in protein loading buffer or 50 μl of phosphatase buffer.

For phosphatase treatments, 1000 U of lambda PPase (New England Biolabs, Beverly, MA) were added to the IP complexes resuspended in the phosphatase buffer and incubated at 30°C for 30 min before loading on SDS–PAGE gels (Garceau *et al*., 1997).

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