VIVID interacts with the WHITE COLLAR complex and FREQUENCY-interacting RNA helicase to alter light and clock responses in Neurospora

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The photoreceptor and PAS/LOV protein VIVID (VVD) modulates blue-light signaling and influences light and temperature responses of the circadian clock in Neurospora crassa. One of the main actions of VVD on the circadian clock is to influence circadian clock phase by regulating levels of the transcripts encoded by the central clock gene frequency (frq). How this regulation is achieved is unknown. Here we show that VVD interacts with complexes central for circadian clock and blue-light signaling, namely the WHITE-COLLAR complex (WCC) and FREQUENCY-interacting RNA helicase (FRH), a component that complexes with FRQ to mediate negative feedback control in Neurospora. VVD interacts with FRH in the absence of WCC and FRQ but does not seem to control the exosome-mediated negative feedback loop. Instead, VVD acts to modulate the transcriptional activity of the WCC.

Light, in addition to providing an energy source for many life forms on Earth, acts as a signal that may trigger development or serve as a repetitive cue that marks the passing of external time. External time cues are used by cellular timers such as circadian clocks to lock their periods to that of the external day. The process of period locking is called “entrainment” and ensures that cellular and behavioral activities happen at times of day when their adaptive value is highest (1–3). Blue light plays a central role in the entrainment of circadian clocks. Indeed blue-light photoreceptors and circadian clocks may have coevolved from a mechanism that originally served to detect (photoreceptor) and avoid (timer) harmful radiation (4). Our understanding of the molecular bases of circadian clocks and their responses to light has improved dramatically during the last decade or so, and the eukaryotic model organism Neurospora crassa has become one of the best-studied systems for understanding both processes (4–6).

The key components of the Neurospora circadian clock are the products of the white collar (wc-1 and wc-2), frequency (frq), and frq-interacting helicase (frh) genes (4, 10, 11). The blue-light photoreceptor WC-1, and its interaction partner WC-2, form the transcriptionally and photoactive WHITE COLLAR complex (WCC) that activates frq expression (4, 12). FRQ protein, in turn, complexes with FRH to form an FRQ-FRH complex (FFC) that represses WCC activity (9, 11). Thus, photoreception and temporal organization of gene expression are linked via the WCC (4, 5). Our understanding of the molecular bases of circadian clocks and their responses to light has improved dramatically during the last decade or so, and the eukaryotic model organism Neurospora crassa has become one of the best-studied systems for understanding both processes (4–6).

VVD Regulates frq Transcript Levels at Dusk. Molecular and physiological data have shown that VVD influences clock resetting at dawn and dusk. At dusk VVD’s impact on molecular events is evident when comparing frq mRNA levels of WT and vvd-knockout (vvdko) strains. The frq transcript remains elevated longer in vvdko strains than in the WT, with a delay of about 4 h in reaching basal levels (top two lanes in Fig. 1A (16, 20). To obtain more direct proof of VVD’s role in the regulation of frq transcript levels, we created a strain in which a quinonic acid (QA)-inducible copy of an myc epitope-tagged vvd gene (qa-vvdmyc) was inserted at the his-3 locus. The qa-vvdmyc construct was integrated into WT or vvdko strains, and these strains are referred henceforth as qa-vvdmyc (WT) and qa-vvdmyc (vvdko), respectively. By using this strategy, we were able to uncouple vvd expression from its normal light regulation (Fig. 1B and Fig. S1). Indeed, the ectopic expression of VVD induced by the addition of QA in qa-vvdmyc (vvdko) restores the normal decline in frq levels (compare the −QA and +QA samples in Fig. 1A, fourth panel) and accelerates frq degradation in qa-vvdmyc (WT) beyond that seen in a normal WT (compare the −QA and +QA samples in the first and third lanes in Fig. 1A and see quantification of data in Fig. S1A). The observation that frq levels are somewhat lower in QA medium was expected, because full expression of frq is dependent on glucose (27). Taken together, these data illustrate an inverse correlation between frq transcript levels and VVD protein.

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VVD Interacts with FRH. Because the FFC plays a key role in frq negative feedback (11, 28, 29), it was possible that VVD directly modulates the activity of this complex to influence frq levels at dusk. To test whether VVD interacts with the FFC, we performed coimmunoprecipitation (Co-IP) experiments on Neurospora whole-cell lysates using FRH or FRQ antisera, respectively. To facilitate detection of VVD, we used a strain that expresses MYC-tagged VVD in a vvd background. We have shown previously that this strain rescues all known vvd mutant phenotypes, thus demonstrating that VVDMYC is fully functional (21). Henceforth all references to VVD protein levels are based on VVDMYC expression. Lysates from the vvd myc-tagged strain exposed to 30 min of LL were incubated with either FRH or FRQ antiserum before probing the blotted immunoprecipitates with MYC antiserum to test for the presence of VVD. Unfortunately, the FRQ antiserum proved too unspecific in our Co-IP experiments, so we were unable to judge whether VVD interacts with FRQ. However, when we used FRH antiserum, VVD was specifically immunoprecipitated (Fig. S2A). No signal was detected in frqko and vvdko strains that lacked a tagged copy of the vvd gene, indicating that the identified signal is VVDMYC and not an unspecific signal.

To test whether a functional WCC or FFC is necessary for the interaction of VVD with FRH, we exploited the QA-inducible system as outlined above and expressed a qa-vvdko in a background in which the wc-1, wc-2, frq, and vvd genes were deleted (strain 127-11) (Fig. S2B). In this qa-vvdko-ko strain, in which only the central clock gene frq remains intact, VVD still interacts with FRH, indicating that neither a functional FFC nor WCC is necessary for the interaction. The interaction occurs at both dawn and dusk transitions (Fig. S2B). As expected, extracts show a significant depletion of FRH after immunodepletion (top two lanes in Fig. S2A). However, no significant depletion of VVD is seen, suggesting that only a small fraction of total VVD interacts with FRH.

VVD Affects the Transcriptional Limb of FFC-Mediated Negative Feedback. Next, we investigated the mechanism by which VVD mediates frq RNA turnover. Two distinct pathways that regulate levels of frq message have been described. First, a negative feedback loop involving FRQ and FRH is important for rhythmic down-regulation of frq at the level of transcription (10, 11). Second, the FFC also functions at the posttranscriptional level to control frq mRNA degradation via the exosome (28).

If VVD influences frq transcription, inhibition of transcription should abolish the differences in frq levels that exist between WT and vvdko strains. To test this possibility, we used the transcriptional inhibitor thiolutin (28) to inhibit transcription 1 h before or directly after the transfer of Neurospora cultures from light-to-dark (Fig. 3). When transcription was inhibited before the light-to-dark transition, we saw no or very little frq transcript present in either WT or vvdko strains, suggesting effective repression of transcription by the drug (second and fourth lanes in Fig. 3A). When frq transcription was allowed to proceed to the light–dark boundary before thiolutin was added, the kinetics of frq transcript decline in a vvdko strain were no longer slowed and resembled that of an untreated WT strain (compare fifth lane with top and third lanes of Fig. 3A). These data show that VVD targets frq transcription.

This conclusion was confirmed by an experiment in which we placed frq under the control of the QA-inducible promoter. The qa-frq construct was integrated into a frqko strain or a frqko vvdko double-mutant strain to generate qa-frq (frqko) and qa-frq (frqko vvdko) strains, respectively. In analogy to our QA-inducible VVDMYC expression system described above, this experiment allowed us to uncouple frq expression from its normal light-induced transcriptional regulation and study the reduction in frq transcript levels in a controlled manner after release from the inducer. If VVD targets frq transcription, replacing the frq promoter with the QA promoter should result in a similar drop in frq transcript levels in both the qa-frq (frqko) and qa-frq (frqko vvdko) strains, respectively. From the expression data on VVD levels, we observed some variability in QA-induced frq levels immediately following release from the inducer, but the kinetics of the decline of frq are similar in all strains and conditions tested (Fig. S3C). These data therefore support the conclusion that VVD influences frq RNA at the level of transcription and not via the exosome-mediated function of the FFC. Interestingly, the presence or absence of light had no significant influence on frq transcript levels in these strains.
and are used to control for loading of Northern blots. (Top) Total (T), nuclear (N), or cytoplasmic (C) extracts were prepared from Neurospora tissue grown for 24 h in DD (0) or with exposure to LL for the indicated times (h). Western blots were probed with an MYC antibody to detect VVD\textsuperscript{MYC}. The amido black-stained membrane serves as a loading control. (Bottom) Graph showing the percent ratio of nuclear to cytoplasmic signal using the Western blot data shown in A. (C) Neurospora extracts of strains expressing GFP-tagged VVD (under ccg-1 promoter control) grown for 24 h in DD or 4 h in LL. Western blots were probed with a GFP antibody to detect VVD\textsuperscript{GFP}, CP, cytoplasmic protein; NP, nuclear protein. (D) Subcellular localization of VVD\textsuperscript{GFP} (under ccg-1 promoter control) in Neurospora conidiospores fixed at time points DD24 and LL4. Each subpanel shows confocal images of fluorescence from DAPI-stained spores (Upper Left), fluorescence from the GFP signal (Upper Right), an overlay of both (Lower Right), and corresponding bright-field image (Lower Left). (Scale bar, 1 μm.)

Fig. 4. VVD is both a cytoplasmic and nuclear protein. (A) Total (T), nuclear (N), or cytoplasmic (C) extracts were prepared from Neurospora tissue grown for 24 h in DD (0) or with exposure to LL for the indicated times (h). Western blots were probed with an MYC antibody to detect VVD\textsuperscript{MYC}. The amido black-stained membrane serves as a loading control. (B) Graph showing the percent ratio of nuclear to cytoplasmic signal using the Western blot data shown in A. (C) Neurospora extracts of strains expressing GFP-tagged VVD (under ccg-1 promoter control) grown for 24 h in DD or 4 h in LL. Western blots were probed with a GFP antibody to detect VVD\textsuperscript{GFP}, CP, cytoplasmic protein; NP, nuclear protein. (D) Subcellular localization of VVD\textsuperscript{GFP} (under ccg-1 promoter control) in Neurospora conidiospores fixed at time points DD24 and LL4. Each subpanel shows confocal images of fluorescence from DAPI-stained spores (Upper Left), fluorescence from the GFP signal (Upper Right), an overlay of both (Lower Right), and corresponding bright-field image (Lower Left). (Scale bar, 1 μm.)

Fig. 3. VVD represses frq transcription. (A) Northern blots showing frq transcript levels in WT (top two lanes) and vvd\textsuperscript{ko} strains (lanes three, four, and five from top) in cultures grown in LL for 24 h (time point 0) or for different lengths of time (h) in DD. Cultures were grown in the presence (+) or absence (−) of the transcriptional inhibitor thiolutin. Thiolutin was added either 1 h before (−1 h) or immediately after (+0 h) liquid cultures were transferred from light to dark. Ethidium bromide-stained ribosomal RNA was used to control for loading of Northern blots. (B) Quantitative analysis of Northern blots (shown in A) of untreated WT (●) and untreated (▲) and thiolutin-treated (△) vvd\textsuperscript{ko} strains. Within each experiment maximum frq RNA levels were set to 100%.

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highly dynamic. In conclusion, our combined data confirmed that a substantial proportion of VVD localizes to the nucleus.

VVD Interacts with the WCC. The nuclear localization of VVD and VVD’s interaction with FRH led us to test whether VVD represses *frq* transcription by binding to the WCC. Such an activity would be consistent with the observation that VVD interacts with FRH, because FRH mediates the phosphorylation and inactivation of WCC (15, 32, 33). Moreover, VVD has been shown to influence WC-1 phosphorylation and thus WCC activity (16, 23). We performed Co-IP experiments in which Neurospora whole-cell lysates were incubated with WC-1 antiserum before probing for the presence of VVD. As can be seen in Fig. 5, VVD was immunoprecipitated in both LL (Fig. 5A and B) and DD (Fig. 5D).

Because the PAS/LOV domains of VVD and WC-1 are similar, it was important to control for the specificity of the WC-1–VVD interaction. Therefore we created strains in which *vvd* would could be expressed in a wc-1Δ background. Because *vvd* expression is dependent on WC-1, a QA-driven *vvd* gene was expressed to uncouple VVD expression from WC-1 expression. The qa-*vvd* construct was integrated into a *vvd*Δ or a *vvd*Δ wc-1Δ double mutant to generate qa-*vvd*ΔN (vre) and qa-*vvd*ΔC (vco) strains, respectively. In these strains VVDMYC is expressed at comparable levels, although VVD levels appeared slightly lower in the qa-*vvd*ΔN (vre) strain (Fig. 5C). In these experiments VVD was immunoprecipitated only in strains that express WC-1 protein (Fig. 5B). In another control experiment using qa-*vvd*ΔN (WT) and qa-*vvd*ΔC (127-11) strains, we observed that VVD is immunoprecipitated only in the qa-*vvd*ΔN (WT) strain (Fig. 5D). If VVD interacts with WC-1 via the WCC, we expected VVD also to interact with WC-2. However, our efforts to test this interaction by Co-IP were hampered by unspecific cross-interactions of the WC-2 and MYC antisera with VVDMYC and WC-2, respectively.

To test whether VVD must be signaling active for the interaction with WC-1, we tested two mutant strains that lack light-induced activity: *vvd*ΔC71S, which is biologically inactive because of its inability to homodimerize (26), and *vvd*ΔC71A, a strain in which the formation of a cysteinyl adduct that is critical for light signaling is impaired (18) (Fig. S2C). Both mutant proteins interacted with WC-1, suggesting that VVD does not need to be in a signaling-active state to interact with WC-1.

To investigate further whether VVD is part of higher molecular weight complexes, as suggested by our immunoprecipitation experiments, we performed sucrose gradient experiments (Fig. 5E). In agreement with previous results, we detected WC-2 in two peaks reported to be about 60 kDa and 200 kDa in size and found WC-1 to co-migrate with the higher molecular peak identified for WC-2 (33). FRH migrated in a single peak that overlaps both WC-1 and WC-2. The broad peak of VVDMYC spanning the fractions where monomeric WC-2 migrates is consistent with VVD forming a homodimer, as was suggested recently (24–26). The presence of VVD in fractions that contain the much higher molecular weight complexes of WC-1, WC-2, and FRH is consistent with our Co-IP data that showed an interaction of VVD with WC-1 and FRH.

Does VVD interact with DNA-bound WCC? We tested this possibility in EMSA (Fig. S5A). Nuclear proteins (LL 4 h) were extracted from *vvd*Δ, *vvd*ΔN, or *vvd*ΔC strains and incubated with a previously identified proximal light-responsive element (pLRE) located in the *frq* promoter (12). In line with published results (12, 34, 35), the free probe was caught in a high molecular weight complex when incubated with the nuclear extracts, and the complex was supershifted when incubated with WC-2 antiserum (Fig. S5). However, we observed no supershift when extracts were incubated with either MYC antibody (Fig. S5A) or GFP antibody (Fig. S5B), suggesting that VVD binds free rather than DNA-bound WC-1.

**Discussion**

We have shown that VVD interacts with central components of the Neurospora circadian clock and with components of blue-light signaling to inhibit the transcriptional activity of the WCC at dawn and dusk. The model shown in Fig. 6 depicts how VVD may exert its function at the dawn and dusk transitions. We know that WC-1 and WC-2 form multimers that can bind to proximal and distal GATN cis elements in the *frq* promoter (12). The complex formed in the dark (WCCp) is faster migrating (i.e., smaller) than the one at levels similar to the control strain *vvd*Δ, suggesting that VVD acts with FRH, because FRH mediates the phosphorylation and inactivation of WCC (15, 32, 33). Moreover, VVD has been shown to influence WC-1 phosphorylation and thus WCC activity (16, 23). We performed Co-IP experiments in which Neurospora whole-cell lysates were incubated with WC-1 antiserum before probing for the presence of VVD. As can be seen in Fig. 5, VVD was immunoprecipitated in both LL (Fig. 5A and B) and DD (Fig. 5D).

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formed in the light (WCC\textsuperscript{4}). The latter mediates transcription of light-induced genes, such as \textit{vvd} (12, 35). Light induction of \textit{frq} is regulated mainly through the proximal light-responsive element (LRE), whereas the distal element, although light responsive, is necessary for sustained rhythmicity of \textit{frq} in DD (12). We have shown here that VVD binds WC-1 both in LL and upon release into DD. It thus seems likely that VVD disrupts the efficient formation of the WCC complex, which leads to rapid inactivation of \textit{frq} transcription. WCC\textsuperscript{L} and WCC\textsuperscript{D} have different preferences for proximal and distal promoter elements, with WCC\textsuperscript{D} preferentially engaging at the distal clock box where negative feedback regulation by \textit{frq} takes place (11). (B) Simplified schematic of the \textit{frq} mRNA profile after light induction and in constant darkness in WT (thick line) and in a \textit{vvd}\textsuperscript{ko} strain (thin line). The gray area depicts the difference in \textit{frq} activation between the two strains and is a result of a change in equilibrium between WCC\textsuperscript{D} and WCC\textsuperscript{L}. Prolonged activation in the dark leads to a characteristic phase delay in the onset of \textit{frq} transcript oscillations and overt circadian rhythmicity.

Consequently, we favor the idea that the interaction is short-lived but not necessarily weak. This situation is reminiscent of the observation that only very small amounts of FRQ seem to interact with the WCC (15). Similarly, the observation that \textit{frq} does not appear to be part of the promoter-bound WCC (12) resembles our observation that antisera against VVD\textsuperscript{Myc} or VVD\textsuperscript{GFP} do not supershift the WCC. However, it is possible that these antibodies are less efficient in EMSA experiments or that a DNA-bound interaction is too weak or transient to be detected by EMSA; therefore, we cannot rule out a DNA-bound interaction at this stage. Interestingly, VVD mutants that are thought to be defective in photosignaling still can interact with WC-1 and FRH, suggesting that the repressive functions of VVD in light signaling may occur as part of a complex, with light-activation of VVD affecting the conformation of the entire complex. VVD’s role in repressing light responses aside, there is evidence that VVD promotes some aspects of light signaling (18), and a more recent microarray study suggests that VVD has a role in modulating late light responses in Neurospora (22). It therefore is likely that VVD may have functions that do not require its interaction with the WCC or FFC.

Finally, our data show that VVD can interact with FRH in the absence of a functional FFC or WCC, and it is tempting to speculate that FRH is the primary platform upon which the various complexes assemble.

Materials and Methods

Plasmids and Strains. Strain S4–3 (bd, a) was used as the WT in all experiments. A detailed description of how strains were created can be found in SI Materials and Methods. All strains were verified using PCR or Southern blot analysis. Homokaryons were generated as previously described (37).

Growth Conditions. Race tube and liquid culture experiments were carried out in Sanyo MLR-350 light- and temperature-controlled chambers as described in SI Materials and Methods. For transcription inhibitor experiments, thiolutin (Tocris Biosciences) was added at a final concentration of 12 μg/mL. Control samples were treated with DMSO.

Co-IP. Co-IP experiments and subsequent Western blot analysis were performed as described in SI Materials and Methods.

Cell Fractionation and EMSA. Cellular fractions were isolated essentially as previously described (38), and details are given in SI Materials and Methods. For EMSA, the \textit{frq} proximal LRE oligonucleotides (Eurofins MWG Operon) sequences described previously (12) were used to make the dsDNA probe. Binding conditions and gel electrophoresis are described in SI Materials and Methods.

Sucrose Gradients. Protein extracts were obtained using standard protein extraction buffer as described previously (30), and sucrose gradient experiments were carried out as detailed in SI Materials and Methods.

RNA Extraction and Northern Blot Analysis. RNA was extracted using the Qiagen RNAeasy mini kit according to the manufacturer’s instructions. Northern blot analysis was carried out as previously described (20) and as detailed in SI Materials and Methods.

Confocal Microscopy. Conidia were fixed in 4% formaldehyde for 1.5 h, washed in distilled H₂O, incubated in 50 μg/mL DAPI (Sigma) for 10 min at room temperature, and then washed and resuspended in 25% glycerol. Details for image collection are given in SI Materials and Methods.

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