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MicroCommentary

A kinase for light and time

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Summary

The eukaryotic filamentous fungus *Neurospora crassa* has proven to be a dependable model system for the analysis of light-regulated gene expression and circadian rhythmicity. The molecular basis of the core circadian clock is a transcription/translation-based feedback loop in which a heterodimer of the white-collar 1 (WC-1) and white-collar 2 (WC-2) proteins act as a transcription factor to promote expression of the negative elements in the clock, *frq* mRNA and FRQ protein. Additionally, WC-1 is a flavoprotein that acts as the primary responder to environmental light and, in conjunction with WC-2, is the major photoreceptor for the clock as well as other light-regulated processes. Protein kinase C acts as a light-dependent regulator of the WC-1 protein.

Introduction

Two common and tightly interconnected forms of organismal regulation are the ability to see environmental light in conjunction with the ability to tell the time of day. The capacity to gauge time of day, called circadian rhythmicity, is based at the cellular level; in organisms with clocks (most eukaryotes and the cyanobacteria), most or all cells of the organism have their own molecular clock. In fungi the ability to sense light using photoreceptive molecules is also common to all cells of the organism. The molecular bases of the photoreceptors have been the focus of intense study, using a combination of classic genetic and modern molecular and biochemical techniques. White-collar 1 (WC-1) is the *Neurospora* photoreceptor for the circadian clock as well as all other blue light-regulated genes and is also a central component of clock feedback mechanism. In the article by Franchi *et al*. in this edition, Guiseppe Macino's group reports another important advance in a story that has continued to provide fundamental insight into how this critical protein relays information, in this case to the cell via the regulation of WC-1 activity by protein kinase C (PKC). However, to appreciate this advance we will first need to provide some background to develop the context.

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Photobiology in *Neurospora*

All known light responses in *Neurospora* are specific to blue light. Responses to either red or far-red light have not been documented, although the *Neurospora* genomic sequence has revealed the possible existence of several photoreceptors including several bacteriophytochromes and a cryptochrome (Dunlap and Loros, 2004). It has been known for several decades that the blue light responses can be blocked by mutations in either of two genes, white-collar 1 (*wc-1*) or white-collar 2 (*wc-2*). Macino and colleagues cloned both genes (Linden *et al*., 1999), showing that the encoded proteins interact *in vitro* (to form the white-collar complex or WCC heterodimer), and speculated that these proteins might act together as the transcription factor directly regulating light-inducible genes with WC-1 as the actual photoreceptor. This scenario was verified by showing that WC-1 is the photoreceptor for the circadian clock in *Neurospora* (Froehlich *et al*., 2002; He *et al*., 2002) and that WC-1 and WC-2 interact *in vivo* at the *frequency* (*frq*) promoter (Froehlich *et al*., 2003). Independent of its role in the clock, the WCC heterodimer, as the primary photoreceptor in the fungal cell, is responsible for light regulation of several per cent of the genes in the genome (Lewis *et al*., 2002).

WC-1, a GATA-like transcription factor, is an 1167-amino-acid protein regulated both transcriptionally and post-transcriptionally at the level of synthesis and additionally through phosphorylation and protein–protein interactions. WC-1 contains a single, class 4 DNA-binding Zn finger domain, a poly-glutamine activation tract, two PAS (Per–Arnt–Sim) domains (see below) that are important in protein–protein contacts required for both light and clock functions (Lee *et al*., 2000), and an additional domain called LOV, for light, oxygen and voltage sensing,

a subclass of PAS domains. WC-1 binds flavin adenine dinucleotide (FAD) as its chromophore. The 530-amino-acid WC-2 protein is related to WC-1 at the sequence level and has single activation, Zn finger and PAS domains. WC-1 and WC-2 form the WCC via their PAS domains (see below). The WCC binds to specific consensus sequences, the light responsive elements (LREs), in promoters of light-regulated genes such as *frq*. The WCC Zn finger domains are thought to be sites of DNA interaction. A model for regulation proposes that when the FAD absorbs blue light it undergoes a transient covalent interaction with a cysteine in the WC-1 LOV domain, inducing a conformation change in the protein and leading to activity of the complex. This activation is thought to occur by the formation of quaternary interactions between WCCs to form a dimer of heterodimers or tetrameric complex with enhanced transcriptional activation ability.

The *Neurospora* clock is a transcription/translation feedback loop requiring the WCC

A true circadian rhythm is an oscillation in a molecular, biochemical, physiological or behavioural function that is generated by a genetically based clock. In the real world, these biological clocks are entrained to exactly 24 h by their ability to sense the rhythmic changes of light and temperature that occur from the daily rotation of the planet. In the absence of these environmental cues (constant temperature and dark), circadian rhythms will continue with an endogenous period length close but usually not exactly equal to 24 h (Dunlap and Loros, 2004).

A circadian oscillator, like any self-sustaining oscillator, must have both a positive element or elements to drive the loop forward (alter the existing condition) and a negative element or elements to slow or block the rate of response to the change. Eukaryotic circadian systems described to date actually operate using multiple interlocked positive and negative regulatory connections, with possibly the simplest system being that described in *Neurospora*. The WCC fulfils the roll of positive central component of the clock, while both the mRNA and the two overlapping proteins, formed from alternative splicing at the *frequency* (*frq*) locus (H.V. Colot, J.J. Loros and J.C. Dunlap, unpubl.), operate as negative components. The WCC promotes expression of the *frq* locus by direct binding to sequence-specific elements in the *frq* promoter, resulting in the production of two distinct FRQ proteins that feed back to block WCC activity (Froehlich *et al*., 2003; Dunlap and Loros, 2004). This results in the rhythmic expression of *frq* mRNA and FRQ proteins in a daily pattern. The FRQ proteins act to limit the abundance of its own transcript through its physical interactions with the WCC, thereby generating a negative feedback loop. After synthesis, FRQ is progressively and continuously phosphorylated, leading to its eventual turnover via the ubiquitin ligase pathway, allowing the WCC to reinitiate the next cycle of *frq* expression. All points within the molecular cycle of *frq* and FRQ, even in constant conditions, correspond to actual times within the physical day (reviewed in Liu, 2003; Dunlap and Loros, 2004).

The *Neurospora* clock entrains to light via the WC-1 photoreceptor

A classic property of circadian systems is the bidirectional response to entraining agents (reviewed in Liu, 2003). In order for a clock to be functional it must respond to light seen late at night or predawn by advancing the clock forward to the coming day. Conversely, if light is seen just past dusk or early in the night, it needs to delay the clock back to the previous day. The molecular basis of resetting by light requires opposite effects on the timing mechanism that are dependent on the time of day when the light is perceived. Additionally, in *Neurospora* and many other circadian systems, the light response system is regulated by the clock such that the incoming light signal is rhythmically modified or 'gated', even though the WC-1 photoreceptor protein is capable of seeing light at all times of day. This gating is conferred in part by VIVID (VVD), another photoreceptor and member of the PAS protein superfamily that binds a flavin chromophore and modulates the WC-1 signal (Heintzen *et al*., 2001; Schwerdtfeger and Linden, 2003).

In the *Neurospora* clock, *frq* mRNA cycles with a peak during the subjective day. FRQ protein expression lags by about 4 h, peaking late in the day, near dusk. In constant conditions, therefore, subjective day is defined as the time when these components are at their highest levels. Conversely, subjective night corresponds to the troughs in the cycles of *frq* and FRQ abundance. By these criteria it is clear that photic induction of the components would result in readjustment of the phase of the rhythm, a process called entrainment. In *Neurospora* this mechanism of light-driven entrainment is fairly well understood. Light acts through the WCC to induce *frq* expression rapidly, resulting in a large and transient increase in *frq* and FRQ that, depending on the time the light falls in the *frq*/FRQ cycle, will push the peak of the rhythm either forward (an advance) or backward (a delay). During the evening or early night, *frq* and FRQ levels fall such that *frq* induction will result in a phase delay while a pulse of light during the late night or predawn, when *frq* and FRQ are low, will advance the phase of the clock.

Photoadaptation is the temporary insensitivity of the organism to respond to a second light pulse or to an increase in light intensity. Post-translational modification of WC-1 that correlates with the light-induced turnover of WC-1 (see below) is thought to be part of the basis of

photoadaptation. VVD is induced in response to light and acts as a repressor of WC-1 activity, also altering subsequent responses to light.

Control of the WC-1 photoreceptor

Many blue light-inducible, WCC-regulated genes are known in *Neurospora*. The initial burst of gene expression induced by light is transient, regardless of whether the light stimulus is delivered as a pulse or is continuous. Additionally, both the rapidity and duration of the response is gene dependent. The response can be of the immediate type with a peak at 15 min, as with the *frq* gene, or take up to 30 min to be detectable with a peak of transcript abundance appearing between 90 min and 2 h, as with the *eas* (*ccg*-2) gene. The WC-1 photoreceptor is also transiently hyperphosphorylated in response to light and this corresponds to the brief induction of gene expression in its targets. The hyperphosphorylation of WC-1 is correlated with turnover of the photoreceptor and the downregulation of the light-induced activation of transcription. WC-1 is positively autoregulatory, meaning that its own transcription is induced by light because increasing WC-1 feeds back to increase *wc-1* mRNA abundance further (reviewed in Linden *et al*., 1999; Liu, 2003).

In the dark, FRQ is a major post-transcriptional regulator of WC-1 although the mechanism of this regulation is not understood (Lee *et al*., 2000). Levels of WC-1 begin to rise sometime after FRQ appears, resulting in a rhythm in WC-1 protein that is close to anti-phase with FRQ. WC-1 levels reach their peak in the late night as FRQ levels reach their trough. WC-1 is also regulated via protein–protein interactions and WC-1 influences WC-2 levels and visa versa. Importantly, though, all of these proteins are subject to hitherto poorly characterized phosphorylation events that might have profound effects on the activities or interactions of each protein. This is the major contribution of the most recent study from the Macino lab.

Previous work using pharmacological inhibitors by Guiseppina Arpaia and colleagues from the Macino lab suggested PKC as a possible player in light-induced gene expression and the phosphorylation of the WC-1 protein (Arpaia *et al*., 1999). Now, Franchi and colleagues have dramatically confirmed and extended this by demonstrating a physical interaction between the WC-1 protein and PKC through an epitope tag strategy. This association is found in the dark but, remarkably, is lost immediately after light exposure, only to return again 2 h after the initial light pulse. Association was never seen within the first 20 min after light exposure, even when modification of WC-1 could be detected, indicating that PKC is not associated with WC-1 during light-activated transcription and is not responsible for the WC-1 modifications seen at 20 min. The ability of the Zn finger region of WC-1 to act as a phosphorylation substrate of immunopurified PKC was also demonstrated.

However, the most important question is what is the role of the phosphorylation? To answer this question, Franchi *et al*. developed and employed a sturdy set of tools and set about using them to dissect the role of PKC-driven phosphorylation of WC-1. These tools included a kinase-inactive mutant that was still expressed and whose protein could interact with WC-1, but would not phosphorylate it (in this regard the mutation is a dominant negative). The second construct encoded a constitutively active kinase. Altogether, the application of these tools yielded a much clearer understanding of the role of PKC phosphorylation in WC-1 regulation. Both proteins, when overproduced in a wild-type strain, conferred the ability to alter levels of WC-1. Overproduced catalytically inactive PKC resulted in increased levels of WC-1 protein, while the constitutively active kinase causes the levels of the photoreceptor to decrease in light and dark. Surprisingly, in neither case was the transient hyperphosphorylation of the photoreceptor altered specifically in response to light. This surprising result suggests the existence of a further kinase(s), possibly involved in WC-1 stability and turnover or even transcriptional activity. Because WC-1 is autoregulatory, the authors predicted and then confirmed that a change in PKC activity exerts an additional effect on *wc-1* expression. Also as predicted, changes in PKC indirectly lead to changes in FRQ levels by regulating WC-1. All of these observations lead the authors to conclude that light decreases the stability of the PKC/WC-1 interaction and that PKC acts in conjunction with light to destabilize the WC-1 protein (as modelled in Fig. 1), although they do caution that the data are also consistent with phosphorylation of WC-1 by PKC reducing its activity as a transcription factor.

Several important questions remain. What is the other kinase and what is its role in WC-1 regulation? Where are the phosphorylation sites on WC-1 and which of them are the most important? Do these phosphorylation sites independently affect protein–protein interactions and stability or is stability affected as a by-product of changed interactions? Does the action of PKC on WC-1 protein contribute to the photoadaptation response and, finally, what is the role of VVD in WC-1 regulation?

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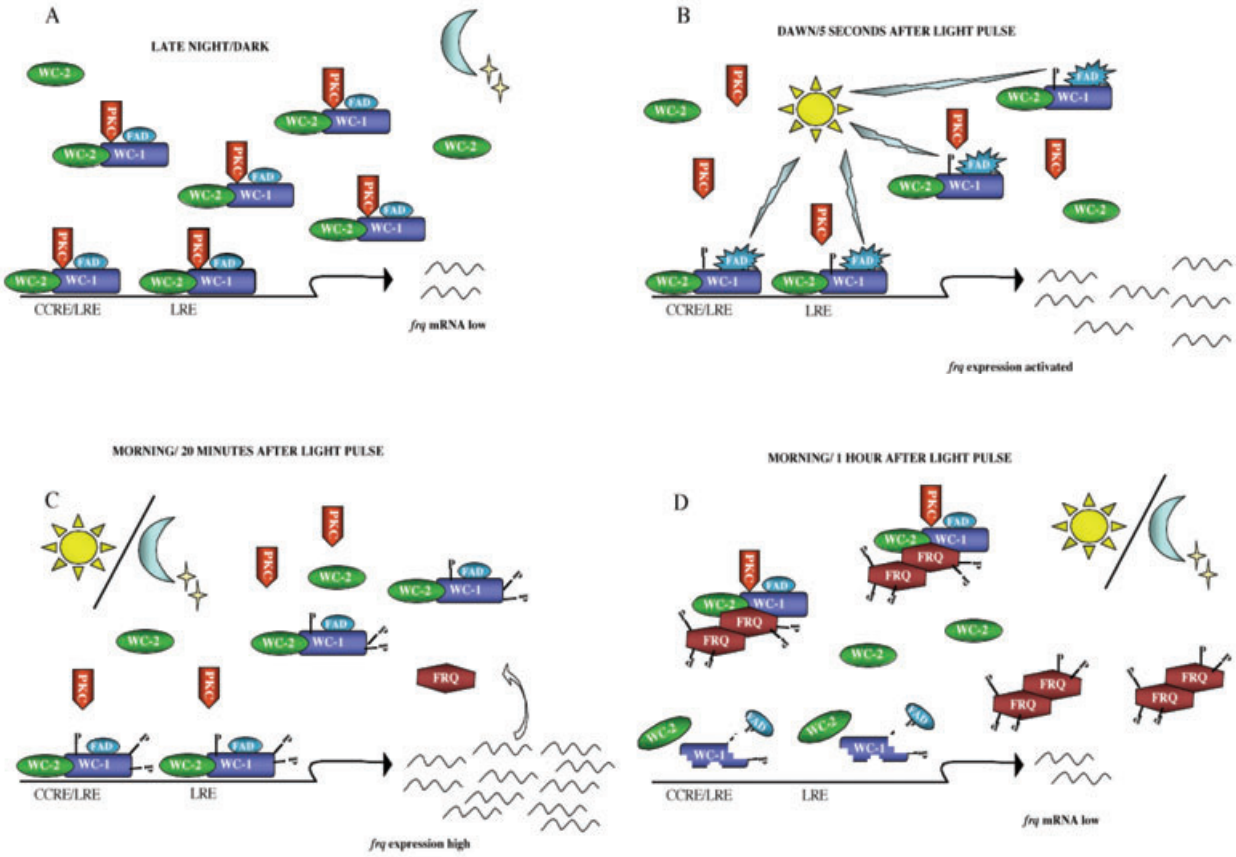

Fig. 1. A model for the action of the white-collar complex (WCC) and PKC during photoinduction of the *frq* gene.
A. In darkness, or late at night, high levels of WCC are associated with PKC and *frq* mRNA levels are low. WC-2 levels are always high.
B. Five seconds after a light pulse, or at dawn, FAD on WC-1 absorbs a photon, WC-1 is phosphorylated by PKC, PKC disassociates from the WCC and *frq* expression is highly induced.
C. Twenty minutes after a light pulse, or constant light, *frq* expression is very high, FRQ protein begins to appear, PKC remains disassociated from the WCC, WC-1 becomes increasingly phosphorylated by an unknown kinase and new *wc-1* mRNA is expressed from light activation of the *wc-1* promoter.
D. One hour after a light pulse or under constant light, WC-1 is increasingly phosphorylated and is turned over, WCC made from newly synthesized WC-*1* is complexed with the high amounts of newly synthesized FRQ, preventing binding to the *frq* promoter and *frq* expression drops to basal levels.

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