Light-Dependent Sequestration of TIMELESS by CRYPTOCHROME

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Most organisms have circadian clocks consisting of negative feedback loops of gene regulation that facilitate adaptation to cycles of light and darkness. In this study, CRYPTOCHROME (CRY), a protein involved in circadian photoperception in Drosophila, is shown to block the function of PERIOD/TIMELESS (PER/TIM) heterodimeric complexes in a light-dependent fashion. TIM degradation does not occur under these conditions; thus, TIM degradation is uncoupled from abrogation of its function by light. CRY and TIM are part of the same complex and directly interact in yeast in a light-dependent fashion. PER/TIM and CRY influence the subcellular distribution of these protein complexes, which reside primarily in the nucleus after the perception of a light signal. Thus, CRY acts as a circadian photoreceptor by directly interacting with core components of the circadian clock.

Many physiological processes display daily fluctuations that accompany cycles of light and dark. The persistence of these rhythms under constant conditions points to the existence of an endogenous timekeeping mechanism. Light and temperature are the major environmental signals responsible for synchronizing this endogenous clock to the ambient environmental conditions. In Drosophila, as well as in other organisms (1), some of the molecules involved in sustaining this cellular oscillation are known, namely PERIOD, TIMELESS, CLOCK (CLK), BMAL/MOP3/CYCLE, and DOUBLETIME (2–4). CLK and CYCLE (CYC) are basic helix-loop-helix–PAS proteins that act together to bind an E-box element in the promoters of the circadian clock genes period and timeless (4, 5). In Drosophila, per and tim mRNA and protein levels cycle every 24 hours, the proteins lagging a few hours behind the mRNA. PER and TIM proteins accumulate within the cytoplasm, form heterodimers, and together they translocate to the nucleus where they repress their own transcription. This negative feedback is required for the cycling of their corresponding mRNAs. Under entrained conditions, such repression is released the following morning when light signals are perceived, followed by a rapid disappearance of TIM protein (1).

A gene product required for circadian photoperception has been recently identified in Drosophila (6–8). This gene, cryptochrome, is involved in transducing photic information from the environment to the core oscillator in plants (9) and flies (6–8), whereas in mice CRY may play a role in clock function as mutations in CRY lead to period or arrhythmic aberrations, with no direct evidence for photoreceptor function (10, 11). Whether CRY functions as the photoreceptor or elsewhere in the input pathway is controversial, and nothing is known of the transduction events that connect photoperception to clock resetting. In support of CRY having a direct role as a photoreceptor, CRYs are closely related to DNA photolyases, molecules that transduce blue light energy into DNA repair activity (12). Some CRYs bind the same chromophores as photolyases, namely pterin and flavin, and participate in a light-dependent redox change of the chromophore (13, 14). Since the levels of Drosophila TIM are regulated by light (15, 16), it is likely that TIM is a downstream target of CRY. Spectral response curves for both TIM degradation and phase shifts in locomotor activity show maximal responses at 400 to 450 nm (17), directly overlapping the CRY absorption spectrum (18). However, the arrhythmicity in double CRY/CRY2 knockout mutant mice suggests that CRY may be still more intimately associated with the core clock machinery (11). In no species has a light-dependent biological activity of CRY been shown in vitro or in a heterologous system. We therefore investigated whether CRY could interact directly with core clock proteins, in an effort to reconstitute the initial events of circadian phototransduction.

CRY Plus Light Blocks PER/TIM

To understand how light resets the clock, we used our previously established cell-based assay for PER/TIM biological activity (4). The rationale behind this approach lies in the fact that Drosophila TIM protein is the only clock component known to rapidly change in abundance in response to light in vivo. The CLK/CYC complex induces the tim promoter, and CLK/CYC activation is greatly reduced in the presence of the PER/TIM complex (4). A Drosophila embryonic cell line was transiently transfected with a reporter construct carrying the luciferase gene under the control of the Drosophila tim promoter. Constructs expressing clk, per, and tim from a Drosophila actin promoter (19) were cotransfected in different combinations together with the reporter construct to assess the role of the CRY protein in the regulation of the tim promoter (20). We found that CLK/CYC’s positive activity on the tim promoter was not directly modified by the presence of CRY (Fig. 1). Likewise, PER/TIM inhibition of such activity was observed irrespective of illumination when...
CRY was absent in the cells. However, whenever CRY was present in the transfected cells under constant light conditions, the PER/TIM complex no longer exerted its negative effect on CLK/CYC’s activity, allowing full activation of the tim promoter (Fig. 1). Both CRY and light were required to elicit this effect. Similar results were obtained with the per E-box (4) as a target (21). Since TIM disappearance in the presence of light can occur in the absence of PER (16), CRY’s effect on the PER/TIM complex most likely involves the TIM protein itself.

When a construct containing the cryb mutation was used, CRYb no longer affected PER/TIM activity (Fig. 1). This single point mutation maps to a highly conserved region involved in chromophore binding and renders the protein at least partially inactive in vivo (6). Thus the light dependent effect of CRY on PER/TIM function is likely to be a result of the normal activity of CRY.

CRY and TIM in the Same Complex

The molecular nature of phototransduction steps between CRY and its target TIM (or the PER/TIM complex) are unknown. One potential mechanism is the formation of protein complexes between CRY and TIM. To test whether CRY and TIM can be found in the same protein complex, we performed coimmunoprecipitation (coIP) assays in S2 cells. In transient transfection assays, S2 cells stably expressing TIM (22) were further transfected with cry-gfp (23), which acts to inhibit PER/TIM functions in the same manner as CRY alone (21). Forty-eight hours after transfection, coimmunoprecipitations assays were performed (24). By using either antibody to TIM or antibody to green fluorescent protein (GFP) in the immunoprecipitation step, we were able to detect both proteins by protein immunoblot (Fig. 2). This interaction was specific for CRY and TIM because we were unable to precipitate GFP together with TIM when only free GFP was transfected.

The light-dependent effect of CRY on PER/TIM action can be explained in two ways: (i) CRY and TIM could interact in darkness and only upon illumination would CRY undergo a conformational change that leads eventually to abrogation of PER/TIM function; alternatively, (ii) light could be the key event that allows these two proteins to come into physical contact. To distinguish between these possibilities we performed coIP assays on transfected cells that had been maintained in continuous darkness, or in continuous light, or were given a 1-hour light pulse immediately before harvesting. We detected CRY/TIM specific interaction in dark-cultured cells, and this interaction was not enhanced after a 1-hour light pulse (Fig. 2). In cells exposed to continuous light, we detected less CRY-GFP, which points to the light-labile nature of the CRY protein, in agreement with previous data (7). The same result was observed in the absence of TIM, indicating that CRY-GFP is unstable in the light (21).
Importantly, TIM protein does not appear to be degraded under the same conditions (Fig. 2). Taken together, these results demonstrate that light-dependent TIM degradation can be uncoupled from the light-dependent inhibition of PER/TIM heterodimer function by CRY.

**Light Promotes CRY and TIM Interaction**

The coIP assays suggest that CRY and TIM reside in the same protein complex. To determine whether CRY and TIM interact in the absence of other *Drosophila* proteins or any recognizable homologs of circadian clock components, we employed a yeast two-hybrid assay as a heterologous system. Because human CRY proteins acquire the flavin and pterin chromophores after expression in bacteria (15), we reasoned that *Drosophila* CRY would likely acquire the chromophores in yeast, permitting detection of potential light-dependent interactions between CRY and TIM or other circadian clock proteins. All two-hybrid experiments were, therefore, carried out in parallel sets in which yeast transformants were divided into two pools, one grown in constant light and the other in darkness (25).

A positive control interaction between PER and TIM was indistinguishable in yeast grown under light or dark conditions (Fig. 3A), as was the interaction between CLK and CYC (26), indicating that in yeast cells the two-hybrid assay itself is insensitive to light. CRY showed a robust interaction with TIM that was dependent on light, and that was also observed when TIM was bound in a complex with PER. No interactions were detected between CRY and PER (Fig. 3A) (27) or between CRY and CLK, CYC, or the CLK/CYC complex under light or dark (26). These findings show that CRY functions as a photoreceptor.

We found that the CRY fusion protein was unable to interact with TIM in yeast cells under our conditions (Fig. 3A). This could not be ascribed to an intrinsic instability of the fusion protein in yeast, as it has been proposed for the mutant protein in the fly (6), since the yeast fusion protein was easily detected in protein immunoblots (Fig. 3B) (25). The most likely explanation is that a photochemically functional CRY is required for a direct interaction between CRY and TIM after photoreception by CRY. We conclude that the ability of CRY to block the negative feedback action of the PER/TIM complex in a light-dependent manner reflects its light-dependent binding to TIM.

The sensitivity to light of the CRY/TIM interaction in yeast contrasts with the results obtained in the coIPs (Fig. 2). This discrepancy may be due to the fact that the experiment in Fig. 3 (as well as in Fig. 1), reflects phenomena taking place within the nucleus, whereas the coIP detects interactions in all cellular compartments. Additionally, we may not be achieving quantitative recovery of complexes in the coIP assay and the detected interaction in darkness may well be overrepresented. It is also possible that additional fly proteins present in S2 cells contribute to the detection of CRY and TIM in the same protein complex in darkness, but that light is still required for a direct physical interaction between these two components.

**CRY Localization Depends on PER/TIM and Light**

To determine the subcellular localization of the CRY protein, S2 cells expressing a CRY-GFP fusion protein were visualized by fluorescence microscopy (28). In most cells some fluorescence was distributed throughout the cytoplasm, but most of the signal accumulated in the nucleus and perinuclear region as confirmed by DAPI (Figs. 4, A and B) or propidium iodide (21) staining. Optical sections obtained by confocal microscopy clearly demonstrated that CRY-GFP signal was indeed found within the nucleus (21). The nuclear and cytoplasmic localization of CRY is in agreement with the notion that CRY is likely to be exerting its role in both compartments (16). A small proportion of the transfected cells (~5%) showed large accumulations of aggregated protein in the form of cytoplasmic globular structures (21). Cells stained with MitoTracker showed no correlation between CRY-GFP and mitochondrial localization (21), in contrast to what has been reported for mCRY1 (29). Light pulsed cells did not show any change in localization (Fig. 4B), even if kept under that condition for several hours (22).

To investigate CRY-GFP localization in the presence of TIM, we transfected a cry-gfp fusion construct into a stably transformed S2 cell line expressing TIM. Immunostaining with antibody to TIM confirmed the constitutive cytoplasmic accumulation of TIM (21). TIM has been previously demonstrated to be cytoplasmic in the absence of PER (30). No evident changes in the pattern of CRY-GFP localization were observed in the presence of TIM, irrespective of the light treatment (Fig. 4, A and B).

**Fig. 4.** In the presence of PER/TIM the subcellular localization of the CRY protein changes upon illumination. WT or TIM expressing S2 cells were transfected with cry-gfp either alone or together with per, and 48 hours after transfection the subcellular localization of GFP-associated fluorescence was determined. (A) The color-coded boxes represent the main fluorescent patterns of CRY-GFP accumulation at the end of the experiment. DNA was stained with DAPI. Superimposition of GFP and DAPI images clearly demonstrates the nuclear localization of CRY-GFP (overlay). Scale bar, 2 μm. (B) Quantitative analysis of CRY-GFP subcellular accumulation. One hundred cells were analyzed in each treatment. Filled and open bars at the bottom of the figure indicate cells kept under DD conditions throughout the experiment or treated with a 3-hour light pulse, respectively.
Transient expression of CRY-GFP together with PER in the TIM stable cell line showed that in about 10% of the cells CRY-GFP localized to speckles primarily in the nucleus (Fig. 4, A and B), suggesting that the presence of PER and TIM is responsible for such compartmentalization of CRY-GFP. This particular localization pattern was more often observed upon a 3-hour light treatment (about 40% of the cells).

Under these conditions some cells displayed only homogenous nuclear fluorescence without signs of perinuclear or cytoplasmic accumulation (Fig. 4B).

Our results show that CRY confers light sensitivity to a heterologous system, indicating that in the fly CRY acts as a circadian photoreceptor, as opposed to elsewhere within the input pathway or the core clock mechanism. Furthermore, CRY undergoes a photochemical change that allows it to interact directly with the input pathway or the core clock mechanism. Sensitivity to a heterologous system, indicating a possible clock adaptation in the experiments including anti-rat-CRY antibody incubation reaction. CoIP assays were performed as described above, and the whole experiment, and cells were harvested under the same conditions and cells were harvested immediately after.


S. Okano et al., Photocem. Photobiol. 69, 108 (1999); C. P. Selby and A. Sancar, ibid., p. 105.

M. Sonnemann et al., Development 124, 4571 (1997). Expression plasmids contained the complete coding region of the indicated gene fused to the Drosophila actin promoter (pAct-19). The tim-luc reporter plasmid contained 6.5 kb of the tim promoter fused to firefly luciferase (4). As a control for transfection efficiency, we used 0.05 µg of a plasmid consisting of the Drosophila copia promoter inserted into pCDNA3 (Promega), which contains Renilla luciferase or 0.1 µg of a construct containing the minimal hsP70 promoter driving lacZ. CYC is endogenously expressed in S2 cells, so addition of CYC in a construct expressing CYC (19). The result was that the perinuclear or cytoplasmic accumulation of CRY was not required to see the transactivation effect (4). S2 cells in 12-well plates were transfected with Lipofectin (GIBCO-BRL), according to the manufacturer’s recommendations. Each transfection contained 1.0 µg pAct-cry, pAct-cry or pAct-alone (0.01 µg pAct-tim and pAct-per or 0.02 µg pAct; 0.005 µg pAct-clock; and 0.5 µg pAct-cell. Cells were kept at 22°C in constant light (40 µmol). The dark-cultured samples were wrapped in foil and kept in the same chamber. Cells were harvested 48 hours after transfection, and enzyme activity was measured with Dual-Luciferase Reporter Assay (Promega), β-galactosidase Assay System (Promega), and Galacto-Light Plus (Tropis) as appropriate. For each transfection, reporter activity was normalized to control enzyme activity. Reporter activity is plotted relative to activity co-transfected with pAct-clock. Values are the mean ±SEM of 4 to 6 replicate experiments.


S. Okano et al., Photocem. Photobiol. 69, 108 (1999); C. P. Selby and A. Sancar, ibid., p. 105.