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 CRY1/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*, *tim*, and *cry* 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

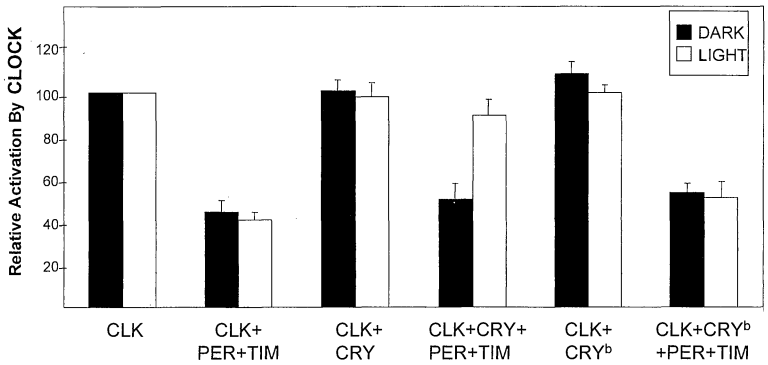

Fig. 1. Light and CRY block PER/TIM inhibition of CLOCK-mediated transcription. *Drosophila* S2 cells were transfected with pAct expression plasmids harboring CLK, PER, TIM, and CRY (or CRY^b) (*20*), together with a reporter plasmid driving firefly luciferase expression from the *timeless* promoter (*tim-luc*). Cells were kept under constant temperature in continuous light (white bars) or continuous darkness (black bars). Data was normalized to a cotransfected reporter plasmid and expressed as percent of CLK activity under the same conditions (dark or light) in the absence of any other protein.

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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 cry^b mutation was used, CRY^b 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.

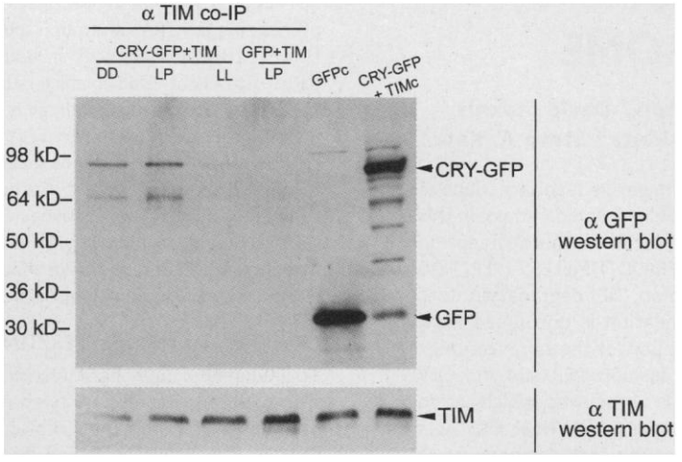

Fig. 2. CRY and TIM are part of the same complex in S2 cells. Protein immunoblot (western blot) analysis of the protein complex immunoprecipitated with rat antibody to TIM and antibody to rat IgG coupled to agarose (Sigma) detected with rabbit antibody to GFP (Molecular Probes), stripped and reprobed with anti TIM. Transiently expressed proteins in each case are noted on the top of each lane, as well as the light treatment received: continuous dark (DD), 1-hour light pulse (LP) and continuous light (LL). "c" indicates samples that were not subjected to immunoprecipitation and are used as positive controls. The same amount of total protein was loaded in each lane. CoIP experiments were repeated four times with antibody to TIM and four times with antibody to GFP. A representative blot is shown.

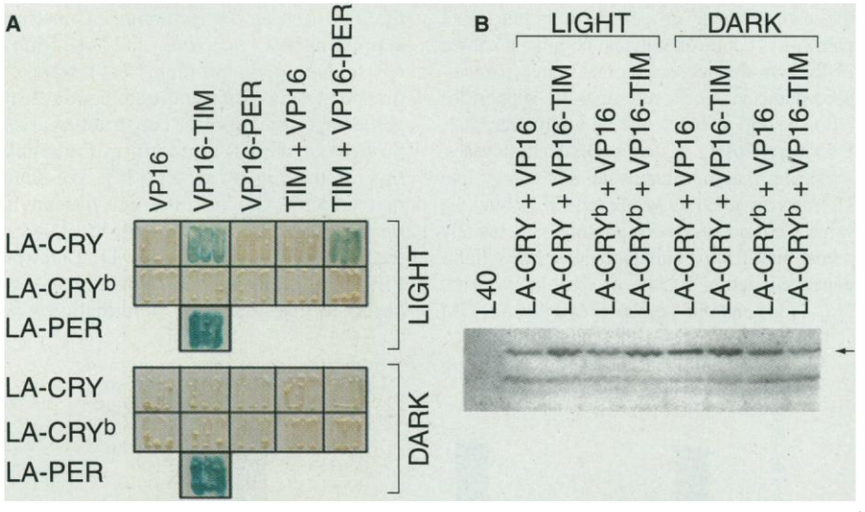

Fig. 3. Light-dependent interaction of CRY with TIM and with the PER/TIM complex. (**A**) Yeast two-hybrid assay (*25*). Blue precipitate represents cumulative β-galactosidase activity, resulting from activation of the *lacZ* reporter gene by protein-protein interaction. Triplicate yeast patches expressing the indicated LEXA hybrid (rows) and the indicated VP16 hybrid (columns) were derived from three independent transformants. Native full-length TIM was additionally expressed in some yeast patches, as indicated (columns). CRY, CRY^b, PER, and TIM hybrid proteins were full length, except for VP16-PER, which was PER (233-685) in this experiment (*27*). Yeast were grown in constant light or in darkness from the time of transformation with plasmid DNA (*25*). (**B**) No effect of light, the cry^b mutation, or interaction with TIM on the steady-state level of LEXA-CRY protein in the yeast two-hybrid strain. Protein immunoblot showing extracts of yeast expressing the indicated LEXA and VP16 proteins probed with antiserum to LEXA (*25*). Light or dark as in (A). Arrow marks the expected size of the LEXA-CRY hybrid protein. The second band corresponding to a smaller protein likely represents a truncation or degradation product of LEXA-CRY, since neither it nor the band corresponding to the correct fusion protein was detected in the parental L40 yeast two-hybrid strain (first lane). No other bands were observed. The same amount of total protein was loaded in each lane.

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 (*13*), 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^b fusion 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 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 photoperception 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 (*21*).

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).

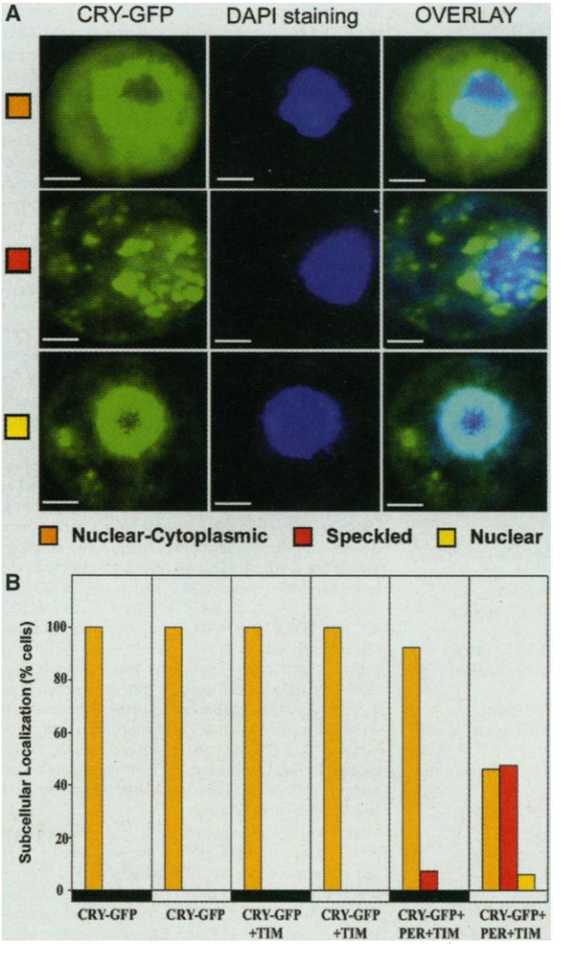

Fig. 4. In the presence of PER/TIM the subcellular localization of the CRY protein changes upon illumination. WT or TIM expressing S2 cells were transfected with *cry-gfp* either alone or together with *per*, and 48 hours after transfection the subcellular localization of GFP-associated fluorescence 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, 2μm. (**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 TIM both in the cytoplasm and the nucleus. This interaction represents the initial step in circadian phototransduction, and renders the PER/TIM complex inactive and unable to participate in negative feedback. In the intact animal, the rise in *tim* transcription 3 to 4 hours after dawn (*31*) must be a consequence of activated CRY preventing feedback inhibition. TIM degradation is not absolutely required for inhibition of PER/TIM complex function. TIM breakdown may, therefore, be a downstream consequence of the light-dependent complex formation between CRY and PER/TIM heterodimers.

References and Notes

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20. Expression plasmids contained the complete coding region of the indicated gene fused to the *Drosophila actin 5C* promoter in 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 pRL-null (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 a construct expressing CYC is 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 0.1 μg pAct-*cry*, pAct-*cry*^{b} or pAct alone; 0.01 μg pAct-*tim* and pAct-*per* or 0.02 μg pAct; 0.0005 μg pAct-*clock*; and 0.5 μg pAct. Cells were kept at 22°C in continuous 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 Enzyme Assay System (Promega), and Galacto-Light Plus (Tropix) as appropriate. For each sample, reporter activity was normalized to control enzyme activity. Reporter activity is plotted relative to activity when co-transfected with pAct-*clock*. Values are the mean ± SEM of 4 to 6 replicate experiments.
21. M. F. Ceriani, T. K. Darlington, P. Más, S. A. Kay, unpublished observations.
22. A stable S2 cell line expressing full-length *tim* under the *Drosophila actin 5C* promoter was generated essentially as described (*30*) with Lipofectin (GIBCO-BRL) as the transfection reagent and 20 to 1 ratio pAct-*tim* to pCopHyg. This TIM stable cell line was then transiently transfected with Effectene (Qiagen) with 0.4 μg of pAct-*cry-gfp* alone or together with 0.4 μg of pAct-*per* (pAct was used to keep the amount of DNA per well constant). Free GFP expressed under the same promoter served as negative control. Cells were kept at 22°C under constant light condition (~50 μmol); the dark control plates were wrapped in foil during the whole experiment, and cells were harvested under red safe light; the 1-hour light pulse was performed under the same conditions and cells were harvested immediately after.
23. In order to detect CRY, we tagged it with GFP, generating a CRY-GFP fusion (*32*). When used in place of pAct-*cry* in experiments such as those of Fig. 1, pAct-*cry-gfp* generated indistinguishable results, thus arguing that the fusion protein can adopt a functional conformation (*21*).
24. We pooled 3.2 × 10^{6} cells for each immunoprecipitation reaction. CoIP assays were performed as in (*33*) whenever Gammabind (Pharmacia) was used, or following the manufacturer's recommendation in the experiments including anti–rat-sepharose (Sigma). After 48 hours, transfected cells were washed twice in PBS and lysed in 20 mM Hepes pH 7.5; 100 mM KCl; 0.05% Triton X-100; 2.5 mM EDTA; 5 mM DTT; 5% glycerol; aprotinin (10 μg/ml); leupeptin (10 μg/ml); and pepstatin (2 μg/ml). Each coIP experiment was repeated four times. Proteins were loaded into a precast 4 to 15% acrylamide gel (BioRad). Protein immunoblots were performed with 1/2000 dilution of rat antibody to TIM (*16*) or rabbit antibody to GFP (Molecular Probes), followed by HRP-conjugated secondary antibodies. The signal was visualized with the ECL kit (Amersham).
25. Yeast two-hybrid experiments and β-galactosidase filter-lift assays were carried out as described (*3*). We constructed LEXA-CRY hybrids with a hydrophilic 10–amino acid linker [$(Gly_5Ser)_2$] separating the LEXA and CRY sequences; a direct amino-terminal fusion of LEXA to CRY showed the same results but produced distinctly weaker signals. Yeast were cotransformed with three selectable expression plasmids, a LEXA plasmid (*TRP1* marker), a VP16 plasmid (*LEU2* marker), and pMET25::ADE2 (*ADE2* marker), either nonrecombinant or containing a full-length *tim* cDNA. pMET25::ADE2 was derived from p426MET25 (*34*) by removing the NdeI-KpaI *URA3* fragment and replacing it with a 3.6-kb Bam HI *ADE2* fragment by blunt-ligation. For light and dark growth conditions, each yeast transformation reaction was split onto two plates of selection medium. The plates were incubated next to one another at 30°C under a NEC 8-W Cool White fluorescent light source (38-cm distance), but one was wrapped in foil to block the light. After 3 days, yeast colonies from each condition were patched in duplicate onto two fresh selection plates and grown for 1 to 2 days in light or dark as above. From each duplicate pair of plates from each condition, one was used for two-hybrid interaction assays and one for immunoblot analysis. For immunoblot analysis, light- and dark-grown patches were inoculated into 2 ml of liquid selection medium and shaken overnight in light or dark as above. Cultures were diluted into 20 ml of selection medium, grown in light or dark as above to an optical density of ~0.7, and total protein was determined (Bio-Rad). 3-ml aliquots were pelleted, dissolved in 70 μl of Laemmli buffer, and diluted in Laemmli buffer to equalize the total protein concentration of the different samples. Samples were run on 7.5% SDS-PAGE, blotted onto nitrocellulose, and immunodetection performed with rabbit anti-LEXA antiserum (1:2000) and goat anti-rabbit IgG–HRP (1:5000). Signals were detected by enhanced chemiluminesence (Amersham).
26. D. Staknis and C. Weitz, unpublished observations.
27. In additional experiments of slightly different design, no interaction was detected between LEXA-PER (full length) and VP16-CRY under conditions in which interactions were detected between LEXA-PER (full length) and VP16-TIM and between LEXA-TIM and VP16-CRY.
28. Cells in a 12-well plate setting were transfected as above (*22*) and kept under constant conditions covered in foil until the end of the experiment. In a blind experiment 100 cells corresponding to each treatment were analyzed. For nuclear staining, cells were embedded in a solution containing 100 nM DAPI (Molecular Probes) and 200 nM propidium iodide (Molecular Probes). Confocal and conventional fluorescence microscopy were performed in an Olympus IX70 inverted microscope equipped with a 100× objective. Fluorescence was filtered with FITC filter sets (Olympus).
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