Cryptochromes are a highly conserved class of UV-A/blue light photoreceptors. In *Drosophila*, cryptochrome is required for the normal entrainment of circadian rhythms to light dark cycles. The photocycle and molecular mechanism of animal cryptochrome photoreception are presently unknown. *Drosophila* cryptochrome undergoes light-dependent degradation when heterologously expressed in Schneider-2 cells. We have generated *Drosophila* luciferase-cryptochrome fusion proteins to more precisely monitor light-dependent cryptochrome degradation. We found that the luciferase-cryptochrome fusion protein undergoes light-dependent degradation with luciferase activity declining ~50% within 5 min of light exposure and ~85% within 1 h of light exposure. Degradation is inhibited by MG-132, consistent with a proteasomal degradation mechanism. Irradiance-response curves yield an action spectrum similar to absorption spectra for prokaryotic and eukaryotic cryptochromes with highest sensitivity in the UV-A. A luciferase-cryptochrome fusion protein lacking the terminal 15 amino acids is stably expressed in the dark but demonstrates increased photosensitivity (6).

When expressed heterologously in yeast, dCRY interacts with the circadian clock protein TIMELESS (TIM) in light but not dark; this effect is lost in the cry mutant, which has a missense mutation in the flavin binding domain (7, 8). In *Drosophila* Schneider-2 (S2) cells transfected with both dCRY and TIM expression constructs, light induces degradation of both proteins in a dCRY-dependent manner (9). These results strongly suggest that dCRY is a photopigment that undergoes a light-dependent conformational change, allowing it to bind to TIM and also initiating its own degradation. The photocycle and molecular mechanisms of *Drosophila* cryptochrome photoreceptor function are not presently understood.

Here we show that *Drosophila* cryptochrome can confer light-dependent degradation to a fused heterologous protein. By fusing firefly luciferase to dCRY, we are able to measure the kinetics of light-dependent degradation of cryptochrome as a function of luciferase activity. This has allowed the generation of an action spectrum for *Drosophila* cryptochrome.

**EXPERIMENTAL PROCEDURES**

**Construction of the pAc-luc and pAc-luc-dCRY and pAc-BlastR Constructs**—The pAc-Myc-dCRY plasmid (generously provided by the Patrick Emery, University of Massachusetts) consists of a cry cDNA with a 5′ Myc tag, which has been introduced into the EcoRI and Xhol sites of pAcV5/HisB (Invitrogen) (9). The QuikChange II XL site-directed mutagenesis kit (Stratagene) was used to insert NotI and Xhol, between Myc and CRY, generating the pAc-Myc-dCRY-SDM plasmid. The following primers were used for the site-directed mutagenesis: 5′-AGG ACC TGA ACG CCG CTA ACT ATC CGC TCG AGA TGG CCA CGC GCT-3′ (dCRYSDFP) and 5′-GCC TGG CCA TCT CGA GCG GAT AGT TAG CGG CCG CGT TCA GTT CCT-3′ (dCRYSDSR). For cloning of the luciferase gene, the pGL3-Basic vector (Promega) was used as template for PCR with high fidelity *Pfu* taq polymerase. The following primers were designed to add NotI and Xhol restriction sites (underlined) and remove the stop codon from luciferase to generate the fusion protein: 5′-AGC GCC CGC ATG GAA GAC GCC AAA AAC-3′ (NotI-Luc) and 5′-CTC GAG CAT GCC GAT TCC GCC CCT-3′ (Luc-Xhol). The pAc-luc vector containing full-length luciferase was constructed using the same NotI-Luc forward primer and the following reverse primer, which includes the stop codon: 5′-TCT AGA ATT CAC GCC GAT CTT TCC GCC CCT-3′ (Luc-Xbal). For stable cell line selection, the blasticidin resistance gene was cloned using the pLenti6/V5D-TOPO vector as a template for PCR with high fidelity *Pfu* taq polymerase. The following primers were used to add appropriate restriction sites (underlined): 5′-GGA ATT CCA TGG CCA AGC CTT TGT C-3′ (EcoRI-BlastR) and 5′-GCT CTA GAG CTT AGC CCT CCC ACA C-3′ (BlastR-Xbal). The three PCR products were cloned into the pCR-Blunt II-TOPO vector (Invitrogen) and transformed into TOP10 cells. The pAc-luc-dCRY plasmid was generated by digesting pCR-Blunt II-TOPO-NotI-Luc-Xhol and pAc-Myc-dCRY-
**Action Spectrum of Drosophila Cryptochrome**

SDM with NotI and XhoI restriction enzymes, gel extraction, and ligation of the products. The pAc-luc plasmid was generated by digesting pCR-Blunt II-TOPO-NotI-Luc-Xbal and pAc-Myc-dCRY-SDM with NotI and Xbal restriction enzymes, gel extraction, and ligation of the products. pAc-Blastk was generated by digesting pCR-Blunt II-TOPO-EcoRI-Blas t Xbal and pAc-Myc-dCRY-SDM with EcoRI and Xbal restriction enzymes, gel extraction, and ligation of the products. Positive clones were sequenced prior to development of stable cell lines. The C-terminal deletion mutant was incidentally generated during the course of cloning and verified by sequencing.

**Generation of Stable Cell Lines**—D.Mel-2 Schneider S2 insect cells (Invitrogen) were cultured in Drosophila serum-free medium until they reached 80–90% confluence in a 75-cm² flask. Cells were co-transfected with pAc-luc-dCRY and pAc-Blas tk or pAc-luc and pAc-Blastk at the recommended 19:1 ratio using Effectene transfection reagent (Qiagen). Cells were allowed to recover for 48 h at 28 °C and transferred to Schneider’s complete Drosophila medium for selection with blasticidin at a concentration of 25 μg/ml. After 2 weeks under selection, the cultures were subjected to serial dilutions to obtain clonal stable cell lines. The resulting clonal lines were grown in 25-cm² flasks until confluent and screened for the highest levels of luciferase activity in the dark.

**Luciferase Assays**—Under dim red 10-W safety light, cells were divided into 1.5-ml aliquots in clear Eppendorf tubes and foil-wrapped. Cells were centrifuged and resuspended in 700 μl of Drosophila M3 medium (without supplement) to maximize light transmission through the cell culture medium. Cells were transferred to 0.5-ml clear PCR tubes and placed in a 28 °C PCR block. A xenon lamp was placed above the PCR block, and the light intensity at each point in the block was measured by calibrated radiometer prior to light pulse. Tube lids were open during light treatment, allowing direct irradiation of cells in medium. At each time point, 50 μl of cells were removed from the tube and centrifuged, and the pellets were frozen until all samples were collected. Pellets were resuspended in 1× cell lysis reagent (Stratagene), and luciferase levels were measured using the Luciferase assay kit (Promega).

**Western Blotting**—D.Mel-2 Schneider S2 insect cells were cultured in a 25-cm² flask and co-transfected with pAc-Myc-dCRY and pAc-luc-dCRY at the recommended 1:1 ratio using Effectene transfection reagent (Qiagen). Cells were allowed to recover for 48 h at 28 °C, transferred to 0.5-ml clear PCR tubes and placed in a 28 °C PCR block. A xenon lamp was placed above the PCR block, and the light intensity at each point in the block was measured by calibrated radiometer prior to light pulse. Tube lids were open during light treatment, allowing direct irradiation of cells in medium. At each time point, 50 μl of cells were removed from the tube and centrifuged, and the pellets were frozen until all samples were collected. Pellets were resuspended in 1× cell lysis reagent (Stratagene), and luciferase levels were measured using the Luciferase assay kit (Promega).

**RESULTS**

Buza et al. (9) previously demonstrated light-dependent dCRY degradation by transfecting S2 cells with a Myc-tagged cryptochrome expression construct and quantifying dCRY protein levels by Myc immunoprecipitation and Western blot, probing with an α-dCRY antibody. This technique is limited in its sensitivity and temporal resolution. To establish a more readily quantifiable assay for light-dependent cryptochrome degradation, we fused a full-length firefly luciferase cDNA to the N terminus of full-length Drosophila cryptochrome. The N terminus was chosen because of the established effects of C-terminal deletions and mutations on dCRY degradation efficiency (8–9, 11). Additionally, we generated a fusion protein with a partial truncation of the C terminus of dCRY (deleting amino acids 528–542), as well as a control construct containing luciferase only (pAc-luc) (Fig. 1). Stable S2 cell lines were selected for pAc-luc and pAc-luc-dCRY constructs.

We initially subjected the pAc-luc and pAc-luc-dCRY stable cell lines to a 1-h light treatment with a Xenon light source at an irradiance of 250 W/m² (Fig. 2A). Temperature was held constant during light exposure. The LUC-dCRY fusion protein exhibited a rapid decrease in luciferase activity with 49.03 ± 3.89% activity remaining after 5 min and a maximal loss of activity to 16.62 ± 0.29% of original activity within 1 h. In contrast, samples maintained in the dark exhibited 98.45 ± 1.97% activity to 16.62 ± 0.29% of original activity within 1 h.
and 84.17 ± 1.23% of original levels at 5 and 60 min, respectively. The light-treated pAc-luc luciferase-only construct behaved as dark-treated LUC-dCRY and did not show light-dependent degradation, indicating that the luciferase itself is not light-sensitive. Viability assays demonstrated that no cell death was associated with light exposure, indicating that protein degradation was not due to nonspecific photic toxicity (data not shown). Luciferase activity recovered following placement of cells in the dark with >85% of initial activity seen 2 h after replacement in the dark (Fig. 2B).

To determine whether the luciferase-cryptochrome fusion proteins recapitulated native degradation after light pulse, we transiently co-transfected S2 cells with pAc-Myc-dCRY and pAc-luc-dCRY and collected cells every 10 min following light exposure of 250 W/m². Western blots were prepared and probed with anti-dCRY antibody. Signal for both dCRY and LUC-dCRY showed time-dependent attenuation (Fig. 2C). Loss of LUC-dCRY signal occurred ∼10 min earlier than loss of dCRY signal (40 min after exposure versus 50 min after exposure), suggesting that the fusion protein is slightly less stable under light exposure than native dCRY protein.

Previous studies have strongly suggested that dCRY is degraded by proteasomal mechanisms in vivo and in vitro (9, 12). Degradation of LUC-dCRY could be partially blocked by the addition of 100 μM MG-132 (a proteasome inhibitor (13)), prior to the light pulse (Fig. 2A). Proteasome-mediated protein degradation can occur in cell-free extracts that preserve proteasome function (14). Light treatment of whole cell extracts showed light-dependent loss of luciferase activity (Fig. 3), consistent with a proteasomal mechanism. The slower time course of degradation observed in cell-free conditions is consistent with that seen in other in vitro proteasome assays (14).

Previous studies have suggested that the C terminus of dCRY plays an important role in light-dependent degradation of TIM and in the intrinsic stability of dCRY protein (7–9, 11). A 19-amino-acid deletion in the cryW mutation, for example, results in constitutively lowered expression in vivo and in vitro (9). We generated a construct expressing luciferase fused to a C-terminal truncated dCRY protein lacking the final 15 amino acids of cryptochrome (LUC-dCRYΔ528) and studied its light-dependent degradation in transient transfection experiments. We found that this protein can be expressed in the dark in S2 cells and undergoes light-dependent loss of luciferase activity (Fig. 4). Interestingly, this mutant protein appeared more sensitive to light than the full-length version; the truncated mutant showed ∼30%
more degradation to 5 mW/cm² white light intensity after 5 min than the full-length fusion protein (Fig. 4).

The action spectrum of a photopigment describes the phototactivity of the pigment as a function of the wavelength of light (15). In a simple system, the action spectrum corresponds with the absorption spectrum of the photoactive pigment. The full action spectrum for animal cryptochrome has not been established. pAc-luc-dCRY stably transfected S2 cells were treated for 30 min with 10 nm bandpass monochromatic light of different irradiances. Irradiance was corrected for medium absorption at each wavelength (Fig. 5). Wavelengths ranging from 350 to 550 nm were tested (Fig. 6). For each wavelength, the irradiance-response curve for protein degradation was fitted via the Zettler-modified Naka-Rushton equation (16–18). The set of irradiance-response curves (Fig. 7A) and corresponding action spectrum (Fig. 7, B and C) for light-dependent dCRY function showed maximum loss of luciferase activity in the UV-A wavelengths from 350 to 400 nm with a plateau in activity between 430 nm and 450 nm and rapid fall-off in sensitivity at wavelengths longer than 500 nm, which were nearly 4 orders of magnitude less effective than UV-A wavelengths.
DISCUSSION

We have demonstrated that a fusion protein of luciferase and Drosophila cryptochrome undergoes light-dependent loss of luciferase activity. Given the previous demonstration by Busza et al. (9) that light induces degradation of cryptochrome and given the reduction of light-dependent loss of luciferase activity following proteasome inhibition, we believe that light induces proteasome-dependent degradation of the luciferase-cryptochrome fusion protein. Because of the high sensitivity and precision of measurement of luciferase activity, the kinetics and photoresponsiveness of light-dependent protein degradation can be assessed with high resolution. We find that light-dependent degradation of the luciferase-dCRY fusion protein is rapid with 50% loss of luciferase activity observed within 5 min of bright light exposure and 80% degradation seen after 1 h. This is more rapid and complete than that observed by Busza et al. (9), who noted ~20% degradation at 10 min and 30% degradation at 1 h. The more rapid degradation observed in our assay may be a function of lighting or culture conditions. In our direct comparison of native dCRY and LUC-dCRY, we found 25% faster degradation of the larger fusion protein after light exposure but found nearly complete degradation of both by 50 min after light exposure. It is possible that the larger fusion protein is more unstable than the native dCRY (in both light and dark); alternatively, it is possible that the fusion protein undergoes more rapid degradation only following light exposure. We do not know at present whether other aspects of dCRY function (such as binding to TIM) are retained by the LUC-dCRY fusion protein.

Busza et al. (9) generated an equal intensity action spectrum for light-dependent degradation of dCRY in S2 cells, measuring the relative efficacy of single light irradiance for each wavelength. This yielded a relatively flat spectrum from 400 to 500 nm with a sharp drop-off between 500 and 650 nm. UV wavelengths were not tested. Ahmad et al. (19) generated an action spectrum for Arabidopsis cryptochrome function by comparing the action spectra of cryptochrome mutant and overexpressing lines. These showed peak activity at ~440 nm but a relatively flat spectrum from 380 to 450 nm. However, the presence of potential screening pigments in plants may have shifted the relative action spectrum in this study. The present study provides the first complete action spectrum for Drosophila cryptochrome function in vitro. This spectrum strongly resembles reported action spectrum for entrainment of the circadian activity rhythm in adult so1;gl60j flies (20). In these flies, loss of all opsin-mediated photoreception reveals the contribution of cryptochrome to circadian entrainment. The in vitro action spectrum also strongly resembles the reported absorption spectra for heterologously expressed cryptochrome from Vibrio cholerae (21) and human (22). In both absorption spectra, maximal absorption is seen in the UV with a significant plateau in the near blue region of 400–450 nm. The action spectrum for light-induced degradation of the luciferase-dCRY fusion protein similarly shows maximal activity in the UV; indeed, the IR50 for light-induced degradation is in the range of 1011 photons/cm²/s. Bouly et al. (24) have recently determined the absorption spectrum of Arabidopsis cry1 protein. Consistent with the present work, they find increasing absorption with short wavelength. In Arabidopsis cry1, the magnitude of “plateau” phase seen between 400 and 500 nm was strongly dependent on the length of time the protein was irradiated with broad spectrum light, suggesting that the redox state of the flavin chromophore may determine the contour of this region of the spectrum. Availability of an action spectrum for animal crypto-
chrome function may thus help constrain and identify the redox state of the native chromophore in situ.

Although we were not able to generate sufficiently bright light to measure full irradiance-response curves for light of wavelengths longer than 510 nm, the slope of the irradiance response functions for light-mediated degradation of luciferase-dCRY appeared to flatten at longer wavelengths (for example, 510 nm). The principle of univariance for photopigments suggests that activation of a photopigment by a photon should be identical regardless of the wavelength of that photon; the action spectrum results from the relative probability of photon absorption. For a univariant pigment, the slope of the irradiance-response function will thus remain constant across wavelengths. The current action spectrum is potentially consistent with a pigment that absorbs multiple photons. The recent work of Bouly et al. (23) on Arabidopsis cryptochrome 1 has demonstrated that green light antagonizes blue light activation of cryptochrome. These authors suggest a three signaling state model for cryptochrome function wherein the inactive cryptochrome binds the FAD chromophore, blue light leads to a radical FADH-containing signaling state, and subsequent absorption of a longer wavelength photon results in conversion to an FADH− state, which does not signal and reverts to FAD in the dark. Further studies are necessary to determine whether animal cryptochrome shows a similar, multiphoton absorption photocycle.

This action spectrum should be useful in assessing the potential role for cryptochromes in other light-induced phenomena in animals. The embryonic chicken ocular iris sphincter muscle, for example, shows light-dependent constriction even when dissected from the retina (24). This activity is vitamin A-independent, but sensitivity decreases with siRNA- or antisense-mediated knock-down of chicken cryptochromes 1 and 2. The action spectrum for this activity closely matches the action spectrum found for light-dependent degradation of Dro sophila cryptochrome. Similarly, the action spectrum for light-mediated Per2 induction in zebrafish also shows significant sensitivity in the UV with a reduced sensitivity at wavelengths greater than 450 nm and nearly complete loss of activity by 500 nm, consistent with the current observed cryptochrome-dependent action spectrum (25).

The role of the C terminus in Drosophila cryptochrome remains somewhat obscure. In plants, the C terminus by itself mediates a constitutive light response (26); conversely, in Drosophila, C-terminal deletions result in constitutive light activity (12). In yeast two-hybrid experiments, C-terminal deleted protein. The cryMM mutation was discovered in a screen for flies exhibiting free-running behavior in constant light conditions (9). In S2 cells, the CRYM protein is expressed at ~20-fold lower levels than wild type; the CRYM protein is nearly undetectable even in the dark in fly eyes. Although CRYM can bind TIM, it does so in both light and dark. The LUC-dCRYΔ528 mutant is a slightly shorter deletion than CRYM (15 versus 19 amino acids) but could be expressed success fully in S2 cells. It is possible that the luciferase fusion confers additional stability to the combined protein when compared with CRYM alone. However, LUC-dCRYΔ528 was more light-sensitive than the full-length protein, supporting the notion that the C terminus does confer additional stability to the cryptochrome protein.

In addition to being a valuable reagent for studying the photobiology of insect cryptochrome, the luciferase-CRY fusion protein also demonstrates that light-dependent degradation can be conferred upon a light-insensitive protein by fusion to cryptochrome. This may be a useful technique for studies of protein function as it may allow for specific proteins to be reversibly and rapidly degraded using ultraviolet or visible blue light.

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