Identification of a Novel Cryptochrome Differentiating Domain Required for Feedback Repression in Circadian Clock Function*§

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Circadian clocks in mammals are based on a negative feedback loop in which transcriptional repression by the cryptochromes, CRY1 and CRY2, lies at the heart of the mechanism. Despite similarities in sequence, domain structure, and biochemical activity, they play distinct roles in clock function. However, detailed biochemical studies have not been straightforward and Cry function has not been examined in real clock cells using kinetic measurements. In this study, we demonstrate, through cell-based genetic complementation and real-time molecular recording, that Cry1 alone is able to maintain cell-autonomous circadian rhythms, whereas Cry2 cannot. Using this novel functional assay, we identify a cryptochrome differentiating α-helical domain within the photolyase homology region (PHR) of CRY1, designated as CRY1-PHR(313–426), that is required for clock function and distinguishes CRY1 from CRY2. Contrary to speculation, the divergent carboxyl-terminal tail domain (CTD) is dispensable, but serves to modulate rhythm amplitude and period length. Finally, we identify the biochemical basis of their distinct function; CRY1 is a much more potent transcriptional repressor than CRY2, and the strength of repression by various forms of CRY proteins significantly correlates with rhythm amplitude. Taken together, our results demonstrate that CRY1-PHR(313–426), not the divergent CTD, is critical for clock function. These findings provide novel insights into the evolution of the diverse functions of the photolyase/cryptochrome family of flavoproteins and offer new opportunities for mechanistic studies of CRY function.

In mammals, many aspects of behavior and physiology, most notably the sleep-wake cycle, are regulated by endogenous circadian clocks and are subject to daily oscillations (1, 2). The mammalian circadian time-keeping system is a hierarchical, multioscillator network with the central clock in the suprachiasmatic nucleus (SCN)2 synchronizing and coordinating peripheral oscillators elsewhere in the body (3). Although virtually all cells in the body have circadian clocks (4–6), the SCN clocks are qualitatively more robust because of functional intercellular coupling mechanisms that are present in the SCN, but absent in most, if not all, peripheral oscillators (7, 8). As a result, peripheral tissues or cells, when cultured in vitro, display cell-autonomous circadian rhythms.

The various clock cells in different tissues share a remarkably similar biochemical mechanism, the autoregulatory negative feedback loop, consisting of negative and positive molecular components (1, 9, 10). The positive components include the two basic helix-loop-helix/PAS-domain-containing transcription factors, BMAL1 and CLOCK, that form a heterodimeric transcriptional complex to activate target gene expression via E/E′-box enhancer elements. Periods (Per1, -2, and -3) and cryptochromes (Cry1 and -2) constitute the negative components of the loop. The PERs and CRYs repress transcription of target genes, by directly interacting with and inhibiting BMAL1-CLOCK complex activity. In particular, the Per and Cry genes themselves are targets of the BMAL1-CLOCK and in turn repress their own transcription, thereby forming the autoregulatory negative feedback loop (11–13). Genetic studies established that CRYs are essential clock components (8, 14, 15). This observation, together with the finding that CRYs are much more potent repressors than PERs for BMAL1-CLOCK complex activity (16, 17), placed the CRYs at the heart of the core clock mechanism.

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** The abbreviations used are: SCN, suprachiasmatic nucleus; PHR, photolyase homology region; CTD, C-terminal tail domain; RRE, ROR/REV-ERB-binding element; CC2, coiled-coil 2.
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The CRYs belong to the photolyase/cryptochrome (PHL/CRY) superfamilly of flavoproteins. All CRYs from different species share a highly conserved core domain at the N terminus, the photolyase homology region (PHR), whereas the C-terminal tail domain (CTD), on the other hand, has diverged during evolution (18, 19). Although photolyases lack this tail region, CRYs from plants to animals contain an extended CTD, but of variable length and amino acid composition. Despite similarity in sequence and domain structures, these flavoproteins play diverse biological roles. Bacterial photolyases, upon activation by light, are DNA repair enzymes that revert UV-induced photoproducts to normal bases to maintain genetic integrity (20). In eukaryotes, however, the CRYs do not exhibit photolyase activity, and the CRYs in plants and *Drosophila* are photoreceptors. Although CRYs in plants function to mediate phototropism, growth, and development (21, 22), CRYs in animals are involved in core clock function (35). In contrast, rhythmic expression of *Cry1* is required for cell-autonomous circadian oscillation (29). In addition to the E/E′-box (responsible for morning-time phase of gene expression, e.g., *Rev-erba*) at the core of the clock mechanism, at least two other circadian cis-elements are involved: the DBP/E4BP4 binding element (D-box; daytime phase, e.g., *Per3*) and the ROR/REV-ERB-binding element (RRE; nighttime phase, e.g., *Bmal1*). In a recent study, we showed that *Cry1* expression is mediated by all three circadian elements (i.e., E/E′-box and D-box elements in the promoter and RREs in the first intron of the *Cry1* gene), giving rise to the distinct *Cry1* evening time phase. Furthermore, through genetic complementation, we showed that this distinctive delayed phase of *Cry1* expression is required to restore circadian rhythmicity in arrhythmic *Cry1−/−:Cry2−/−* fibroblasts (29).

In the present study, we took advantage of the *Cry1* rescue assay to dissect the differential functions of *Cry1* and *Cry2*. First, we confirmed that *Cry1* is required for cell-autonomous circadian rhythms, whereas *Cry2* is dispensable. Through systematic analyses of protein domain structure-function relationships, we identified a highly conserved α-helical domain within the PHR that distinguishes CRY1 from CRY2. Contrary to previous speculation, the least conserved CTD is dispensable for circadian oscillation, but serves to modulate rhythm amplitude and period length. Finally, we demonstrated that CRY1 is a much stronger repressor than CRY2, and that repression strength positively correlates with rhythm amplitude. Thus, our data demonstrate that CRY1-specific repression is necessary for normal clock function.

**MATERIALS AND METHODS**

**Plasmid Construction**—The *Cry1* expression vector, pMU2-P(*Cry1*)-intron-*Cry1*, was made in a previous study (29). To generate pMU2-P(*Cry1*)-intron-*Cry2*, the full-length coding region of mouse *Cry2* was amplified using HiFi-DNA polymerase (Invitrogen) with forward primer (5′-TCTAGATGGCCAA-ACAGCTATTAGGTATATGGTGCGCCGCTGCTGTGGTG-3′; underline, XbaI restriction site) and reverse primer (5′-GTCAGTTCATCTTTCTTGCTGACCCAGATAGGTTAGGAGGCTGCTGTGGTG-3′; underline, Sall). The PCR product was cloned into pCR2.1-TOPO vector (Invitrogen) and the digested Xbal/Sall fragment was then subcloned into pMU2 vector (36) in place of the *Cry1* gene.

Domain swap constructs were generated by overlapping PCR. The primers (supplemental Table S1) were designed so that swap junctions reside in highly conserved or identical sequences, so as to minimize major structural changes and protein folding problems. Site-directed mutagenesis using overlapping PCR was performed to generate single mutations within...
the Cry1-PHR(313–426). Similarly, the PCR products were cloned into pCR2.1-TOPo and subsequently into the pMU2 vector, as described above. For construction of pMU2-P(CMV)-Cry2, the full-length coding sequence of Cry2 was digested from pMU2-P(SV40)-Cry2 (29) with Pl-PspI and Pl-PscI, and the Cry2 fragment was cloned into the Pl-PspI-PI-PscI sites immediately downstream of the CMV promoter.

Each Cry construct (1 μg) was co-transfected with either empty vector (0.4 μg) or Bmal1/Clock (0.2 μg each) in 293T cells or in Cry1−/−·Cry2−/− fibroblasts in a 12-well plate. Forty-eight hours after transfection, cells were lysed in RIPA buffer containing protease inhibitors. The lysates were cleared by centrifugation and supernatants were used for Western blot analysis with guinea pig polyclonal antibodies against CRY1 or CRY2 as described previously (16, 30, 31, 37) or against FLAG tag according to the manufacturer’s protocol (Sigma).

Kinetic Bioluminescence Recording and Data Analysis—Real-time circadian reporter assays were performed using a Lumicycle luminometer (Actimetrics, Inc.) as previously described (8, 29). Briefly, Cry1−/−·Cry2−/− mouse embryonic fibroblasts were maintained in DMEM supplemented with 10% fetal bovine serum (FBS) and antibiotics (100 units/ml of penicillin and 100 μg/ml of streptomycin). One day prior to transfection, 4 × 105 cells were plated onto 35-mm culture dishes. Cells were cotransfected using FuGENE 6 (29) with 3.95 μg of pGL3-P(Per2)-dLuc reporter plasmid (38) and 0.075 μg of a Cry expression plasmid. For the Cry1 dose-response experiment, the amount of plasmid was adjusted to 5.45 μg with empty vector. Three days post-transfection, the medium was replaced with HEPES-buffered recording medium supplemented with B-27 and containing 0.1 mm luciferin and 10 μM forskolin as previously described (29). Bioluminescence from each dish was continuously recorded with a photomultiplier tube for ~70 s at intervals of 10 min at 36 °C. Raw data (counts/sec) were plotted against time (days) in culture and are presented in the figures.

For analysis of rhythm parameters, we used the LumiCycle Analysis program (version 2.31, Actimetrics, Inc.). Raw data were baseline fitted, and the baseline-subtracted data were fitted to a sine wave (damped), from which the period was determined. For samples that showed persistent rhythms, goodness-of-fit of >80% was usually achieved. Due to high transient luminescence upon medium change, the first cycle was usually excluded from rhythm analysis. Amplitude of bioluminescence rhythms was determined as described previously (29). First, a moving average of the linearly detrended bioluminescence was calculated. The window size of the moving average was set to half of the estimated period. The moving average was smoothed by the smoothing spline method, resulting in an amplitude trend, which was then removed by dividing by the trend curve of the original time series.

Transcription Repression Assay—Cry1−/−·Cry2−/− fibroblasts were grown and transfected as described above with the following modifications. In transfection, 1 μg of reporter plasmid, pGL3–3xE-box-P(SV40)-dLuc, pGL3–3xE-box-P(SV40)-dLuc, or pGL3–3xE-box-P(SV40)-dLuc (39) was used together with 2 μg of a Cry expression plasmid. In some assays as presented in supplemental Fig. S5, 0.5 μg each of Bmal1 and Clock plasmid DNA (40) was also included. Empty vector was used to make up the total amount of DNA to 4.1 μg/well. As an internal control, 50 ng of a pRL-SV40 plasmid expressing Renilla luciferase (RLuc) (Promega) was added in each transfection. Forty-eight hours after transfection, cells were harvested and assayed with the Dual Luciferase Reporter Assay System (Promega). Luciferase activity was normalized by RLuc activity.

For evaluation of correlation between rhythm amplitude and repression activity, linear fit of a first-order polynomial was performed by the least square method. Statistical significance was evaluated by Pearson’s correlation. Analysis was performed using Microsoft Excel or R version 2.8.1.

Protein Structure Homology Modeling—Homology models for full-length mCRY1 and mCRY2 were generated using the I-TASSER protein structure prediction server (41–43). This server first threads fragments of the target sequence to representative PDB structure templates with matched sequence identity greater than 70%. The fragments are then assembled into a full-length model, whereas the unmatched regions are built via ab initio modeling. Hence, unlike other homology modeling software, this server predicts the structure even when there are no matched sequences in known PBD structures. The quality of predicted structure was assessed with a scoring method, and five atomistic models with the highest scores were obtained for each input protein sequence. Images of predicted structures were created using PyMOL software, version 1.2r3pre (Schrödinger, LLC).

RESULTS

Cry1, But Not Cry2, Can Restore Circadian Clock Function in Cry1−/−·Cry2−/− Fibroblasts—To confirm the differential functions of Cry1 and Cry2 in clock function, we first tested their ability to restore circadian rhythms in otherwise arrhythmic Cry1−/−·Cry2−/− fibroblasts through genetic complementation and kinetic bioluminescence recording. In this assay, expression of Cry is under control of a composite Cry1-phase promoter containing E/E′-box and D-box elements in the promoter and RREs in the first intron of the Cry1 gene (Fig. 1A).

As expected, Cry1 was able to restore rhythms in these cells (Fig. 1B), consistent with previous results (29), and the rescued cells showed longer period lengths than wild type, characteristic of Cry2−/− cells (8). In contrast, however, Cry2 was unable to restore circadian oscillation to Cry1−/−·Cry2−/− fibroblasts, confirming results found for cells from Cry1−/− mice (8) (Fig. 1B). As the Cry expression level in these fibroblasts was below the detection limit, the ability of P(Cry1)-Intron-Cry constructs to express CRY proteins was tested by Western blot in transfected 293T cells (supplemental Fig. S1A). Additionally, to compare their relative expression in Cry1−/−·Cry2−/− fibroblasts, we determined that 3xFlag-Cry1 and 3xFlag-Cry2 (functionally comparable with Cry1 and Cry2, respectively, in the rescue assay; supplemental Fig. S1B, left panel) are expressed to similar levels in these cells (supplemental Fig. S1B, right panel). Interestingly, rescue of rhythmicity is largely independent of the dose of Cry1, ranging from nanograms to micrograms of DNA used in the transfection (Fig. 1C, left panel). On the other hand, Cry2 of any amount failed to rescue circadian rhythmicity.
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FIGURE 1. Cry1, but not Cry2, restores circadian rhythmicity in arrhythmic Cry1−/−:Cry2−/− fibroblasts. A, schematic representation of expression vectors and general experimental design. In the Cry expression vector, Cry is under control of a composite Cry1-phase promoter that contains all three circadian elements: E′-box, D-box from the Cry1 promoter, and RRE from a Cry intron. The reporter vector contains the destabilized Luciferase (dLuc) gene driven by the Per2 promoter. Transfected Cry1−/−:Cry2−/− fibroblasts are either harvested for a transcription repression assay, or synchronized for kinetic bioluminescence recording. B, representative bioluminescence records from Cry1−/−:Cry2−/− fibroblasts expressing Cry1 or Cry2. Genetic complementation of Cry1 (red), but not Cry2 (blue), restored circadian rhythms in these cells. Each expression construct was cotransfected with the P(Per2-RRE)Luc into the cells. C, Cry1 of different amounts of plasmid DNA restored circadian rhythms in Cry1−/−:Cry2−/− fibroblasts. Experiments were done as in B.

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in these cells (Fig. 1C, right panel). Thus, our data establish that Cry1 and -2 play differential roles at the level of core clock function: whereas Cry1 is essential for generation of cell-autonomous circadian clock function, Cry2 is dispensable.

Unlike the high-amplitude rhythmic expression of Cry1 in various tissues and cells, Cry2 expression is either not rhythmic or rhythmic at very low amplitude (16, 30, 31, 37). It is thus possible that this differential rhythmic expression contributes to functional differences in vivo. In our in vitro rescue assay, the same Cry1-phase promoter is used to control both Cry1 and Cry2 expression, so this strategy eliminates confounding effects of differential transcriptional regulation. Thus, our data showing that Cry1 (but not Cry2) restores circadian rhythms in Cry1−/−:Cry2−/− fibroblasts suggest that CRY1 and -2 possess different intrinsic biochemical properties at the protein level that call for further investigation.

CRY1-PHR(313–426) Is Critical for CRY1 Function—To probe the biochemical origin of the differential functions of CRY1 and -2, we set out to identify the critical structural region that differentiates the two. Based on known structure and domain functions of PHL/CRY proteins (18, 19, 44), we divided CRY1 and -2 proteins into four regions, namely A, B, C, and D (Fig. 2A; supplemental Fig. S2). Using an overlapping PCR strategy, we generated a series of Cry swapping chimeras by systematically substituting different regions of CRY1 with the corresponding sequences from Cry2 (Fig. 2A). To minimize major structural changes and protein folding problems, we selected highly conserved or identical sequences at swap junctions (supplemental Fig. S2). The ability of these chimeras and the mutant Cry constructs to express CRY proteins was tested by Western blot (supplemental Fig. S1). These chimeras were then tested for their ability to restore circadian rhythms in Cry1−/−:Cry2−/− fibroblasts. Cry1 chimeras that harbor A, C, or D regions of Cry2 were able to generate cellular rhythms, suggesting that these regions of Cry1 and -2 have comparable clock function (Fig. 2B). However, when the B region in Cry1 (Cry1-B) is replaced with the corresponding Cry2-B, the A1B2C1D1 chimera failed to restore rhythms, suggesting that Cry1-B is required for clock function (Fig. 2B).

To further confirm the role of Cry1-B, we generated a Cry2 chimera, A2B1C2D2, in which the B region of Cry2 is replaced by the corresponding Cry1-B, designated as Cry*. Similar to Cry1, Cry* was also able to generate rhythms, indicating that the B region of Cry1 is sufficient to render Cry2 able to perform the role of Cry1 in clock function (Fig. 2C). In fact, all chimeras that harbor A1B1-C1D1 were able to sustain circadian oscillation, whereas those containing Cry2-B failed to do so (Fig. 2A). Interestingly, a previous mutagenesis study also hinted that this region likely differentiates CRY1 and CRY2 (45). Thus, we have identified a critical region within the highly conserved α-helical domain of CRY1 PHR (from amino acid 313 to 426) that can differentiate CRY1 from CRY2 and is critically required for Cry1 function. We name this region as CRY1-PHR(313–426).

Identification of Critical Amino Acid Residues within the CRY1-PHR(313–426)—Because the CRY1-PHR(313–426) underlies functional divergence of Cry1 and Cry2, we performed site-directed mutagenesis to identify the critical amino acid residues. Among the ~100 residues within the CRY1-PHR(313–426), 12 are divergent between Cry1 and Cry2, major structural changes are unlikely to occur. We then tested individual mutants for their ability to rescue rhythms in Cry1−/−:Cry2−/− fibroblasts. Among 12 mutants, six restored circadian rhythms in these cells, similar to Cry1 and Cry*, whereas the other 6 failed to do so: Cry*-V316I, K322R, I372V, I392V, S404A, and N425S (Fig. 2D), indicating that these six residues within the CRY1-PHR(313–426) are critical for CRY function in the clock mechanism.
We further performed protein homology modeling to determine the locations of the 6 critical residues in the modeled CRY1 structure. CRY1 and CRY2 have conserved structures for regions A–C, with a root mean square deviation less than 2.0 Å among structures predicted by different programs using different templates. Most homology modeling programs failed to predict a structure for the CTD, except for I-TASSER, which placed it in many different orientations, implying intrinsic flexibility for this region. In a model excluding the CTD, the identified critical residues are all solvent exposed with the exception of Ile-392 (Fig. 2E), which is located near the FAD-binding cavity. Asn-425 is located within a loop motif between helix α18 in region B and α19 in region C, and is potentially involved in protein-protein interactions. The other four residues (i.e. Val-316, Lys-322, Ile-372, and Ser-404) are readily available for potential interaction with the CTD (see below), CC2, or other clock factors. Ser-404 is accepted that the CTD is critical for CRY function (47). To test the functional importance of the CTD, we generated a CRY1 CTD-deletion construct, CRY1ΔCTD (Fig. 3A). To our surprise, CRY1ΔCTD was able to rescue circadian rhythms in Cry2−/− fibroblasts. Thus, contrary to expectation, our data suggest that the CTD is not absolutely essential for CRY1 function (Fig. 3B). This result is consistent with a previ-
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FIGURE 3. CTD is dispensable for CRY function, but modulates period length. A, schematic diagram of various CRY constructs, including the truncation construct Cry1(ΔCTD) in which the CTD is deleted. Period length corresponding to each construct is shown on the right. Mean ± S.D. (error bar) of two independent experiments are shown. Raw data are presented in Figs. 2, B and C, 3B, and supplemental Fig. S3. B, representative bioluminescence records from Cry1+/−:Cry2+/− fibroblasts expressing different CRY constructs. Deletion of CTD did not render Cry1 unable to generate circadian rhythms, suggesting that the CTD is dispensable for Cry1 function. Cry1(ΔCTD), green. C, three-dimensional homology model structure of full-length CRY1. The model was generated using the I-TASSER protein structure prediction server. Color scheme: region A, blue; B, CRY1-PHR(313–426), green; C, cyan; and D, CTD, orange. The CTD assumes a flexible structural configuration, and one of the predicted orientations is shown. In this configuration, the CTD resides in close proximity with the core CRY1-PHR(313–426), particularly with the 4 critical residues (red spheres).

Our homology models for full-length CRY1 and CRY2 suggested plausible interactions between the CTD and the identified cryptochrome differentiating domain involving the above identified critical residues. Consistent with previous observations, the CTD assumes flexible structural configurations (48). Among possible arrangements of the CTD, those involving interactions with CRY1-PHR(313–426) are energetically favored, especially interactions with the side chains of Val-316, Lys-322, Ile-372, and Ser-404 (Figs. 2E and 3C), each shown to be critical for CRY function. The observation that these residues reside in critical regions (e.g. Ile-392 and Ser-404) and/or at an interface (e.g. Val-316, Lys-322, Ile-372, and Ser-404) available for potential protein-protein interaction explains why mutating them impairs normal clock function.

CRY1 and CRY2 Display Differential Transcriptional Repression Activity—In kinetic rhythm assay experiments, we noticed low expression levels of the P(Per2)-dLuc reporter in rhythmic cells and high levels in arrhythmic cells, suggesting that rhythm amplitude may be related to potency of repression of BMAL1-CLOCK transcriptional activity. To examine this correlation more quantitatively, we measured P(Per2)-dLuc expression in the presence of Cry1 or Cry2 in transiently transfected, nonsynchronized cells. When assayed under arrhythmic conditions in which Cry expression is controlled by a strong, constitutive promoter such as CMV or SV40, Cry1 and -2 both displayed slightly different but strong levels of repression (Fig. 4A and supplemental Fig. S4), consistent with previous studies (16, 17, 38, 45). To test for differences in repression activity of CRY1 and CRY2, we measured Cry repression under our conditions of genetic complementation in Cry1−/−:Cry2−/− fibroblasts, in which Cry is regulated by the Cry1-phase promoter. Under these conditions, CRY1 still displayed strong repression on the P(Per2)-dLuc reporter. CRY2, however, did not repress transcription to the same extent as CRY1, showing a repression activity 10 times weaker than CRY1 (Fig. 4A). This difference in repression by CRY1 and CRY2 was independent of the reporter used in the assay, as similar results were obtained with 3xE-box-P(SV40)-dLuc or 3xE-box-P(SV40)-dLuc (supplemental Fig. S4). Similar differential repression was also observed when Bmal1 and Clock were co-transfected in these cells (supplemental Fig. S4). Therefore, we conclude that CRY1 is a much more potent transcriptional repressor than CRY2 when expressed under control of a Cry1-phase promoter.

CRY Transcriptional Repression Positively Correlates with Rhythm Amplitude—These differential repression data prompted us to analyze the dependence of rhythm generation on transcriptional repression. To do this, we determined the repression activity of a subset of Cry chimera and mutants used in our rescue studies. Under control of the Cry1-phase promoter, these Cry constructs showed various strengths of repression activity (Fig. 4B). Importantly, we observed that all constructs that were able to rescue the rhythms exhibited stronger repression activities, similar to Cry1, whereas those that failed to rescue have much weaker repression, similar to Cry2 (Fig. 4C). For example, Cry1 (A1B1C1D1) and chimera A2B1C1D1 exhibited low but similar P(Per2)-dLuc expression,
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FIGURE 4. Transcriptional repression positively correlates with rhythm amplitude. A, dual luciferase reporter assay in Cry1−/−:Cry2−/− fibroblasts. For Cry1 expression, three different promoters were tested. Each Cry construct was cotransfected with P(SV40)-dLuc (control) or P(Per2)-dLuc reporter. A Renilla luciferase (RLuc) was added in each transfection to normalize transfection efficiency. Under the control of the Cry1-phase promoter, Cry1 acted as a much more potent repressor than Cry2. Mean ± S.D. (error bars) of two independent experiments are shown (n = 3 for each experiment). B, repression activities of various Cry chimeras and mutants. Dual luciferase reporter assay was done as in A. The constructs that rescued rhythms exhibited stronger repression, similar to Cry1, whereas those that failed to rescue rhythms exhibited much weaker repression, similar to Cry2. Mean ± S.D. (error bars) of two independent experiments are shown (n = 3). C, representative bioluminescence records from Cry1−/−:Cry2−/− fibroblasts expressing various Cry chimeras and mutants. The Cry rescue assay was performed as described in the legend to Fig. 1B. D, relative amplitudes of rescued rhythms in C. Mean ± S.D. (error bar) of two independent experiments are shown (n = 3). E, relative rhythm amplitude (x axis) is plotted against relative repression activity (y axis). Rhythm amplitude bears a positive correlation with transcriptional repression by various CRYs. Mean ± S.D. (error bar) of two independent experiments are shown (n = 3).

DISCUSSION

Unlike hourglass-type timers, oscillator-type timers such as the circadian clock regulate cyclic processes that repeat upon completion of a cycle. The mechanism underpinning this circadian oscillation in mammals is an autoregulatory transcriptional-translational negative feedback loop (1, 10), in which transcriptional repression by the CRYs lies at the heart of this mechanism (16, 38, 39). To gain basic understanding of this biochemical mechanism, we sought to investigate the unique biochemical and structural aspects of the CRYs. Through a systematic analysis of protein structure-function relationships, we identified the distinct sequences that distinguish Cry1, and demonstrated that Cry1-specific transcriptional (strong) repression is required for mammalian clock function. This study provides insights into the unique biochemical and structural properties of Cry1, and presents new opportunities for future dissection of its precise role in the circadian clock mechanism.

Genetic Complementation of Cry1 in Cry-deficient Cells Provides a Functional Clock Model for Mechanistic Studies—In a recent study, we identified the full set of cis-elements responsible for the circadian expression pattern of Cry1, including pri-
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Dramatically the E/E'-box and D-box elements in the promoter and RREs in the first intron of the Cry1 gene. This allowed us to engineer a synthetic composite promoter that is both necessary and sufficient for establishing the Cry1-phase. Importantly, we demonstrated, through genetic complementation in Cry1-/-: Cry2-/- fibroblasts, that Cry 1 expression at the evening phase is required for generation and maintenance of cell-autonomous circadian rhythms.

This Cry rescue assay provided us with a unique opportunity to study Cry function in clock cells and confirmed that Cry 1 and Cry 2 indeed have differential functions in clock regulation. This assay also enabled us to uncover for the first time the different potency in transcriptional repression exhibited by Cry 1 and Cry 2, which underlie their differential roles in clock function. In several prominent structure-function studies in which Cry expression was under a strong constitutive promoter (45, 47, 49, 50), CRY protein (likely saturated) was assayed at steady-state levels, masking differences in repression activity between Cry 1 and Cry 2. Consistent with this notion, we show that, compared with the stronger CMV promoter, SV40-driven Cry 1 and Cry 2 exhibited a more noticeable difference in transcriptional repression (Fig. 4D). In our study, Cry expression, under the control of the Cry1-phase promoter, is properly connected to the negative feedback loop involving both the E/E’-box and D-box elements and the RREs; under this condition, Cry expression levels would not reach saturation.

Sequence and Domain Structural Features that Distinguish Cry1 from Cry2—In this study we demonstrated that the functional difference between Cry 1 and Cry 2 lies primarily at the Cry1-PHR(313–426) and secondarily at the CTD. Mechanistically, the level of appropriately timed Cry 1 repression is the key to generating robust rhythms. The Cry1-PHR(313–426) is critical for potent transcriptional repression. We observed a significant positive correlation between Cry repression activity and amplitude of the rhythms (Fig. 4). As the repression activity goes up, so does the amplitude of the rhythms. Thus, from the evolutionary point of view, it is the elaboration of a new function for the conserved core domain of Cry that rendered it a core clock component.

Although the CTD is not absolutely required for circadian clock function, it participates in modulating basic clock function. Compared with wild type Cry 1, the Cry1 chimera harboring the CTD of Cry2 (A1B1C1D2) shortened the period length (Fig. 3), indicating its role in period length regulation. Compared with Cry 1, Cry* (A2B1C2D2) displayed slightly reduced, but by and large similar repression activity. Interestingly, however, compared with the full-length Cry 1, Cry1(ΔCTD) displayed less transcriptional repression and generated lower amplitude rhythms, whereas Cry* (ΔCTD) exhibited dramatically reduced repression activity and failed to generate rhythms, similar to Cry 2. Thus, our data suggest that CTD1 and CTD2 (from Cry 1 and Cry 2, respectively) play differential roles in fine-tuning the clock function, and that there might be a mechanism for signal transduction from the identified cryptochrome differentiating domain to CTD to accomplish the fine-tuning.

However, the mechanism of repression by Cry and potential signal transduction from the Cry1-PHR(313–426) to the CTD remain unknown. Current structural data on the CTD are confined to limited proteolysis and qualitatively interpreted solution NMR spectra (48), confirming predictions that CTD is largely disordered. A recent study described the crystal structure of full-length Drosophila Cry in which the CTD is found to interact with the FAD binding core domain (i.e. region B in our study). The CTD of dCRY contains only 20 residues, whereas CTDs of mCRYs are much longer (80–100 residues) and diverge from dCRY, and thus, structurally more flexible. Our homology models of mCRYs confirmed the potential for interactions between CTD and the cryptochrome differentiating domain. However, future structural and functional studies are required to elucidate the mechanism of coordinated function of CTD and the cryptochrome differentiating domain of CRY proteins.

CRY1-specific Transcriptional Repression Is Required for Circadian Clock Function—The basic concept of a circadian negative feedback loop in mammals was established in the late 1990s (1, 9, 10), and feedback repression is mediated primarily by CRYs, not PERs (16, 17). Through studies of Bmal1 and Clock mutants that interfere with CRY interaction, it was later demonstrated that CRY-mediated repression of BMAL1-CLOCK activity is required for clock function and maintenance of circadian rhythmicity (38). A hallmark of circadian clock function is the rhythmic expression of clock genes. Recently, we demonstrated that Cry 1 expression at the evening time phase (i.e. not morning or day time) and therefore proper phasing in feedback repression by Cry 1 is important for normal circadian clock function (29). Here we further demonstrate that CRY 1-specific repression is the key to generating circadian rhythms; Cry 1 was able to rescue the rhythms in Cry1-/-: Cry2-/- fibroblasts, but Cry 2 failed to do so. In addition, Cry1-/- cells are largely arrhythmic, suggesting that endogenous Cry 2 alone is unable to support clock function (8, 27). Thus, experimental data from both gain-of-function (this study) and loss-of-function studies in cellular clock models (8, 51), as well as in circadian behavior of composite knockout mice (14, 28), establish that Cry 1 plays a more prominent role in clock function than Cry 2. Despite the essential role of Cry 1 in cell-autonomous models, Cry1-/- mice, nevertheless, display persistent free-running rhythms (14, 15, 28). Therefore, there exists a gap in knowledge as to how transient rhythms in individual Cry1-/- neurons are organized into coherent rhythms in the SCN.

Future Perspective—Importantly, the mechanism by which Cry 1 represses BMAL1-CLOCK complex activity remains elusive. Our findings that Cry 1, but not Cry 2, plays an essential role in clock function, and that Cry 1 possesses unique biochemical features, especially within the key Cry1-PHR(313–426) domain, suggest that Cry 1 holds the key to our understanding of the feedback repression mechanism. A recent study showed that Cry 1 and Cry 2 bind to the CLOCK-BMAL1-E-box complex with the same affinity (52). Thus, it is possible that their functional difference lies at their different intrinsic repression activities or differential post-translational mechanisms, and future studies need to focus on the precise biochemical mechanism by which CRYs repress BMAL1-CLOCK transcriptional activity. The functional assay established in this study provides new opportunities for future investigations into CRY1
structure-function relationships. Our findings shed new light on the functional importance of the CRY1-PHR (313–426) and the CTD in the clock mechanism. Several previous studies identified a subset of common motifs and sites, including nuclear localization sequences, coiled-coils, phosphorylation sites of CK1ε, GSK3β, MAP kinase, and AMP-activated protein kinase (44, 45, 47, 49, 50, 53–56), and surely additional motifs remain yet to be identified. The functional significance of these various sequences and structural features in CRY function will need to be tested using the assays developed in this study. These future studies will ultimately provide important insights into the biology of CRYs and their role in the negative feedback mechanism, as well as the functional evolution of the PHL/CRY family of flavoproteins.

Acknowledgments—We thank Dr. Choogon Lee for CRY antibodies and Drs. Koji Ode and David Welsh for critical reading and comments on the manuscript.

REFERENCES


Differential Functions of Cryptochromes 1 and 2


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Differential Functions of Cryptochromes 1 and 2

role for Bmal1 cycling in transcriptional regulation of intracellular circadian rhythms. PLoS Genet. 4, e1000023
Supplemental Data

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Identification of a Novel Cryptochrome Differentiating Domain Required for Feedback Repression in Circadian Clock Function

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   Figure S4. CRY1 and CRY2 display differential repression activity.
   Figure S5. Mutation of each of the six critical residues within the CRY1-PHR(313-426) of CRY* abolished transcriptional repression activity.
Table S1. Primer list for generation of domain swapped chimeric constructs.

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Figure S1

(A) HEK-293T cells were transfected with different Cry constructs in the absence or presence of Bmal1/Clock as indicated. Total cell extracts were probed with either anti-CRY1 (left panel) or anti-CRY2 (right panel) antibody. Compared to CMV promoter, P(Cry1)-Intron drives low levels of protein expression, which can be further induced by cotransfected Bmal1/Clock to a higher level. Detection of CRY proteins indicates that all Cry constructs are able to express the proteins. Because the regulation of Cry expression in functional clock cells (e.g., those that are rescued by P(Cry1)-Intron-Cry1) is different from that in arrhythmic cells (e.g., those expressing Cry2), to directly compare Cry expression, transfected 293T cells provide a better means to test these constructs for their ability to express proteins. 3xFlag-CRY1 and CRY2, positive controls. Arrow, non-specific (NS) band.

(B) Cry1 and Cry2 are expressed to similar levels in Cry1–/–:Cry2–/– fibroblasts in the rescue assay. Cry1–/–:Cry2–/– fibroblasts were transfected with 3xFlag-Cry1 or 3xFlag-Cry2 constructs in the absence or presence of Bmal1/Clock as indicated. 3xFlag-Cry1 and 3xFlag-Cry2 are functionally comparable to Cry1 and Cry2, respectively, in the rescue assay (left panel). Western blot analysis with anti-Flag antibody shows that CRY1 and CRY2 are expressed to similar levels in these cells (right panel).

Figure S1. Western blot analysis of CRY proteins. (A) HEK-293T cells were transfected with different Cry constructs in the absence or presence of Bmal1/Clock as indicated. Total cell extracts were probed with either anti-CRY1 (left panel) or anti-CRY2 (right panel) antibody. Compared to CMV promoter, P(Cry1)-Intron drives low levels of protein expression, which can be further induced by cotransfected Bmal1/Clock to a higher level. Detection of CRY proteins indicates that all Cry constructs are able to express the proteins. Because the regulation of Cry expression in functional clock cells (e.g., those that are rescued by P(Cry1)-Intron-Cry1) is different from that in arrhythmic cells (e.g., those expressing Cry2), to directly compare Cry expression, transfected 293T cells provide a better means to test these constructs for their ability to express proteins. 3xFlag-CRY1 and CRY2, positive controls. Arrow, non-specific (NS) band. (B) Cry1 and Cry2 are expressed to similar levels in Cry1–/–:Cry2–/– fibroblasts in the rescue assay. Cry1–/–:Cry2–/– fibroblasts were transfected with 3xFlag-Cry1 or 3xFlag-Cry2 constructs in the absence or presence of Bmal1/Clock as indicated. 3xFlag-Cry1 and 3xFlag-Cry2 are functionally comparable to Cry1 and Cry2, respectively, in the rescue assay (left panel). Western blot analysis with anti-Flag antibody shows that CRY1 and CRY2 are expressed to similar levels in these cells (right panel).
Figure S2. Sequence alignment and key structural features of mCRY1 and mCRY2.

mCRY1 and mCRY2 share ~70/80% sequence identity/similarity. Amino acid sequence alignment was generated using Vector NTI (Invitrogen). Above the sequence alignment, a schematic diagram of domain structure of CRY proteins, as in Figure 2A. Amino acid sequence alignment: blue, identical; green, similar; red, divergent. Domain assignment: region A (blue), B (green), C (cyan), and D (yellow). Secondary structures: arrow, alpha helix (numbers are assigned based on Arabidopsis UVR3); barrel, beta strand; CC2: coiled coil 2. Key amino acid residues: black star, FAD-binding residues; gray star, Trp triad; red star, arrhythmic when mutated in Cry*; green star, rhythmic when mutated.

Khan et al. Amino acid sequence alignment of mouse CRY proteins
Figure S3. Roles of coiled-coil 2 (CC2)-containing C region and the CTD of CRY in clock function. 

(A) In the truncation construct, Cry1(ΔCC2-CTD) (top panel), both the CTD and the C region containing CC2 are deleted from the full-length Cry1. This Cry1 truncation mutant failed to restore cellular rhythms in Cry1−/−:Cry2−/− fibroblasts (bottom panel; representative bioluminescence records).

(B) Representative bioluminescence records from Cry1−/−:Cry2−/− fibroblasts expressing various Cry chimeras. The Cry rescue assay was performed as in Figure 1B. While the chimeras that contain the CTD from CRY1 (Cry1 and A2B1C1D1) restored rhythms with a period length of ~27 hr (Figure 3A), those that contain the CTD from CRY2 (A1B1C1D2, A1B1C2D2 and Cry*) restored rhythms with a period length of ~24 hr (Figure 3A), implicating the CTD in regulating period length.

Khan et al. The roles of CC2 and CTD of CRY in clock function.
Figure S4. Cry1 and Cry2 display differential repression activity.

Dual Luciferase reporter assay in Cry1–/–:Cry2–/– fibroblasts was performed to test repression activity of Cry1 and Cry2 in the absence (top panel) or presence (bottom panel) of exogenous Bmal1 and Clock. For Cry expression, three different promoters were tested: P(CMV), P(SV40) or P(Cry1)-Intron. Each Cry construct was cotransfected with P(SV40)-dLuc (control) or the P(Per2)-dLuc reporter. A Renilla Luciferase (RLuc) was added in each transfection to normalize transfection efficiency. Under all three promoters, particularly the Cry1-phase promoter, CRY1 acted as a much more potent repressor than CRY2. Similar results were obtained using 3x E’-box-P(SV40) or 3xE-box-P(SV40) promoter, and when Bmal1 and Clock are ectopically over-expressed. Mean and SD (error bar) of two independent experiments are shown (n = 3 for each experiment).

Khan et al. Differential repression by CRY1 and CRY2.
Khan et al. Mutation of the critical residues within PHR(313-426) of Cry* impaires repression