Quantitative Analyses of Cryptochrome-mBMAL1 Interactions

MECHANISTIC INSIGHTS INTO THE TRANSCRIPTIONAL REGULATION OF THE MAMMALIAN CIRCADIAN CLOCK

Received for publication, March 28, 2011, and in revised form, April 21, 2011 Published, JBC Papers in Press, April 25, 2011, DOI 10.1074/jbc.M111.244749

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The mammalian cryptochromes mCRY1 and mCRY2 act as transcriptional repressors within the 24-h transcription-translational feedback loop of the circadian clock. The C-terminal tail and a preceding predicted coiled coil (CC) of the mCRYs as well as the C-terminal region of the transcription factor mBMAL1 are involved in transcriptional feedback repression. Here we show by fluorescence polarization and isothermal titration calorimetry that purified mCRY1/2Ctail proteins form stable heterodimeric complexes with two C-terminal mBMAL1 fragments. The longer mBMAL1 fragment (BMAL490) includes Lys-537, which is rhythmically acetylated by mCLOCK in vivo. mCRY1 (but not mCRY2) has a lower affinity to BMAL490 than to the shorter mBMAL1 fragment (BMAL577) and a K537Q mutant version of BMAL490. Using peptide scan analysis we identify two mBMAL1 binding epitopes within the coiled coil and tail regions of mCRY1/2 and document the importance of positively charged mCRY1 residues for mBMAL1 binding. A synthetic mCRY coiled coil peptide binds equally well to the short and to the long (wild-type and K537Q mutant) mBMAL1 fragments. In contrast, a peptide including the mCRY1 tail epitope shows a lower affinity to BMAL490 compared with BMAL577 and BMAL490(K537Q). We propose that Lys-537 acetylation enhances mCRY1 binding by affecting electrostatic interactions predominantly with the mCRY1 tail. Our data reveal different molecular interactions of the mCRY1/2 tails with mBMAL1, which may contribute to the non-redundant clock functions of mCRY1 and mCRY2. Moreover, our study suggests the design of peptidic inhibitors targeting the interaction of the mCRY1 tail with mBMAL1.

In mammals many physiological processes are regulated by circadian clocks, which are operated by transcriptional and translational feedback loops. In the central feedback loop, the bHLH-PAS (basic Helix-Loop-Helix-PER-ARNT-SIM) transcription factors mBMAL1 (brain and muscle ARNT-like protein) and mCLOCK (circadian locomotor output cycle kaput) activate the transcription of three period genes (mper1,2,3) and two cryptochromes (mCRY1,2) (1). The mPER proteins and (even more potently) the mCRY proteins feedback-repress their own transcription by regulating the activity of mBMAL1 and mCLOCK (2, 3). Notably, the mBMAL1-mCLOCK transcription factor complex not only regulates the mper and mCRY genes but also a large number of clock-controlled genes, including genes involved in cell cycle regulation, cellular differentiation, and metabolism (4). Hence, the regulation of these transcription factors is of relevance for many body functions and associated diseases (e.g. sleep and depressive disorders, metabolic syndrome, cardiovascular diseases, and tumor formation) that are under the control of the circadian clock (5). The importance of mBMAL1 for clock function is clearly demonstrated by the fact that mBMAL1 +/− knockout mice show an immediate and complete loss of circadian rhythmicity at a behavioral and molecular level (6). Although mCRY1/mCRY2 double knockout mice become totally arrhythmic, mCRY1−/− single knock-out mice exhibit a 1-h shorter period, and mCRY2−/− single knock-out exhibit mice a 1-h longer period (7–9). Hence, the two cryptochromes are partially redundant but also have nonredundant clock functions leading to the opposite effects of mCRY1 and mCRY2 disruption on the period length.

The cryptochromes are composed of an ~500 amino acid photolyase homology region (PHR) (10) and variable C-terminal extensions, the tails (Fig. 1A). The mCRY tails together with a preceding predicted coiled coil (CC) region, which corresponds to the most C-terminal α-helix of the PHR (10, 11), are
involved in the transcriptional repression of mCLOCK and mBMAL1 (12). In the following we will refer to the PHR lacking the C-terminal CC region as the photolyase homology core region (PHCR). Strikingly, the mCRY1Ctail fragment alone does not mediate transcriptional repression when fused to enhanced GFP (12). Moreover, mutations that are expected to destabilize the interface between the PHCR and the coiled coil region inhibit the transcriptional repression activity of both mCRY homologues and reduce the interaction of mCRY2 with mBMAL1, mPER1/2, and mCLOCK (13). Hence, the correct positioning of the coiled coil region with respect to the PHCR is critical for molecular interactions and transcriptional repression activities of the mammalian cryptochromes. Notably, both cryptochromes contain functional bipartite nuclear localization signals within their tails (12, 14). Furthermore, Ser-557 and Ser-553 in the mCRY2 tail are phosphorylated sequentially by DYRK1A (dual-specificity tyrosine-phosphorylated and -regulated kinase 1A) and glycogen synthase kinase-3 (GSK-3β). As a result, the C-terminal CC region acts as a repressing mCRY-bound “off” mode.

The interaction of the mCRY/mBMAL1 complex with mCLOCK represents a regulatory switch that cycles in a day-time-dependent manner between an activating coactivator-bound “on” mode and a repressing mCRY-bound “off” mode. Although literature reports about direct interactions of mCRY1 and mCRY2 with mCLOCK are inconsistent, there is no doubt that mCLOCK stabilizes the mBMAL1-mCRY interactions in a ternary mCRY-mBMAL1-mCLOCK complex (17, 20). Importantly, mCLOCK acetylates mBMAL1 in vivo specifically on Lys-537 (21) (Fig. 1B). Acetylation of Lys-537 in the mBMAL1-(577–625) region occurs in a daily regulated manner with a peak at about 15:00 h (CT15) or in a fluidizer, and insoluble material was removed by centrifugation. The supernatant was loaded onto a GSH Sepharose 4B column (GE Healthcare) with a 3–10-kDa molecular weight cutoff which involves electrostatic interactions predominantly with the mCRY1 tail. Our study also suggests the design of specific peptidic or small molecule ligands targeting the nonconserved interaction of the mCRY1/2 tails with mBMAL1.

**EXPERIMENTAL PROCEDURES**

**Recombinant Expression and Purification of mCRY and mBMAL1 Proteins—C-terminus fragments of the mouse cryptochromes (mCRY1-(471–606) and mCRY2-(489–592)) and mouse mBMAL1 (mBMAL1-(577–625) and mBMAL1-(490–625)) were cloned into a pGEX-6P2 expression vector using restriction sites 5’ BamHI (mCRY1, both mBMAL1 fragments) or SmaI (mCRY2) and 3’ NotI (all 4 fragments). The K537Q mutation was introduced into the mBMAL1-(490–625) construct using the QuickChange site-directed mutagenesis kit (Stratagene). The proteins were overexpressed as GST fusions in the *Escherichia coli* strain BL21(DE3) and purified via GSH affinity and size exclusion chromatography. For purification, 5–10 liters of mCRY or mBMAL1 expression cultures in TB (Terrific Broth) medium were induced with 0.1 mM isopropyl-β-D-galactopyranoside at an A₆₀₀ of ~1. Expression was carried out for 5 h at 30 °C or overnight at 18 °C. Pellets were thawed on ice and homogenously resuspended in lysis buffer containing 50 mM Tris-Cl, pH 7.8, 250 mM NaCl, 10 mM β-mercaptoethanol, 10% glycerol, 2 mM EDTA, and 1 mM phenylmethylsulfonyl fluoride. Cells were lysed by sonication or in a fluidizer, and insoluble material was removed by centrifugation. The supernatant was loaded onto a GSH affinity column. The GST tag was removed by cleavage with PreScission protease either on the GSH column (mBMAL1) or in batch (mCRY1/2) after elution of the GST-fused proteins with a buffer containing 50 mM Tris-HCl, pH 7.8, 150 mM NaCl, 10 mM β-mercaptoethanol, 5% glycerol, and 20–30 mM glutathione. Tag removal yielded recombinant mCRY and mBMAL1 proteins with the N-terminal extensions GPLGS (BamHI) or GPLGS/PGIPG (Smal, mCRY2) leading to the following molecular weights and isoelectric points (pis) of the recombinant proteins: mCRY1-(471–606), 14,432.7 Da, pl = 8.91; mCRY2-(489–592), 12,087.0 Da, pl = 6.94; mBMAL1-(490–625), 14,263.8 Da, pl = 4.18; mBMAL1-(577–625), 5,482.0 Da, pl = 3.44. Fractions containing cleaved mCRY or mBMAL1 proteins were concentrated using an Amicon Ultra-15 filter device (Milipore, Bedford, MA) with a 3–10-kDa molecular weight cut off and loaded onto a Superdex HiLoad 5/75 16/60 size exclusion column (GE Healthcare) with a running buffer containing 25 mM Hepes, pH 7.8, 40 mM NaCl, 2 mM DTT, and 5% glycerol.

**Cryptochrome-mBMAL1 Interactions**

To quantitatively analyze the mCRY-mBMAL1 interactions underlying the transcriptional regulation of the mBMAL1-mCLOCK complex, we have purified mCRY1/2Ctail proteins as well as two C-terminal mBMAL1 fragments of 5.5 and 14.3 kDa, the latter including the in vivo acetylated Lys-537. We show that mCRY1 (but not mCRY2) exhibits a lower affinity to the longer than to the shorter mBMAL1 fragment and compared with a mutant version of the longer mBMAL1 fragment, in which Lys-537 is exchanged to an acetyl mimetic glutamine. Using peptide scan analysis, we identify two mBMAL1 binding epitopes in mCRY1 and mCRY2 corresponding to the coiled coil region and a more C-terminal region within the tails. Isothermal titration calorimetry (ITC) experiments with mCRY coiled coil and tail epitope peptides revealed different mBMAL1 interactions of the mCRY1 and mCRY2 tails, which may contribute to the non-redundant clock functions suggested by mCRY1⁻/⁻ and mCRY2⁻/⁻ knock-out studies (7–9). Furthermore, we propose a molecular mechanism for the regulation of mCRY1 binding by Lys-537 in mBMAL1 acetylation, which involves electrostatic interactions predominantly with the mCRY1 tail. Our study also suggests the design of specific peptidic or small molecule ligands targeting the nonconserved interaction of the mCRY1/2 tails with mBMAL1.
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The remaining GST was removed by applying mBMAL1- or mCRY-containing fractions onto a second GSH column. Fractions containing highly purified mCRY and mBMAL1 proteins were pooled, concentrated to typically 5 mg/ml (350–400 μM), and snap-frozen in liquid nitrogen. Samples were stored at −80 °C until measured.

Analytical Ultracentrifugation-Sedimentation Coefficient Velocity Experiments—Sedimentation velocity experiments were performed with an Optima XL-1 analytical centrifuge (Beckman Inc., Palo Alto, CA) using an An 60 Ti rotor with double-sector formation with an Optima XL-I analytical centrifuge (Beckman Instruments). Sedimentation coefficient distributions were computed using the SEDFIT software package (23), resulting in a c(s)-distribution corrected for diffusion by means of a signal-average friction function (24). Experimental hydrodynamic radii (Rh) were calculated with the SEDFIT software package (23), resulting in a c(s)-distribution corrected for diffusion by means of a signal-average friction function, which was optimized during fitting. The combination of s and f0 allows an estimate of the molar mass Mf and apparent sedimentation coefficients used for subsequent calculations were determined by integration of the area under the c(s) curve for the species of interest. Experimental hydrodynamic radii (Rh) were calculated with SEDNTERP. Expected Rh values for folded and unfolded proteins of equal molar mass were obtained using the empirical formulae for globular and guanidinium-HCl-unfolded proteins (Equations 1 and 2) (25).

\[
\log(R_h^W) = - (0.204 \pm 0.023) + (0.357 \pm 0.005) \cdot \log(M) \\
(Eq. 1)
\]

\[
\log(R_h^{GdmCl}) = - (0.723 \pm 0.033) + (0.543 \pm 0.007) \cdot \log(M) \\
(Eq. 2)
\]

Circular Dichroism (CD) Spectroscopy—Purified protein samples were diluted to final concentrations between 13 and 60 μM in 25 mM NaH2PO4, pH 7.8, 5 mM Tris(2-carboxyethyl)phosphine buffer. CD spectra were measured by a Jasco J-715 spectropolarimeter using a 0.1-cm path length quartz cuvette and represent the mean molar ellipticity per amino acid residue of protein after buffer correction. Measurements were performed at 4 °C in a wavelength range from 190 to 250 nm with 0.1-nm intervals collecting data for 0.5 s at each point. For each measurement 10 spectra were used for accumulation. Analysis was performed using the CONTIN algorithm (26) with the reference dataset SMP56 (27, 28).

Fluorescence Polarization—mBMAL1 fragments were fluorescently labeled with Fluorolink™ Cy3.5 monoreactive Dye (GE Healthcare), which reacts with free amine groups (N-terminal amino groups and lysine side chain amino groups) of proteins. For labeling, a 15–20 mg/ml concentrated solution of purified mBMAL1 protein in a sodium carbonate buffer (0.1 mM Na2CO3, pH 8.5) was incubated with the Cy3.5 dye for 2 h at 4 °C. For the fluorescence polarization measurement, the protein was transferred into a buffer containing 25 mM Hepes, pH 7.8, 50 mM NaCl, 2 mM dithioerythritol, 2.5% (v/v) glycerol with a desalting HiTrap column (GE Healthcare). Fluorescence polarization spectra were recorded with excitation of the Cy3.5 fluorophor at 581 nm and emission at 596 nm. A FluoroMax II spectrofluorimeter (Spex Industries, Edison, NJ) was used in the polarization mode at 10 °C. 500 nM Cy3.5-labeled mBMAL1 was titrated with increasing amounts of mCRY1 or mCRY2 proteins (concentrations 500 nM–300 μM) until saturation was reached. For each titration step, 30 measurements were accumulated and buffer-corrected. To obtain the dissociation constants (Kd) for the mBMAL1-mCRY interactions, the concentration-dependent binding curve was fitted using a nonlinear regression function (Single Rectangular I, 3 Parameter, Hyperbola, SigmaPlot 10.0) provided by the program SigmaPlot.

ITC—The ITC experiments were performed using an ITC 200 MicroCalorimeter (MicroCal, Northampton, MA). All reagents were extensively dialyzed against a buffer containing 25 mM Bis-tris propane, pH 7.8, 100 mM NaCl, 5 mM Tris(2-carboxyethyl)phosphine, at 22 °C. The concentrations of the binding components in the reservoir solution were experimentally adjusted based on the preliminary knowledge of the interaction range. The concentration of the ligands was chosen between 0.5 and 0.9 mM, whereas the receptor was 10–15 times less. The typical titration consisted of 20 injections of 0.2–2 μl aliquots of the ligand into the receptor solution (250 μl in the cell), at time intervals of 180–360 s. The enthalpy changes ΔH upon binding, the association constant (Kd), and the binding stoichiometry (N) were obtained directly, and the Gibbs energy (ΔG) and entropy (ΔS) changes were calculated according to Equation 3. The dilution heat of the control titration, consisting of the identical titrant solution but with only buffer in the sample cell, was subtracted from each experimental titration. All steps of the data analysis were performed using the ORIGIN (Version 5.0) software provided by the manufacturer (Microcal).

\[
\Delta G^o = \Delta H^o - T\Delta S^o = -RT\ln K_d \\
(Eq. 3)
\]
Ser, Thr, Tyr (tBu); His, Lys, Trp (Boc); Asn, Gln, Cys (Trt); Arg (Pbf). After the last coupling step, the acid-labile protection groups of the amino acid side chains were cleaved using 90% trifluoroacetic acid (TFA) for 30 min and 60% TFA for 3 h.

Binding Studies on Cellulose Membrane-bound Peptides—All primary incubation and washing steps were carried out under gentle shaking at room temperature. After washing the membrane with ethanol once for 10 min and 3 times for 10 min with Tris-buffered saline (TBS: 50 mM Tris-(hydroxymethyl)-amino methane, 137 mM NaCl, 2.7 mM KCl, adjusted to pH 8 with 0.05% HCl), the membrane-bound peptide arrays were blocked for 3 h with blocking buffer (blocking buffer concentrate (Sigma), 1:10 in TBS containing 5% (w/v) sucrose) and then washed with TBS (1 × 10 min). Subsequently, the peptide arrays were incubated with 10 μM analyte solutions (mCRY2-(489–592) or Cy3.5 fluorescence-labeled mBMAL1-(577–625) in TBS blocking buffer at 4 °C overnight. After washing 3 times for 10 min with TBS, analysis and quantification of peptide-bound mBMAL1 was carried out using a Lumi-Imager (Roche Applied Science). For mCRY2, a two-antibody system was used; anti-mCRY2/rabbit antibody (Alpha Diagnostics International, San Antonio, TX) in TBS was incubated at room temperature, resulting in reproducible signal intensities. The spot signal is calculated from a circular region around the spot center detected in the image. The background signal for each analyte solution (mCRY2-(489–592) or Cy3.5 fluorescence-labeled mBMAL1-(577–625)) in TBS blocking buffer at 4 °C overnight. After washing 3 times for 10 min with TBS, detection was done via chemiluminescence of the substrate.

Measurement of Spot Signal Intensities—Analysis and quantification of spot signal intensities were conducted with the Genespotter software package (Microdiscovery, Berlin, Germany). Genespotter has a fully automatic grid-finding routine, resulting in reproducible signal intensities. The spot signal is calculated from a circular region around the spot center detected in the image. The background signal for each spot is determined with a safety margin to the whole membrane background. The fluorescence of Cy3.5-labeled mBMAL1 was measured at 600 nm, and mCRY2 was detected via chemiluminescence.

Peptide Synthesis and Purification—Peptides P1 and P2 corresponding to the predicted coiled coil region of mCRY1 (P1, 472NHAESRLNIERMKIQYQQLSRYRGLALLASVPS365) and the C-terminal mBMAL1 binding epitope in the mCRY1 tail (P2, 564SQQTHSLKQGRSSAGTGSSLGKRPSQE901) were synthesized using standard Fmoc chemistry on solid phase. Purification was performed on a C18 column using a gradient of water, ethanol, 0.08% TFA. The N termini of the peptides were protected by an acetyl group, and the C-termini were protected by amide.

RESULTS

Expression and Purification of mCRY and mBMAl1 Fragments—We have cloned, expressed, and purified C-terminal fragments of the mouse cryptochromes 1 and 2 (mCRY1-(471–606) and mCRY2-(489–592)), which contain the most C-terminal α-helix of the photolyase homology region (predicted CC) and the tail region (Fig. 1). Whereas the coiled coil region is well conserved between mCRY1 and mCRY2, their tails are clearly different. In addition, two C-terminal mBMAL1 fragments, mBMAL1-(490–625) and mBMAL1-(577–625), were constructed based on secondary structure predictions. The mCRYCCtail and mBMAL1 fragments were expressed in E. coli as GST fusion constructs and purified via affinity and size exclusion chromatography. The described purification scheme resulted in overall yields of ~15 mg of highly purified mCRY or mBMAL1 proteins per liter of cell culture (supplemental Fig. S1). The identity of the purified proteins was confirmed by mass spectrometry.

Analysis of Self-oligomerization and Folding by Analytical Ultracentrifugation and CD Spectroscopy—To determine the oligomeric state of the mCRY and mBMAL1 fragments, we have performed analytical ultracentrifugation (AUC) sedimentation velocity experiments (Table 1, supplemental Fig. S2). These experiments showed that all fragments are monomeric at concentrations between 50 and 200 μM. The hydrodynamic (Stokes) radii determined by AUC analyses (Table 1) suggest that the mCRY and mBMAL1 proteins have somewhat elongated shapes and might be at least partially unstructured. Using CD spectroscopy, we have analyzed the secondary structure content of the purified mCRY and mBMAL1 fragments. The CD spectra (supplemental Fig. S3A) and their analysis (Table 2) indicate that all fragments are partially (between 30 and 40%) disordered. This may contribute to their enlarged hydrodynamic radii. Additionally, our CD spectra confirmed the helicity of the synthetic peptide P1 comprising the predicted coiled coil region of the mCRY proteins (12).

Analysis of mCRY-mBMAl1 Interactions by Fluorescence Polarization—To find out if our purified C-terminal mCRYCCtail and mBMAL1 fragments form stable heterodimeric complexes in solution and to determine their binding affinities, we have performed fluorescence polarization experiments. mBMAL1 fragments were labeled with Cy3.5, and mCRY fragments were titrated to a 500 nM solution of fluorescently labeled mBMAL1 (mCRY concentrations ranging from 500 nM to 300 μM). The shorter mBMAL1-(577–625) fragment bound to both mCRYCCtail fragments with a roughly 10 μM affinity (Fig. 2A). In contrast, the longer mBMAL1-(490–625) fragment bound to mCRY1 with an ~40 μM affinity and to mCRY2 with an ~10 μM affinity (Fig. 2B). The different affinities of mCRY1 and mCRY2 to the longer mBMAL1 fragment might be due to the fact that the Cy3.5 dye not only attaches to free N-terminal amino groups but also to side-chain amino groups of lysine residues. Whereas the mBMAL1-(577–625) fragment lacks lysine residues, the mBMAL1-(490–625) fragment contains three lysine residues (Lys-493, Lys-537, Lys-538). Notably, Lys-537 acetylation by mCLOCK enhances mCRY1 binding to mBMAL1 in a cellular environment (21). It is, therefore, conceivable that covalent modification of the mBMAL1-(490–625) fragment by the Cy3.5 dye or the lack of Lys-537 acetylation in the E. coli-expressed mBMAL1-(490–625) fragment specifically weakens mCRY1 binding in our assay.

Analysis of mCRY-mBMAL1 Interactions by Isothermal Titration Calorimetry—To assess the possible influence of a covalent modification of lysine residues or the N-terminal amino group by the Cy3.5 dye, in particular on the mCRY1-mBMAL1-(490–625) interaction, we also determined binding
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In good agreement with the fluorescence polarization data, mCRY1 and mCRY2 bind to the mBMAL1-(577–625) fragment with a roughly 10 μM affinity (Fig. 3A and Table 3). Whereas mCRY2 shows a similar (~10 μM) affinity to both mBMAL1 fragments, mCRY1 binds to the longer mBMAL1-
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TABLE 1
Analysis of the oligomeric state and molecular shape of mBMAL1 and mCRYCtail fragments by analytical ultracentrifugation

| Protein                  | S (nm) | Rf, globular | Rf, GuHCl-unfolded | MW (Da) | Oligomeric state |
|--------------------------|--------|--------------|---------------------|---------|------------------|
| mCRY1CCT                 | 1.146  | 3.26         | 1.91                | 3.43    | Monomeric        |
| mCRY2CCT                 | 1.176  | 2.50         | 1.79                | 3.12    | Monomeric        |
| BMAL490                  | 1.322  | 2.62         | 1.90                | 3.41    | Monomeric        |
| BMAL577                  | 0.667  | 1.98         | 1.35                | 2.03    | Monomeric        |

a The concentration of mCRY1/2 and BMAL490 was adjusted to [mCRY] = 0.5 (corresponds to 0.07 mCry1, 0.17 mCry2, 0.06 mBMAL490). The BMAL577 concentration was 0.2 mM. BMAL490 was also shown to be monomeric at [mBMAL] = 5 corresponding to 0.6 mM BMAL40. mCry1/2CCT = mCRY1/2 coiled-coil-tail fragment.

b BMAL490/577 = mBMAL(490–577/–625) fragment.

c sfs = sedimentation coefficient in Svedberg units. The s values are normalized to 20 °C and water.

d Rf = hydrodynamic (Stokes) radius.

* Sequence molecular weight, calculated as described under "Experimental Procedures."

TABLE 2
CD spectra suggest that the mBMAL1 and mCRY proteins are partially conserved tails and is interrupted. In mCRY1, the second

| Protein                  | % Helix | % Strand | % Turn | % Un-ordered | r.m.s.d. | (N)r.m.s.d. |
|--------------------------|---------|----------|--------|--------------|----------|-------------|
| mCRY1CCT                 | 0.19    | 0.25     | 0.23   | 0.33         | 0.048    | 0.022       |
| mCRY2CCT                 | 0.18    | 0.21     | 0.23   | 0.38         | 0.028    | 0.010       |
| BMAL490                  | 0.09    | 0.26     | 0.24   | 0.41         | 0.018    | 0.008       |
| BMAL577                  | 0.08    | 0.29     | 0.23   | 0.40         | 0.062    | 0.005       |
| Peptide P1               | 0.32    | 0.15     | 0.21   | 0.32         | 0.043    | 0.015       |

For secondary structure analysis the CONTIN algorithm (26) was used with the reference dataset SMP56 (28). Helices include regular and distorted helices. Strands include regular and distorted ß-strand. Values are normalized to 20 °C and water.

* Percent Helix, Strand, Turn, Un-ordered, r.m.s.d. (N)r.m.s.d.

(490–625) fragment with a roughly 20 μM affinity (Fig. 3B and Table 3). Values of 40 μM were never obtained for the mCry1-mBMAL1-(490–625) interaction using ITC. This indicates that the lower affinity of mCRY1 to mBMAL1-(490–625) compared with mBMAL1-(577–625) is an intrinsic feature of the unlabeled proteins and the mCRY1-mBMAL1-(490–625) interaction is additionally weakened by the Cy3.5 dye in the fluorescence polarization experiments. Interestingly, the mutation of Lys-537 to Glu, which mimics the acetylation of Lys-537, increases the affinity of the mBMAL1-(490–625)-mCRY1 interaction to ~10 μM (Table 3). This result suggests that the non-acetylated Lys-537 is indeed responsible for the lower (~20 μM) affinity of mCRY1 to the wild-type mBMAL1-(490–625) fragment. According to our CD spectra, the K537Q mutation does not change the secondary structure content of the mBMAL1-(490–625) fragment (data not shown).
binds to mBMAL1-(577–625) and to the mBMAL1-(490–625)K537Q mutant fragment with an affinity of ~3 μM but to the wild-type mBMAL1-(490–625) fragment with a lower affinity of ~8 μM (Table 3 and Fig. 5, B–D). In contrast to all other mCRY-mBMAL1 interactions that we have analyzed by ITC, the interaction of peptide P2 with mBMAL1-(490–625)K537Q is exothermic and enthalpically as well as entropically favored (Fig. 5D, Table 3). This indicates that the P2-mBMAL1-(490–625)K537Q complex involves a larger number of polar contacts (e.g. hydrogen bonds) than the other mCRY-mBMAL1 interactions, which are entropically but not enthalpically favored (30).

**DISCUSSION**

The C-terminal coiled coil and tail (CCTail) regions of the mammalian cryptochromes (mCRY1/2) and the C-terminal mBMAL1 region critically regulate the activity of the mBMAL1-mCLOCK transcription factor complex within the mammalian circadian clock (12, 16, 17). To provide mechanistic insights into the molecular interactions of the mCRY-
TABLE 3
ITC binding constants and thermodynamic parameters

| Complex             | N  | $K_D$ (μM) | ΔH (kcal/mol) | ΔS (kcal/mol) |
|---------------------|----|------------|---------------|---------------|
| mCRY1CCT-BMAL577    | 0.9| 10.5 ± 2.3 | 2.6           | 9.2           |
| mCRY1CCT-BMAL490    | 0.8| 18.9 ± 6.0 | 1.8           | 8.1           |
| mCRY1CCT-BMAL490(K537Q) | 0.9| 9.3 ± 2.2  | 1.4           | 8.2           |
| mCRY2CCT-BMAL577    | 1.1| 7.8 ± 1.2  | 1.0           | 8.4           |
| mCRY2CCT-BMAL490    | 1.1| 9.5 ± 2.2  | 4.2           | 11.1          |
| P1-BMAL577          | 1.0| 10.7 ± 2.6 | 1.1           | 8.3           |
| P1-BMAL490          | 1.0| 10.6 ± 3.0 | 1.3           | 8.1           |
| P1-BMAL490(K537Q)   | 1.0| 9.6 ± 2.0  | 0.7           | 7.5           |
| P2-BMAL577          | 0.9| 3.3 ± 1.0  | 0.5           | 8.0           |
| P2-BMAL490          | 0.9| 7.7 ± 1.9  | 7.3           | 14.2          |
| P2-BMAL490(K537Q)   | 1.0| 2.9 ± 0.9  | −0.6          | 6.8           |

mBMAL1 fragments (Table 3). We conclude that the mBMAL1 region between residues 490 and 576 and of the Lys-537mBMAL1Gln mutation on the mBMAL1-mCRY1 interaction. The increased affinity of the mCRY1Ctail protein and the P2 tail peptide to the K537Q mutant version of mBMAL1-(490–625) likely mimics the effect of Lys-537 acetylation in vivo, which enhances mCRY1 binding to mBMAL1 and thereby down-regulation of mBMAL1-mCLOCK dependent transcription (21).

Our substitution analysis (Fig. 4D) revealed that alanine mutations of positively charged residues in both mCRY1 peptides weaken the interaction with mBMAL1, whereas alanine mutations of the negatively charged residues Glu-590, Glu-591, and Asp-592, at which the mCRY1 tail epitope is interrupted, strengthen it. Because the mBMAL1 fragments used in this study are negatively charged (pI mBMAL1-(577–625) = 3.4; pI mBMAL1-(490–625) = 4.2), we suggest that binding of the mCRYCCtail fragments is driven by electrostatic interactions. We propose that in its non-acetylated state Lys-537 masks negative charges in mBMAL1 through intramolecular interactions and thereby interferes with mCRY1 binding. Lys-537 acetylation would weaken this masking effect and strengthen electrostatic interactions with positively charged mCRY1 residues predominantly in the tail (Fig. 6).

Notably, Arg-501 and Lys-503 in the coiled coil region are important for the interaction of mCRY2 with mPER2 (31) as well as mBMAL1 (this study). Hence, binding of the mCRY2 coiled tail to mBMAL1 and mPER2 involves very similar molecular surfaces and is likely to be competitive. The functional importance of the coiled coil interaction with mBMAL1 is documented by the reduced efficiency of the R501E/K503R mCRY2 double mutant in transcriptional repression of the mBMAL1-mCLOCK complex (31). Yet the single mutations R501E or K503R weaken the binding of mCRY2 to full-length mPER2 but not to full-length mBMAL1. This is likely due to the fact that mCRY interactions with mPER1 and mPER2 are predominately (if not exclusively) mediated by the coiled coil region and do not require the mCRY tails or the PHCR (12, 31). In the repressive mBMAL1-mCRY complex, the additional and regulated interaction of mBMAL1 with the mCRY tails might facilitate the displacement of mPERs from the common coiled coil binding site.

It is striking that the P2 peptide binds to our mBMAL1 fragments with higher affinities than the mCRY1Ctail fragment (Table 3). This is probably due to the fact that this peptide ends at Glu-591 and, therefore, excludes one of the repulsive residues, Asp-592. Hence, our study suggests the design of tighter binding mCRY1-derived peptides by further elimination of negative charges or the addition of positive charges. Importantly, the K537Q mutation not only leads to an increased affinity of the P2 peptide to mBMAL1-(490–625) but also to an exothermic binding reaction, which is enthalpically and entropically favored (Fig. 5D, Table 3). It is, therefore, conceivable that in the cell, peptide P2 would preferentially bind to mBMAL1, when it is acetylated on Lys-537 by mCLOCK. Peptide P2 and P2-derived potentially tighter binding mCRY1 tail peptides may, therefore, be used in a cell-based system to specifically inhibit the repressive mBMAL1(K543-Ac)-mCRY1 interaction. This could arrest the clock in a state where the mBMAL1-mCLOCK complex is transcriptionally active, possi-
Cryptochrome-mBMAL1 Interactions

It is possible that the full-length mCRY or mBMAL1 proteins contain additional binding regions that further stabilize the mCRY-mBMAL1 complex. For the mCRYs, the correct alignment of the coiled coil region with the PHCR has been shown to be functionally important (12, 13). Because the isolated mCRY-CCtail fragments are partially unstructured and peptide P2 is mostly disordered (supplemental Fig. S3, Table 2), the presence of the PHCR may enhance folding of the tail region. Indeed, a stabilizing interaction between the PHCR and the CCtail frag-

FIGURE 4. Identification of mCRY-mBMAL1 interacting epitopes by peptide scan analysis. mCRY1mCRY1-(471–606) (A) and mCRY2-(489–592) (B) were incubated with Cy3.5-labeled mBMAL1-(577–625). mCRY1mCRY1-(471–606) and mCRY2-(489–592) were dissected into overlapping 10-mer sequences with an overlay of one amino acid (peptide scan). The resulting peptide array was synthesized using SPOT synthesis and probed against mBMAL1-(577–625). Signal intensities (SI) for each membrane spot are plotted against the first amino acid of the corresponding 10-mer peptide. The mCRY1 and mCRY2 membranes were incubated with Cy3.5-labeled mBMAL1-(577–625) (c = 10 μM). Binding to the mCRY peptides was detected by measuring the fluorescence emission of Cy3.5 on each membrane spot at 600 nm. Fluorescence emission of each spot was calculated from a circular region around the spot center detected in the membrane image. The presented results are global background-corrected. C, mBMAL1-(577–625) incubated with mCRY2-(489–592) (c = 10 μM). Binding to the mBMAL1 peptides was detected by measuring the fluorescence emission of Cy3.5 on each membrane spot at 600 nm. Fluorescence emission of each spot was calculated from a circular region around the spot center detected in the membrane image. The presented results are global background-corrected. D, shown is a substitution analysis of the two mBMAL1 binding epitopes of mCRY1. Left, N-terminal epitope (473HAEASRLNIEKQYQQLSRYRGLGLLASVP504) corresponding to the predicted coiled coil region is shown. Significant effects of alanine mutations were only found in the depicted N-terminal peptide region. Right, C-terminal epitope within the mCRY1 tail region (565QQTHSLKQGRSSAGTGLSSGKRPSQEEDAQS595) is shown. Spots in the first row represent the wt mCRY1 sequences. Each spot of the second row corresponds to a mutated peptide in which one residue was replaced by alanine (mutated position as written to the left of the two spot columns). The mCRY1 membranes were incubated with Cy3.5-labeled mBMAL1-(577–625) (c = 10 μM). Signals were measured as described in A and B. Basic and acidic residues, whose substitution by alanine lead to reduced or enhanced mBMAL1-(577–625) binding, are highlighted in red and green, respectively.

bly due to prolonged recruitment of p300/CBP transcriptional coactivators. It is conceivable that a peptide that interferes with the binding of the mCRY1 tail epitope would rather selectively target the mCRY1-mBMAL1 complex. Because our studies revealed different mBMAL1 interactions of the nonconserved mCRY1 and mCRY2 tails, an mCRY1-tail-derived peptide should not significantly affect mBMAL1-mCRY2 interactions. Furthermore, the tails are not required for the interactions of mCRY1 and mCRY2 with mPER2 and mPER1 (12, 31).
FIGURE 5. ITC analysis of the binding of mCRY peptides to mBMAL1. Shown are ITC experiments for binding of peptide P1 (ligand, 0.73 mM) to mBMAL1-(490–625) (receptor, 0.055 mM) (A), peptide P2 (receptor, 0.04 mM) to mBMAL1-(577–625) (ligand, 0.34 mM) (B), peptide P2 (receptor, 0.04 mM) to mBMAL1-(490–625) (ligand, 0.46 mM) (C), and peptide P2 (receptor, 0.025 mM) to mBMAL1-(490–625)K537Q (ligand, 0.34 mM) (D). Binding reactions are dominated by favorable entropy changes. A–C, binding events are endothermic and entropically favored. D, binding is exothermic and entropically and enthalpically favored. The top panels show the time response of the heat change upon addition of the ligand. The best fits (lower panels) were obtained by using a single site binding model (best $\chi^2$ statistic) resulting in a 1:1 stoichiometry ($N$ close to 1). At the used concentrations, receptor and ligand are monomeric according to our AUC measurements (Table 1).
nuclear localizationsignals (585KRP epitopes of mCRY1 and mCRY2 both contain bipartite
or be affected by mBMAL1 binding. Furthermore, the tail
enhanced by mPER interactions with the mBMAL1-mCLOCK
repressive mPER-mCRY complex their binding might be
mBMAL1 interacts with the mCRY2 PHCR in a mammalian
8-hybrid system (31), and mCRY1 binds weakly to the PAS-B
mCRY1 residues predominantly in its tail region.
ment of hCRY2 has been reported previously (32). Moreover,
mBMAL1 interacts with the mCRY2 PHCR in a mammalian
C-terminal residues as well as a preceding predicted
585KRKP13KVKQR602 in mCRY1, 559KRX13KRAR578 in mCRY2) (12, 14) (Figs. 1B and 4A and
K537 in mCRY2 (12, 14) (Figs. 1B and 4A and B), which might also be affected by the mBMAL1 interaction.
We have mapped the mCRY binding epitopes of mBMAL1 to the most C-terminal residues as well as a preceding predicted
α-helix (Figs. 4C and 1B). Although it is tempting to speculate
about a helical interaction between the mCRY coiled coil region
and the predicted helical epitope of mBMAL1, deletions or
mutations within the last 15 mBMAL1 residues have been
reported to interfere with mCRY-mBMAL1 interactions and mCRY-dependent transcriptional repression (16, 17). Presum-
ably, both epitopes are relevant to mCRY interactions, and it
remains to be seen which part of the mCRYCCtail region binds
to which mBMAL1 epitope.

With the presented work we have shown that the mCRY
collapsed coil and tail regions directly interact with the C-terminal
27 amino acids of the transcription factor mBMAL1. The mCRY1tail-mBMAL1 interaction is specifically affected by Lys-537, whose acetylation enhances mCRY1-mBMAL1 inter-
actions in vivo. Our study suggests the design of peptide ligands
targeting the interface between the mCRY1 tail region and
mBMAL1. By inhibiting the repressive mBMAL1-mCRY1
interaction, such peptides may affect the transcriptional regu-
lation of clock genes (and, hence, the circadian clock) and
clock-controlled genes (and hence the circadian regulation of
body functions).

Acknowledgments—We thank S. Luebel and E. Weyer-Stingl in the
Core facility of the MPI of Biochemistry for help with biophysical
experiments.

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