Quantitative analyses of Cryptochrome - mBMAL1 interactions: mechanistic insights into the transcriptional regulation of the mammalian circadian clock

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ABSTRACT
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 polarisation and isothermal titration calorimetry that purified mCRY1/2CCtail proteins form stable heterodimeric complexes with two C-terminal mBMAL1 fragments. The longer mBMAL1 fragment (BMAL490) includes Lys537, 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 to BMAL577 and BMAL490(K537Q). We propose, that Lys537 acetylation enhances mCRY1 binding by affecting electrostatic interactions predominantly with the mCRY1 tail. Our data reveal different molecular interactions of the mCRY1 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.
INTRODUCTION
In mammals, many physiological processes are regulated in a day-time dependent manner. These circadian (24 h) rhythms are generated 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 detoxification 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 behavioural and molecular level (6). While mCRY1/mCRY2 double knockout mice become totally arrhythmic (7-9), mice with single mCRY1−/− or mCRY2−/− single knockout mice exhibit a 1 h shorter period and mCRY2−/− single knockout 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 a ~500 amino acids 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 photolyase homology core region (PHCR). Strikingly, the mCRY1Ctail fragment alone does not mediate transcriptional repression when fused to EGFP (12). Moreover, mutations which 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 localisation signals within their tails (12,14). Furthermore, Ser557 and Ser553 in the mCRY2 tail are phosphorylated sequentially by DYRK1A (Dual-specificity tyrosin-phosphorylated and regulated kinase 1A) and GSK-3β (Glycogen synthase kinase-3β) (15). Ser553/557 phosphorylation triggers the proteasomal degradation of mCRY2 and thereby delays its accumulation and nuclear entry.

In the C-terminal region of mBMAL1, the mutations Ala610Ser, Ala610Thr and Gly611Glu were shown to reduce sensitivity to mCRY1/2 repression (16). Moreover, deletion of the last 8 mBMAL1 residues reduces the mBMAL1-mCRY1 interaction in coimmunoprecipitation experiments and insertions C-terminal to Ala600 or Leu606 severely affect circadian core oscillations and transcriptional activation (17). Interestingly, the most C-terminal 43 amino acids of mBMAL1 also mediate transcriptional activation by recruiting coactivators such as p300/CBP in a daily regulated manner, with a maximum efficiency around CT (circadian time) 6 (18,19). Furthermore, mCRY1 and mCRY2 inhibit the p300 induced transcriptional activation of mBMAL1/mCLOCK by about 80% (19). Since mCRY1/2 protein levels in the SCN (suprachiasmatic nucleus) are highest between CT12 and CT16 and low at CT6 (3), it is conceivable that mCRY proteins displace transcriptional coactivators in a daily regulated manner. Collectively, the published data suggest that the C-terminal mBMAL1 region 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. While 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 Lys537 (21) (Fig. 1B). Acetylation of Lys537 of mBMAL1 occurs...
in a daily regulated manner with a peak at about CT15, i.e. during the repressive phase. Moreover, Lys537 acetylation enhances the mCRY1–mBMAL1 interaction and thereby transcriptional repression (21). To quantitatively analyse 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 kDa and 14.3 kDa, the latter including the in vivo acetylated Lys537. We show that mCRY1 (but not mCRY2) exhibits a lower affinity to the longer- than to the shorter mBMAL1 fragment and compared to a mutant version of the longer mBMAL1 fragment, in which Lys537 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 -/- knockout studies (7-9). Furthermore, we propose a molecular mechanism for the regulation of mCRY1 binding by Lys537 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.

**EXPERIMENTAL PROCEDURES**

**Recombinant Expression and Purification of mCRY and mBMAL1 proteins** - C-terminal fragments of the mouse cryptochromes (mCRY1[471-606] and mCRY2[489-592]) and mouse mBMAL1 (mBMAL1[577-625] and mBMAL1[490-625]) were subcloned into a pGEX-6P2 expression vector using restriction sites 5’ BamHI (mCRY1, both mBMAL1 fragments) or Smal (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 litres of mCRY- or mBMAL1 expression cultures in TB (Terrific Broth) medium were induced with 0.1 mM IPTG at an OD₆₀₀ of about 1. Expression was carried out for 5 h at 30°C or over night at 18°C. Pellets were thawed on ice and homogeneously resuspended in a lysis buffer containing 50 mM Tris-HCl pH 7.8, 250 mM NaCl, 10 mM β-Mercaptoethanol, 10% Glycerol, 2 mM EDTA and 1mM phenylmethylsulfonylfluoride (PMSF). 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 Prescision 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 Glutathion. Tag removal yielded recombinant mCRY and mBMAL1 proteins with the N-terminal extensions GPLGS (BamHI) or GPLGSPGIPG (Smal, mCRY2) leading to the following molecular weights and isoelectric points (pIs) of the recombinant proteins: mCRY1[471-606]: 14432.7 Da, pI = 8.91; mCRY2[489-592]: 12087.0 Da, pI = 6.94; mBMAL1[490-625]: 14263.8 Da, pI = 4.18; mBMAL1[577-625]: 5482.0 Da, pI = 3.44. Fractions containing cleaved mCRY- or mBMAL1 proteins were concentrated using an AmiconUltra-15 filter device (Millipore, Bedford, MA, USA) with a 3-10 kDa molecular weight cut off and loaded onto a Superdex HiLoad S75 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. 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 concentrated using an Amicon Ultra-15 filter device (Millipore, Bedford, MA, USA) with a 3-10 kDa molecular weight cut off and loaded onto a Superdex HiLoad S75 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. 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 velocity experiments** – Sedimentation velocity experiments were performed with an Optima XL-I analytical centrifuge (Beckman Inc., Palo Alto, Ca, U.S.A.) using an An 60 Ti rotor with double-sector epon centrepieces or titanium centrepieces of 12 mm pathlength (Nanolynics, Germany), capped with sapphire windows. The
proteins were kept in 25 mM Bis-tris propane pH 7.8, 100 mM NaCl, 5 mM TCEP at concentrations of 0.07 mM for mCRY1 [471-606], 0.17 mM for mCRY2 [489-592], 0.2 mM for mBMAL1 [577-625] and 0.06 or 0.6 mM for mBMAL1 [490-625]. The buffer density was measured with a DMA 5000 densitometer. All other auxiliary parameters were calculated from the buffer composition using SEDNTERP (22).

The protein concentration distribution during sedimentation was monitored by absorption or interference detection. 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 frictional coefficient f/f₀, which was optimised during fitting. The combination of s and f/f₀ allows an estimate of the molar mass Mₛ 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 (Rₑ) were calculated with SEDNTERP, expected Rₑ-values for folded and unfolded proteins of equal molar mass were obtained using the empirical formulae for globular and guadinium-HCl-unfolded proteins (equations (2) and (6), respectively of references (24,25)).

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 TCEP 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 nm to 250 nm with 0.1 nm intervals collecting data for 0.5 s at each point. For each measurement ten spectra were used for accumulation. Analysis was performed using the CONTIN algorithm (26) with the reference dataset SMP56 (27,28).

Fluorescence Polariation - mBMAL1 fragments were fluorescently labelled 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 labelling, a 15-20 mg/ml concentrated solution of purified mBMAL1 protein in a sodium carbonate buffer (0.1 M Na₂CO₃ pH 8.5) was incubated with the Cy3.5 dye for 2 h at 4°C. For the fluorescence polarisation measurement, the protein was transferred into a buffer containing 25 mM Hepes pH 7.8, 50 mM NaCl, 2 mM DTE, 2.5% (v/v) glycerol with a desalting HiTrap column (GE Healthcare). Fluorescence polarisation 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, USA) was used in the polarisation mode at 10°C. 500 nM Cy3.5-labelled 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 (Kₐ) 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.

Isothermal Titration Calorimetry (ITC) - The ITC experiments were performed using an ITC 200 MicroCalorimeter (MicroCal, Northampton, MA). All reagents were extensively dialysed against a buffer containing 25 mM Bis-tris propane pH 7.8, 100 mM NaCl, 5 mM TCEP, 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 mM and 0.9 mM while the receptor was 10 to 15 times less. The typical titration consisted of 20 injections of 0.2 to 2 µl aliquots of the ligand into the receptor solution (250 µl in the cell), at time intervals of 180 to 360 sec. The enthalpy changes ΔH upon binding, the association constant (Kₐ), 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 (V5.0) software provided by the manufacturer (Microcal).

\[\Delta G^\circ = \Delta H^\circ - T\Delta S^\circ = - RT \ln K_A\]

SPOT synthesis - Cellulose-bound peptide arrays were prepared according to standard SPOT synthesis protocols using a SPOT synthesizer (Intavis, Köln, Germany) as described in detail.
in (29). The peptides were synthesized on amino-functionalized cellulose membranes (Whatman, Maidstone, Great Britain) of the ester type prepared by modifying cellulose paper with Fmoc-b-alanine as the first spacer residue. In the second coupling step, the anchor position Fmoc-b-alanine-OPfp in dimethylsulfoxide (DMSO) was used. Residual amino functions between the spots were capped by acetylation. The Fmoc group was cleaved using 20% piperidine in dimethylformamide (DMF). The cellulose-bound peptide arrays were assembled on these membranes by using 0.3 M solutions of Fmoc-amino acid-OPfp in 1-Methyl-2-pyrrolidone (NMP). Side-chain protection of the Fmoc-amino acids used was as follows: Glu, Asp (OtBu); 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% trifluoro-acetic 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 three times for 10 min with Tris buffered saline (TBS: 50 mM Tris-(hydroxymethyl)-aminomethane, 137 mM NaCl, 2.7 mM KCl, adjusted to pH 8 with HCl/0.05%), the membrane bound peptide arrays were blocked 3 h with blocking buffer (blocking buffer concentrate (Sigma-Aldrich, Steinheim, Germany), 1:10 in TBS containing 5% (w/v) sucrose), and then washed with TBS (1x10 min). Subsequently, the peptide arrays were incubated with 10 µM analyte solutions (mCRY2[489-592] or Cy3.5 fluorescence labelled mBMAL1[577-625]) in TBS blocking buffer at 4°C overnight. After washing three times for 10 min with TBS, analysis and quantification of peptide-bound mBMAL1 was carried out using a Lumi-Imager (Roche, Indianapolis, USA). For mCRY2, a two-antibody system was used: Anti-mCRY2/rabbit antibody (Alpha Diagnostics International, San Antonio, USA) in TBS was incubated at room temperature (1 h) and after washing three times with TBS, peroxidase labeled anti rabbit IgG was used as secondary antibody and also incubated for 1 h. After washing three times 10 min with TBS, detection was done via chemiluminescence of the substrate.

Measurement of spot signal intensities - Analysis and quantification of spot signal intensities (SI) 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-labelled 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: 472NHAEASRLNIERMKQIYQQLSRYRGLLASVPS505) and the C-terminal mBMAL1 binding epitope in the mCRY1 tail (P2: 564SQQTTHSLKQGRSSAGTLSSGKRPSQEE591) 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 by amid.

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 coiled coil, 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 mCRYCtail- 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 about 15 mg of highly purified mCRY1 or mBMAL1 proteins per litre of cell culture (Supplementary Fig. S1). The identity of the purified proteins was confirmed by mass spectrometry.

Analysis of selfoligomerisation 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 (SV) experiments (Table 1, Supplementary 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 disordered. Using circular dichroism (CD) spectroscopy, we have analysed the secondary structure content of the purified mCRY- and mBMAL1 fragments. The CD spectra (Supplementary 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 polarisation.** 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 polarisation experiments. mBMAL1 fragments were labelled with Cy3.5 and mCRY fragments were titrated to a 500 nM solution of fluorescently labelled 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 a - 40 µM affinity and to mCRY2 with a - 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 (Lys493, Lys537, Lys538). Notably, Lys537 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 Lys537 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 have also determined binding affinities by ITC using unlabelled mCRY and mBMAL1 proteins. In good agreement with the fluorescence polarisation 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[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 to mBMAL1[577-625] is an intrinsic feature of the unlabelled proteins and the mCRY1-mBMAL1[490-625] interaction is additionally weakened by the Cy3.5 dye in the fluorescence polarisation experiments. Interestingly, the mutation of Lys537 to Gln, which mimics the acetylation of Lys537, increases the affinity of the mBMAL1[490-625]-mCRY1 interaction to about 10 µM (Table 3). This result suggests, that the non-acetylated Lys537 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).

**Identification of mCRY-mBMAL1 interacting epitopes by Peptide scan analysis.** To map the mCRY-mBMAL1 interaction sites more accurately, we have performed peptide scan analyses. For determination of the mBMAL1 binding sites of mCRY1 and mCRY2, we used the Cy3.5 labelled mBMAL1[577-625] fragment as analyte. Mapping of the mCRY2 binding site on the mBMAL1[577-625] fragment was performed with an anti-mCRY2 antibody. The peptide scan analysis revealed two mBMAL1 binding sites in mCRY1 and mCRY2 (Fig. 4A, B). One binding site corresponds to the predicted coiled coil region (Fig. 1). This epitope has been identified in both cryptochromes and comprises residues Ala476 to Pro504 in mCRY1 and residues Thr494 to Pro522 in mCRY2. The second epitope of both cryptochromes lies within their non-conserved tails and is
interrupted. In mCRY1, the second epitope includes amino acids between Ser564 and the C-terminal end and is interrupted at the acidic residues Glu590, Glu591 and Asp592 (Fig. 4A). In mCRY2, the second epitope lies between Ala540 and Thr580 and includes a gap at residues Glu566, Glu567 and Pro568 (Fig. 4B).

Our peptide scan analysis of mBMAL1 revealed two binding sites for mCRY2 (Fig. 4C): one epitope between residues Ala598 and Ala610 including a predicted α-helical mBMAL1 segment (Fig. 1B) and a second epitope between Leu612 and the C-terminal end.

**Substitutional analysis of mBMAL1 binding mCRY epitopes.** To determine, which mCRY residues are most critical for the mBMAL1 interaction, we have SPOT synthesized peptides including the conserved mCRY coiled coil epitope (mCRY1 sequence 473HAEASRLNIER MKQIYQQLSRYRLGGLASVP490) and the major part of the mCRY1 tail epitope (565QQTHSLKGGRSSATGGLSSGKRPSQED AQ585). We have exchanged each amino acid in the mCRY peptides to alanine and measured the binding of the modified peptides to Cy3.5 fluorescently labelled mBMAL1 [577-625] (Fig. 4D). This experiment showed, that single alanine mutations of the negatively charged residues Glu590, Glu591 or Asp592 in the mCRY1 tail peptide raise its affinity to mBMAL1 drastically, whereas the exchange of any single lysine or arginine to alanine lowers it. Similarly, the exchange of positively charged residues in the N-terminal part of the coiled coil epitope (Arg478, Arg483 and Lys485 in mCRY1; Arg496, Arg501 and Lys503 in mCRY2) to alanine significantly reduced the affinity to mBMAL1 [577-625]. We conclude, that the interaction with the overall acidic mBMAL1 fragments is driven predominantly by electrostatic interactions and the interruption of the mCRY1 tail epitope is likely due to electrostatic repulsion effects.

**ITC analysis of mBMAL1 interactions with mCRY peptides.** To quantify the contributions of the two mBMAL1 binding epitopes of mCRY1 and to find out, which epitope is responsible for the different binding affinities of mCRY1 to mBMAL1 [577-625], mBMAL1 [490-625] and mBMAL1 [490-625]K537Q, we have synthesized peptides comprising mCRY1 residues Asn472 to Ser505 corresponding to the predicted coiled coil region (peptide P1) as well as residues Ser564 to Glu591 within the mCRY1 tail region (peptide P2). CD spectroscopy showed that peptide P1 has a high α-helical content (as predicted), whereas peptide P2 is mostly disordered (Table 2 and Supplementary Fig. S3B). Our ITC measurements revealed, that peptide P1 binds to all three mBMAL1 fragments with an affinity of about 10 µM (Table 3 and Fig. 5A). Peptide P2 however, binds to mBMAL1 [577-625] and to the mBMAL1 [490-625]K537Q mutant fragment with an affinity of about 3 µM, but to the wild-type mBMAL1 [490-625] fragment with a lower affinity of about 8 µM (Table 3 and Fig. 5B, C, D). In contrast to all other mCRY-mBMAL1 interactions that we have analysed by ITC, the interaction of peptide P2 with mBMAL1 [490-625]K537Q is exothermic and enthalpically as well as entropically favoured (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-BMAL1 interactions, which are entropically but not enthalpically favoured (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 mCRYCCtail- and C-terminal mBMAL1 regions and their regulation by mBMAL1 acetylation on Lys537 (21), we have purified mCRYCCtail proteins (mCRY1 [471-606] and mCRY2 [489-592]) and two C-terminal mBMAL1 fragments (mBMAL1 [577-625] and mBMAL1 [490-625]) and quantitatively analysed their interactions by fluorescence polarisation and ITC (Figs. 2, 3 and Table 3). While mCRY2 [489-592] bound equally well to both mBMAL1 fragments (K_D = 8 – 10 µM), mCRY1 showed a roughly two times weaker interaction with the longer mBMAL1 [490-625] fragment, which was additionally destabilized by the Cy3.5 dye used in the fluorescence polarisation assay (K_D about 20 µM without- and 40 µM with Cy3.5 dye). We conclude, that the mBMAL1 region between residues 490 and 576 specifically weakens the binding of mCRY1 (but not mCRY2), presumably by masking mCRY1 binding sites located within the shorter mBMAL1 [577-625] fragment. Interestingly, the mutated
mBMAL1[490-625]K537Q fragment, in which Lys537 acetylation is mimicked by a glutamine, binds to the mCRY1CCtail protein with a similar affinity ($K_D$ about 10 $\mu$M) as mBMAL1[577-625] (Table 3). The K537Q mutation therefore appears to unmask the mCRY1 binding sites in the mBMAL1[577-625] fragment. We have identified two mBMAL1 binding epitopes in the predicted coiled coil region and within the tails of mCRY1- and mCRY2 (Fig. 4A, B). Whereas a synthetic peptide corresponding to the mCRY coiled coil region (P1) bound equally well to the short and to the long (wild-type and K537Q mutant) mBMAL1 fragments ($K_D$ about 10 $\mu$M), peptide P2 including the mCRY1 tail epitope showed a roughly two times lower affinity to mBMAL1[490-625] than to mBMAL1[577-625] ($K_D$s about 8 $\mu$M vs 3 $\mu$M) and bound to the mBMAL1[490-625]K537Q mutant fragment with a similar affinity as to mBMAL1[577-625] ($K_D$ about 3 $\mu$M). Hence, the relative affinities of peptide P2 to our mBMAL1 fragments reflect those of the mCRY1CCtail fragment (Table 3). We conclude, that the mCRY1 tail epitope accounts for the effects of the mBMAL1 region between residues 490 and 576 and of the Lys537mBMAL1Gln mutation on the mBMAL1-mCRY1 interaction. Furthermore, the non-conserved mCRY tail epitopes are responsible for the different binding affinities of mCRY1 and mCRY2 to the mBMAL1[490-625] fragment. The increased affinity of the mCRY1CCtail protein and the P2 tail peptide to the K537Q mutant version of mBMAL1[490-625] likely mimics the effect of Lys537 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 mCRY epitopes weaken the interaction with mBMAL1, whereas alanine mutations of the negatively charged residues Glu590, Glu591 and Asp592, at which the mCRY1 tail epitope is interrupted, strengthen it. Since 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 mCRY1CCtail fragments is driven by electrostatic interactions. We propose, that in its non-acetylated state, Lys537 masks negative charges in mBMAL1 through intramolecular interactions and thereby interferes with mCRY1 binding. Lys537 acetylation would weaken this masking effect and strengthen electrostatic interactions with positively charged mCRY1 residues predominantly in the tail (Fig. 6).

Notably, Arg501 and Lys503 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 mCRY coiled coil 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 Arg501Gln/Lys503Arg mCRY2 double mutant in transcriptional repression of the mBMAL1/mCLOCK complex (31). Yet, the single mutations Arg501Gln or Lys503Arg weaken the binding of mCRY2 to full-length mPER2 but not to full-length mBMAL1. This is likely due the fact that mCRY interactions with mPER1 and mPER2 are predominantly (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 mCRY1CCtail fragment (Table 3). This is probably due to the fact, that this peptide ends at Glu591 and therefore excludes one of the repulsive residues, Asp592. Hence, our study suggests the design of tighter binding mCRY1-derived peptides by further elimination of negative charges or 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 favoured (Fig. 5D, Table 3). It is therefore conceivable, that in the cell peptide P2 would preferentially bind to mBMAL1, when it is acetylated at Lys537 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, possibly 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. Since
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).

It is possible, that the full-length mCRY- or mBMAL1 proteins contain additional binding regions, which 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). Since the isolated mCRYCCtail fragments are partially unstructured and peptide P2 is mostly disordered (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 Ctail fragment of hCRY2 has been reported previously (32).

Moreover, mBMAL1 interacts with the mCRY2 PHCR in a mammalian-2-hybrid system (31) and mCRY1 binds weakly to the PAS-B domain of mBMAL1 (20). Finally, mCRYs might bind more tightly to the mBMAL1-mCLOCK heterodimer and, in a repressive mPER-mCRY complex their binding might be enhanced by mPER interactions with the mBMAL1-mCLOCK PAS domains (33). Additionally, posttranslational modifications such as acetylation (e.g. on Lys537mBMAL1, see above) or phosphorylation (e.g. Ser553/Ser557 in the mCRY2 tail region (15)) may influence the mCRY-mBMAL1 interactions. Since Ser553 and Ser557 are located within the C-terminal mBMAL1 binding epitope of mCRY2 (Fig. 4B), their phosphorylation may influence or be affected by mBMAL1 binding. Furthermore, the tail epitopes of mCRY1 and mCRY2 both contain bipartite nuclear localisation signals (585KRP-X15-KVQR602 in mCRY1, 559KRXX13KRAR578 in mCRY2) (12,14) (Figs. 1B, 4A, 4B), 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, 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). Presumably, 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 coiled coil and tail regions directly interact with the C-terminal 27 amino acids of the transcription factor mBMAL1. The mCRYtail - mBMAL1 interaction is specifically affected by Lys537, whose acetylation enhances mCRY1-mBMAL1 interactions 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 regulation of clock genes (and hence the circadian clock) and clock controlled genes (and hence the circadian regulation of body functions).
REFERENCES


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FOOTNOTES

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The abbreviations used are: CRY, cryptochrome; PER, Period; PAS, PER-ARNT-SIM; PH(C)R, photolyase homology (core) region; CC, coiled coil; bHLH, basic Helix-Loop-Helix; BMAL, brain and muscle ARNT-like protein; CLOCK, circadian locomotor output cycle kaput; m, mouse/mammalian; h, human; ITC, isothermal titration calorimetry; CD, circular dichroism; AUC, analytical ultracentrifugation; CT, circadian time;

FIGURE LEGENDS

Fig. 1: Domain architecture and secondary structure prediction of mCRY1/2 and mBMAL1

A: Domain architecture of full-length mCRY1/2 and mBMAL1. mCRY1 and mCRY2 are composed of a conserved photolyase homology region (PHR) and nonconserved C-terminal tails. The PHR consists of an N-terminal αβ-domain and an α-helical domain, which includes a predicted coiled coil (CC) region at its C-terminal end. In the PHR of photoreceptor-type cryptochromes, the chromophores methenyltetrahydrofolate (MTHF) and flavine adenine dinucleotide (FAD) are non-covalently bound (34). In mBMAL1, the two PAS (PER-ARNT-SIM) domains (PAS-A and PAS-B) and the bHLH domain are shown. The C-terminal mCRYCCtail- and mBMAL1 fragments used in our biochemical studies are represented as black arrows.

B: Secondary structure prediction of the C-terminal mCRY1/2CCtail- and mBMAL1 fragments. The amino acid sequences and secondary structure predictions (PSIPRED v3.0; (35)) of the mCRY1[471-606], mCRY2[489-592], mBMAL1[577-625] and mBMAL1[490-625] fragments studied herein are shown. Numbering of mBMAL1 residues refers to Isoform b/2, which contains 625 amino acids. mCRY1 peptides P1 and P2, which have been synthesized for interaction studies are shown as yellow boxes. CC: predicted coiled coil, Ac: in vivo acetylated Lys537. mCRY1/2CCT: mCRYCCtail fragments mCRY1[471-606] and mCRY2[489-592] including the coiled coil and tail regions.

Fig. 2: Fluorescence polarisation spectra for mBMAL1-mCRY1/2 interactions. Polarisation values are plotted against the concentration of the mCRY titrant. mCRY1[471-606] and mCRY2[489-592] proteins are titrated into 500 nM solutions of Cy3.5 fluorescently labelled mBMAL1[577-625] (A) and mBMAL1[490-625] (B) proteins. mCRY concentrations are stepwise increased from 500 nM to 300 µM. The increasing polarisation values document the formation of mBMAL1-mCRY complexes. K_D values are in the µM range (see insets). The experiments were reproduced at least three times with similar results. mCRY1/2CCT: mCRYCCtail fragments. BMAL577/490: mBMAL1[577-625] and mBMAL1[490-625] fragments.

Fig. 3: Analysis of mCRY-mBMAL1 interactions by isothermal titration calorimetry (ITC). Representative ITC experiments for the interactions of (A) mCRY2[489-592] (R, 0.052 mM) with mBMAL1[577-625] (L, 0.6 mM) and (B) mCRY1[471-606] (R, 0.06) with mBMAL1[490-625] (L, 0.8 mM). For both titrations the binding events are endothermic (heat is absorbed) and entropically 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-squared statistics) resulting in a 1:1 stoichiometry (N close to 1). At the used concentrations, receptor (R) and ligand (L) are monomeric according to our AUC measurements (Table 1).
**Fig. 4: Identification of mCRY-mBMAL1 interacting epitopes by peptide scan analysis**

(A) mCRY1[471-606] and (B) mCRY2[489-592] incubated with Cy3.5 labelled mBMAL1[577-625]: mCRY1[471-606] and mCRY2[489-592] were dissected into overlapping 10mer 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 for each membrane spot are plotted against the 1st amino acid of the corresponding 10mer peptide. The mCRY1 and mCRY2 membranes were incubated with Cy3.5-labelled 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 is 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]: Signal intensities for each membrane spot are plotted against the 1st amino acid of the corresponding 10mer peptide. The membrane spots contain an array of 10mer peptides covering the entire mBMAL1[577-625] sequence with a shift of one acid. The mBMAL1 membrane was incubated with a 10 µM mCRY2 solution and a two-antibody system was used to detect mCRY2 binding to the mBMAL1 peptides via chemoluminescence. The spot signal measured by means of chemiluminescence is calculated from a circular region around the spot center detected in the image. The presented results are global background corrected.

**D:** Substitutional analysis of the two mBMAL1 binding epitopes of mCRY1. Left: N-terminal epitope (473HAEASRLNIERMKQIQYQQLSRYRGLLASSV504) corresponding to the predicted coiled coil region. Significant effects of alanine mutations were only found in the depicted N-terminal peptide region. Right: C-terminal epitope within the mCRY1 tail region (585QQTHSLKQGRSSAGTGGLSSGKPSQEDAQS595). 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 2 spot columns). The mCRY1 membranes were incubated with Cy3.5-labelled mBMAL1[577-625] (c = 10 µM). Signals were measured as described in 4 A/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.

**Fig. 5: ITC analysis of the binding of mCRY-peptides to mBMAL1.** ITC experiments for binding of (A) Peptide P1 (L, 0.73 mM) to mBMAL1[490-625] (R, 0.055 mM), (B) Peptide P2 (R, 0.04 mM) to mBMAL1[577-625] (L, 0.34 mM) (C) Peptide P2 (R, 0.04 mM) to mBMAL1[490-625] (L, 0.46 mM) and (D) Peptide P2 (R, 0.025 mM) to mBMAL1[490-625]K537Q (L, 0.34 mM). Binding reactions are dominated by favorable entropy changes. A,B,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-squared statistics) resulting in a 1:1 stoichiometry (N close to 1). At the used concentrations, receptor (R) and ligand (L) are monomeric according to our AUC measurements (Table 1).

**Fig. 6: Model for the regulation of mCRY1 binding by Lys537BMAL1 acetylation**

Top: In its non-acetylated state, Lys537 masks negative charges in mBMAL1 through intramolecular interactions and thereby interferes with mCRY1 binding. Bottom: Lys537 acetylation unmasks negative mBMAL1 charges and allows for electrostatic interactions with positively charged mCRY1 residues predominantly in its tail region.
Table 1: Analysis of the oligomeric state and molecular shape of mBMAL1- and mCRYCCtail fragments by analytical ultracentrifugation

<table>
<thead>
<tr>
<th>Protein</th>
<th>s [S] 2)</th>
<th>R_H [nm] 3) globular</th>
<th>R_H [nm] GuHCl-unfolded</th>
<th>MW [Da] 4)</th>
<th>oligomeric state</th>
</tr>
</thead>
<tbody>
<tr>
<td>mCRY1CCT</td>
<td>1.146</td>
<td>3.26</td>
<td>1.91</td>
<td>3.43</td>
<td>14433</td>
</tr>
<tr>
<td>mCRY2CCT</td>
<td>1.176</td>
<td>2.50</td>
<td>1.79</td>
<td>3.12</td>
<td>12087</td>
</tr>
<tr>
<td>BMAL490</td>
<td>1.322</td>
<td>2.62</td>
<td>1.90</td>
<td>3.41</td>
<td>14264</td>
</tr>
<tr>
<td>BMAL577</td>
<td>0.667</td>
<td>1.98</td>
<td>1.35</td>
<td>2.03</td>
<td>5482</td>
</tr>
</tbody>
</table>

1) The concentration of mCRY1/2 and BMAL490 was adjusted to OD_{280} = 0.5 (corresponds to 0.07 mM mCRY1, 0.17 mM mCRY2, 0.06 mM BMAL490). The BMAL577 concentration was 0.2 mM. BMAL490 was also shown to be monomeric at OD_{280} = 5 corresponding to 0.6 mM BMAL490. mCRY1/2CCT = mCRY1/2 coiled-coil-tail fragment. BMAL490/577 = mBMAL1[490/577-625] fragment.
2) s[S] = sedimentation coefficient in Svedberg. The s values are normalized to 20 °C and water.
3) R_H = hydrodynamic (Stokes) radius.
4) Sequence molecular weight, calculated as described in Mat & Meth.
Table 2: CD spectra suggest that the mBMAL1 and mCRY proteins are partially disordered and confirm the predicted helicity of the mCRY coiled coil region

<table>
<thead>
<tr>
<th>Protein</th>
<th>Fractions*</th>
<th>Helix [%]</th>
<th>Strand [%]</th>
<th>Turn [%]</th>
<th>Unordered [%]</th>
<th>RMSD</th>
<th>NRMSD</th>
</tr>
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<tr>
<td>mCRY1CCT</td>
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<td>0.25</td>
<td>0.23</td>
<td>0.33</td>
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<td></td>
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<tr>
<td>mCRY2CCT</td>
<td>0.18</td>
<td>0.21</td>
<td>0.23</td>
<td>0.38</td>
<td>0.028</td>
<td>0.010</td>
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<tr>
<td>BMAL490</td>
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<td>0.26</td>
<td>0.24</td>
<td>0.41</td>
<td>0.018</td>
<td>0.008</td>
<td></td>
</tr>
<tr>
<td>BMAL577</td>
<td>0.08</td>
<td>0.29</td>
<td>0.23</td>
<td>0.40</td>
<td>0.062</td>
<td>0.005</td>
<td></td>
</tr>
<tr>
<td>Peptide P1</td>
<td>0.32</td>
<td>0.15</td>
<td>0.21</td>
<td>0.32</td>
<td>0.043</td>
<td>0.015</td>
<td></td>
</tr>
</tbody>
</table>

*For secondary structure analysis the CONTIN algorithm (26) was used with the reference dataset SMP56 (28). (N)RMSD = (normalized) root mean square deviation. Helices include regular and distorted helices. Strands include regular and distorted β-strand (27). Peptide P1: mCRY peptide comprising the predicted coiled coil region.
Table 3: ITC binding constants and thermodynamic parameters$^{ab}$

<table>
<thead>
<tr>
<th>COMPLEX</th>
<th>N</th>
<th>$K_D$ (µM)</th>
<th>$\Delta H$ (kcal/mol)</th>
<th>$T\Delta S$ (kcal/mol)</th>
</tr>
</thead>
<tbody>
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<td>mCRY1CCT / BMAL577</td>
<td>0.9</td>
<td>$10.5 \pm 2.3$</td>
<td>2.6</td>
<td>9.2</td>
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<tr>
<td>mCRY1CCT / BMAL490</td>
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<td>$18.9 \pm 5.0$</td>
<td>1.8</td>
<td>8.1</td>
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<tr>
<td>mCRY1CCT / BMAL490K537Q</td>
<td>0.8</td>
<td>$9.3 \pm 2.2$</td>
<td>1.4</td>
<td>8.2</td>
</tr>
<tr>
<td>mCRY2CCT / BMAL577</td>
<td>1.1</td>
<td>$7.8 \pm 1.2$</td>
<td>1.0</td>
<td>8.4</td>
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<tr>
<td>mCRY2CCT / BMAL490</td>
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<td>$9.5 \pm 2.2$</td>
<td>4.2</td>
<td>11.1</td>
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<tr>
<td>P1 / BMAL577</td>
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<td>$10.7 \pm 2.6$</td>
<td>1.1</td>
<td>8.3</td>
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<tr>
<td>P1 / BMAL490</td>
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<td>$10.6 \pm 3.0$</td>
<td>1.3</td>
<td>8.1</td>
</tr>
<tr>
<td>P1 / BMAL490K537Q</td>
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<td>$9.6 \pm 2.0$</td>
<td>0.7</td>
<td>7.5</td>
</tr>
<tr>
<td>P2 / BMAL577</td>
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<td>$3.3 \pm 1.0$</td>
<td>0.5</td>
<td>8.0</td>
</tr>
<tr>
<td>P2 / BMAL490</td>
<td>0.9</td>
<td>$7.7 \pm 1.9$</td>
<td>7.3</td>
<td>14.2</td>
</tr>
<tr>
<td>P2 / BMAL490K537Q</td>
<td>1.0</td>
<td>$2.9 \pm 0.9$</td>
<td>-0.6</td>
<td>6.8</td>
</tr>
</tbody>
</table>

$^a$ITC experiments were performed at 22°C in 25 mM Bis-tris propane buffer pH 7.8, 100 mM NaCl, 5 mM TCEP. At the concentrations used in the ITC experiments, all proteins were monomeric as shown by AUC (Table 1).

$^b$Binding was dominated by favorable entropy changes and the contribution of $T\Delta S$ to $\Delta G$ was significantly higher than $\Delta H$. The BMAL490K537Q-P2 interaction was entropically and enthalpically favored. N: number of binding sites (N=Ligand/Receptor). Except for the mBMAL1-P1 complexes, the mBMAL1 fragments were used as ligands. Reported values and standard deviations are the mean of at least three independent titrations.
Fig. 1
Fig. 2
A

Time (min)

\[ K_0 = 7.8 \, \mu M, \, N = 1.04 \]

\[ T \Delta S = 8383 \, \text{cal/mol} \]

\[ \Delta H = 977 \, \text{cal/mol} \]

B

Time (min)

\[ K_0 = 17.9 \, \mu M, \, N = 0.8 \]

\[ T \Delta S = 9294 \, \text{cal/mol} \]

\[ \Delta H = 2649 \, \text{cal/mol} \]

Fig. 3
Fig. 4
**Fig. 5**

**Panel A**

- Time (min)
- Molar Ratio vs. KCal/Mole of Injectant
- $K_d = 11.8 \, \mu M$, $N = 0.82$

**Panel B**

- Time (min)
- Molar Ratio vs. KCal/Mole of Injectant
- $K_d = 3.0 \, \mu M$, $N = 0.87$

**Panel C**

- Time (min)
- Molar Ratio vs. KCal/Mole of Injectant
- $K_d = 7.8 \, \mu M$, $N = 1.0$

**Panel D**

- Time (min)
- Molar Ratio vs. KCal/Mole of Injectant
- $K_d = 3.5 \, \mu M$, $N = 1.0$

**Thermodynamic Parameters**

- **Panel A:**
  - $\Delta S = 7935 \, \text{cal/mol}$
  - $\Delta H = 1370 \, \text{cal/mol}$

- **Panel B:**
  - $\Delta S = 7935 \, \text{cal/mol}$
  - $\Delta H = 484 \, \text{cal/mol}$

- **Panel C:**
  - $\Delta S = 14160 \, \text{cal/mol}$
  - $\Delta H = 7253 \, \text{cal/mol}$

- **Panel D:**
  - $\Delta S = 6667 \, \text{cal/mol}$
  - $\Delta H = -683 \, \text{cal/mol}$
Fig. 6
Quantitative analyses of cryptochrome - mBMAL1 interactions: mechanistic insights into the transcriptional regulation of the mammalian circadian clock
A. Czarna, H. Breitkreuz, C. C. Mahrenholz, J. Arens, H. M. Strauss and E. Wolf

*J. Biol. Chem.* published online April 25, 2011

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