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Cryptochromes impair phosphorylation of transcriptional activators in the clock: a general mechanism for circadian repression

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CLOCK and BMAL1 [brain and muscle ARNT (arylhydrocarbon receptor nuclear translocator)-like protein 1] are central components of the molecular clock in mammals and belong to the bHLH (basic helix–loop–helix)/PAS [PER (Period)/ARNT/SIM (single-minded)] family. Features of their dimerization have never been investigated. Here, we demonstrate that PAS domain function requires regions extending over the short PAS core repeats. Strikingly, while deleting PAS core repeats does not overtly affect dimerization, it abolishes the transcriptional activity of the heterodimer. Interestingly, these deletions also abolish co-dependent phosphorylation of CLOCK and BMAL1, suggesting a link between the phosphorylation status of the heterodimer and its transactivation potential. We demonstrate that NPAS2 (neuronal PAS domain protein 2) and BMAL2 also undergo similar post-translational modifications, thereby establishing the mechanism proposed for CLOCK–BMAL1 as a common feature of transcriptional activators in the circadian clock. The discovery of two novel splice variants of BMAL2 confirms the crucial role of the PAS domain and further strengthens the view that co-dependent phosphorylation is of functional significance. In agreement with this, we demonstrate that CRY1–2 (cryptochromes 1–2) affect transactivation and phosphorylation of transcriptional activators of the clock. Furthermore, CRY proteins stabilize the unphosphorylated forms of BMAL1(BMAL2) thereby shifting the phosphorylated/unphosphorylated ratio towards a predominantly unphosphorylated (transcriptionally inactive) form. In contrast, PER proteins, which are weak repressors, are without effect. From these results, we propose a general mechanism for the inhibition of CLOCK(NPAS2)–BMAL1(BMAL2) circadian transcriptional activation by CRY1–2.

Key words: circadian clock, COS-7 cell, cryptochrome (CRY), PER/ARNT/SIM domain (PAS domain), suprachiasmatic nucleus, transcription activators.

INTRODUCTION

Circadian clocks are a pervasive feature in virtually all Kingdoms of life. They are thought to improve fitness of an organism through resonance between internal time and external cues [1,2]. In animals, circadian clocks are not only present in neural structures, but also in most tissues and cells of the organism [3,4]. Interlocked transcriptional–translational feedback loops involving a set of clock genes are a hallmark of the molecular clock mechanism [2,5]. In mammals, a heterodimer of the transcription factors CLOCK and BMAL1 [brain and muscle ARNT (arylhydrocarbon receptor nuclear translocator)-like protein 1] plays a major role in driving rhythmic gene transcription. Both proteins belong to the bHLH (basic helix–loop–helix)/PAS [PER (Period)/ARNT/SIM (single-minded)] family of transcription factors [6]. Among the target genes are the three *Per* (*Per1–3*) and two *Cry* (*Cryptochrome*) (*Cry1–2*) genes, which are themselves clock components and code for proteins that dimerize and repress CLOCK–BMAL1 transcriptional activity through unknown mechanisms [7,8].

Other molecular components of circadian clocks have been identified [9,10]. Among these are two other bHLH/PAS proteins, NPAS2 (neuronal PAS domain protein 2) and BMAL2, which display extensive similarity to CLOCK and BMAL1 respectively [6,11]. Furthermore, NPAS2 forms transcriptionally active heterodimers with BMAL1 [6,12–14], while there is evidence that BMAL2 forms transcriptionally active heterodimers with CLOCK [6,15]. CLOCK and BMAL1 appear to be crucial for the maintenance of circadian rhythmicity, as mutants and knockout mice display arrhythmic phenotypes [7,8]. This view has, however, been recently challenged with the finding that *Clock* null-mutant mice are rhythmic [16].

Consensus bHLH motifs are known to play two roles: the basic region binds to specific DNA sequences (E-box motifs), while the HLH mediates dimerization with another bHLH protein, which is a requirement for DNA binding [17,18]. The PAS domains have been assigned the role of sensors and integrators of external cues, as exemplified by the dioxin or hypoxia response pathways [6]. PAS domains are themselves ligand-binding pockets that can accommodate small molecules such as dioxin or haem [6,19]. The PAS domain sequence is conserved between members of the family. It comprises two core repeats (PAS A and B) spaced by a linker region of variable length and regions flanking the core repeats [6].

The presence of PAS and bHLH domains in CLOCK and BMAL1 suggested that they interact through these domains [6,20]. While there is evidence for dimerization of BMAL1 with the CLOCK homologue NPAS2 through the bHLH domains [21], we found very little information in the literature regarding the role of PAS and bHLH domains in CLOCK–BMAL1 dimerization. We thus sought to clarify the roles of these domains using complementary approaches. The results of these experiments prompted us to investigate the role of post-translational modifications of CLOCK and BMAL1 as well as that of their counterparts NPAS2 and BMAL2. This is of the utmost importance, as there is growing evidence that post-translational mechanisms play

Abbreviations used: 3-AT, 3-amino-1,2,4 triazole; ARNT, arylhydrocarbon receptor nuclear translocator; bHLH, basic helix–loop–helix; BMAL1, brain and muscle ARNT-like protein 1; CKIε, casein kinase Iε; CRY, cryptochrome; DTT, dithiothreitol; HA, haemagglutinin; PER, Period; PAS domain, PER/ARNT/SIM domain; NPAS2, neuronal PAS domain protein 2; SD, synthetic dropout; SIM, single-minded.

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major roles in setting the pace and general functioning of the clock [22–27]. Our results indicate that CLOCK(NPAS2)–BMAL1(BMAL2) undergo co-dependent phosphorylation and that all four combinations of heterodimers are transcriptionally active. The use of deletion mutants for BMAL1 and novel splice variants for BMAL2 demonstrates that the integrity of the PAS domain is required for this process and supports a functional significance of phosphorylation of transcriptional activators. Furthermore, our results provide a mechanistic explanation for the CRY-mediated transcriptional repression.

EXPERIMENTAL

Yeast two-hybrid assay

Fragments for PAS domains were generated by PCR on expression vectors (FLAG-mClock and 5 × Myc-mBmal1b; [28]) as templates. PCR was carried out as follows: 95 °C for 2 min, followed by 30 cycles of amplification at 95 °C for 30 s, 54 °C for 30 s and 68 °C for 1 min, with a final extension of 10 min at 68 °C (Taq DNA polymerase High Fidelity; Invitrogen). PCR fragments of the expected sizes were purified by gel extraction, digested with BamHI and XhoI, purified by phenol/chloroform extraction and then cloned in either BamHI/XhoI-digested pGADT7 vector (Clontech) or BamHI/SalI-digested pGBKT7 vector (Clontech). BMAL1 PAS domains were cloned in pGBKT7, while CLOCK PAS domains were cloned in pGADT7 (Clontech). The resulting fusion proteins are depicted in Figure 1(A). These and all other clones were sequenced by Genome Quebec (Montreal, QC, Canada).

Two hybrid assays were done using the Matchmaker 3 system (Clontech), according to the manufacturer's instructions. Negative and positive controls were empty vectors (pGBKT7 and pGADT7) and pGBKT7-P53 and pGADT7-T plasmids respectively. Two reporter systems were used: the *HIS3* gene (AH109 strain), which allows growth on a medium lacking histidine when expressed, and the *lacZ* gene (Y187 strain), whose product β-galactosidase can be assayed in yeast extracts. pGBKT7 (0.25 μg) or a BMAL1 derivative, and pGADT7 (0.25 μg) or a CLOCK derivative were co-transformed in yeast by the PEG [poly(ethylene glycol)]–lithium acetate technique, and the cells were spread on SD (synthetic dropout) medium lacking leucine and tryptophan (SD − LW). For the growth assays a few colonies of transformed AH109 cells were resuspended in SD medium, the D_{600} (attenuance) was measured and equivalent amounts of cells were spread on either SD − LW plates or on plates also lacking histidine (SD − LWH). For the 3-AT (3-amino-1,2,4-triazole) assays, 3-AT (Sigma) was added to the medium at different final concentrations. In all cases, plates were incubated at 30 °C for 3–5 days.

For β-galactosidase assays, transformed Y187 yeast colonies were picked from the SD − LW plates and inoculated in liquid SD − LW. After overnight growth at 30 °C, cells were diluted in YPD (yeast extract/peptone/dextrose) medium [1 % (w/v) yeast extract, 2 % (w/v) peptone and 2 % (w/v) glucose], grown to early exponential phase, lysed and tested in the ONPG (*o*-nitrophenyl α-D-galactopyranoside) assay (procedure and Miller unit calculation according to Clontech's standard protocol). All procedures were repeated at least three times.

Yeast protein extracts were performed according to Clontech's SDS/urea protocol. In brief, cells transformed with expression vectors were grown to early exponential phase, pelleted, washed in water and resuspended in a buffer containing 8 M urea, 5 % (w/v) SDS, 40 mM Tris/HCl (pH 6.8), 0.1 mM EDTA, 0.4 mg/ml Bromophenol Blue, 1 % 2-mercaptoethanol, Complete™ Mini protease inhibitor cocktail (Roche) and 1 mM PMSF. Cells were lysed by vortex-mixing in the presence of glass beads and then boiled and vortex-mixed again.

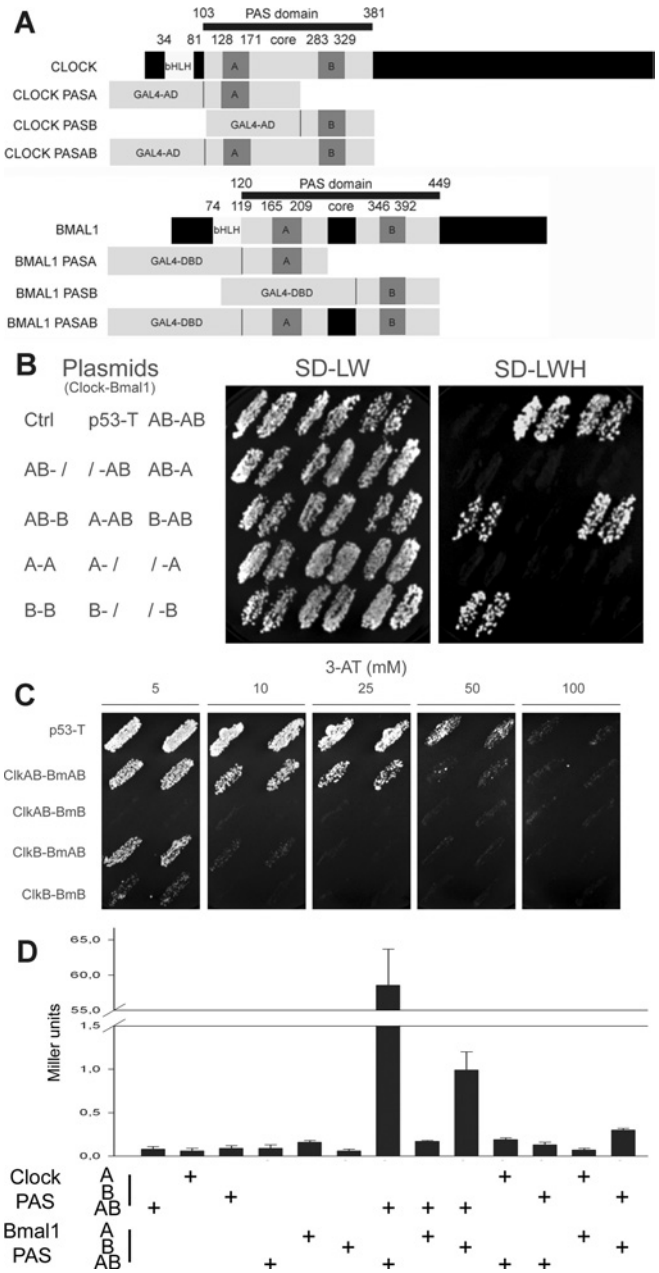

Figure 1 Single PAS domains cannot sustain dimerization with a bipartite PAS

(**A**) Schematic diagrams depicting the extent of the CLOCK and BMAL1 PAS domains and limits of PAS A and PAS B core repeats. Domains broader than these PAS core repeats were used to generate the different GAL4 fusion proteins as indicated. Numbers correspond to positions of amino acids; DBD, DNA-binding domain; AD, activation domain. (**B**) Yeast two-hybrid assays in AH109; left panel is a summary of the different combinations used; the '/' symbols stand for the control plasmids encoding GAL4 domain alone. Middle and right panels depict growth on media lacking leucine/tryptophan (LW) and leucine/tryptophan/histidine (LWH) respectively. (**C**) The four combinations exhibiting growth on LWH medium were tested on a medium supplemented with 3-AT, a competitive inhibitor of the *HIS3* gene product. (**D**) Yeast two-hybrid assays in Y187; results of β-galactosidase assay with the different combinations. Note that the *y*-axis is broken. Only the PASAB + PASAB gave strong and significant activation (different from all other conditions, $P < 0.0002$; $P > 0.5$ for all other pairwise comparisons).

PAS- or bHLH-deleted *Clock* and *Bmal1* clones

Mutants lacking either PAS A (ΔPAS A), PAS B (ΔPAS B), or PAS A and PAS B (ΔPAS AB) core repeats or bHLH (ΔbHLH) were generated by PCR using FLAG-mClock and 5 × Myc-mBmal1b plasmids as templates. The putative bHLH domain of CLOCK covers amino acids 34–81, while that of BMAL1 covers amino acids 74–119 [NCBI (National Center for Biotechnology Information) Conserved Domain Search tool]. Primers were phosphorylated with T4 kinase (Invitrogen) for 20 min at 20 °C and purified on Sephadex G25 columns. Conditions for PCR (Taq DNA polymerase High Fidelity) were as follows: 95 °C for 2 min, followed by 30 cycles of amplification at 95 °C for 30 s, 54 °C for 30 s and 68 °C for 8 min with a time increment of 20 s per cycle and a final extension at 68 °C for 10 min. PCR products were purified on gel (gel extraction kit; Qiagen) and ligated [25 min at room temperature (22–23 °C); T4 DNA ligase; New England Biolabs]. Constructs lacking both PAS core repeats were generated using clones lacking one PAS domain as PCR templates. Because deletions are likely to affect protein folding, the limits of PAS domains used for these mutants were different from those for the two-hybrid assays and only encompass what is thought to be the core of the domains [6]. Thus ΔPAS AB constructs retain the linker between PAS A and PAS B core repeats (see Figure 2A). Larger deletion mutants retaining only the bHLH and the C-terminal region were also generated (CLOCKΔ103–381 and BMAL1Δ120–449, see Figure 2E).

Npas2 and *Bmal2* clones and *Bmal2a* site-directed mutagenesis

The complete coding sequence of *Npas2*, *Bmal2a* and two novel *Bmal2* splice variants (see Figure 4A; hereafter referred to as *Bmal2sv1* and *Bmal2sv2*; deposited in GenBank® database under accession numbers DQ322241 and DQ355143 respectively) were amplified by RT (reverse transcription)–PCR. *Npas2* was cloned in the backbone vector used for the *Clock* clone described above (giving FLAG-mNpas2) and the different *Bmal2* cDNAs were cloned in the backbone vector of the *Bmal1* clone (giving 5 × Myc-Bmal2a, 5 × Myc-Bmal2sv1 and 5 × Myc-Bmal2sv2). The different point mutants of *Bmal2a* (S365A/S365D, T371A/T371D, S365A/T371A and S365D/T371D) were generated according to standard procedures.

Cell culture and luciferase assay

COS-7 cells were grown in Dulbecco's modified Eagle's medium supplemented with 10 % (v/v) fetal bovine serum and 2 mM L-glutamine in a humidified atmosphere with 5 % CO_2 at 37 °C. COS-7 cells are an adequate model as they endogenously express low levels of clock genes [27]. Cells were plated in 24-well plates and transfected, as recommended by the manufacturer, with Lipofectamine™ 2000 (Invitrogen) using 200 ng of each expression construct (except for *Cry1–2*; 50 ng), 50 ng of the reporter gene construct (*mPer1* promoter reporter; −1803 to +40, see [28]) and 25 ng of a β-galactosidase reporter construct. Total DNA amount was set to 600 ng by addition of empty vector. On the day following transfection, medium was replaced and the cells were allowed to grow for another 24 h. Cells were then rinsed twice in cold PBS and lysed by shaking for 15 min in lysis buffer [25 mM Tris, 2 mM EDTA, 1 mM DTT (dithiothreitol), 10 % (v/v) glycerol and 1 % Triton X-100]. The luciferase assay was carried out using an Orion II microplate luminometer (Berthold, Pforzheim, Germany). Luciferase buffer contained 20 mM Tris/phosphate, pH 7.8, 1 mM $MgCl_2$, 2.7 mM $MgSO_4$, 0.1 mM EDTA, 33.3 mM DTT, 470 μM D-Luciferin, 530 μM ATP and 270 μM CoA. Results were normalized to β-galactosidase activity and protein levels (DC Protein Assay; Bio-Rad).

Data (in relative luminescence units) represent fold induction over the control condition (empty vectors) once normalized to β-galactosidase and total amount of proteins and are expressed as the means ± S.E.M. For all other transfection studies, various amounts of each construct were used (as indicated in the Figure legends) and total amount was kept constant by adding an appropriate empty vector. Experiments were done in triplicate and repeated three to five times. Results of a representative triplicate are shown.

Western blot and immunoprecipitation

Cells were plated in six-well plates and transfected as described above (1–4 μg of plasmid DNA/well depending on the experiment). In protein synthesis inhibition experiments, 100 μg/ml cycloheximide (Calbiochem no. 239764) was added and cells were harvested after 1, 2 or 4 h. Cells were rinsed twice in cold PBS and resuspended in lysis buffer (10 mM Hepes, pH 8, 0.1 mM EDTA, pH 8, 50 mM NaCl, 50 mM KCl, 5 mM $MgCl_2$, 4 mM spermidine, 0.7 mM DTT, 100 μg/ml BSA and 17 % (v/v) glycerol) supplemented with 0.5 mM PMSF, 0.1 % Nonidet P40 and a cocktail of protease inhibitors (Complete™ Mini; Roche) and incubated for 30 min on ice. After vortex-mixing, the extract was centrifuged for 2 min at 16000 ***g***. Supernatants were incubated overnight at 4 °C with anti-Myc affinity resin in lysis buffer (Sigma). The beads were then washed three times in lysis buffer, resuspended in Laemmli buffer [50 mM Tris/HCl pH 6.8, 2 % (w/v) SDS, 0.1 % Bromphenol Blue, 10 % (v/v) glycerol and 100 mM dithiothreitol], denatured at 95 °C for 5 min and the supernatant was resolved on an SDS/6 % (v/v) polyacrylamide gel.

Membranes were then probed with antibodies directed against Myc (Roche), FLAG, HA (haemagglutinin; Sigma) and V5 epitope (Invitrogen). Anti-actin was used to ascertain equal loading (Sigma). Anti-mouse horseradish peroxidase-conjugated antibody (Sigma) and the ECL® (enhanced chemiluminescence) detection reagent (Amersham) were used for revelation. Crude supernatant not submitted to immunoprecipitation procedure was used to check the input. *mPer1–3* (V5 tag) and *mCry1–2* (HA tag) expression vectors were kindly provided by Dr S. M. Reppert (Department of Neurobiology, University of Massachusetts Medical School, Worcester, MA, U.S.A.) and Dr D. Knutti (Department of Neurobiology, Harvard Medical School, Boston, MA, U.S.A.) respectively. All proteins, including actin, were revealed on the same nitrocellulose membrane, unless otherwise stated. In all cases, representative experiments (out of at least three) are shown.

Statistics

Data were analysed (Statistica and Sigma-Plot softwares) by a one-way ANOVA followed, when appropriate, by a Newman–Keuls post-hoc test.

RESULTS

Interaction of PAS domains of CLOCK and BMAL1

The yeast two-hybrid assay was used to study the interaction of CLOCK and BMAL1 PAS domains. Information related to the extent of PAS domains used for the yeast two-hybrid assay constructs was kindly provided by Dr Georges Mer (Mayo Clinic, Rochester, MN, U.S.A.). CLOCK PAS A, B and AB domains encompass amino acids 103–265, 262–381 and 103–381

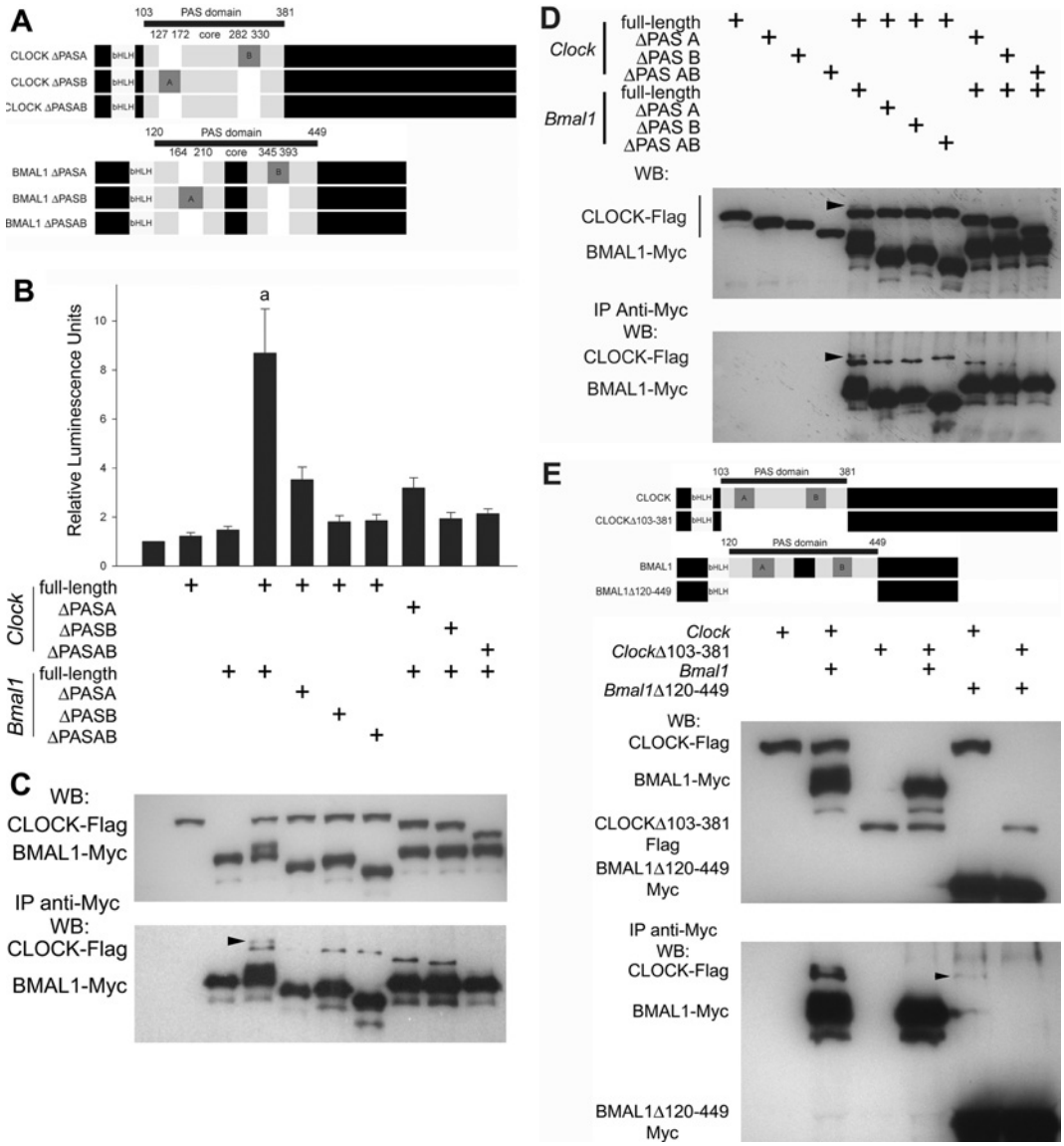

Figure 2 Role of the PAS domains in transactivation and post-translational modifications

(**A**) Schematic diagrams depicting the deletion mutants (lacking PAS A, PAS B or both PAS A and PAS B core repeats). (**B**) CLOCK and BMAL1 deleted for core PAS repeats do not transactivate at an E-box. Full-length CLOCK and BMAL1 or proteins deleted for either PAS A, PAS B or PAS A and PAS B core repeats were assessed for transactivation of a *Per1*-luciferase reporter. Significant transactivation was observed only when *Clock* and *Bmal1* plasmids encoding full-length proteins were co-transfected ('a', different from all other conditions, $P < 0.0002$; $P > 0.1$ for all other pairwise comparisons). The first bar corresponds to co-transfection of *Clock* and *Bmal1* empty vectors. The '+' sign indicates presence of the corresponding constructs (applies to both **B** and **C**). (**C**) Upper panel is a Western blot (WB) with extracts from cells transfected with the different combinations of plasmids, with anti-Myc antibody for BMAL1 and anti-FLAG for CLOCK. Note the appearance of a slower migrating band for BMAL1 only when full-length *Clock* and *Bmal1* vectors were co-transfected. Lower panel is the result of BMAL1 immunoprecipitation (IP) with anti-Myc and WB with anti-Myc (for BMAL1) and anti-FLAG (for CLOCK). Note that a slow-migrating form of CLOCK is observed (arrowhead) only when full-length *Clock* and *Bmal1* vectors were co-transfected. (**D**) This is a replicate experiment of the IP shown in (**C**) including the different CLOCK ΔPAS control lanes that were omitted in (**C**) for the sake of clarity. (**E**) bHLH domains are not sufficient for strong dimerization of CLOCK and BMAL1. *Clock* and *Bmal1* plasmids or large deletion mutants lacking the whole PAS domain (see schematic diagrams) were co-transfected and interaction was assessed by co-IP (as in **C**). While BMAL1Δ120–449 was found to sustain minimal dimerization with CLOCK (arrowhead), CLOCKΔ103–381 could not been pulled down with BMAL1.

respectively. BMAL1 PAS A, B and AB domains encompass amino acids 120–260, 309–449 and 120–449 respectively. These are thus much broader than the limits of the PAS A and PAS B core repeats [6]. All these data (position of the whole PAS domain and limits of PAS A and PAS B core repeats and bHLH of both CLOCK and BMAL1) are recapitulated in Figure 1(A).

Yeast growth on histidine-free medium revealed interaction between PAS AB domains of CLOCK and BMAL1 as well as interaction between various combinations of shorter PAS constructs, all of which contain PAS B subdomain (Figure 1B). No growth was observed for yeast transformed with a PAS A and a PAS AB domain. The interaction between PAS AB of CLOCK and BMAL1 conferred growth even in the presence of high concentrations of 3-AT (a competitive inhibitor of the *HIS3* gene product), while growth was either severely impaired or abolished for other PAS combinations at low 3-AT concentrations (Figure 1C). Assessment of a second reporter gene (*LacZ*) confirmed these results. High β-galactosidase activity was only observed when yeast expressed PAS AB domains of both CLOCK and BMAL1 ($P < 0.0002$; $P > 0.5$ for other pairwise comparisons, Figure 1D). Similar levels of expression of the fusion proteins in both yeast strains were confirmed by Western

blotting (Supplementary Figure 1 at http://www.BiochemJ.org/bj/402/bj4020525add.htm).

Altogether these results show that PAS AB domains of CLOCK and BMAL1 can interact and that isolated PAS A or B repeats do not, or only weakly, associate.

Integrity of the PAS domains is required for transcriptional activity and phosphorylation of CLOCK and BMAL1

Constructs deleted for PAS A, PAS B or both PAS A and PAS B core repeats (Figure 2A) were co-transfected in COS-7 cells along with a *Per1*-luciferase reporter gene (Figure 2B). As expected, luminescence was increased by approx. 8-fold on co-transfection of *Clock* and *Bmal1* ($P < 0.0002$). In contrast, none of the various combinations of deleted mutants gave any statistically significant transactivation (pairwise comparisons, $P > 0.1$).

This transcriptional defect is not attributable to impaired heterodimerization ability of the mutant constructs, as demonstrated by co-immunoprecipitation (Figures 2C and 2D). Not only full-length CLOCK and BMAL1, but also other combinations (except full-length BMAL1/CLOCKΔPASAB), were found to heterodimerize. Thus dimerization of CLOCK and BMAL1 can still occur even when both PAS A and PAS B core repeats of BMAL1 are deleted, which indicates that other parts of the protein can support dimerization. To investigate this, we generated CLOCK and BMAL1 mutants (CLOCKΔ103–381 and BMAL1Δ120–449) that lack both PAS core repeats, the linker region and flanking sequences and thus only retain the bHLH and the C-terminal region (Figure 2E). Only very minute amounts of CLOCK co-purified with BMAL1Δ120–449 in co-immunoprecipitation assays, and no CLOCKΔ103–381 was detected in immunoprecipitations of BMAL1 (Figure 2E). Because BMAL1ΔPASAB still interacted with CLOCK, but the larger deletion Δ119–449 strongly reduced interaction, we conclude that the interaction of CLOCK and BMAL1 also encompasses the linker region and sequences directly upstream and downstream of PAS A and PAS B respectively. Collectively, these results demonstrate that the functional definition of CLOCK and BMAL1 PAS domains encompasses regions broader than the PAS core repeats.

Interestingly, from Figures 2(C) and 2(D), it was obvious that co-transfection of CLOCK and BMAL1 led to the appearance of a phosphorylated form of BMAL1 (also see Figure 3D, top panel; phosphatase treatment in Figure 3E). Furthermore, a slow-migrating form of CLOCK, barely noticeable in the crude input, was enriched in immunoprecipitation of BMAL1 (Figures 2C–2E). This band most likely represents phosphorylated CLOCK (see the Discussion). We interpret the data as evidence that CLOCK phosphorylation is dependent on dimerization with BMAL1. These results are in agreement with previous findings [24]. Importantly, slow-migrating forms of BMAL1 and CLOCK were never detected when we expressed deletion variants (Figures 2C and 2D).

This indicates that post-translational modifications of both partners are impaired when the integrity of the PAS domain is compromised and suggests that proper phosphorylation may be a prerequisite for transcriptional activation by the heterodimer. To investigate this further and to decipher whether phosphorylation is a DNA-binding-related event, we generated bHLH-deleted constructs for CLOCK and BMAL1.

Role of DNA binding in phosphorylation of CLOCK and BMAL1

We tested CLOCK and BMAL1 variants lacking the bHLH domains (Figure 3A) for their ability to transactivate a *Per1*-luciferase reporter gene. CLOCK and BMAL1 significantly enhanced luminescence (Figure 3B; $P < 0.0002$). As expected, the transactivation capability of bHLH-deleted constructs was severely impaired (Figure 3B). Pairwise comparisons revealed that only full-length BMAL1 combined with bHLH-deleted CLOCK was slightly different from CLOCK or BMAL1 alone ($P < 0.05$; all other comparisons $P > 0.05$).

Dimerization of the CLOCK and BMAL1 variants was not disturbed, as demonstrated by consistent co-immunoprecipitation of all combinations (Figure 3C). This supports our previous finding that the bHLH domains do not significantly contribute to the dimerization (Figure 2E).

Western-blot analysis also revealed that phosphorylation of BMAL1 varied among the different combinations (Figures 3C–3E). Importantly, bHLH-deleted CLOCK still induced phosphorylation of BMAL1 in a dose-dependent manner (Figure 3D, second panel). Because bHLH proteins bind DNA as obligate dimers [17,18], this result most likely indicates that BMAL1 phosphorylation is not a DNA-binding-related event. However, we cannot absolutely rule out the possibility that BMAL1 phosphorylation would be altered to a minor extent, not readily noticeable by our Western-blot procedure.

In contrast, deleting BMAL1 bHLH impaired BMAL1 phosphorylation. When expressed with full-length CLOCK, a phosphorylated form of bHLH-deleted BMAL1 was not observed (Figures 3C and 3D, third panel), whereas with bHLH-deleted CLOCK, it was much less abundant than for full-length BMAL1 (Figure 3C, upper panel, Figure 3D, bottom panel, and Supplementary Figure 2A at http://www.BiochemJ.org/bj/402/bj4020525add.htm). Even though CLOCK still interacted with bHLH-deleted BMAL1 (Figure 3C), the slow-migrating form was only observed in the presence of full-length BMAL1 (Figure 3C; see also Figures 2C–2E and Supplementary Figure 2B). This suggests that CLOCK phosphorylation may be a DNA-binding-related event. Finally, contrary to what was reported in HEK-293 cells (human embryonic kidney cells) [24], we have never noticed any BMAL1-dependent CLOCK degradation (Supplementary Figure 3 at http://www.BiochemJ.org/bj/402/bj4020525add.htm).

CLOCK(NPAS2)–BMAL1(BMAL2) undergo co-dependent phosphorylation and display transcriptional activity

We then wished to test whether co-dependent phosphorylation mechanisms observed for CLOCK–BMAL1 may also be important for the activity of the other combinations of transcriptional activators (CLOCK–BMAL2; NPAS2–BMAL1 and NPAS2–BMAL2) of the circadian clock. While isolating *Bmal2a* cDNA, we identified two novel splice variants (Figure 4A; hereafter referred to as *Bmal2sv1* and *Bmal2sv2*). *Bmal2sv1* and *Bmal2sv2* arise from the use, in distinct exons, of an alternative splicing 3′ acceptor and 5′ donor site respectively. The resulting BMAL2sv1 protein lacks amino acids 362–371 (VLQSKEKILT) that are in the PAS B core repeat, while BMAL2sv2 lacks amino acids 490–498 (VNGGNAYGP), located downstream of the PAS domain.

Luciferase assays demonstrate that all four combinations of heterodimers are transcriptionally active (Figure 4B). Interestingly, the transactivation efficiency of CLOCK–BMAL2sv2 is indistinguishable from that of CLOCK–BMAL2, while CLOCK–BMAL2sv1 is not transcriptionally active (Figure 4B). Furthermore, NPAS2–BMAL2 also seems to be able to induce minimal transactivation from the *Per1-luc* reporter (not statistically significant). Similar to what was found with the combinations with CLOCK, the transactivation induced by NPAS2–BMAL2sv2 is indistinguishable from that of NPAS2–BMAL2, while NPAS2–BMAL2sv1 is not transcriptionally active (Figure 4B).

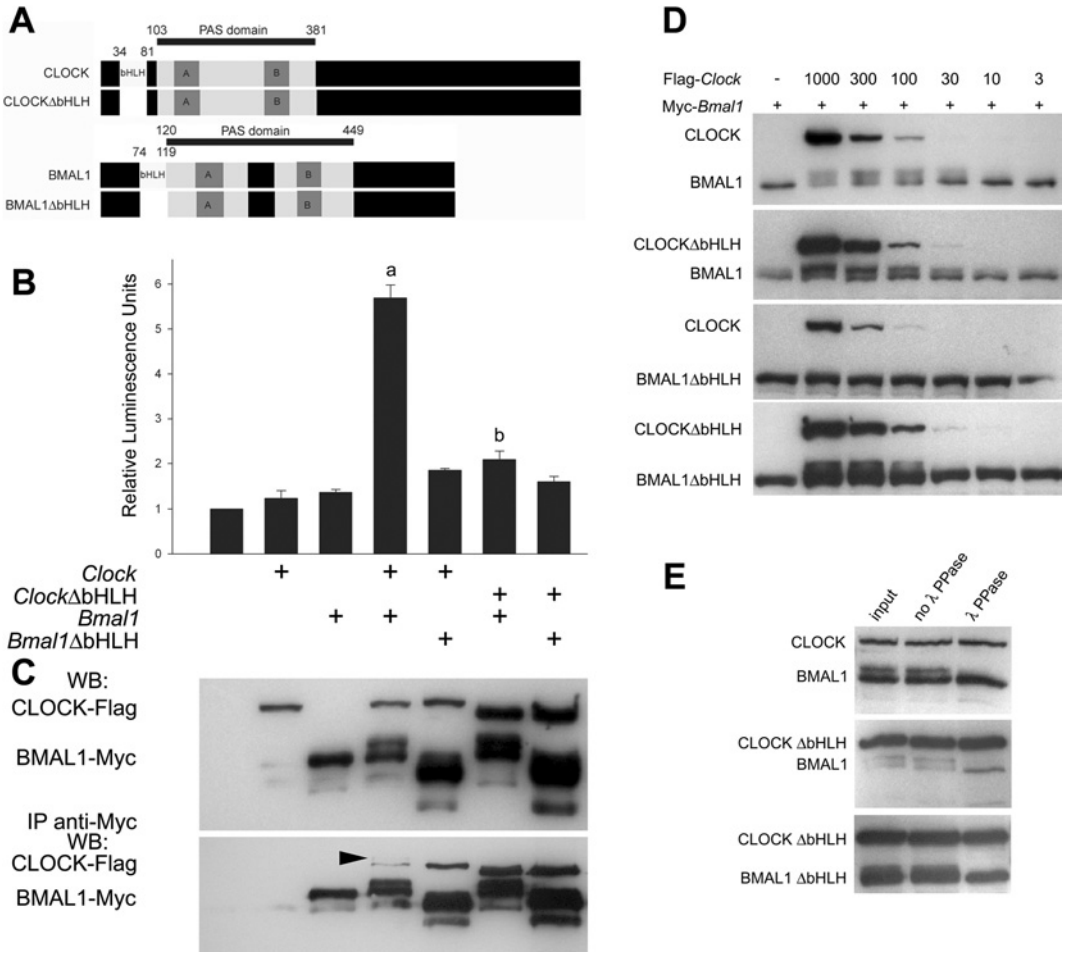

Figure 3 bHLH domains are necessary for transactivation by the CLOCK–BMAL1 heterodimer but dispensable for dimerization

(**A**) Mutant CLOCK and BMAL1 full-length or deleted for bHLH domain were assessed for transactivation of a *Per1*-luciferase reporter gene. (**B**) Significant transactivation was observed when *Clock* and *Bmal1* plasmids encoding full-length proteins were co-transfected ('a', different from all other conditions $P < 0.0002$). Full-length BMAL1 along with bHLH-deleted CLOCK also gave slightly higher luciferase levels than controls ('b', $P < 0.05$) All other pairwise comparisons are non significant ($P > 0.05$). The first bar corresponds to co-transfection of *Clock* and *Bmal1* empty vectors. The '+' sign indicates presence of the corresponding constructs (applies to both **B** and **C**). (**C**) Upper panel is a Western blot with extracts from cells transfected with the different combinations of plasmids, with anti-Myc antibody for BMAL1 and anti-FLAG for CLOCK. CLOCK was found to interact with full-length and bHLH-deleted BMAL1. A slower migrating form of CLOCK (arrowhead) was only observed when full-length *Clock* and *Bmal1* vectors were co-transfected. Another independent experiment is shown in Supplementary Figure 2(A). (**D**) Differential roles of bHLH domains in BMAL1 phosphorylation (Western blot with antibodies as in **C**). Deleting bHLH of CLOCK did not induce any modification in the CLOCK-dependent BMAL1 phosphorylation, while deleting bHLH of BMAL1 abolished it. Deleting bHLH of both CLOCK and BMAL1 resulted in a mild effect (amounts of *Clock* DNA transfected in ng are indicated on the top; for *Bmal1*, bHLH-deleted or wild-type, 100 ng were added). (**E**) Treatment with λ protein phosphatase (λ PPase) confirmed that the shifted bands truly represent phosphorylated BMAL1 (Western blot with antibodies as in **C**). Samples were incubated (30 min at 30 °C) with 600 units of λ PPase. As a control, a sample was incubated with λ buffer (50 mM Tris/HCl, pH 7.5, 100 mM NaCl, 2 mM dithiothreitol, 0.1 mM EGTA and 0.01 % Brij 35) and $MnCl_2$ but without λ PPase ('no λ PPase' lane).

BMAL1 undergoes phosphorylation when in combination with either CLOCK or NPAS2 (Figure 4C, upper panels). Likewise, a slow-migrating band corresponding to phosphorylated BMAL2 is observed when CLOCK is co-expressed (Figure 4C, upper panels; phosphatase treatment in Figure 4D). The same holds true when NPAS2 is co-expressed, although the amount of the phosphorylated form is very low, as demonstrated by the longer exposure time required to see it. Interestingly, BMAL2sv1 was not phosphorylated in the presence of either CLOCK or NPAS2, while the BMAL2sv2 does not display any gross modification in phosphorylation (Figure 4C, upper panels).

Results obtained by co-immunoprecipitation experiments clearly parallel what is observed for phosphorylation of BMAL1 and BMAL2. Slow-migrating forms of CLOCK and NPAS2 (not observable in the input extract; Figure 4C, upper panels) were observed when co-transfected with either BMAL1 or BMAL2 (Figure 4C, lower panel). Only minor amounts of putatively phosphorylated NPAS2 were recovered by immunoprecipitation against BMAL2, however, paralleling the minute amounts of phosphorylated BMAL2 observed in the crude extract and the weak transcriptional activity of this heterodimer. Furthermore, while dimerization of CLOCK or NPAS2 with BMAL2sv1 did not seem to be severely impaired, phosphorylated forms were not recovered (Figure 4C, lower panels). On the other hand, the slow-migrating form of CLOCK was observed when CLOCK was co-expressed either with BMAL2 or BMAL2sv2. Finally, no slow-migrating band was observed for NPAS2 when co-expressed with BMAL2sv2, probably because it only represents a very small fraction (Figure 4C: see NPAS2/BMAL2 lane).

Defects in phosphorylation/transactivation of BMAL2sv1 may be the consequence of impaired folding of the PAS domain. Alternatively, this may result from the absence of phosphorylation sites. Two potential sites (Ser^{365} and Thr^{371}) are present in the ten-amino-acid stretch missing in this splice variant. To test this hypothesis

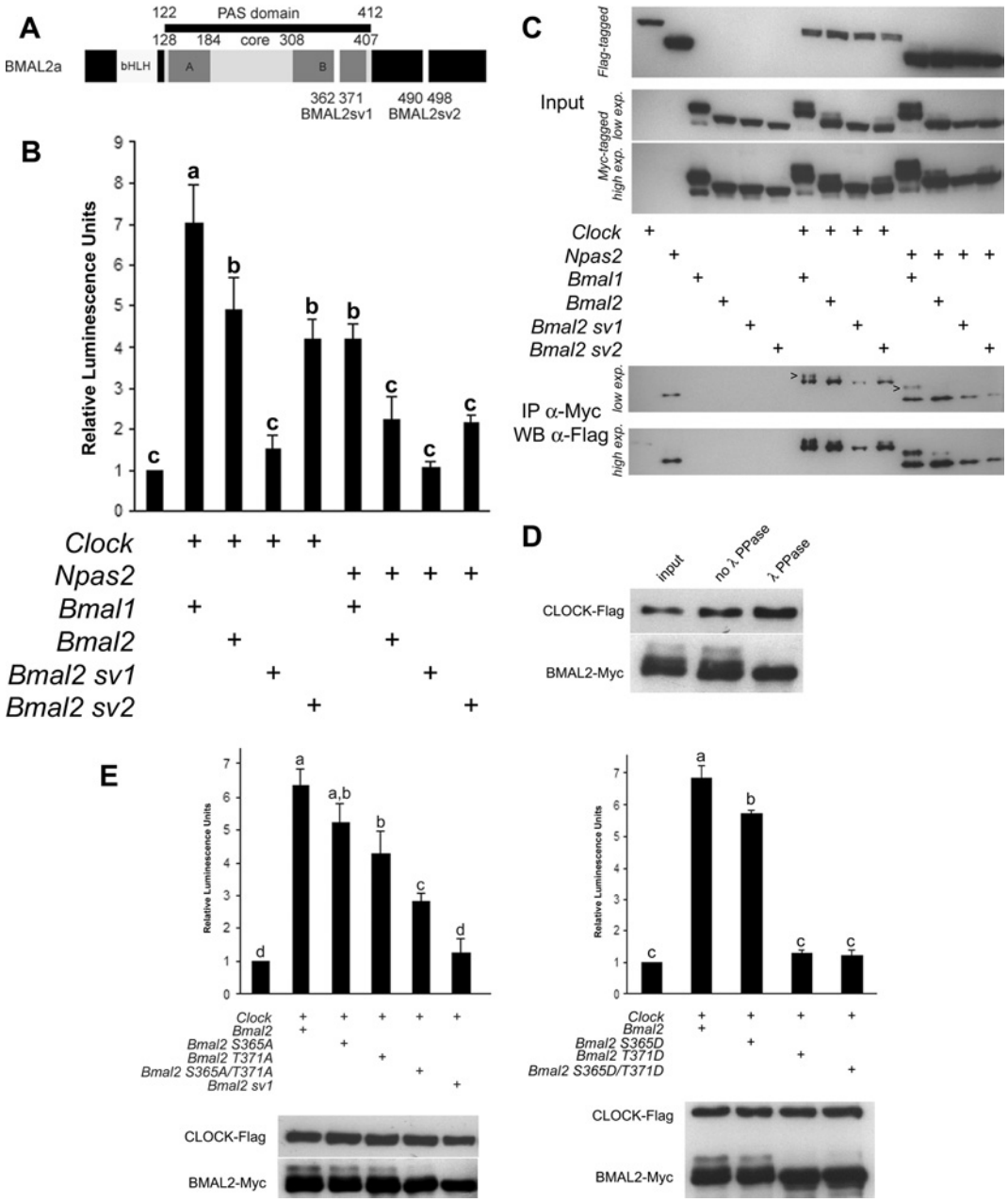

Figure 4 Transcriptional activity and phosphorylation of CLOCK(NPAS2)–BMAL1(BMAL2) heterodimers

(**A**) Schematic diagrams depicting differences in the sequence of the two novel BMAL2a splice variants, BMAL2sv1 and BMAL2sv2. Numbers below the schematics indicate the amino acid stretches lacking in BMAL2sv1 and BMAL2sv2. Note that BMAL2sv1 lacks amino acids in the PAS B core repeat. (**B**) The different combinations of heterodimers are able to transactivate at a *Per1-luc* reporter gene. Note that the BMAL2sv1 splice variant does not transactivate with CLOCK(NPAS2), while transactivation with BMALsv2/CLOCK(NPAS2) is not impaired and is similar to that obtained for BMAL2a/CLOCK(NPAS2). Different letters indicate statistical differences ($P < 0.05$ between two consecutive letters). (**C**) Upper panels depict a Western blot with extracts from cells transfected with the different combinations of plasmids, with anti-Myc antibody for BMAL1 and 2 and anti-FLAG for CLOCK and NPAS2. Note the appearance of slower migrating bands for BMAL1(BMAL2) when co-transfected with *Clock* or *Npas2* vectors. Also note that the phosphorylation pattern of BMAL2sv2 is indistinguishable from that of BMAL2a, while BMAL2sv1 is not phosphorylated. Lower panels are the result of BMAL1(BMAL2) immunoprecipitation (IP) with anti-Myc and Western blot with anti-FLAG (to detect CLOCK and NPAS2). Even though NPAS2 when expressed alone was also pulled down by anti-Myc beads to some extent (see control lane), note that the putative phosphorylated form of CLOCK(NPAS2) can only be detected (arrowheads) with BMAL1(BMAL2). CLOCK(NPAS2) are not phosphorylated when BMAL2sv1 is co-expressed. (**D**) Treatment with λ protein phosphatase (λ PPase) confirmed that the shifted BMAL2 band represents a phosphorylated form (Western blot with anti-Myc antibody for BMAL2 and anti-FLAG for CLOCK). Samples were incubated (30 min at 30 °C) with 600 units of λ PPase. As a control, a sample was incubated with λ buffer (see Figure 3) and $MnCl_2$ but without λ PPase (no λ PPase). (**E**) Analysis of point mutants of BMAL2. Ser^{365} and Thr^{371} of BMAL2 (both absent from BMAL2sv1) were converted into either alanine (left panel) or aspartic acid (right panel). Shown are results of luciferase assays and Western blot (with anti-Myc antibody for BMAL2 and anti-FLAG for CLOCK) with the different mutants when co-expressed with *Clock*. All of these mutants show, to different degrees, impaired transactivation activity paralleled by impaired phosphorylation patterns.

we performed site-directed mutagenesis of *Bmal2a* to generate *Bmal2* S365A and *Bmal2* T371A clones. Consistent with our hypothesis, when co-expressed with CLOCK these clones demonstrated reduced transcriptional activation, which correlated with impaired phosphorylation (Figure 4E, left panel). We then wished to test whether mimicking constitutive

phosphorylation would lead to the reverse phenotype, that is, enhanced transactivation. We generated *Bmal2* S365D and *Bmal2* T371D clones. Contrary to our expectations, these clones also show impaired transactivation ability. This is again correlated with impaired phosphorylation (Figure 4E, right panel). Actually, the single point mutant BMAL2 T371D does not have any transcriptional activity and is not phosphorylated. From these results, we conclude that impaired phosphorylation/transactivation of BMAL2sv1 is better explained by a compromised folding of the PAS domain than by the lack of crucial phosphorylation sites.

In conclusion, all four combinations of transcriptional activators show co-dependent phosphorylation and the extent of the latter correlates with transcriptional capabilities. Data obtained with BMAL2sv1 further strengthen this notion and illustrate the fact that the integrity of the PAS domain, while not an absolute requirement for heterodimerization, is key to proper subsequent phosphorylation and transactivation.

CRY1–2 affect post-translational modification of CLOCK and BMAL1

Because CLOCK and BMAL1 appear to be co-dependently phosphorylated and phosphorylation and transactivation are impaired with specific mutant constructs, it is tempting to speculate that phosphorylation plays a role in transactivation by CLOCK–BMAL1. PER and CRY proteins (PER1–3 and CRY1–2) repress transcriptional activity of the CLOCK–BMAL1 heterodimer through unknown mechanisms [7,8]. We thus tested whether their effect could be mediated by a modification in the phosphorylation status of CLOCK and BMAL1.

While co-expression of PER1–3 proteins had no obvious effect on the phosphorylation status of BMAL1 (Figure 5C), CRY2, but not CRY1, dose-dependently blunted the slow-migrating phosphorylated form of BMAL1 (Figure 5B). Interestingly, expression of either CRY1 or CRY2 led to increased levels of the unphosphorylated form of BMAL1, indicating its stabilization (Figures 5A and 5B). Further evidence for an increased half-life of BMAL1 in the presence of CRYs was obtained by using cycloheximide: BMAL1 levels quickly decreased after stopping protein synthesis in the absence of CRY1, while levels remained high when CRY1 was co-expressed (Figure 5D).

We next tested whether the effect of CRYs on BMAL1 can occur independently of CLOCK. Co-transfection of CRYs did not appear to have any stabilizing effect on BMAL1 when CLOCK was absent (Figure 6, upper panel). Again, co-transfection of CLOCK and BMAL1 with CRY1–2 led to a strong stabilizing effect on the non-phosphorylated form of BMAL1 (Figure 6, upper panel). Immunoprecipitation from these extracts revealed that CLOCK, BMAL1 and CRY1–2 are in a complex (Figure 6, lower panel). From these results, we conclude that the effect of the CRYs on BMAL1 is CLOCK-dependent and occurs when CLOCK and BMAL1 are dimerized. Importantly, the immunoprecipitation revealed that CRY1–2 severely blunted the slow-migrating CLOCK band, similar to the effect of CRY2 on BMAL1 (Figure 6, lower panel, and Figure 5B).

Thus CRY1–2 impinge on CLOCK–BMAL1 co-dependent phosphorylation, indicating a mechanism for inhibition of circadian transcriptional activation. Since CRY1–2 appear to have no effect on CLOCK–BMAL1 interaction as judged by the co-immunoprecipitation, we also conclude that the CRYs impact on the phosphorylation status of the dimer without disrupting it. We thus sought to test the hypothesis that the CRYs can repress all of the other three combinations of transcriptional activators, in a manner similar to CLOCK–BMAL1.

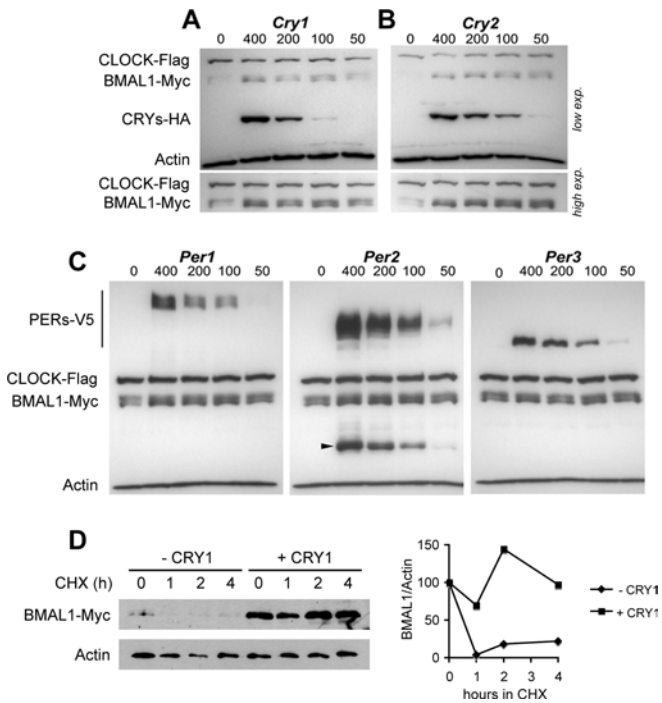

Figure 5 Differential post-translational effects of CRYs and PERs on CLOCK–BMAL1

(**A**) Western blot with anti-Myc antibody for BMAL1, anti-FLAG for CLOCK, anti-HA for CRY1 and anti-actin. Upper and lower panels depict the same membrane with short and long exposure times respectively; for clarity only CLOCK and BMAL1 are depicted in the lower panel. The differential effect of CRY1 and CRY2 on BMAL1 phosphorylation is more obvious in the lower panel. (**B**) Same as (**A**) but with cells transfected with CRY2. Note the trend towards higher levels of unphosphorylated BMAL1 with increasing doses. (**C**) PER1–3 have no effect on either CLOCK or BMAL1 levels and phosphorylation status (Western blot with the same antibodies as in (**A**) except that anti-V5 is used to detect PERs). The second PER2 band (~60 kDa; arrowhead) probably reflects partial proteolysis. Amount of *Per*/*Cry* DNA transfected (in nanograms) is indicated on top. *Clock* and *Bmal1* were kept constant at 400 ng each. (**D**) Western blot with anti-Myc and anti-actin antibodies (left panel) on extracts from cells expressing CLOCK, BMAL1 and CRY1, with or without cycloheximide treatment (CHX) for 1, 2 or 4 h. The right panel shows a quantification of the Western blot, with BMAL1 level values normalized for actin levels.

CRY1–2 can repress transcriptional activation induced by CLOCK(NPAS2)–BMAL1(BMAL2) and impact on phosphorylation and stability of BMAL2: a common mechanism for repression?

Luciferase assays were done to test the effect of the CRYs on the different heterodimers of transcriptional activators. Data show that CRY1–2 can repress all four combinations of transcriptional activators (Figure 7A). We then assessed the effects of PER1–3 and CRY1–2 on levels and phosphorylation status of CLOCK(NPAS2)–BMAL1(BMAL2). As described above, CRYs were found to have a strong stabilizing effect on the unphosphorylated form of BMAL1 when co-transfected with CLOCK, while PER1–3 were without effect (Figure 7B). A similar effect was observed with the combination CLOCK–BMAL2: PER1–3 had no obvious effect on levels and phosphorylation status of BMAL2, while CRY1–2 clearly stabilized the unphosphorylated form of BMAL2 (Figure 7B). Higher exposure time, necessary to observe the phosphorylated form of BMAL2, further revealed that the CRYs severely blunted the phosphorylated form of BMAL2, while PER1–3 were without effect (Figure 7B). Even though the effect was more modest, we observed that CRY1–2, but not PER1–3, also stabilized the unphosphorylated form of BMAL1 when it was co-expressed with NPAS2 (Figure 7C, left). In contrast with what was observed when in combination with

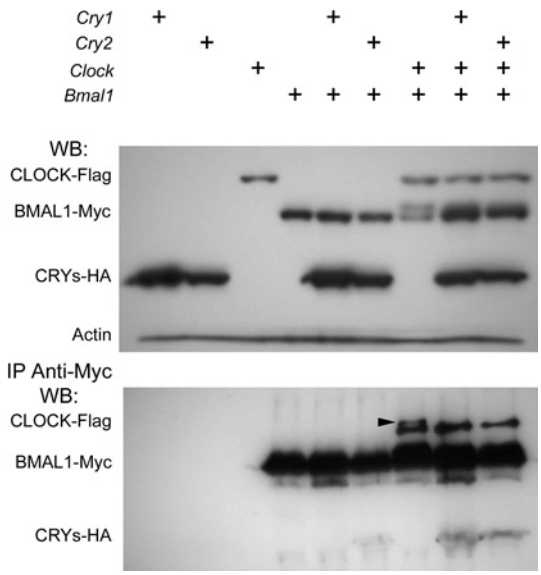

Figure 6 CRYs bind to BMAL1 when in a complex with CLOCK and blunt phosphorylation of both proteins

Upper panel: cells were transfected with the indicated vectors (1 μg each) and input was assessed by Western blot with anti-Myc antibody for BMAL1, anti-FLAG for CLOCK, anti-HA for CRY1 and anti-actin. Note the potent CLOCK-dependent stabilizing effect of CRY1–2 on non-phosphorylated BMAL1. Lower panel: same extracts, but first subjected to immunoprecipitation (IP) with anti-Myc. CRY1–2 are both recovered when CLOCK is present. While the slower migrating CLOCK band is obvious in the control lane (arrowhead), only non-phosphorylated CLOCK is found in extracts of cells co-transfected with the CRYs.

CLOCK, neither CRY1–2 nor PER1–3 had a significant effect on BMAL2 when it was co-expressed with NPAS2 (Figure 7C, right). Notably, the stabilizing effect of CRYs also occurs for the mutant BMAL2 with Ser^{365} and Thr^{371} changed to alanine (Figure 7D). This suggests that phosphorylation and stabilization of BMALs by CRY proteins are mostly independent processes, which is supported by the effect of CRY1 on BMAL1 stabilization but not on its phosphorylation (Figure 5A).

Altogether, these results suggest that the repressive effect of CRY1–2 towards CLOCK(NPAS2)–BMAL1(BMAL2)-induced transactivation may result from the decrease in the phosphorylated forms of BMAL1(BMAL2) and CLOCK and a shift in the ratio phosphorylated (transcriptionally active) form/unphosphorylated (transcriptionally inactive) form towards predominance of unphosphorylated proteins.

DISCUSSION

The exact role of CLOCK and BMAL1 PAS domains in dimerization has never been evaluated. The results from our yeast two-hybrid assays with isolated PAS domains (either PAS A, PAS B or the bipartite PAS AB) clearly demonstrate that strong interaction occurs only between two bipartite PAS domains. The lack of interaction between the isolated PAS A and B subdomains is unlikely to be caused by a disturbed folding of the isolated domain, because the fragments used for this assay also contain sequences adjacent to the core repeats of the PAS domain [6]. Surprisingly though, co-immunoprecipitation demonstrates that deleting one PAS core repeat or even both (at least in the case of BMAL1) did not severely impair dimerization. The use of broader deletion mutants that retain only the bHLH and C-terminal regions clearly indicates that bHLH motifs do not make a significant contribution to dimerization. As similarity is not restricted to the PAS core repeats but extends to the linker and regions directly upstream and downstream of PAS A and PAS B core repeats respectively [6], we conclude that CLOCK–BMAL1 dimerization involves multiple interaction surfaces in each protein PAS domain, including the PAS core repeats. Recent crystallographic data for *Drosophila* PER PAS repeat [29] are consistent with this notion.

Levels of expression, subcellular localization and phosphorylation status of BMAL1 and CLOCK are at great variance in the literature [24,25,30–32]. Phosphorylation of CLOCK has been reported in the liver [24,25] and in fibroblasts [24,30] but is much less obvious in the suprachiasmatic nuclei [24,31]. In our experiments in COS-7 cells, the putative phosphorylated form of CLOCK was barely noticeable in crude extracts but was enriched in extracts first immunoprecipitated with BMAL1, which may explain why some studies failed to detect it. This also indicates that CLOCK phosphorylation is induced upon its dimerization with BMAL1. Furthermore, our results with NPAS2 and BMAL2 extend the model of co-dependent phosphorylation to all four combinations of transcriptional activators and imply that, similarly to CLOCK, the phosphorylated NPAS2 pool represents only a small fraction of the total NPAS2 pool, which appears to be bound to either BMAL1 or BMAL2.

Phosphorylated forms of CLOCK(NPAS2) and BMAL1 are mostly found in the nucleus [24,25,33] and at times corresponding to the expected maximal transcriptional activity of the heterodimer [22,34,35]. It is thus tempting to link the phosphorylation status of CLOCK(NPAS2) and BMAL1(BMAL2) to transcriptional activity. Indeed, Eide et al. [36] showed that BMAL1 can be phosphorylated by CKIε (casein kinase Iε), which likely results in enhanced transcriptional activity. As deletion of a single PAS core repeat in either CLOCK or BMAL1 did not severely impair dimerization, it came as a surprise that it abolished the transcriptional activity of the heterodimer. Further analysis revealed that all core PAS repeat deletions apparently abolished phosphorylation of both partners, clearly indicating that these repeats play a crucial role in co-dependent phosphorylation. Furthermore, the isolation of two novel *Bmal2* splice variants, lacking a short stretch of amino acids in the PAS domain (*Bmal2sv1*) or downstream (*Bmal2sv2*), strengthens this view. While *Bmal2sv1* is not transcriptionally active with CLOCK(NPAS2), *Bmal2sv2* displays levels of transcriptional activity that are indistinguishable from *Bmal2a* when ectopically co-expressed with CLOCK(NPAS2). Similar to the *Bmal1* mutants lacking PAS core repeats, *Bmal2sv1* cannot be co-dependently phosphorylated in the presence of CLOCK(NPAS2), while this process is not impaired for *Bmal2sv2*. Altogether, these results underline a clear correlation between phosphorylation of the heterodimers and their transcriptional activity.

Our experiments with bHLH-deleted mutants, which heterodimerize but do not transactivate, owing to impaired DNA binding, suggest that BMAL1 phosphorylation occurs independently of DNA binding while CLOCK phosphorylation may be DNA-binding-dependent. Phosphorylation of the bHLH-deleted BMAL1 was notably impaired though. It cannot be excluded that bHLH domains themselves harbour phosphorylation sites. An alternative explanation is that bHLH deletion, which eliminates a putative nuclear localization signal of BMAL1, prevents the nuclear translocation of BMAL1 [23]. However, fractionation (cytosol/nucleus) of transfected cells revealed that bHLH-deleted BMAL1 could be found in both compartments (results not shown).

Kinases responsible for the co-dependent phosphorylation of CLOCK(NPAS2) and BMAL1(BMAL2) are unknown. While there is evidence for CKIε phosphorylation of BMAL1 [36], no site has been mapped. The importance of phosphorylation in determining the pace of the clock has been underpinned in rodents

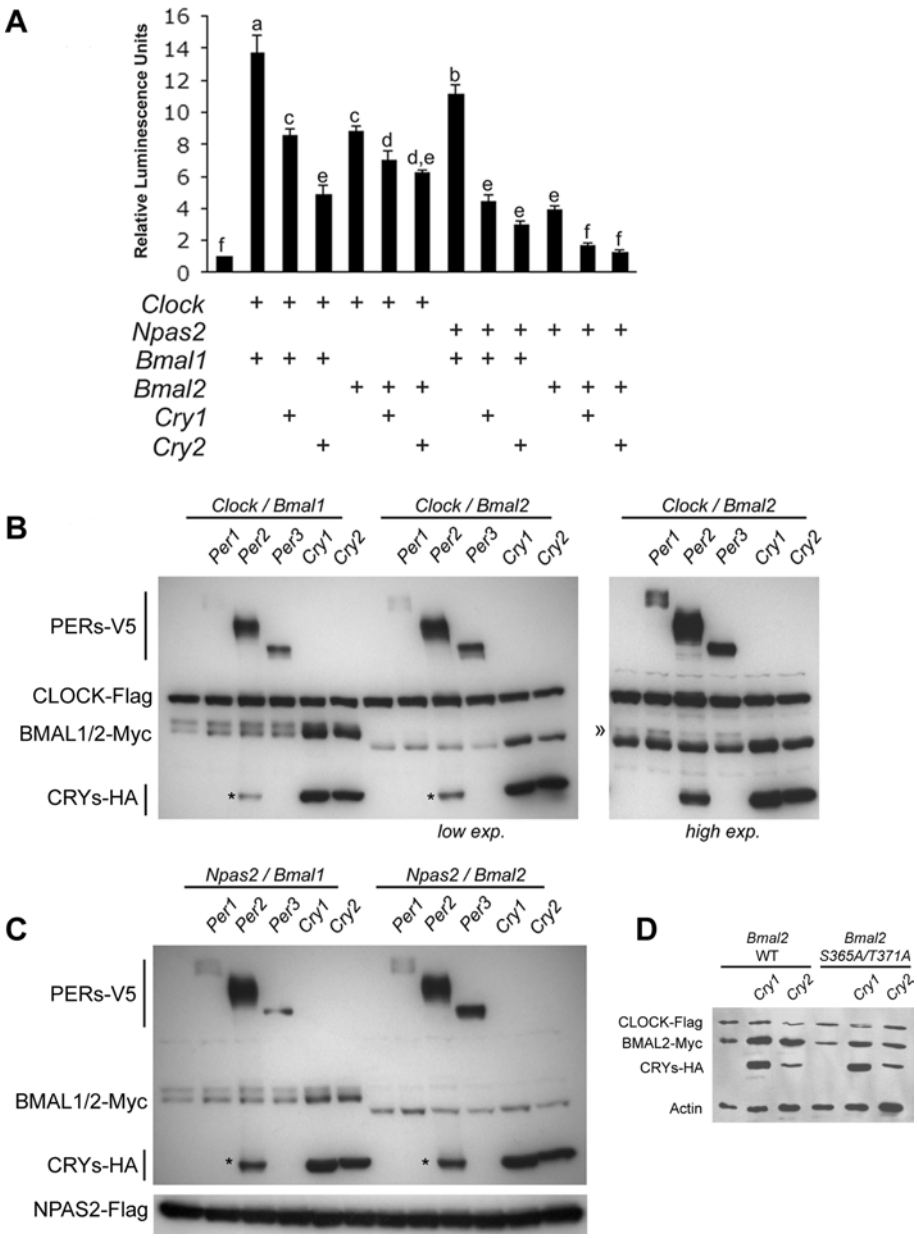

Figure 7 Transcriptional and post-translational effects of CRY1–2 and PER1–3 on CLOCK(NPAS2)–BMAL1(BMAL2)

(**A**) The effects of CRY1–2 on CLOCK(NPAS2)–BMAL1(BMAL2)-induced transcriptional activation were tested by luciferase assay with a *Per1-luc* reporter. All four combinations activate transcription and are repressed by addition of CRY1–2. (**B**) CRY1–2 but not PER1–3 shift the ratio of unphosphorylated/phosphorylated forms of BMAL1(BMAL2) towards predominantly unphosphorylated forms when co-expressed with CLOCK (Western blot with anti-Myc antibody for BMAL1 and 2, anti-FLAG for CLOCK, anti-HA for CRYs and anti-V5 for PERs). The second PER2 band (~60 kDa; asterisk) probably reflects partial proteolysis. Longer exposure time (right panel) reveals that CRY1–2 but not PER1–3 blunt the phosphorylated forms of BMAL2. (**C**) CRY1–2 but not PER1–3 shift the ratio of unphosphorylated/phosphorylated forms of BMAL1 towards predominantly unphosphorylated forms when co-expressed with NPAS2 (left panel) while having no obvious effect on NPAS2–BMAL2 (Western blot with antibodies as in **B**, except that anti-FLAG detects NPAS2); 400 ng of each construct was transfected. (**D**) CRY1 stabilizes the S365A/T371A BMAL2 mutant protein (Western blot with anti-Myc antibody for BMAL2, anti-FLAG for CLOCK and anti-HA for CRYs and anti-actin).

[26] and humans [37]. At least three kinases, CKIε, CKIδ and GSK-3 (glycogen synthase kinase-3), seem to be at play in circadian clocks in mammals, regulating the stability and subcellular localization of clock proteins, especially the PERs and CRYs [25,36,38–42]. PER and CRY proteins play a central role in the clock as repressors of CLOCK–BMAL1 transcriptional activity. CRY1–2 proteins exhibit strong transcriptional repression while PER1–3 are weak repressors [12,13,43,44].

The mechanism(s) by which PERs and CRYs repress CLOCK–BMAL1 is unknown. CLOCK–BMAL1 appear to be constitutively bound to E-boxes (*Per1*, [25]; *Per2*, [45]; however, see recent data by Ripperger and Schibler [35] on a clock-controlled gene, *dbp*) so that rhythmic transcription is conferred by the rhythm in PER/CRY repression, perhaps through chromatin remodelling [46]. All these proteins can be co-immunoprecipitated from tissue extracts, indicating that they are all part of a large multimeric complex [25]. However, while direct interactions between PERs and CRYs as well as interactions between CRYs and CLOCK or BMAL1 have been observed, there is no proof for a direct interaction between the PERs and either CLOCK or

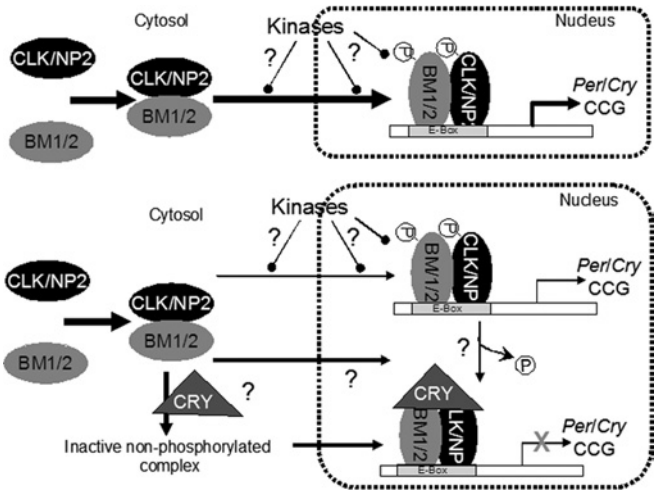

Figure 8 Model for CRY repression of CLOCK(NPAS2)–BMAL1(BMAL2)-induced transactivation

Top panel: CLOCK(NPAS2) ('CLK/NP2') and BMAL1(BMAL2) ('BM1/2') heterodimerize in the cytoplasm and undergo co-dependent phosphorylation that may occur either in the cytoplasm or in the nucleus. The dimer is then active. Its binding to the E-box results in transcription of clock (*Pers* and *Crys*) and clock-controlled genes (CCG). Bottom panel: after a time lag, CRY1–2 proteins accumulate and bind to CLOCK(NPAS2)–BMAL1(BMAL2), which results in escape from kinases and/or active dephosphorylation by phosphatases. This may occur in the cytoplasm and/or nucleus. Because data for CLOCK–BMAL1 heterodimer suggest that the heterodimer may be constitutively bound to DNA, the repression effect of the CRYs likely occurs as the consequence of a progressive replacement or conversion of active (phosphorylated) by inactive (non-phosphorylated) heterodimers, ultimately leading to a maximum repression when CRY proteins reach peak levels.

BMAL1 [13,14,43,47,48]. We reasoned that if phosphorylated forms of CLOCK and BMAL1 are functionally meaningful for transactivation, as suggested by our results, then PERs and CRYs may exert their repression on the heterodimer through modification of their phosphorylation status.

We first examined this hypothesis with the CLOCK–BMAL1 heterodimer. In agreement with our hypothesis, CRY2 dose-dependently blunted the phosphorylated forms of BMAL1 while CRY1 and PER1–3 proteins did not have any obvious effect. However, we cannot exclude that CRY1 and PER1–3 affect phosphorylation in a way not noticeable in BMAL1 gel mobility. Perhaps mechanistically more relevant, CRY1–2 led to substantially higher levels of the non-phosphorylated form of BMAL1, suggesting a stabilization process. This effect on BMAL1 appears to be CLOCK-dependent as we did not notice any obvious stabilizing effect of CRY1–2 on BMAL1 when CLOCK was absent. Furthermore, CLOCK could still be co-immunoprecipitated with BMAL1, indicating that CRY proteins do not disrupt the heterodimer.

We then wished to determine whether similar mechanisms could apply to other combinations of transcriptional activators. CRY1–2 but not PER1–3 co-transfection blunted the phosphorylated forms of BMAL2 and led to a strong stabilization of its unphosphorylated form when CLOCK was present. Albeit less dramatic, CRY1–2 also stabilized the unphosphorylated BMAL1 when it was co-expressed with NPAS2. In contrast, no effect could be observed for the NPAS2–BMAL2 combination.

Altogether these results demonstrate that the presence of CRY proteins dose-dependently diminishes the phosphorylated/unphosphorylated BMAL1(BMAL2) ratio, impairs the BMAL1-dependent CLOCK apparent phosphorylation, but does not disrupt the heterodimer. In agreement with our results, Cardone et al. [22] and Tamaru et al. [30] reported that it is mainly the phosphorylated form of BMAL1 that can be detected in *Cry1/Cry2* double knockout mice. Furthermore, Akashi et al. [34] recently demonstrated with pull-down assays that both phosphorylated and non-phosphorylated BMAL1 can bind E-boxes. Collectively, our results and the aforementioned studies support a model (Figure 8) in which co-dependently phosphorylated CLOCK(NPAS2) and BMAL1(BMAL2) heterodimers are transcriptionally active and CRYs exert their repression through a dose-dependent decrease in the BMAL1(BMAL2) phosphorylated/unphosphorylated ratio and impairment of CLOCK phosphorylation, ultimately locking the heterodimer in an inactive state. Future work should decipher whether CRYs inhibit the recruitment of kinases and/or recruit phosphatases to achieve their effect and how these events modulate CLOCK(NPAS2)–BMAL1(BMAL2) transcriptional activity.

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