COMMENTARY

Stabilizing daily clock proteins

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Biological timekeeping is determined by internal temporal programmes and the resetting of these programmes or clocks by external stimuli. Many of the core genes of the mammalian daily or circadian clock are known, but the factors regulating so-called ‘clock’ gene proteins are unclear. In this issue of the *Biochemical Journal*, Gallego and colleagues show for the first time that protein phosphatase 1 plays a major role in the stability of mammalian PER2, a key protein in the core clock works. This contrasts somewhat with circadian rhythm control in the fruitfly *Drosophila* and

the fungus *Neurospora* where current evidence supports a role for protein phosphatase 2A in core timekeeping. The mechanisms underpinning these actions of phosphatase 1 are unclear, and future investigations will need to identify the regulatory subunit that targets phosphatase 1 to mammalian PER2 (Period 2).

Key words: circadian clock, clock gene protein, *Drosophila*, kinase, *Neurospora*, phosphatase.

Biological clocks are endogenous timekeeping mechanisms present in both prokaryotes and eukaryotes. Different clocks have different periods and those with a near-daily or 24 h periodic oscillation are called circadian (circa = about; dian = a day) clocks. In mammals, circadian clocks control a wide variety of physiological and behavioural processes, including the onset of sleep, patterns of hormone release, metabolism and cognitive performance. The first circadian clock identified in mammals is in the SCN (suprachiasmatic nuclei), located in the hypothalamus of the brain [1]. Experimental destruction of the SCN renders an adult laboratory rodent such as the Syrian hamster arrhythmic, and behavioural rhythms can be rescued through transplantation of fetal hamster SCN tissue into the brain of an SCN-lesioned adult hamster. The observation in the 1980s that some Syrian hamsters spontaneously developed an abnormally short circadian clock (≈ 20 h period; the ‘tau’ mutant hamster) enabled researchers to conduct a key experiment. Researchers tested whether the genotype of the host or donor tissue determined the period of restored rhythms in the SCN grafting paradigm. Wild-type SCN-lesioned hamsters (normal period when intact ≈ 24 h) receiving fetal tau-mutant SCN tissue expressed a 20 h rhythm, whereas SCN-lesioned adult tau-mutant hamsters implanted with fetal wild-type SCN grafts expressed near 24 h rhythms. Thus intrinsic rhythmicity is determined by the genotype of the donor tissue [2]. Studies *in vitro* have revealed that in fact individual SCN neurons function as cell autonomous oscillators such that single tau mutant SCN neurons oscillate every 20 h, whereas wild-type hamster SCN neurons cycle with a near 24 h period [3].

Complementing these physiological and behavioural investigations, genetic approaches have revealed a remarkable conservation in the molecular and biochemical basis of circadian clocks. Several circadian mutants of the fruitfly *Drosophila* have been described and the ‘clock genes’ responsible identified. Thus flies with mutant *period* alleles can exhibit short, long or arrhythmic periods. Building on the identification of *period* (*dper*), *cryptochrome* (*dCRY*), *clock* (*dCLK*) and *double-time* (*dbt*) (and their respective dPER, dCRY, dCLK and DBT proteins) in *Drosophila*, researchers in several laboratories cloned the mammalian orthologues and paralogues [*mper1*, *mper2*, *mcry1*, *mcry2*, *mClock*,

Bmal1, *ck1ε* (cf. *dbt*), etc.] and determined that these played key roles in the molecular basis of the mammalian circadian clock. The current formulation of the molecular clock contains feed-forward and feedback loops (see [1,4] for reviews) in which mCLOCK–BMAL1 heterodimers drive positive transcription of *mper* and *mcry*, whereas mPER–mCRY complexes in the cytoplasm translocate to the nucleus to down-regulate their own transcription. Through monitoring reporter constructs (luciferase and green fluorescent protein) driven by clock gene promoters, clock-like activities are now known to be present in brain areas outside the SCN, and importantly in many peripheral tissues, including the retina, heart, lungs and liver.

Over the last 4 years, identification of new mammalian clock genes has slowed down (indeed, the *mClock* gene is possibly not required for the molecular oscillator in the SCN [5]). Instead, focus is now shifting to understanding the factors that determine the stability and interactions of the protein products of the clock genes. Kinases play key roles in regulating clock function. For example, activation of the MAPK (mitogen-activated protein kinase) pathway in SCN neurons is important for resetting of this circadian clock via light stimulation of the retina [6]. Furthermore, CK1ε (casein kinase 1ε; also known as protein kinase CK1ε) (and possibly CK1δ), which associates with and phosphorylates mPER in the cytoplasm, influences the translocation of mPER–mCRY–mCK1ε multimers to the nucleus and hence the period of the clock. Indeed, this post-translational control is altered in the tau mutant hamster; specifically, the phosphorylation activities of CK1ε are altered in a substrate-specific manner [7]. Polymorphisms in *hPer2* (human *Per2*) can also accelerate the endogenous core body temperature and sleep–wake cycles, an effect attributable to alterations in the CK1ε-binding site on hPER2 (human Period 2).

Implicit to protein phosphorylation by kinases is that dephosphorylation of such proteins is accomplished by protein phosphatases. However, in contrast with kinases, knowledge of the role of phosphatases in circadian timekeeping is impoverished. A principal class of serine/threonine phosphatases in cells is the PPP family whose canonical members are PP1 (protein phosphatase 1) and PP2A (protein phosphatase 2A), and inhibitors of PP1 or

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PP2A alter circadian rhythms in dinoflagellates [8]. In *Neurospora crassa*, a fungus widely used in circadian biology, the degradation of FREQUENCY, a key molecule in the *N. crassa* circadian clock, is regulated by PP1 [9,10]. In *Drosophila*, PP2A is implicated in the stabilization of dCLK, and RNAi (RNA interference) knockdown of the *widerborst* and *twins* transcripts (which encode regulatory subunits of PP2A) increases dPER degradation via DBT phosphorylation and destabilization, and *twins* mutant flies have lengthened circadian rhythms [11,12].

In this issue of the *Biochemical Journal*, Gallego and colleagues [13] present the first evidence that PP1 plays a key role in the stabilization of mPER2 via dephosphorylation. The authors used a variety of techniques and models to determine that PP1, but not PP2A, is key for mPER2 stability. First, using mPER2 extracted from *Xenopus* eggs, they show that the PP1 specific inhibitor Inhibitor-2 causes enhanced degradation of mPER2. Secondly, they show that mouse PP1 dephosphorylates CK1 ϵ -mediated phosphorylation of mPER2. Thirdly, they show that PP1 and full-length mPER2 co-immunoprecipitate, whereas a PP2A catalytic subunit and mPER2 do not. Fourthly, using a dominant negative PP1 (the D95N substitution in which a mutation of aspartic acid to asparagine at amino acid position 95 greatly impairs the catalytic activity of PP1) they show that, although PP1 D95N and mPER2 interact, the P95N mutation leads to a reduction of mPER2 abundance. Furthermore, in transfected cells overexpressing the specific PHI-1 (PP1 holoenzyme inhibitor 1) or Inhibitor-2, levels of mPER2 are reduced significantly the day following transfection; tests with SV40 (simian virus 40) small T-antigen, which inhibits many PP2A holoenzymes, has no effect on mPER2 stability. Dominant negative PP1 (D95N) shortens the half-life of mPER2 to approx. 3 h compared with 6 h under control conditions. Finally, since *Drosophila* Slimb, a member of the F-box/WD40 protein family of the ubiquitin ligase SCF complex, targets dPER to the proteasome, and because the mammalian Slimb homologue β -TrCP (β -transducin repeat-containing protein) is involved in proteasomal degradation of hyperphosphorylated mPER2, they tested whether PP1 regulated β -TrCP-degraded mPER2. They found that co-expression of dominant negative β -TrCP increased abundance of mPER2, and inhibition of 26S proteasome with MG132 prevented the induction of mPER2 degradation by D95N PP1. Finally, dominant negative β -TrCP and MG132 blocked dominant negative PP1-induced degradation of mPER2, indicating that PP1-regulated phosphorylation is required for the ubiquitin- and proteasome-mediated degradation. In the course of these studies, the authors found (as had other researchers) that expression levels of PP1 isoforms do not appear to vary across the circadian cycle, suggesting that PP1 interacts indirectly with mPER2 via regulatory proteins.

This is the first investigation to show that a balance of the activities of CK1 ϵ and PP1 determines stability of mammalian PER2. Unlike the situation in *N. crassa* and *Drosophila*, it appears that, in mammals, PP1 and not PP2A is important for mPER2 stability, raising the possibility of subtle species-specific fine-tuning of the molecular circadian clock. It is, however, possible that PP2A regulates dephosphorylation of PP1, and further investigations are necessary to evaluate the potential influence of protein phosphatase cascades on the regulation of mPER2 stability. Similarly, the identity of the regulatory subunit that targets

PP1 to mPER2 is unclear. The regulatory domain of mPER2 is located between amino acids 450 and 763 and contains CK1 ϵ - and β -TrCP-binding sites, but the RVXF motif, the best-characterized PP1-binding site, is absent. This suggests that PP1 regulatory proteins mediate the association of PP1 with mPER2. Hence circadian expression of regulatory proteins could contribute to the stability of mPER2 by determining PP1 binding to mPER2. No doubt this represents the first of many investigations into the factors and processes that mediate the stability of clock gene protein products. In concert with such approaches will be research strategies aimed at establishing how such proteins regulate cellular activity. The recent observation that hyperpolarizing mouse and fly clock neurons through transgenic alteration of ion channels and G-protein-coupled receptors ablates or blunts clock activities indicates that clock regulation is also achieved by ionic events in the membrane [14,15]. Future models and experimental strategies will have to consider both ionic and nuclear-cytoplasmic events in order to dissect the relationships between the molecular clock and its cellular environment.

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