PERspective on PER phosphorylation

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Period (PER) proteins are essential parts of the molecular clocks that control circadian rhythms in flies and mammals. Phosphorylation regulates PER’s stability and subcellular localization; however, the physiologically relevant sites have been difficult to identify in spite of knowing the relevant kinase. In this issue of Genes & Development, Chiu and colleagues (pp. 1758–1772) identify a key phosphorylation site on PER that recruits the F-box protein Slimb to trigger PER degradation and set clock speed.

In the beginning there was per. Or at least three mutant alleles of the Drosophila period gene: a null mutation (per^null) that made flies behaviorally arrhythmic, and two period-altering alleles (per^short and per^long) that, respectively, gave flies 19-h and 29-h rhythms in constant darkness (Konopka and Benzer 1971). These mutants were surprising to many when described in 1971 since there were doubts that animal behavior in this case, the ~24-h circadian rest/activity cycles of fruitflies) would be so dependent on a single gene. Humans and other mammals have three Per genes, which suggests some redundancy. However, humans heterozygous for a point mutation in hPer2 that causes a single amino acid change suffer from Familial Advanced Sleep Phase Syndrome, and two other direct CLK/CYC targets and, like per, a rhythmically expressed gene. PER and TIM then enter the nucleus in a time-gated manner. In the nucleus, PER represses further expression of per, tim, and many other CLK/CYC direct targets by removing CLK and CYC from DNA. Negative feedback ends with PER degradation, allowing CLK/CYC to reactivate transcription of per and tim, and a new cycle begins. In this way, per RNA and PER protein levels oscillate taking 24 h for one complete cycle, paralleling flies’ behavioral rhythms.

PER is progressively phosphorylated with time over the course of the night and early morning and is then degraded [Edery et al. 1994]. Mutations in double-time (dbt), which encodes a CK1 family member, alter rates of PER phosphorylation. In turn, this changes the timing of molecular clocks and the resulting behavior, suggesting that DBT regulates PER phosphorylation and stability [Price et al. 1998]. Subsequently, it was shown that PER and DBT stably associate in flies [Kloss et al. 2001]. However, the precise sites on PER that are phosphorylated by DBT have proved elusive, for several reasons: the large number of serine and threonine residues in PER (>250) and the inactivity of bacterially produced DBT, as well as the difficulty in obtaining enough PER from flies to map phosphorylation sites. In this issue of Genes & Development, Chiu et al. (2008) report how they surmounted these problems by using a cell culture system to identify DBT-dependent phosphorylation sites on PER. They identify a key phosphorylation site at the N terminus of PER that helps recruit the F-box protein Slimb in vitro. Importantly, altering this one amino acid in PER in vivo lengthened behavioral rhythms by almost 7 h, emphasizing its importance in setting clock speed.

PER phosphorylation

Many of the genes that regulate circadian timing alter PER phosphorylation and/or PER stability. Together, they have led to the idea that the rate of PER phosphorylation determines the speed of the clock. These relevant

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The phosphorylation of Ser47 is a relatively late event in the life of a PER protein. This makes sense since once Ser47 is phosphorylated, it is only a matter of time before Slimb is recruited and PER is degraded. So to allow PER to endure for many hours, it seems that DBT phosphorylation of Ser47 is deliberately poor. Or, perhaps that PP2A-mediated dephosphorylation of Ser47 is very fast—or both. Similarly the Slimb-binding site surrounding Ser47 also seems “deliberately bad”—it is not a good Slimb consensus sequence, and again this may be important in stretching out the half-life of a PER protein to help fill a 24-h cycle.

More PER phosphorylation

Chiu et al. (2008) also used the cell culture system to identify additional PER phosphorylation sites via mass
spectrometry. Among the 25 sites identified, the DBT-mediated PER phosphorylation site at Ser589 stood out. Ser589 is the amino acid mutated in the original per\textsuperscript{short} allele identified by Konopka and Benzer (1971). By this point, you will no doubt be predicting that in per\textsuperscript{short}, Ser589 is mutated to a phospho-mimetic Asp or Glu to speed up the clock just as Chiu et al. (2008) had done when engineering the S47D mutation to give 22-h rhythms. Unfortunately, this is not the case—per\textsuperscript{short} has a Ser-to-Asn change at position 589. Why, then, is per\textsuperscript{short} short and not long? Clearly not all phosphorylation events on PER have the same effect. While phosphorylation of Ser589 and the surrounding residues seems to normally slow down the clock, phosphorylation around Ser47 normally speeds up the clock. Similarly, the hPer2 mutation that underlies Familial Advanced Sleep Phase Syndrome is the loss of a phosphorylatable Ser that speeds up the clock (Toh et al. 2001), in an analogous manner to the per\textsuperscript{short} mutation.

The phospo-mapping by Chiu et al. (2008) for Drosophila PER and by Vanselow et al. (2006) for mPER2 indicates that PER phosphorylation occurs in discrete clusters. Together, they provide excellent support for the idea that PER proteins have different domains whose phosphorylation has different effects. Some domains could affect PER stability in the nucleus and others in the cytoplasm. Other regions of PER potentially regulated by phosphorylation include domains that help time nuclear entry [Meyer et al. 2006] and that repress transcription [Chang and Reppert 2003]. These different clusters could then regulate different aspects of PER function—even if phosphorylated by the same kinase (DBT/CK1\epsilon in flies and mammals, respectively). This idea helps to explain some confusing phenotypes that arise from mutations that alter DBT/CK1\epsilon activity since the mutant enzymes seem to differentially affect phosphorylation at different domains of PER [Gallego et al. 2006; Xu et al. 2007; Meng et al. 2008].

Indeed, it is striking that Chiu et al. (2008) show that altering Ser47 stabilizes PER but PER still becomes hyperphosphorylated. Until relatively recently, the dogma was that PER phosphorylation simply preceded degradation. Now it is clear that PER proteins are phosphorylated in numerous domains to regulate PER activity and help PER accurately track time. Elucidation of the function of these different PER domains and their regulation by phosphorylation now seems tractable.

PERspective: transcriptional or post-translational regulation?

Last year, the circadian rhythms field was invited to share its ideas at the Cold Spring Harbor Symposium for the first time since 1960. One of the questions discussed was whether transcriptional regulation or post-transcriptional regulation is more important in the clock. A case can be made for each side. For example, one could argue that post-transcriptional regulation is more important since the most dramatic period length changes in circadian mutants tend to affect post-transcriptional regulation (e.g., Smith et al. 2008 and the study from Chiu et al. 2008). However, the animal clock transcriptional regulatory loops are so intertwined [for review, see Hardin 2005] that mutations in clock transcription factors may be buffered by the connectivity of the system as long as they allow sufficient gene expression to take place. Again supporting the post-translational camp, Yang and Sehgal (2001) found that constitutive expression of per and tim could rescue the rhythmic behavior of per\textsuperscript{short}; tim\textsuperscript{01} double null mutants. However, a recent reanalysis of this experiment indicates that the extent of rhythmicity was probably overestimated and most of the rhythms detected were weak [Hall et al. 2007]. However the strongest piece of evidence for a post-translational clock comes from Cyanobacteria, where circadian rhythms in phosphorylation of the KaiC clock protein can be seen for several days in a test tube when mixing three proteins together—with no DNA or RNA present [Nakajima et al. 2005]. Thus a purely post-translational clock can function. However, a subsequent study revealed that robust and accurate in vivo circadian rhythms of Cyanobacteria require transcriptional feedback and can even proceed without rhythmic KaiC phosphorylation [Kitayama et al. 2008].

So is transcriptional or post-transcriptional regulation more important? I believe the answer is “No.” Instead, it seems that each tier of regulation adds to the robustness and accuracy of these internal molecular clocks that help animals stay synchronized with their environment and keep their internal organs working together in harmony.

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