A PERIOD inhibitor buffer introduces a delay mechanism for CLK/CYC-activated transcription

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Abstract We investigated the functions of clock genes *period* (*per*) and *timeless* (*tim*) in establishing negative feedback on circadian transcription factors *clock*/*cycle* (*Clk*/*cyc*) in *Drosophila*. We show that PER protein persists for several hours after rapid degradation of TIM in the morning. We observed in cell culture that isolated PER inhibits CLK/CYC-activated transcription in the absence of TIM and we further demonstrated for the first time in vivo that PER accumulation in a *tim* loss-of-function mutant background causes efficient inhibition of CLK/CYC-dependent transcription. These results identify PER to be the main inhibitor for CLK/CYC and they suggest a delay mechanism during early morning, when PER protein, after degradation of TIM, forms an inhibitor buffer for CLK/CYC that attenuates the restart of the next cycle of CLK/CYC-activated transcription. While TIM likely enhances the inhibition of CLK/CYC by PER in the dark, our results suggest a reduction of PER-mediated inhibition by TIM in light.

Key words: Circadian clock; Behavior; Gene expression; Regulation of transcription; Period; Biological rhythm

1. Introduction

The circadian clock regulates a broad range of behavioral and physiological activities in many species, such as cyanobacteria, plants, insects, and mammals [1–3]. A common theme in circadian clock function is a positive–negative feedback loop formed between a positive element that activates transcription of its own inhibitor [4–6]. The constitution of a self-sustained circadian oscillator on the basis of such feedback regulation requires delay mechanisms that separate and precisely time the inhibition and activation events. Such delay mechanisms prevent dampening of the oscillation and allow the maintenance of a constant period length under free running conditions that is in the absence of environmental cues such as light/dark cycles (LD).

In *Drosophila*, the positive element of the circadian clock is formed by transcription factors *clock* (*Clk*) and *cycle* (*cyc*). CLK/CYC heterodimeric complexes activate transcription of their inhibitors *period* (*per*) and *timeless* (*tim*) (for review see [4,6,7]). In the cytoplasm, PER is rapidly degraded and does not accumulate in the absence of TIM due to phosphorylation of PER protein by the casein kinase Iε homolog DOUBLETIME (DBT) [8]. TIM however is light sensitive, which prevents accumulation of TIM and PER during the light phase [9,10]. PER and TIM form heterodimeric complexes that accumulate in dark and migrate into the nucleus [11], where they bind to CLK/CYC and negatively feed back on their own transcription. Inhibition of CLK/CYC is released after degradation of TIM and PER in the morning and CLK/CYC-activated *per* and *tim* transcription restarts at dusk. CLK mRNA and protein concentrations cycle too, due to a second feedback loop involving *vrille* [12–14].

Important delay mechanisms in the clock oscillation were identified with a lag in peak accumulation of clock gene mRNA and protein levels [15–17]. Although feedback regulation of PER/TIM on CLK/CYC is well established, the identification of the main CLK/CYC inhibitor remains unclear. In cell culture, a deletion construct of PER that lacks the cytoplasmic localization domain (CLD) showed TIM-independent inhibition of CLK/CYC activity [18,19], while wild-type PER under different expression conditions acts dependent [17,19] or independent [20] of TIM. However, PER and TIM as well as PER/TIM complexes inhibit the DNA-binding activity of CLK/CYC in vitro [21], suggesting that PER, TIM and heterodimeric complexes of both may act independently as CLK/CYC inhibitors. The identification of the main CLK/CYC inhibitor is important, since PER and TIM protein levels respond differently to light during the morning. While TIM is rapidly degraded upon exposure to light [9,10,22,23], PER protein appears to be more stable [16,19,24]. We therefore tested the hypothesis that PER forms a titration buffer that allows continued inhibition of CLK/CYC in the absence of TIM for several hours during the morning. Such PER-mediated inhibition would constitute a delay mechanism for the restart of the next cycle of CLK/CYC-activated transcription.

2. Materials and methods

2.1. Cotransfection assays

ddbt, *dClk*, *dper* and *dtim* were expressed from pAc5.1/V5-HisA constructs (Invitrogen, Carlsbad, CA, USA). A pAc5.1 plasmid expressing a MHHHHHHEPRYFQS peptide was used as pHT control. Cotransfection assays were performed as described in [17], in short: 400 μl culture (10^6 cells/ml) were transfected with 25 ng pRLcopia (for *Renilla* luciferase control), 10 ng pGL3-(4-*per*-E-box)hs::luc (for expression of firefly luciferase from a minimal heat shock promoter with four *per*-E-box elements), 0.5 ng pAc-CLK (or 0.25 ng pAc-CLK in Fig. 2), amounts of pAc-PER and pAc-TIM indicated in figures (10 ng pAc-PER and 10 ng pAc-TIM in Fig. 2A), 200 ng pAc-DBT (Fig. 3B), and pHT control vector to give 235 ng of total DNA (260 ng in

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Fig. 3B). Identical controls were performed without pAc-CLK. Cell lysates were prepared after 40–48 h and firefly luciferase (FF-luc) and *Renilla* luciferase (R-luc) activities were measured using a dual luciferase reporter assay (Promega, Madison, WI, USA). Luciferase activity is calculated from ratios of FF-luc/R-luc of each lysate sample to control for transfection efficiency and lysate concentration. Averages of at least three independent experiments are shown as percent of control.

2.2. Real-time luciferase luminescence measurement and Western blot analysis

Bioluminescence from 1–4 days old live flies, of genotypes indicated in Fig. 4, was measured after a 24-h incubation in constant light at 25°C as described in [25]. For Western blot analysis 1–7 days old flies were harvested either after incubation for 24 h in constant light (Fig. 4, insert) or after 7 days of entrainment in 12 h:12 h LD cycles either in LD or in constant darkness (DD) at times indicated in Fig. 1. Fly head extracts were prepared by homogenization in H1 buffer (125 mM Tris–HCl pH 6.8, 4% sodium dodecyl sulfate, 0.02% β-mercaptoethanol, 20% glycerol) and heated for 5 min at 95°C prior to centrifugation. Equal amounts of total protein samples were analyzed by Western blotting with anti-PER (Alpha Diagnostics, San Antonio, TX, USA) and anti-TIM (generous gift of M.W. Young, Rockefeller University) antibody.

3. Results

3.1. PERIOD is present in the absence of TIMELESS

We first determined changes in the relative concentrations of PER and TIM proteins in fly head extracts at different times before and after the transition from dark to light (Fig. 1). Flies were entrained in 12 h:12 h LD cycles prior to harvesting at different times of day during an additional cycle in LD (Zeitgeber time, ZT) or DD (circadian time, CT). Approximately the same amounts of total protein of fly head extracts

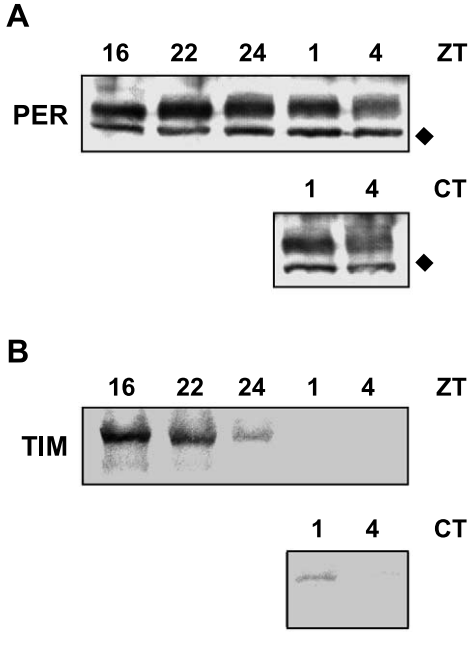

Fig. 1. Presence of PER and TIM proteins before and after transition from dark to light. Western blot analysis of fly head extracts was performed with anti-PER (A) or anti-TIM (B) antibody to determine the relative concentrations of PER and TIM proteins during a cycle in LD (ZT) or in DD (CT). ZT and CT samples shown are from the same Western blot and exposure time. Western blots were reprobed with anti-TIM and anti-PER antibody respectively to confirm changes in relative concentrations. Loading of equal amounts of lysate samples is shown by an unspecific band stained with anti-PER antibody marked with ♦.

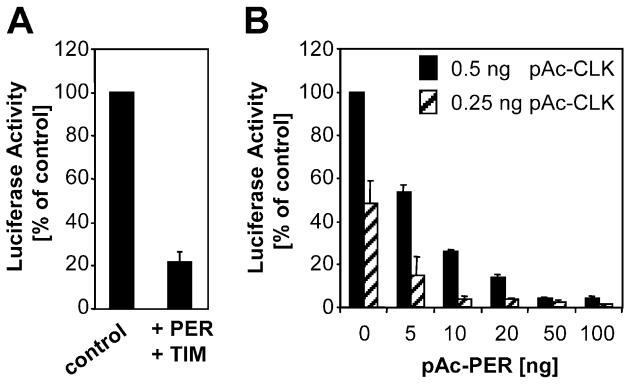

Fig. 2. Inhibition of CLK/CYC-activated transcription by PER in *Drosophila* S2 cells. Luciferase activity was determined in cotransfection assays as described in Section 2. A: CLK/CYC-activated luciferase reporter gene expression in the absence (control) or presence of PER and TIM coexpression. B: CLK/CYC-activated luciferase reporter gene expression either with 0.5 ng (black bars) or 0.25 ng (hatched bars) CLK expression vector pAc-CLK and indicated amounts of PER expression plasmid pAc-PER. Mean luciferase activities ± S.E.M. are shown as percent of control, which is luciferase activity in a transfection with 0.5 ng pAc-CLK in the absence of pAc-PER and pAc-TIM set to 100%.

were subjected to Western blot analysis for each time point, which was confirmed by Ponceau S staining after protein transfer to nitrocellulose membranes and by staining of an unspecific band by anti-PER antibody (Fig. 1). Western blots probed for the detection of PER and TIM proteins show that TIM is rapidly degraded after lights on, while PER is more stable (Fig. 1). The shift of relative amounts between PER and TIM towards an excess of PER after lights on, indicates that PER protein is present in the absence of TIM during morning hours (Fig. 1, ZT 1 and 4). This result is in agreement with previous studies, which have shown that TIM is rapidly degraded in the light [9–11,16,19,22,23]. Degradation of TIM is thought to induce the decomposition of the PER/TIM inhibitor that accumulates during late night in the nucleus, thus releasing inhibition of CLK/CYC during the day. Head extracts from flies, which were not transferred to light after 7 days of entrainment but incubated in DD show a slower degradation of TIM during subjective morning (Fig. 1, CT) [9,10,22]. However, also under free running conditions TIM is degraded faster than PER leaving an excess of PER protein over TIM during subjective morning hours.

3.2. PERIOD inhibits CLOCK/CYCLE in the absence of TIMELESS

We next asked whether PER is a functional inhibitor of CLK/CYC in the absence of TIM. Previously it was shown that CLK/CYC-activated transcription can be reconstituted in *Drosophila* S2 cell culture by use of a reporter plasmid expressing luciferase from a four *per*-E-box containing promoter [17,26]. CLK, PER and TIM show cyclic expression in vivo but they are not expressed to functional levels in S2 cells [2,17,19]. Therefore, endogenous CYC allows activation of the luciferase reporter if cotransfected with a CLK expression vector. In this assay it was shown that coexpression of PER together with TIM causes inhibition of CLK/CYC-activated transcription [17,19,26], reflecting the dependence of PER on TIM for accumulation of functional PER/TIM inhibitor in vivo [27]. However, when we expressed clock proteins in S2

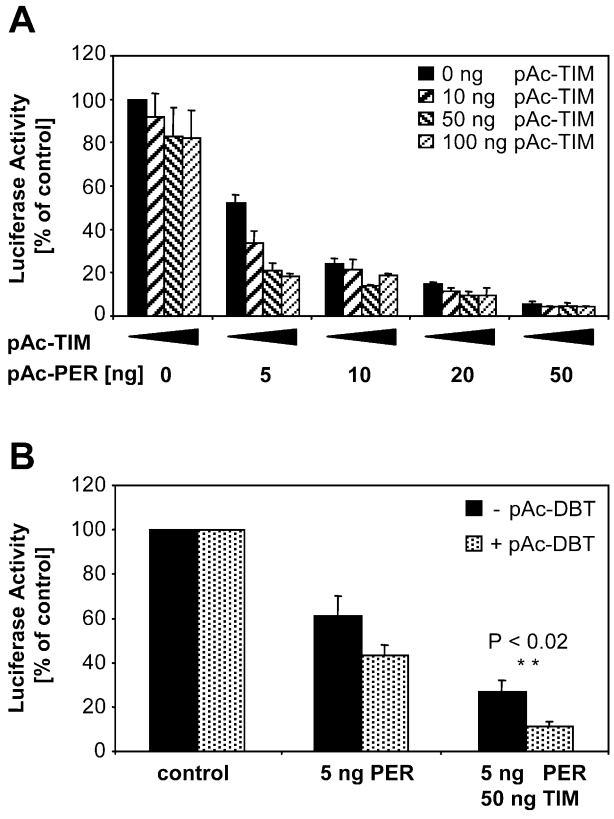

Fig. 3. TIM and DBT enhance PER-mediated inhibition of CLK/CYC activity. Cotransfection assays were performed as in Fig. 2. A: CLK/CYC-activated LUC expression with increasing concentrations of pAc-TIM expression construct and indicated amounts of PER expression vector pAc-PER. B: CLK/CYC-activated LUC expression in cotransfection assays with indicated amounts of PER and TIM expression vector either in the absence (black bars) or presence (hatched bars) of coexpressed DBT. Mean luciferase activities ± S.E.M. are shown as percent of control, which is luciferase activity in a transfection with 0.5 ng pAc-CLK either in the absence or presence of pAc-DBT set to 100%.

cell culture we observed that not only coexpression of PER and TIM together but also PER expression alone resulted in efficient inhibition of CLK/CYC activity (Fig. 2). Increasing inhibition of CLK/CYC-activated luciferase expression was observed with increasing amounts of PER expression construct (Fig. 2). This result shows that wild-type PER is able to efficiently inhibit CLK/CYC activity in the absence of TIM. The difference between our results and previous reports may be due to the use of different expression vectors that may alter expression levels. However, consistent with previous reports we observed that coexpression of TIM enhances the inhibitory function of PER. This is best observed under conditions with moderate inhibition of CLK/CYC by PER alone. At 5 ng of PER expression construct, CLK/CYC-activated luciferase expression is reduced to ~55% of control. Increasing concentrations of TIM expression enhanced the inhibitory activity of PER, while no significant inhibition of CLK/CYC was observed in the presence of TIM alone (Fig. 3A). PER is phosphorylated by the casein kinase Iε homolog DBT [8], which associates with PER in the cytoplasm and in the nucleus [28]. We therefore tested whether DBT modulates PER inhibitor function. Coexpression of DBT did not significantly affect PER-mediated inhibition of CLK/CYC but DBT significantly enhanced the inhibitory function of PER/TIM complexes (Fig. 3B). These results indicate that TIM enhances the inhibitory function of PER but PER itself is an efficient inhibitor for CLK/CYC in the absence of TIM.

3.3. PERIOD forms a CLOCK/CYCLE inhibitor buffer in vivo

Next we aimed to confirm the inhibitory function of PER in the absence of TIM in *Drosophila*. PER is stable for a few hours after degradation of TIM in the morning (Fig. 1). At this time PER is predominantly nuclear [11]. During night, nuclear localization of PER has been observed prior to nuclear accumulation of TIM [11], indicating that PER may not depend on TIM to enter the nucleus and function as a CLK/CYC inhibitor. However, efficient accumulation of PER depends on the presence of TIM, and PER protein accumulation is not observed in tim^0 loss-of-function mutants [27]. After translation in the cytoplasm, PER degradation is initiated by phosphorylation through DBT [8]. Recently a partial *dbt* loss-of-function mutation, dbt^{ar}, has been characterized that allows the accumulation of hypophosphorylated PER even in the absence of TIM due to inefficient phosphorylation of PER (Fig. 4, insert) [24]. We made use of this mutation to investigate the effect of PER on CLK/CYC-activated transcription in vivo and in the absence of TIM. The dbt^{ar} mutation was crossed into *tim:luc* flies that express luciferase from a *tim* promoter [25]. We determined CLK/CYC-dependent *tim* expression in *tim:luc* flies by luciferase bioluminescence measurements in live flies after 24 h in constant light. Since light induces TIM degradation and PER does not accumulate in the absence of TIM, both PER and TIM levels are low in constant light and CLK/CYC-activated LUC expression is maximal in *tim:luc* flies. When *tim:luc*;; dbt^{ar} flies were assayed under the same conditions a slight but statistically significant reduction in LUC expression levels was observed (Fig. 4, columns 1 and 2; Student's *t*-test, $P < 0.005$).

To exclude that residual amounts of TIM, which may be present during the light phase, affect the inhibitory function of

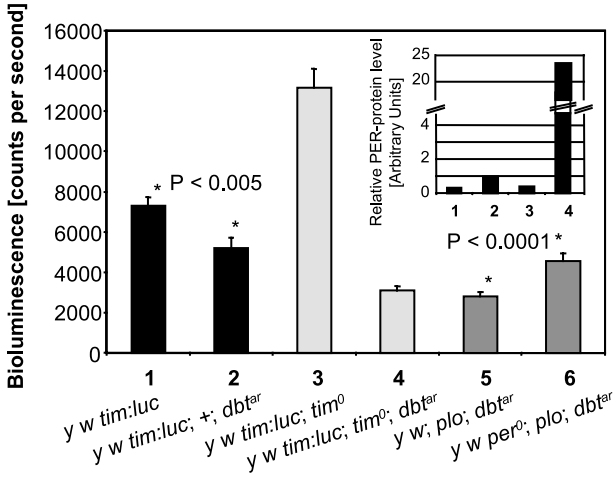

Fig. 4. PER inhibits CLK/CYC-activated transcription in *Drosophila* in the absence of TIM. CLK/CYC-dependent luciferase expression from a *tim* promoter (*tim:luc*) (columns 1–4) or a *per* promoter (*plo*) (columns 5, 6) is shown as mean bioluminescence ± S.E.M. over all *Drosophila* flies of indicated genotypes (26–35 flies) from three independent experiments. Flies were incubated for 24 h in constant light prior to bioluminescence measurement. The insert shows a quantification of PER protein levels in these genotypes after 24 h in constant light, determined by Western blot analysis relative to an unspecific band stained with anti-PER antibody (as in Fig. 1).

PER, we repeated the experiment in a *tim* loss-of-function (tim^0) genetic background. Under the same conditions as above *tim:luc*; tim^0 flies show high CLK/CYC-activated luciferase expression due to the absence of TIM and PER inhibitor (Fig. 4, column 3). Importantly, the dbt^{ar} mutation causes a strong reduction in luciferase expression in this genetic background (Fig. 4, compare columns 3 and 4), suggesting that PER accumulation in the absence of TIM constitutes a functional inhibitor for CLK/CYC-activated transcription in vivo. Surprisingly, PER-induced inhibition of CLK/CYC is more pronounced in tim^0 flies than in *tim* wild-type flies (Fig. 4, compare differences between columns 1 and 2 with columns 3 and 4, also compare columns 2 and 4). The inhibitor effects on CLK/CYC correlate with the levels of PER protein accumulation (Fig. 4, insert) in the various genetic backgrounds. In constant light, PER accumulation in dbt^{ar} flies is decreased in the presence of wild-type *tim* compared to tim^0 flies. Since luciferase expression in *tim:luc*;; dbt^{ar} flies compared to *tim:luc*; tim^0; dbt^{ar} flies appears to be affected to a lesser extent than PER protein levels, the effect of TIM on PER expression may be posttranscriptional.

In order to confirm that the inhibition of CLK/CYC-dependent transcription in dbt^{ar} flies is due to PER, we tested the effect of the *per* loss-of-function mutation, per^0, on CLK/CYC-dependent reporter gene expression in dbt^{ar} flies. For this experiment we made use of the *plo* reporter that expresses luciferase from a *per* promoter [29]. Expression of the *plo* luciferase reporter is activated by CLK/CYC and oscillates in wild-type flies [29]. In *dbt* wild-type flies the per^0 mutation causes an increase in *plo* expression due to the loss of CLK/CYC inhibition (data not shown). When we tested the effect of the per^0 mutation on *plo* expression in a dbt^{ar} background after 24 h in constant light (Fig. 4, compare columns 5 and 6) we observed an increase in *plo* expression in *y w* per^0; *plo*; dbt^{ar} flies (Student's *t*-test, $P < 0.0001$), consistent with the loss of *per*-mediated inhibition of CLK/CYC. Since dbt^{ar} enhances *tim*-independent inhibition of CLK/CYC in tim^0 flies but inhibition of CLK/CYC in dbt^{ar} flies is reduced by the per^0 mutation, the results strongly suggest that increased PER accumulation in dbt^{ar} flies in the absence of TIM causes inhibition of CLK/CYC.

4. Discussion

In the present study, we have shown that PER-mediated inhibition of CLK/CYC is enhanced but not dependent on TIM. A partial loss-of-function mutation in the PER kinase *dbt*, allowed us to investigate PER inhibitor activity in the absence of TIM in *Drosophila*. We found that accumulation of PER in flies correlates with inhibition of CLK/CYC-dependent transcription. This observation indicates that the prolonged presence of PER after degradation of TIM during the morning constitutes an active CLK/CYC inhibitor that introduces a delay mechanism for the progression of the clock oscillation by attenuating the restart of the next cycle of CLK/CYC-activated transcription.

Previously a deletion construct of PER, which lacked the CLD, was shown to reduce CLK/CYC-activated transcription in cell culture independent of TIM [19]. However, PER and TIM as well as PER/TIM complexes inhibit the DNA-binding activity of CLK/CYC in vitro [21], suggesting that PER, TIM and heterodimeric complexes of both may act independently as CLK/CYC inhibitors after translocation into the nucleus. TIM lacked individual inhibitory function and under certain expression conditions only the presence of both, PER and TIM, reduced CLK/CYC-mediated expression in cell culture [17,19]. In *Drosophila*, TIM accumulation in the absence of PER in per^0 flies during dark did not show inhibition of CLK/CYC activity [9,10]. These experiments indicated that both proteins are required for constitution of an efficient CLK/CYC inhibitor, due to the requirement of TIM for accumulation of PER in vivo [8,27] and possibly facilitation of PER nuclear localization through a TIM-regulated mechanism [18]. However, these experiments were not conclusive towards the identification of the main CLK/CYC inhibitor, which could either be PER/TIM complexes or PER alone. We first investigated PER inhibitor activity in *Drosophila* cell culture. Consistent with a previous report [20], we made the interesting observation that wild-type PER acts as an efficient inhibitor of CLK/CYC even in the absence of TIM (Fig. 2). No significant inhibitor activity was found for wild-type TIM in the absence of PER (Fig. 3), which is consistent with the observation that accumulation of TIM in the absence of PER during dark does not inhibit CLK/CYC activity in *Drosophila* [9,10]. The presence of TIM, however, enhanced the inhibitory activity of PER (Fig. 3), which is consistent with previous results [17,19]. The inhibitory function of isolated PER on CLK/CYC is physiologically important during the morning, when PER may form an active inhibitor for CLK/CYC after degradation of TIM (Fig. 1). Such a PER inhibitory buffer would delay the advance of the clock oscillation for several hours before the next cycle of CLK/CYC-activated transcription can start.

Since the inhibitor activity of isolated PER was not observed in previous cell culture experiments we sought to confirm such activity of PER in vivo. As mentioned above, in *Drosophila*, TIM is required for stabilization and accumulation of PER in the cytoplasm during the night [27] and PER does not accumulate in wild-type flies in the absence of TIM. Accumulation of TIM in the absence of PER in per^0 flies during dark did not show inhibition of CLK/CYC activity [9,10]. Therefore, both proteins are required for efficient inhibition of CLK/CYC. On the other hand, degradation of TIM is faster during morning than the degradation of PER, both in LD after lights on as well as in DD during subjective morning (Fig. 1) [11,16]. The prolonged presence of PER in the absence of TIM during morning, when both proteins are nuclear [11], suggests that nuclear PER degradation is not directly coupled to the degradation of TIM. The different time frames for the presence of PER and TIM also suggest specialized functions of both proteins in establishing negative feedback on CLK/CYC. The presence of PER in the absence of TIM during morning supports that PER may be the main inhibitor for CLK/CYC [19]. To directly address this question we tested the function of isolated PER on CLK/CYC-activated transcription in *Drosophila* using the partial *dbt* loss-of-function mutation dbt^{ar}. dbt^{ar} flies were previously characterized and shown to allow accumulation of PER in the absence of TIM due to inefficient PER phosphorylation (Fig. 4, insert) [24]. We found that the dbt^{ar} mutation causes a strong reduction in CLK/CYC-activated reporter gene expression in flies that carry a loss-of-function mutation in the *tim* gene (tim^0) (Fig. 4). This experiment shows that increased inhibition of CLK/CYC in dbt^{ar} flies is not mediated by TIM and may be

due to the accumulation of PER. A loss-of-function mutation in the *per* gene (per^0) releases inhibition of CLK/CYC in dbt^{ar} flies (Fig. 4), indicating that PER is a functional CLK/CYC inhibitor and the increased accumulation of PER in dbt^{ar} flies causes inhibition of CLK/CYC also in the absence of TIM. These results identify PER to be the main inhibitor for CLK/CYC. Our experiments indicate that isolated PER constitutes a titration buffer that allows continued inhibition of CLK/CYC after degradation of TIM during the morning, which delays the restart of the next cycle of CLK/CYC-activated transcription for several hours. This delay mechanism is important for the precise timing of the clock oscillation and the maintenance of a ~24 h period.

As to the function of TIM in establishing negative feedback on CLK/CYC, it is well known that TIM is required for the accumulation of PER [8,27]. Phosphorylation of TIM by GSK3 kinase has also been linked to the regulation of PER/TIM nuclear transport [30], although the mechanism for this regulation is not yet understood. Nuclear localization of PER precedes the accumulation of TIM by several hours [11], indicating a TIM-independent nuclear import of PER. This interpretation is supported by observations in cell culture, which found some nuclear PER in the absence of TIM despite a primary cytoplasmic localization of PER [20]. Also for mammalian clock proteins, where mCRY performs some of the functions of TIM, a CRY-independent nuclear import of mPER1 and mPER2 has been reported [32,33]. Although nuclear import of PER may not depend on TIM, the recent observation of a rapid and PER-independent nucleocytoplasmic shuttling of TIM [31] is consistent with a function of TIM to facilitate and regulate the nuclear accumulation of PER [18]. Such function of TIM for the regulation of PER inhibitor activity on CLK/CYC is consistent with our observation that overexpression of DBT in cell culture enhances PER/TIM-mediated, but not PER-mediated inhibition of CLK/CYC (Fig. 3B). This finding indicates that DBT does not only affect PER stability (Fig. 4) but together with TIM and possibly GSK3 also PER activity (Fig. 3B). Consistent with this observation it was found that the short period mutant dbt^S affects feedback of PER on its own mRNA [34]. TIM-regulated accumulation and nuclear import of PER may also form the mechanistic basis for the observation that PER accumulation and inhibition of CLK/CYC in the dbt^{ar} background are strongly enhanced in tim^0 flies compared to *tim* wild-type (Fig. 4, columns 2 and 4). Although results in Fig. 4 would be consistent with an inhibitor activity of TIM on CLK/CYC, the observation that in dbt^{ar} flies PER protein levels are much stronger affected by the tim^0 mutation than *tim:luc* expression levels (Fig. 4, compare columns 2 and 4 and insert) suggests a posttranscriptional effect of TIM on PER expression. Unlike in darkness, when TIM likely enhances the inhibition of CLK/CYC by PER (Fig. 3A), the effect of TIM on PER expression reduces PER-mediated inhibition of CLK/CYC in light (Fig. 4). These observations suggest a function of TIM in the regulation of PER inhibitor activity, for which further investigation will need to unravel the molecular mechanism.

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