PER-TIM Interactions in Living Drosophila Cells: An Interval Timer for the Circadian Clock

Pablo Meyer, Lino Saez, Michael W. Young*

In contrast to current models, fluorescence resonance energy transfer measurements using a single-cell imaging assay with fluorescent forms of PER and TIM showed that these proteins bind rapidly and persist in the cytoplasm while gradually accumulating in discrete foci. After ~6 hours, complexes abruptly dissociated, as PER and TIM independently moved to the nucleus in a rapidly and persist in the cytoplasm while gradually accumulating in discrete foci. After ~6 hours, complexes abruptly dissociated, as PER and TIM independently moved to the nucleus in a narrow time frame. The per mutation delayed nuclear accumulation in vivo and in our cultured cell system, but without affecting rates of PER/TIM assembly or dissociation. This finding points to a previously unrecognized form of temporal regulation that underlies the periodicity of the circadian clock.

In Drosophila melanogaster, PER and TIM are two essential proteins of the circadian clock that shift from the cytoplasm of clock cells to the nucleus once a day, promoting ~24-hour oscillations of per and tim transcription. They do this in a regulated manner, and the period length of Drosophila’s circadian rhythm is in part determined by how long these proteins are held in the cytoplasm before entering the nucleus (1–4).

Formation of PER/TIM heterodimers appears to promote the nuclear accumulation of both proteins. In vivo, a 4- to 6-hour delay in PER nuclear accumulation may be influenced by the slow cytoplasmic assembly of PER/TIM heterodimers, such that once formed, the PER/TIM heterodimer is rapidly transferred from the cytoplasm to the nucleus. It is thought that in the nucleus PER physically interacts with CLOCK and CYCLE, transcriptional activators of per and tim, inhibiting CLOCK/CYCLE activity and hence closing a delayed feedback loop that contributes to oscillating RNA and protein levels (1–4).

Recently, the proposal that PER and TIM translocate to the nucleus as obligate heterodimers, and even the necessity of TIM for PER’s nuclear accumulation, have been questioned (5–8). To follow PER and TIM during their passage from the cytoplasm to the nucleus and to determine the role of PER/TIM interaction in the regulation of nuclear accumulation, we developed a single-cell, fluorescent, live-imaging assay using a Drosophila cell line (Schneider’s line 2, S2). Although S2 cells do not express several clock genes and are not rhythmic, this cultured cell system has become an important tool for investigating intracellular mechanisms contributing to Drosophila’s circadian clock (9–14).

We constructed C-terminal fusions of PER and TIM with cyan fluorescent protein (CFP) and yellow fluorescent protein (YFP), respectively, and monitored these separately or together in S2 cells (fig. S1). Expression of per-cfp (without TIM) was followed in two cell lines. In one line, PER-CFP production was controlled by a heat shock promoter. These cells were constantly monitored for 10 hours after induction (~100 cells in 10 independent experiments). In the second line, an actin promoter drove per-cfp expression, and 40 cells in two experiments were followed for 10 hours after transfection. PER-CFP was detected only in the cytoplasm of live S2 cells in both studies. In a third study, we followed cells in which tim-yfp was driven by a heat shock promoter in the absence of PER (130 cells in 10 experiments). TIM was retained in the cytoplasm in most but not all cells 10 hours after induction (123 cytoplasmic, 7 nuclear). In contrast, when cotransfected, per-cfp and tim-yfp gave predominantly nuclear fluorescence for both proteins in most cells (209 nuclear out of 265 cells monitored in 39 experiments at 8 hours after induction). The behavior of the proteins in our S2 cell system is therefore concordant with in vivo findings (1–4) and indicates that the fluorescent tags do not detectably interfere with either cytoplasmic retention of the individually expressed proteins or with interactions that promote nuclear accumulation.

To evaluate the validity of an existing model in which rates of PER/TIM interaction affect the timing of their nuclear translocation (1–4), we compared PER-CFP/TIM-YFP fluorescence resonance energy transfer (FRET) measurements dynamically by continuously imaging CFP and YFP in live single cells. We calculated FRET within subcellular compartments on a pixel by pixel basis (Fig. 1A; fig. S2 and Movie S1A) and by averaging over the whole cell as a function of time after PER and TIM induction (Fig. 1B). Temporal profiles of changing FRET levels were then compared to contemporaneous nuclear accumulation profiles of PER calculated for each image as the ratio of mean pixel value in the nucleus to mean pixel value in the whole cell (Fig. 1B). We found that maximum levels of FRET were reached during the earliest stages of PER-CFP and TIM-YFP accumulation (within 30 min of PER and TIM production), indicating that physical interaction followed PER-CFP/TIM-YFP synthesis without a measurable delay (Fig. 1B left). Moreover, high levels of FRET were maintained for several hours preceding the onset of nuclear accumulation of PER and TIM (Fig. 1, A and B). Unexpectedly, FRET declined rapidly as PER and TIM proteins were transferred from the cytoplasm to the nucleus. As PER and TIM became predominately nuclear, FRET levels remained low in all subcellular compartments, which were typically monitored for a further 100 min (Fig. 1, A and B; fig. S2).

We further observed that immediately following coinduction, PER and TIM were always diffusely present in the cytoplasm. However, this largely uniform distribution was followed by a gradual accumulation in prominent cytoplasmic foci (Figs. 1 and 2; Movies S1A and S2). These foci remained in the cytoplasm until PER and TIM translocated to the nucleus (Figs. 1 and 2; Movies S1A and S2). Notably, we did not detect the formation of foci when either PER or TIM was expressed alone (15). In addition, when PER and TIM were coexpressed, the foci often disappeared...
Fig. 1. FRET and nuclear translocation of PER and TIM. (A) Time-lapse images of PER-CFP (top), TIM-YFP (middle), and FRET (bottom) from a single cell. Left to right, images taken at 20-min intervals. First (leftmost) image was taken 200 min after heat shock. (B) (Left) Whole-cell FRET (thin blue line) of cell shown in (A) over ~400-min interval (note break in time axis), relative to nuclear accumulation of PER-CFP (thick blue line). Orange line (with error bars), whole-cell FRET for six control cells expressing PER-CFP and YFP. (Right) Whole-cell FRET of five different cells (five colored thin lines), compared to nuclear accumulation of PER-CFP (thick lines respective colors). (C) Onset of nuclear accumulation in single cells for PER-CFP (blue dots) and TIM-YFP (red dots) as a function of their mean fluorescence levels. The onset is determined as the inflexion point of the nuclear accumulation profile.

Earlier in PER-CFP images than in TIM-YFP images (Fig. 2 red arrows). Thus, formation of these foci may be an important step in the temporal control of nuclear entry. The abrupt decrease in FRET upon nuclear translocation could reflect either dissociation or a change in conformation of the PER-CFP/TIM-YFP complex. To differentiate between these two possibilities, we independently measured the rates of nuclear accumulation for PER and TIM. If PER and TIM undergo a conformational change but remain physically associated as nuclear translocation occurs, individual rates of PER and TIM nuclear accumulation should be equal.

In a survey of 85 cells, we found that the onset of nuclear accumulation, determined as the inflexion point of the nuclear accumulation profile for PER-CFP, occurred in a narrow time frame, 340 ± 70 min after heat shock in our S2 cells (Fig. 1C). Consistent with our observation that PER and TIM associate rapidly and that these association kinetics have no influence on the onset of nuclear translocation in our S2 cell system, we found that the time of onset of nuclear accumulation in these experiments was not correlated with the level of PER-CFP (correlation coefficient $\tau^2 = 0.0523$) or TIM-YFP ($\tau^2 = 0.0038$) expressed in the cytoplasm (Fig. 1C). To determine whether the kinetics of the nuclear accumulations of PER-CFP and of TIM-YFP were similar, we next calculated the rate of each protein’s nuclear accumulation as the coefficient of a first-order linear regression. The latter was taken from the steepest slope of the profile of nuclear translocation, scaled to the mean fluorescence in each cell. We found that the rates of nuclear accumulation of PER-CFP and TIM-YFP were independent ($\tau^2 = 0.0476$) (Fig. 3A). Also, although the rate of accumulation of PER-CFP was positively correlated with the level of PER-CFP ($\tau^2 = 0.5366$), this rate was independent of the level of TIM-YFP produced in the same cell ($\tau^2 = 0.0004$) (Fig. 3B). Similarly, TIM-YFP accumulation rates were correlated with the TIM-YFP level ($\tau^2 = 0.4243$), but not with the PER-CFP level in the same cell ($\tau^2 = 0.0057$) (Fig. 3C). To control for a possible effect of the fluorescent protein tags on these results, we reversed the associations of CFP and YFP and followed the nuclear accumulation of PER-YFP and TIM-CFP. This study confirmed that the kinetics of accumulation were determined by PER and TIM and not by their tags (fig. S3A).

One issue that is not resolved by measuring these accumulation rates is whether the PER/TIM complex dissociates before or after traveling to the nucleus. As shown in Fig. 2, comparisons of PER and TIM nuclear translocations within individual cells reveal that onset of PER nuclear accumulation often precedes that of TIM, as recently reported in vivo (5). Earlier work has shown that, in the absence of PER, TIM shuttles between the nucleus and cytoplasm through the action of both nuclear localization and nuclear export signals (16). Possibly, TIM transports PER to the nucleus in a complex, after which the proteins separate, allowing TIM to return to the cytoplasm to transport more PER.

To determine whether this property of TIM contributes to the independent rates of PER and TIM nuclear translocation observed in our studies, we used leptomycin B to block TIM-YFP nuclear export (16). In the presence of this inhibitor of nuclear export, for cells expressing only TIM-YFP, the protein was constitutively localized to the nucleus in most cells (45 cells out of 50 surveyed). In contrast, in cells expressing only PER-CFP, PER remained in the cytoplasm (50 out of 50 cells) in the presence of the drug (17, 18). Intriguingly, addition of leptomycin B to cells coexpressing PER-CFP and TIM-YFP suppressed the rapid transfer of TIM-YFP to the nucleus. Instead, both proteins were sequestered in the cytoplasm for several hours before nuclear translocation (369 ± 58 min, 29 cells), as previously observed in the absence of drug (fig. S3B). Evidently, even in the presence of leptomycin B, TIM is retained by its interaction with PER. Addition of leptomycin B also failed to modify the divergent profiles of PER and TIM nuclear accumulation; rates of PER and TIM nuclear accumulation remained uncorrelated ($\tau^2 = 0.0372$) in a study of these cells (Fig. 3A). The latter finding indicates that although we have confirmed TIM shuttling between the nucleus and cytoplasm, this mechanism cannot explain the independent rates of PER-CFP and TIM-YFP nuclear accumulation that we have observed. Our measurements hence favor an alternative mechanism for nuclear translocation wherein most of the cytoplasmically derived complexes dissociate in the cytoplasm as the proteins translocate to the nucleus (19).

The per$^L$ mutation produces a delayed nuclear translocation phenotype in pacemaker cells of the Drosophila brain (20). This results in long-period behavioral rhythms of ~28 hours (21). per$^L$ involves a single amino acid substitution (22), and it also depresses the physical interaction of PER$^L$ and TIM when the proteins are coexpressed in yeast (23). The tim$^{UL}$ mutation is associated with a distinct single-amino acid substitution that delays PER and TIM nuclear turnover, resulting in a 33-hour behavioral rhythm (24). In contrast to
per\textsuperscript{L}, tim\textsuperscript{UL} has no effect on the timing of nuclear translocation in vivo (24).

The mean onset of PER-CFP nuclear accumulation in cells coexpressing PER-CFP and TIM\textsuperscript{UL}-YFP is 299 ± 33 min (20 cells), and it is also independent of PER-CFP and TIM\textsuperscript{UL}-YFP levels (Fig. 4, A and B). Furthermore, we found no persistent FRET when PER-CFP and TIM\textsuperscript{UL}-YFP moved to the nucleus: FRET decay was not delayed when compared to the onset of nuclear accumulation (Fig. 4C). We observed a loss of FRET with TIM\textsuperscript{UL} in parallel with nuclear translocation, which suggests that, as for wild-type TIM, TIM\textsuperscript{UL}/PER heterodimers dissociate as nuclear translocation proceeds in this mutant. Previous studies have shown that, in tim\textsuperscript{UL} mutants, PER is found in high molecular weight complexes late at night when it is presumably nuclear (24). We cannot rule out the possibility that, following translocation, PER and TIM form new associations that do not support FRET in the nucleus in both wild-type and TIM\textsuperscript{UL}-expressing cells.

S2 cells reproduced the delay in nuclear translocation onset when PER\textsuperscript{L} was expressed in place of PER. In PER\textsuperscript{L}-expressing cells, the mean onset of nuclear accumulation was at 492 ± 97 min after induction, as compared with 340 ± 70 min in PER-expressing cells (25 cells, Wilcoxon test \( P = 10^{-8} \)) (Fig. 4, A and D). The onset of PER and TIM nuclear accumulation remained independent of PER\textsuperscript{L}-CFP and TIM-YFP levels (Fig. 4A). The profiles of nuclear accumulation of these proteins also indicated significant independence in their rates of translocation (15). FRET decayed as PER\textsuperscript{L}-CFP and TIM-YFP were transferred to the nucleus (Fig. 4E), and as previously seen from PER/TIM combinations, maximum levels of FRET arose without a measurable delay in cells expressing PER\textsuperscript{L} (Fig. 4E). This result was not predicted by earlier models, which assumed that an altered rate of PER\textsuperscript{L} and TIM physical association chiefly determines the temporal delay found in nuclear accumulation. Because nuclear translocation instead followed a protracted interval of maximum FRET in PER\textsuperscript{L}-expressing cells, a step distinct from PER/TIM assembly appears to trigger nuclear translocation in S2 cells and is likely also responsible for delayed nuclear translocation in vivo.

Our studies indicate that cytoplasmically formed PER/TIM complexes are not translocated to the nucleus: FRET disappears in parallel with PER and TIM nuclear accumulation, suggesting a dissociation of the complex, and measurements of PER and TIM nuclear accumulation rates show that, for a given cell, these are different and independent for each protein. Because PER/TIM associations are not sufficient to initiate nuclear accumulation, these results point to a mechanism in which physical interaction precedes an activity that precisely times nuclear translocation of both proteins. In this respect, PER and TIM appear to act as constituents of an intracellular interval timer. A better understanding of this timer might be sought in the discrete cytoplasmic foci we have observed to routinely precede nuclear translocation (Figs. 1 and 2). These foci may reflect condensations of cytoplasmic PER/TIM complexes together with additional factors responsible for their posttranslational modifications. Such factors could include the kinases SGG, DBT, and CK2 or the phosphatase PP2A, each known to affect the phosphorylation of PER.
Fig. 3. Nuclear translocation rates. (A) Single-cell rates of TIM-YFP nuclear accumulation against rates of PER-CFP nuclear accumulation (black circles) and when leptomycin B was added (red circles). Rates were calculated as the linear regression for the maximum steepness of the nuclear accumulation profile and scaled through multiplying by the mean pixel fluorescence for the cell. Rates were calculated per image frame; one frame corresponds to 4 min. The dotted line represents the diagonal for visual support. (B) Single-cell rates of nuclear accumulation for PER-CFP (blue) and TIM-YFP (red) plotted against PER-CFP fluorescence levels for each cell. (C) Same rates as in (B) but plotted against TIM-YFP fluorescence levels. Colored dotted lines represent the linear regression for points of the same color.

Fig. 4. FRET and nuclear translocation in per¹ and tim²⁶. (A) Onset of nuclear accumulation of PER proteins in single cells coexpressing either PER¹ and TIM (blue dots) or PER and TIM²⁶ (red dots) as a function of their mean fluorescence level in the cell. Open blue and red circles show onset of PER nuclear accumulation in the corresponding experimental controls (cells expressing wild-type PER and TIM). (B) Profiles of nuclear accumulation for TIM²⁶-YFP (thin colored lines) and PER-CFP (thick colored lines). Each cell is represented by a different color. (C) Whole-cell FRET (thin lines) for the same cells as in (B) relative to PER-CFP nuclear accumulation (thick lines). (D) Profiles of nuclear accumulation for PER¹-CFP (thick colored lines) and TIM-YFP (thin colored lines). Each cell is represented by a different color. (E) Whole-cell FRET (thin lines) for the same cells as in (D) relative to PER¹-CFP nuclear accumulation (thick lines). Note the break in the time axis, to show early time points.

References and Notes
16. Although our results are consistent with in vivo responses to leptomycin B (16), they differ from another study showing PER in nuclei of S2 cells in the presence of this drug (6). In the latter study, PER was coexpressed with CLOCK (6), which is a transcriptional activator of tim in S2 cells (18). In the presence of TIM, nuclear rather than cytoplasmic localization of PER would be expected with or without leptomycin B.
18. Because only full-length nuclear PER and TIM fusion proteins are recognized in Western assays using antibodies to green fluorescent protein (GFP), loss of FRET cannot reflect proteolytic separation of CFP and/or YFP during nuclear translocation (15).
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Supporting Online Material
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Figs. S1 to S3
Movies S1 and S2
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