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Block in Nuclear Localization of *period* Protein by a Second Clock Mutation, *timeless*

Leslie B. Vosshall,* Jeffrey L. Price, Amita Sehgal,† Lino Saez, Michael W. Young‡

In wild-type *Drosophila*, the *period* protein (PER) is found in nuclei of the eyes and brain, and PER immunoreactivity oscillates with a circadian rhythm. The studies described here indicate that the nuclear localization of PER is blocked by *timeless (tim)*, a second chromosome mutation that, like *per* null mutations, abolishes circadian rhythms. PER fusion proteins without a conserved domain (PAS) and some flanking sequences are nuclear in *tim* mutants. This suggests that a segment of PER inhibits nuclear localization in *tim* mutants. The *tim* gene may have a role in establishing rhythms of PER abundance and nuclear localization in wild-type flies.

Mutations in the Drosophila period (per) gene disrupt circadian rhythms of pupal eclosion and adult locomotor behavior (1). Although per has been cloned and sequenced and its pattern of expression has been analyzed (2, 3), the biochemical function of the PER protein is unknown. PER shares some homology with a family of transcription factors (4-6) that possess a common sequence motif called the PAS domain. The PAS domain consists of two repeats of approximately 50 amino acids within a homology region of 258 to 308 amino acids (7).

Immunocytochemical experiments demonstrated that PER is a nuclear protein in a variety of Drosophila tissues (8, 9). In cells of the adult fly visual and nervous systems, the amount of PER protein fluctuates with a circadian rhythm (10), the protein is phosphorylated with a circadian rhythm (10), and PER is observed in nuclei at night but not late in the day (8). The expression of per RNA is also cyclic. However, peak mRNA amounts are present late in the day, and the smallest amounts are present late at night (11, 12). Three mutant alleles—per^o, per's, and per^L-cause arrhythmic behavior or shorten or lengthen periods, respectively (1). These mutations also produce corre-

†Present address: Department of Neuroscience, University of Pennsylvania School of Medicine, Philadelphia, PA 19104, USA.

‡To whom correspondence should be addressed.

sponding changes in the rhythms of per RNA and protein amounts (10-12) and PER immunoreactivity in nuclei (8). This suggests a possible role for molecular oscillations of per in the establishment of behavioral rhythms (11). Recently, a new mutation, timeless (tim), was isolated that produces arrhythmic behavior and suppresses the circadian oscillation of per RNA (12). Here, we examine the effect of tim on the expression and localization of PER protein.

We compared PER protein expression in wild-type, per^o mutant, and tim mutant flies by staining head sections with PER antibody. Because the amounts of PER protein staining in eye and brain nuclei fluctuate daily (8), sections were prepared at four time points. Nuclear staining in wild-type photoreceptor cells was most prominent at Zeitgeber times 2 and 20 (ZT2 and 20) (13), intermediate at ZT7, and absent at ZT13 (Fig. 1A). Because the per^o mutation introduces a stop codon in the PER reading frame (2, 3), PER antibody specificity was demonstrated by the absence of staining in sections from this null mutant at each time point (Fig. 1C). In tim mutants, nuclear staining was not seen at any time point (Fig. 1B).

PER- β -galactosidase (PER- β -gal) fusion proteins have been used extensively to study patterns of *per* locus expression (9, 14, 15). Such fusion proteins have also allowed the functional dissection of the gene and the encoded protein (9, 14, 15). To explore the effect of *tim* on PER protein expression and to map elements of the *per* locus responding to *tim*, we compared patterns of expression of a PER- β -gal fusion protein (PER-SG) (9) in transgenic flies with a wild-type or *tim* mutant genetic background. PER-SG contains

Howard Hughes Medical Institute, National Science Foundation Science and Technology Center for Biological Timing, and Laboratory of Genetics, The Rockefeller University, New York, NY 10021, USA.

^{*}Present address: Center for Neurobiology and Behavior and Howard Hughes Medical Institute, Columbia University College of Physicians and Surgeons, New York, NY 10032, USA.

the NH₂-terminal half of PER (amino acids 1 to 636) (2) fused to B-gal. The expression of PER-SG in transgenic flies in vivo (9) closely matches that of the endogenous protein (8). Whereas PER-SG localized to nuclei in wild-type flies, we found that nuclear localization was blocked in tim mutants. Although no nuclear staining was observed in tim mutants, PER-SG accumulated in the same tissues in both tim and wild-type flies (Fig. 2, A and D, and Fig. 3B). Specificity of the β -gal antibody used in these studies was indicated by the complete absence of staining in sections derived from wild-type or tim flies lacking the transgene that encodes PER-SG (16).

To determine whether a single mutation causes both aberrant intracellular localization of PER and the arrhythmic behavior of tim flies, we generated recombinants between the second chromosome bearing the PER-SG construct and the tim mutant chromosome. Head sections prepared from each of 36 homozygous tim^+ or tim recombinant lines (17 tim^+ and 19 tim) were stained with β -gal antibody. The PER-SG fusion protein was nuclear in all 17 tim⁺. PER-SG recombinant lines. Nuclear localization was not observed in any of the 19 tim, PER-SG recombinants (16, 17). Therefore, mutant behavioral and localization phenotypes are likely to be the result of the same mutation. Representative sections from three independent tim+, PER-SG recombinants (with nuclear labeling in the photoreceptor, cone, and pigment cells in the eye and in the lamina, medulla, and central brain) are shown in Fig. 2, A through C. Representative sections from tim, PER-SG recombinants show that nuclear localization of the PER-SG protein was not observed in any of these cells (Fig. 2, D through F).

We examined the intracellular localization of the PER-SG fusion protein in tim/ tim⁺ heterozygotes to learn whether the aberrant localization phenotype is a dominant or a recessive trait. PER-SG fusion proteins localize to nuclei in the heterozygotes (17). Therefore, like the behavioral defect (12), the nuclear localization defect of tim is recessive. The nuclear localization of PER-SG in tim+ flies also is not dependent on the presence of endogenous PER protein because its localization was nuclear in a per° genetic background (17). Thus, neither functional PER protein nor behavioral rhythmicity is required to generate correct nuclear localization of the PER-SG fusion protein in tim⁺ flies. As well, the tim mutant localization phenotype was independent of endogenous PER, because PER-SG fusion protein did not localize to nuclei in per^o; tim double mutants (17).

To search for possible differences in the rate of synthesis, stability, or structure of PER-SG proteins isolated from wild-type and *tim* mutant flies, we prepared protein immunoblots from tim^+ , PER-SG and *tim*, PER-SG head extracts. PER- β -gal fusion proteins of the same apparent size and abundance were detected in the two genetic backgrounds (Fig. 2G). Thus, effects on nuclear localization observed immunocytochemically in *tim* flies are not the result of reduced expression or increased proteolysis of the fusion protein.

To explore the specificity of the block in nuclear localization in *tim* mutants, we examined the expression in *tim* mutants of two β -gal marker proteins (containing no PER sequences) that had been modified by the addition of heterologous nuclear localization signals and are expressed in the nuclei of the eye and brain. The addition of such nuclear localization signals is required for nuclear localization of β -gal in wild-type flies (16–18). Nuclear localization of these marker proteins was unaffected by *tim* (Fig. 3, D and E). The *tim* mutation affected neither the morphology nor the number of nuclei visualized by these markers.

We also examined the localization of four additional PER- β -gal fusion proteins in *tim* mutants and wild-type flies. Fusion proteins contained the first 23 amino acids of PER (PER1-23 β -gal), PER amino acids 1 to 95 and 530 to 636 (PER-SG Δ 96–529), the first 95 amino acids of PER (PER1–95 β gal), or the NH₂-terminal half of PER without a putative nuclear localization signal (PER1–636 Δ NLS[66–79] β -gal) (2).

The pattern of PER1–23 β -gal expression in wild-type and *tim* genetic backgrounds was identical to that of PER-SG in *tim* mutants (16, 17). Because no sequences resembling a nuclear localization signal are found in this segment of the PER protein, cytoplasmic accumulation of this fusion protein was predicted for both genetic backgrounds (16-18). PER-SGΔ96-529 was nuclear in wild-type and tim flies (17). The localization of PER1-95β-gal also was nuclear in wild-type (Fig. 3A, left) and tim backgrounds (Fig. 3A, right), which indicates that sequences in this NH₂-terminal region of PER would promote nuclear localization of a full-length PER protein but are unable to do so in tim mutant flies unless another, more centrally located region of PER is deleted. PER1-636ANLS[66-79]Bgal localized to nuclei in wild-type tissue (Fig. 3C, left), which indicates that PER contains additional nuclear localization signals. PER1-636 Δ NLS[66-79] β -gal failed to enter nuclei in a tim background (Fig. 3C, right). Together, these results suggest that wild-type tim activity is required for nuclear localization of proteins that contain a specific region of PER. Because both PER1-95B-gal and PER-SG Δ 96–529 are nuclear in tim flies, the tim-sensitive region of PER must be located between amino acids 96 and 529. This interval includes the site of the per^L mutation and the PAS domain (2, 3), but would not include the per' domain, a region in which mutations predominantly confer short-period phenotypes (19).

We have drawn four principal conclusions from this work. (i) The *tim* gene is required for nuclear localization of PER. (ii) Aberrant behavioral and localization phenotypes appear to be a result of the same mutation. (iii) Regulation of nuclear localization by *tim* may be specific for PER. (iv) PER contains sequences that somehow inhibit PER nuclear localization in the absence of *tim*. A protein encoded by *tim* might interact with PER to facilitate nuclear entry, or *tim* may regulate factors responsible for cytoplasmic retention of PER pro-



Fig. 1. Expression of PER protein in (**A**) wild-type, (**B**) *tim*, and (**C**) *per*^o mutant backgrounds at four time points. The nuclear layer is marked with open arrows. Structures underlying the lens frequently stain nonspecifically and do not reflect PER antibody staining in nuclei (black arrowheads). Flies were raised at 25°C in a 12-hour light:12-hour dark cycle (LD 12:12). Frozen sections (14 μ m) from adult fly heads collected at the indicated times were incubated with PER antibody (1:30 dilution of immune serum with PER antibody 19-06 raised against full-length PER protein and preabsorbed against *per*^o head lysate) for 16 hours at room temperature. Antibody staining was continued as described (ϑ) with the use of the Vectastain ABC kit (Vector Labs).

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tein. The arrhythmic behavior and lack of per RNA rhythms observed in *tim* mutants (12) are likely to be a direct consequence of the failure of PER to enter the nucleus. Thus, PER must be present in the nucleus at some time of day for expression of circadian rhythms. Our detection of large amounts of cytoplasmic PER- β -gal fusion protein, but not full-length cytoplasmic PER protein, in *tim* mutants may also indicate that PER is unstable in the cytoplasm and stabilized by transport to the nucleus. Thus, the observed cycling of PER protein abundance (10) might reflect

cycling of PER nuclear localization.

The region implicated in *tim* control of nuclear localization of PER includes the PAS domain, nonconserved sequences upstream of PAS (amino acids 96 to 232) (20), and 33 amino acids downstream of PAS (amino acids 497 to 529) (4, 14). Regulation of nuclear localization has been observed for two other proteins containing the PAS domain. The dioxin receptor is a ligand-activated transcription factor (21) that accumulates in the cytoplasm until dioxin ligand interacts with the PAS domain of the AHR subunit (6). This induces the formation of a complex with the ARNT subunit and promotes nuclear localization of the receptor complex (22). In the absence of either ligand or ARNT, the receptor fails to enter nuclei and has no transcriptional activity (5, 23). No ligands or binding partners



Fig. 2. Immunocytochemistry and protein immunoblot analysis of a PER-β-gal fusion protein (PER-SG) (9) in tim+ and tim recombinant lines. Shown is expression of PER-SG in head sections from tim+ (A through C) and tim mutant (D through F) recombinant lines. For (A) through (C), staining of nuclei was observed in photoreceptor cells (ret), lamina (lam), medulla (med), and some cells of the central brain. Open arrows indicate nuclear labeling. In (D) through (F), staining of photoreceptor cells and cells of the lamina, medulla, and central brain was also observed, but there is no indication of nuclear localization of the stain. Frozen sections were incubated with affinity-purified β-gal polyclonal antibody (Cappel/Organon Technika, Durham, North Carolina) (1:1000 dilution preabsorbed against adult fly acetone powder) and processed as described (Fig. 1). (G) Lines of tim+ and tim mutant flies containing PER-SG or a control line (Canton-S) lacking the trans-



gene, were raised as described (Fig. 1). Flies (6 to 12 days old) were collected from ZT18 to ZT22. Extracts were prepared from the heads of flies as described (15). Extract containing 1× (10 μ g by Bradford assay), or the indicated fraction of total protein, was electrophoresed in each lane of an SDS-polyacrylamide gel (24) and analyzed for β -gal antigen levels as described by Saez and Young (8). The primary antibody was a mouse β -gal monoclonal antibody, and the secondary antibody was goat antibody to mouse immunoglobulin G (heavy and light chains) alkaline phosphatase conjugate (both from Promega). Extracts from *tim*, PER-SG flies were loaded in the first four lanes; *tim*⁺, PER-SG extract in the fifth lane; and extract from a line that does not contain the PER-SG transgene (Canton-S) in the sixth lane. Molecular size markers (Amersham) are indicated to the left of the gel.



Fig. 3. Localization of PER- β -gal and β -gal marker proteins in wild-type (left column) and tim mutant (right column) adult head sections. (A) PER1-95β-gal. (B) PER1-636β-gal (identical in amino acid composition to PER-SG, but constructed as described below). (C) PER1-636ANLS[66-79]B-gal. (D) Enhancer trap line S45 (expresses β -gal modified by the addition of a nuclear localization signal in the eye and a few cells in the brain). (E) Eye protein kinase SV40 nuclear localization signal under the control of the eye-specific protein kinase C promoter) (18). Staining with antibodies to β-gal was as described in legend to Fig. 2. PER-β-gal fusion constructs were made by inserting an Xho I linker at nucleotide 3183 (PER1-956-gal; Acc I site) or at nucleotide 4936 (PER1-6366gal and PER1-636ANLS[66-79]β-gal; Sac I site) (2). PER1-636ΔNLS[66-79]β-gal is identical to PER1-636β-gal except that a fragment encoding amino acids 65 to 95 has been deleted in the former (2, 16). The Sph I-Xba I per promoter fragment was added and the per fragment excised with Xho I. All constructs were cloned into pCaSpeR-β-gal (25), altered to create three different frames with Xho I linkers (16).

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analogous to ARNT have been identified for PER. However, it has recently been shown that the PAS domain of the PER protein can function as a protein interaction domain (14). Further mapping of the *tim*-sensitive region of PER should determine whether PAS is centrally involved in the control of PER nuclear localization.

Peak amounts of per RNA expression (11, 12) precede the greatest amount of PER protein staining in nuclei of the photoreceptors and brain (8) by about 8 hours. Thus, nuclear immunoreactivity of PER occurs when the amount of per RNA is small. As suggested above, the entry of PER protein into the nucleus may be under temporal control, so that some of the previously observed oscillation in PER protein amounts and nuclear staining (8, 10) may reflect rhythmic movement between the cytoplasm and the nucleus, with a phase that is distinct from that of per RNA synthesis. In light of these observations and our work with tim, we speculate that formation of an intracellular circadian clock may require nuclear localization of PER to be limited to a particular time of day.

In such a model, constitutively cytoplasmic (as in tim mutants) or constitutively nuclear PER would fail to generate circadian rhythms, and *tim*⁺ activity might play a role in temporal regulation of the access of PER to the nucleus. It was proposed that PER may directly or indirectly regulate its own transcription, because the cycling of per transcription is blocked in per^o mutants (11). Because the tim mutation also abolishes these RNA rhythms (12), a form of feedback regulation may exist in which cycling nuclear localization of PER might produce rhythmic signals influencing per transcription. We wish to emphasize that tim was recovered in a screen for clock mutations that was not biased to afford recovery of new mutations interacting with per (12). Thus, the discovery of a functional interaction between tim and per indicates that a single intracellular mechanism is probably central to the generation of circadian rhythms in Drosophila. The effects of tim on per RNA oscillation and PER nuclear localization would presumably be components of this mechanism.

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Activation of Phosphatidylinositol-3' Kinase by Src-Family Kinase SH3 Binding to the p85 Subunit

Christopher M. Pleiman, W. Marc Hertz, John C. Cambier*

Engagement of antigen receptor complexes induces rapid activation of Src-family kinases and association with phosphatidylinositol-3' kinase (PI-3 kinase). Here it was found that the Src homology 3 (SH3) domain of Lyn and Fyn bound to a proline-rich region (residues 84 to 99) within the 85-kilodalton subunit (p85) of PI-3 kinase. The binding of SH3 to the purified kinase led to a five- to sevenfold increase in the specific activity of PI-3 kinase. Ligand-induced receptor stimulation activated PI-3 kinase, and this activation was blocked by a peptide containing residues 84 to 99 of p85. These data demonstrate a mechanism for PI-3 kinase activation and show that binding of SH3 domains to proline-rich target sequences can regulate enzymatic activity.

The response of lymphocytes to antigen is mediated through a multisubunit cell surface receptor complex. Antigen receptor engagement can initiate multiple intracellular signaling events that lead to activation, differentiation, and tolerance induction, depending on the nature of the stimulus and differentiative stage of the cell (1). The binding of ligand to B cell antigen

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receptors causes activation of the receptorassociated Src-family tyrosine kinases $p55^{blk}$ (Blk), $p59^{fyn}$ (Fyn), and $p53/56^{fyn}$ (Lyn) (2). Lyn associates with the noncatalytic 85-kD subunit of PI-3 kinase in B cells after antigen receptor ligation (3).

The PI-3 kinase is a heterodimeric protein composed of a noncatalytic p85 subunit (4), catalytic 110-kD subunits (p110) (5), and phosphorylated inositol lipids on the D-3 hydroxyl position (6). The exact roles of the phosphoinositide products generated by PI-3 kinase in signaling pathways have not been determined, but they accumulate in cells activated by growth factors (7). PI-3 kinase interacts with SV40 middle

Division of Basic Sciences, Department of Pediatrics, National Jewish Center for Immunology and Respiratory Medicine, 1400 Jackson Street, Denver, CO 80206, USA, and Department of Microbiology and Immunology, University of Colorado Health Science Center, Denver, CO 80206, USA.

^{*}To whom correspondence should be addressed.