Biochemical and Biophysical Research Communications **279,** 131–138 (2000)
doi:10.1006/bbrc.2000.3927, available online at http://www.idealibrary.com on IDEAL®

Molecular Characterization and Nuclear Localization of Rat *timeless*-like Gene Product

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Received October 20, 2000

Among three *period* genes (*per*1, *per*2, *per*3) in mammals, only *per*2 gene was shown to be involved in the core clock mechanism. To elucidate the molecular function of rat PERIOD2 (rPER2), we searched for binding proteins to the PAS domain of rPER2. We isolated a binding protein to this domain and identified it as a TIMELESS-like protein (TLP) on the basis of mass analyses. Then, we isolated a rat TLP cDNA from the rat hypothalamus library. RNA blot analysis and *in situ* hybridization indicates that rTLP mRNA was expressed in all rat tissues from whole brain, the suprachiasmatic nucleus, eye, lung, heart, liver, kidney, placenta, and testis. When rTLP gene product was expressed in COS-1 cells, nuclear localization of rTLP was detected in 99.6% of transfected cells. These results suggest that the interaction of rPER2 with rTLP may influence the regulation of circadian clock components in nucleus after rPER2 is translocated into the nucleus.

Key Words: **circadian rhythm; rat; PERIOD 2; TIMELESS-like protein; nuclear localization; suprachiasmatic nucleus; day/night cycle; RNA blot; Far-Western blot; peptide mapping.**

Circadian rhythms are driven by endogenous biological clocks that regulate many biochemical, physiological and behavioral process in a wide range of life forms (1). In fruit flies, a central clock mechanism involves the dynamic regulation of two clock genes, *period* (*Per*) and *timeless* (*tim*), which participate in an intracellular transcriptional/translational feedback loop (2). The transcription of *per* and *tim* is positively regulated by two basic helix-loop-helix (bHLH PAS proteins, *jrk* (dCLOCK) and *cycle* (dBMAL1), which heterodimerize and bind to E box enhancers of *per* and *tim* genes (3–6). Translated PER and TIM form a heterodimer, which translocate into a nucleus. In the nucleus, the complex functions as a negative regulator of the dCLOCK-BMAL1 dependent E box transactivation. This negative regulation may involve PER and/or TIM binding either dCLOCK or dBMAL1 so that the latter proteins are unable to form functional complexes for transcriptional activation (5). In mammals, the suprachiasmatic nucleus(SCN) in hypothalamus is considered to be a major pacemaker for circadian rhythm phenomena, as demonstrated by many anatomical and physiological studies (7). Recently, three homologues of *Drosophila period* gene and one homologue candidate of *Drosophila timeless* gene were reported in mouse and human (8). Among three *period* genes (*per*1, *per*2, *per*3) in mammals, only m*per*2 gene have been shown to be involved in the core clock mechanism because of the loss of circadian behavior in constant dark(DD) for m*per*2^{brdm1} mutants (9). Unlike Timeless in *Drosophila*, mCry1 and mCry2 instead of mTIM did translocate mPER proteins from cytoplasm to nucleus (10). Furthermore, mTIM mRNA levels did not oscillate in the SCN and mPER-mTIM interactions was not observed using yeast two-hybrid assay (11). mTIM protein is constitutively expressed *in vivo* in the nucleus of the SCN neurons (12). In contrast, it is suggested that mTIM and hTIM have a similar role for dTIM in the circadian gene regulation because mTIM protein can suppress Clock:BMAL1-mediated *trans*-activation *in*

Abbreviations used: PER, PERIOD; TIM, TIMELESS; PPBP, PAS domain of PER binding protein; SCN, suprachiasmatic nucleus; ZT, zeitgebere.

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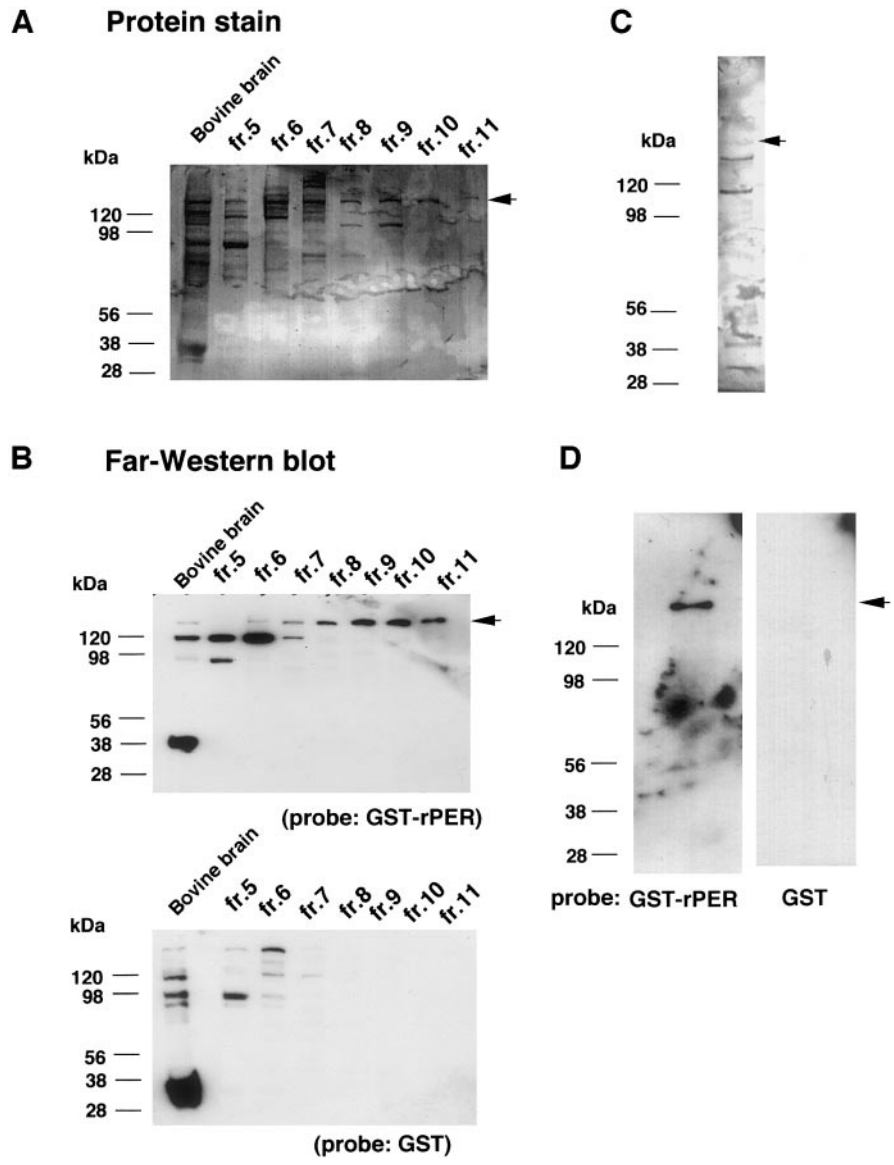

FIG. 1. Purification of a rPER2 PAS domain binding protein from bovine nuclear extracts. Bovine brain nuclear proteins were fractionated by Mono Q chromatography and an aliquot (10 μl) of each fraction was subjected to SDS–PAGE. The gel was stained with Coomassie brilliant blue (CBB) (A). A duplicate gel was transblotted onto a PVDF membrane and Far-Western blot analysis was performed with either POD-labeled GST-rPER 2 or GST as a probe. (B) Nuclear proteins in fraction 9 were separated on SDS polyacrylamide gel and stained with CBB (C). A duplicate gel was blotted onto a membrane and analyzed with either POD-labeled GST-rPER 2 or GST by Far-Western blot analysis (D). An arrow indicates the PPBP protein.

vitro (13, 14). These data did not exclude the possibility that hTIM and mTIM are not a real ortholog of dTIM.

We have cloned a rat *Period*2 (r*per*2) and demonstrated that rPER2 behaves as a mammalian homologue of the *Drosophila* PER (dPER) as mentioned below (15, 16). To clarify whether rPER2 is involved in the circadian rhythm of locomotor behavior of rats, we have made arrhythmic SCN-lesioned rats to monitor circadian rhythms in peripheral tissues and showed that the rhythmic nature of the multiple tissue expression of rPER2 was completely abolished by the SCN lesion. The multiple tissue expression of rPER2 is therefore under the control of the SCN (15, 16). rPER2 seems to be involved in the circadian rhythm of locomotor behavior in mammals, because the loss of circadian expression of rPER2 mRNA in the whole body occurred simultaneously with a loss of the circadian locomotor activity in rats. Although these data suggested that the rPER2 is involved in the core clock mechanism of the circadian rhythm in mammals, its molecular behavior has not been reported yet in rat system. We previously reported dPER/PAS-binding proteins in rat brain SCN nuclear extracts (17). In this paper, we focused the PAS domain of rPER2 to assess

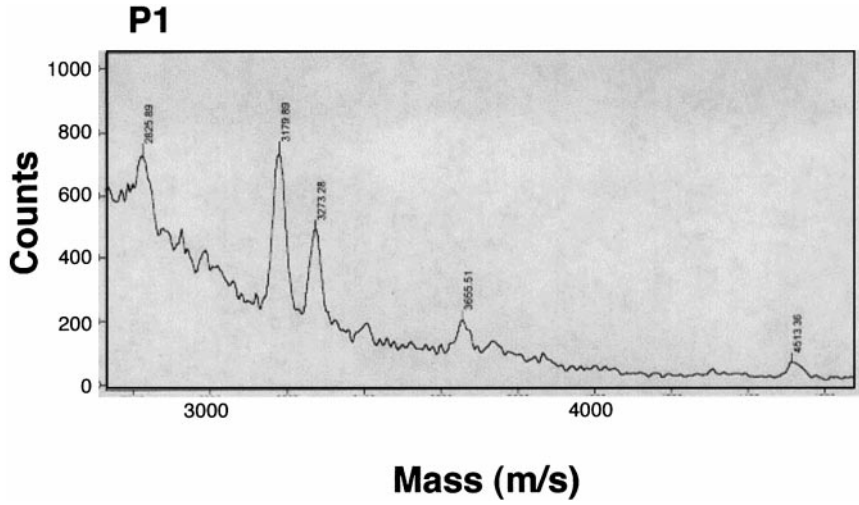

FIG. 2. Characterization of a rPER2 PAS domain binding protein. The PPBP protein on the membrane was digested with endopeptidase Lys-C and a mass of the digested fragments was analyzed by Voyager Elite MALDI-TOF mass spectrometer (PerSeptive Biosystems) as described under Materials and Methods.

the molecular nature of rPER2 and found a binding protein to this domain. We also isolated the binding protein and identified it as TIMELESS-like protein (TLP). Then, we cloned a rat TLP cDNA and examined the expression in multiple tissues of rat. The overexpression of TLP gene was carried out for the determination of cellular localization.

MATERIALS AND METHODS

Preparation of nuclear extracts and isolation of rPER 2 binding proteins. Nuclear extracts were prepared according to the procedure of Dignam *et al.* (18) with some modifications. Bovine brain (100 g) was homogenized with 3 volumes of homogenization buffer (2.5 M sucrose, 11.1 mM $MgCl_2$, 11.1 mM Tris–HCl, pH 7.5) by a Polytron homogenizer for 15 min on ice. A homogenate was centrifuged at 22,500 rpm for 1 h to precipitate nuclei. The crude nuclear pellet was washed with buffer A (10 mM Hepes, pH 7.4, 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM DTT, 1 mM PMSF) twice, then suspended in 4 volume of buffer B (20 mM Hepes, pH 7.9, 25% glycerol, 1 M NaCl, 1 mM EDTA, 1 mM EGTA, 0.5 mM DTT, 1 mM PMSF) and rotated on a tube rotator at 4°C for 1 h. Chromatins were sedimented by centrifugation at 10,000 rpm for 60 min. The supernatant solution was stored at −70°C and used as a brain nuclear extract. Protein concentrations were determined by the Bradford method (19).

The nuclear extract was diluted 10 times with 50 mM potassium phosphate buffer, pH 7.5, and loaded onto the Mono-Q column (SMART system, Pharmacia Biotech), equilibrated by 50 mM potassium phosphate buffer, pH 7.5 containing 0.1 M KCl and washed with same buffer. Proteins were eluted using a KCl gradient (0.1–1 M) in the same buffer. Each fraction was examined by Far-Western blot analysis with horseradish peroxidase (POD)-labeled GST-rPER 2 as a probe.

Construction of GST-rPER2. A rat *per* 2 cDNA (1073–2108 nucleotides) was amplified by PCR using rat *per* 2 cDNA (RG1528) and a pair of primers (upper, ctagaattcctcccgagaa; lower, cggaattcgctgcactggctggtgag). The PCR product was digested with *Eco*RI and inserted into *Eco*RI site of pGEX-2TK vector (Pharmacia Biotech). The resultant pGST-rPER 2 expression vector was transformed into *E. coli* BL21. The fusion protein was purified on a glutathione-Sepharose column from the extract of IPTG-induced transformant.

Far-Western blot analysis. Nuclear proteins were separated on 10% SDS–PAGE and then transferred onto a PVDF membrane. After soaking in 5% skim milk-TBBN (20 mM Tris–HCl, pH 7.5, 50 mM NaCl, 10 mM $MgCl_2$, 0.1% NP-40) for 30 min at 25°C, the membrane was incubated overnight with POD-labeled GST-rPER 2 in 0.1% skim milk-TBBN at 4°C. Finally the band was visualized by a chemiluminescence detection system (ECL) according to the manufacturer's instructions (Amersham).

Mass analysis. Partially purified nuclear proteins in fraction 9 was subjected to SDS–PAGE and blotted onto a PVDF membrane. After staining with Coomassie brilliant blue (CBB), the membrane corresponding to the band P1 was cut and disulfide bond of the protein was carboxymethylated with monoiodoacetate and digested by Endopeptidase Lys-C (Boehringer-Mannheim) in digestion buffer (1 M Tris–HCl, pH 9.0) at 37°C for overnight. The digested sample was analyzed by Voyager Elite MALDI-TOF mass spectrometer (PerSeptive Biosystems).

Cloning of rTLP cDNA An EST clone (01B00053KN06) from mouse early blastocyst cDNA library was kindly provided by Konno H (The Institute of Physical and Chemical Research). The EcoRI fragment (1.9 kb) of the clone was labeled with [α-^{32}P]dCTP and used as a probe to screen the cDNA library (pAP3neo vector) constructed from rat hypothalamus mRNA (TAKARA). Hybridization and washing were performed under high stringent conditions. Positive clones (pAP3neo-rTLP) containing the longest insert size (4.1 kb) were subcloned into pBluescript vector and sequenced using Big Dye terminator cycle sequencing Kit (PE Biosystems).

GST-rPER2 pull-down assay. In vitro transcription and translation was performed with pAP3neo-rTLP as a template by using TNT coupled Reticulocyte Lysate System (Promega) and ^{35}S-labeled Met (ICN) according to the manufacturer's instructions. The ^{35}S-labeled translated product (10 μl) was mixed with 100 μl of a binding buffer (20 mM Tris–HCl, pH 7.5, 50 mM NaCl, 10 mM $MgCl_2$, 0.1% NP-40, 1.5 mM PMSF, 1 mM DTT) and 15 μl of 50% glutathione-Sepharose 4B slurry (Pharmacia Biotech). The mixture was rotated at 4°C for 30 min to remove nonspecific interaction with the affinity beads. After removing the beads, the supernatant was mixed with 300 μl of the binding buffer and 15 μg of either GST-rPER 2 or GST. One hour

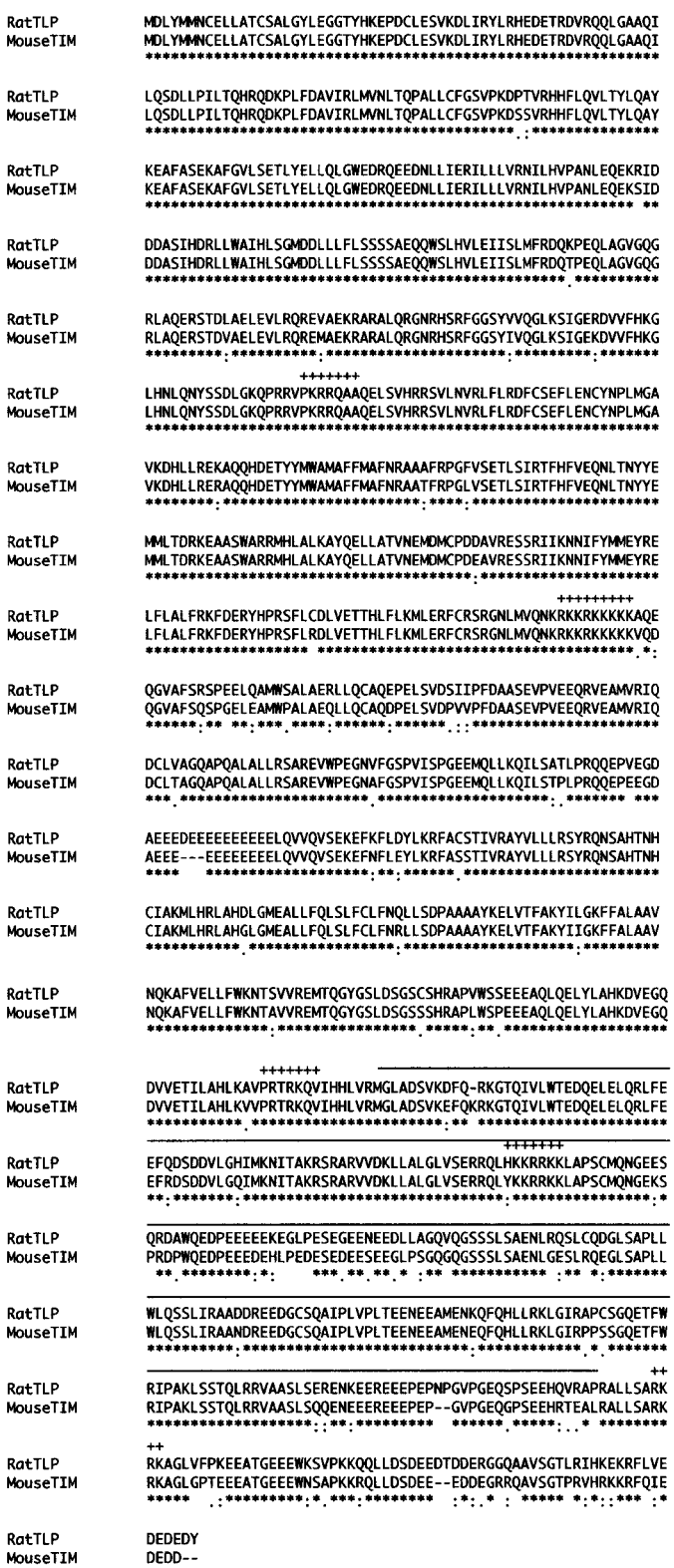

FIG. 3. Comparison of the predicted amino acid sequence of the rat TLP sequence and mouse TIM. * and : correspond to the same amino acids and conserved substitutions, respectively. The overlined region denotes the sequence conserved between *Drosophila* and rat. The putative nuclear localization signal was shown by +. The amino acid sequence of mouse TIM is quoted from GenBank Accession No. AB015598.

after rotation at 4°C, 15 μl of 50% glutathione-Sepharose slurry was added to the mixture and further rotated at room temperature for 1 h. The beads was spun down and washed 5 times with the binding buffer. Proteins associated with GST-rPER 2 was solubilized with SDS-sample buffer and subjected to SDS–PAGE. Fluorography of the gel was performed by the method of Chamberline (20).

In situ hybridization. Animals used for *in situ* analysis were anesthetized with barbiturate and were perfused from the left ventricle with 4% paraformaldehyde in phosphate-buffered saline (pH 7.4). Rat were killed as described above and tissues were fixed with 4% paraformaldehyde in phosphate buffered saline (pH 7.4) for 1 h at room temperature. The care of all rats used in this study was in accordance with institutional guideline. Then tissues were embedded in Tissue-Tek OCT compound (Miles). Eight-micrometer cryosections were cut. Hybridization was done as described (21, 22).

RNA blot analysis. Male Wistar rats (12 weeks old) obtained from Clea Japan, Inc. (Tokyo) were housed in a 12 h light–12 h dark cycle]LD 12:12; lights on at zeitgebere (ZT) 0] for at least 1 week before the day of the experiment. For placenta RNA, female rats aged 8 weeks were impregnated and decapitated at day 20. A white fluorescent lamp was used as a source of light during the day (150–200 lux at the level of the cages). Rats were decapitated at ZT 17, and other tissues were dissected, quickly frozen, and kept in liquid nitrogen until used. In darkness, dissections were carried out under a dim red light. Poly(A)$^+$ RNA was isolated from tissues by using QuickPrep Micro mRNA Purification Kit (Amersham Pharmacia Biotech Inc.) and separated on a 1% agarose/0.7 M formaldehyde gel as described. The poly(A)$^+$ RNA was transferred to a nylon membrane (GeneScreen Plus; DuPont, USA) by passive capillary transfer. Each lane contained 5 μg of poly(A)$^+$ RNA from each tissue. A ^{32}P-labeled random primed probe was generated from a rTLP cDNA fragment (bases: 3190–3749; GenBank Accession No. AB019576). Then hybridization was performed with commercially available hybridization buffer, PerfectHyb (Toyobo Co., Ltd., Osaka, Japan) according to the instruction manual, and detection was performed as described (15).

Transfection and immunocytochemistry. COS-1 cells were grown in Dulbecco's modified Eagle medium (Sigma) supplemented with 10% fetal bovine serum (Filtron), 50 U/ml penicillin, and 0.05 μg/ml streptomycin at 37°C with 5% CO. Cells were cultured on glass coverslips in 12 well dishes and transfected the following day. The coding region of rTLP was ligated into the pcDNA3 (Invitrogen) containing C-Terminal HA tag. For transfections, Superfect (QIAGEN) was used according to the manufacturer's protocol. Amounts of the rTLP constructs transfected was adjusted to 1.5 μg per well. Forty-eight hours after transfection, cells were rinsed with phosphate buffered saline (PBS) and fixed with 4% paraformaldehyde for 30 min. Cells were permeabilized and the nucleus was stained with PBS containing 0.05% Triton X-100 and 300 nM 4′,6-diamidino-2-phenylindole, dihydrochloride (DAPI). After blocking with 5% BSA in PBS, cells were incubated with anti-HA IgG (1:400; Santa Cruz Biotechnology) for 1 h. Cells were rinsed with PBS containing 0.05% Tween 20 and then applied with secondary antibody conjugated with FITC (ICN Pharmaceuticals, Inc.) for 1 h. Cells were washed and then mounted for fluorescence microscopy.

RESULTS

Purification and characterization of a rPER2 PAS domain binding protein (PPBP). Three homologues of d*per* gene were found in mouse and human and we

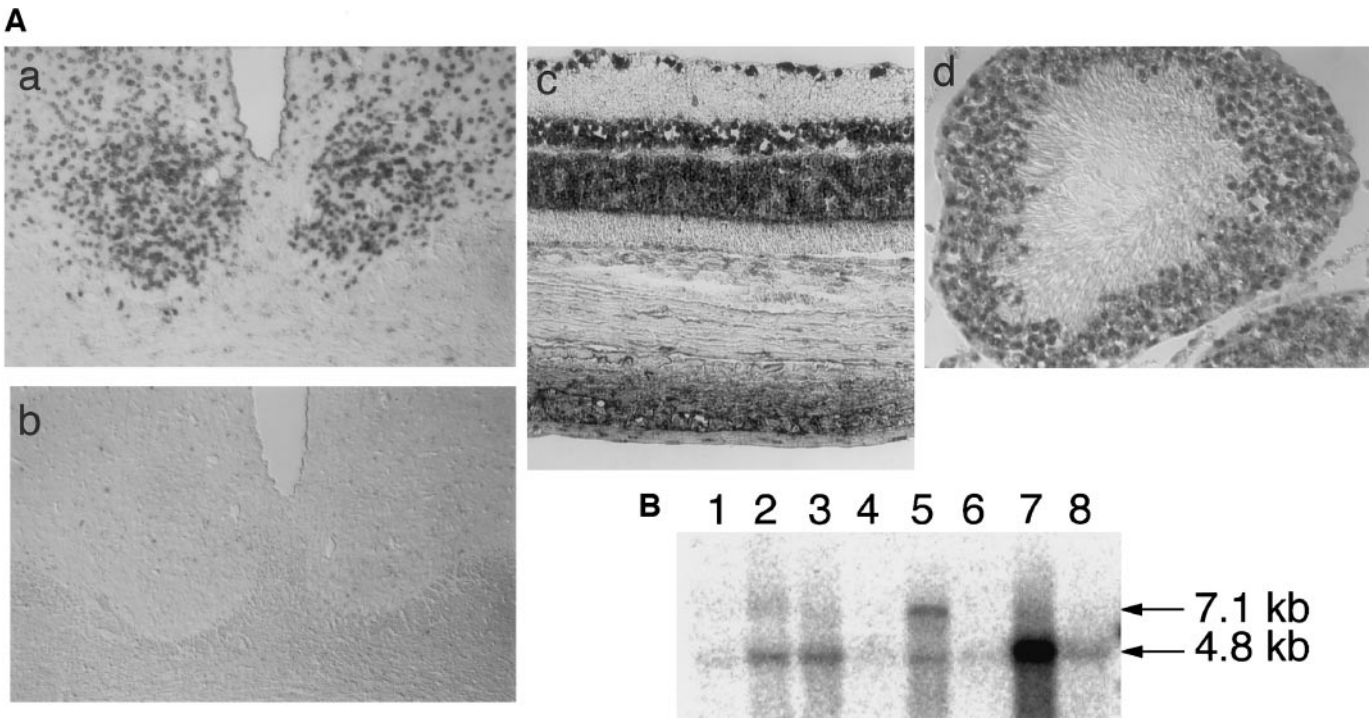

FIG. 4. Tissue distribution of rTLP mRNA in rats. (A) Spatial distribution of rTLP mRNA in rat tissues by *in situ* hybridization. The blue signal shows the strand-specific expression of rTLP mRNA in the suprachiasmatic nucleus (SCN) of rat hypothalamus at ZT14 using antisense cRNA probe (a) and sense probe (b). (c) Rat retina at ZT2 using antisense cRNA probe. The strand-specific signal of rTLP mRNA was detected in ganglion cell layer, inner nuclear layer, outer nuclear layer and layer of photosensitive cells. (d) Rat testis at ZT14 using antisense cRNA probe. (B) Northern blot analysis of rat multitissue RNA probed with the rTLP fragment. Rats were decapitated at ZT17. Each lane contains 5 mg of poly(A)$^+$ RNA. Lane 1, whole brain; lane 2, eye; lane 3, lung; lane 4, heart; lane 5, liver; lane 6, kidney; lane 7, placenta; lane 8, testis. 7.1 kb mRNA were detected as shown in eye and liver.

cloned a rat homologue of d*per* gene, which had the highest homology to mPER2. We compared the secondary structures of these proteins and found that rPER2 had a similar structure of *Drosophila* PERIOD (dPER). Since the PAS domain (230–390) of dPER was responsible for binding to dTIM, we constructed a GST-fusion protein (GST-rPER2), which contained the corresponding part (317–667) of rPER2. We searched a binding protein to the PAS domain of rPER2 in bovine brain nuclear extracts. Far-Western blot analysis probed with POD-labeled GST-rPER2 indicates that a few proteins interacted with the PAS domain of rPER2 in brain nuclear extracts. First, the nuclear extracts were loaded onto a Mono-Q column and proteins were eluted by a linear KCl gradient. Figure 1A shows the protein-stained pattern of the fractions eluted from the Mono-Q column. The majority of PPBP was found in fractions 9–11 using Far-Western blot analysis probed with POD-labeled GST-rPER2 or GST alone (Fig. 1B). Since fraction 9 mostly contained the PPBP, the fraction was subjected to SDS–PAGE, blotted onto a PVDF membrane (Figs. 1C and 1D). The membrane corresponding to PPBP was cut and digested by Endopeptidase Lys-C. The molecular masses of digested fragments were analyzed by MALDI-TOF mass spectroscopy (Fig. 2). The molecular masses of the fragments were 2825, 3173, 3273, 3655, and 4513 Da. Data base search of ProFound (http://prowl.rockefeller.edu/cgi-bin/ProFound) on the bases of these data and the molecular weight of PPBP indicate that the most suitable candidate of PPBP was human TIMELESS (hTIM).

Cloning of rTLP cDNA. To identify cDNA of rat TIM-like protein, we searched the expressed sequence tag database (dbEST) and detect a mouse EST (C88957) and rat EST (01B00053KN06) (23) similar to the amino-terminal portion of dTIM. Using the rat EST clone as a probe, we obtained a full-length cDNA clone for rat TIM-like protein (rTLP) from a rat hypothalamus cDNA library. The rTLP cDNA encodes a protein of 1205 amino acids having 89.3% amino-acid identity to the mouse TIM (mTIM) (Fig. 3) (11). The amino-terminal half corresponding to C1 and C2 regions of mTIM is most conserved between rTLP and mTIM. Analysis using a PSORT II program (http://psort.nibb.ac.jp/form2.html) suggests that rTLP2 could be localized in nucleus with 35% probability based on the presence of nuclear localization signals at amino acid (aa) 319–325 (PKRRQAA), aa 529–537 (RKKRKKKKK), aa 854–860 (PRTRKQV), aa 941–947 (HKKRRKK)

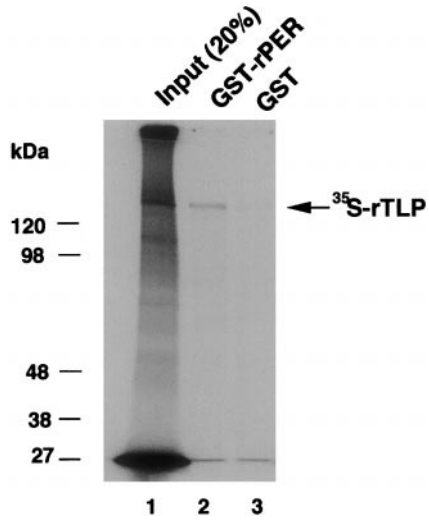

FIG. 5. *In vitro* association of the PAS domain of rPER2 with rTLP. The *in vitro* translated product of rat TIM was incubated either with GST-rPER 2 or GST and then glutathione-Sepharose 4B. A sediment was subjected to SDS–PAGE and visualized by fluorography. Procedures are described under Materials and Methods.

and aa 1138–1141 (RKRK), all of which are highly conserved between rTLP and mTIM.

Tissue distribution of rTLP mRNA. To show the spatial and temporal expression pattern of rTLP mRNA in the brain, we carried out *in situ* hybridization histochemistry. We first examined rTLP mRNA expression in a coronal section of rat brain by *in situ* hybridization at ZT115 (Fig. 4A, a). The expression of rTLP mRNA was detected throughout the SCN, but the level of rTLP mRNA expression was not changed significantly during day and night (data not shown). A strand-specific signal of rTLP mRNA was detected in rat retina (Fig 4A, c) and testis (Fig 4A, d).

RNA blot analysis indicates that a predominant band at 4.8 kb was detected in all rat tissues from whole brain, eye, lung, heart, liver, kidney, placenta, and testis (Fig 4B). High level of rTLP mRNA expression was observed in eye, lung, liver, placenta and kidney. Especially in kidney, 7.1 kb mRNA is evident, but we do not know the biological significance of the different size of mRNA.

In vitro association of the PAS domain of rPER2 with rTLP. To confirm the interaction between the PAS domain of rPER2 and rTLP, rTLP mRNA was transcribed from T7 promoter-driven rTLP cDNA (pAP3neo-rTLP) by T7 RNA polymerase and the message was translated into full length rTLP in reticulocyte lysates as described under Materials and Methods. GST-pull down assay was performed to examine protein-protein interaction between the PAS domain of rPER2 and *in vitro* translated full length rTLP (Fig. 5). The result reveals that GST-rPER2 and rTLP associated each other under the conditions as described above (lane 2), whereas rTLP displayed no binding with GST alone (lane 3). These data indicate direct association between the PAS domain of rPER2 and rTLP at least *in vitro.*

Nuclear localization of rTLP protein in COS-1 cells. To establish the cellular localization of rTLP protein, COS-1 cells were transfected with HA-tagged rTLP. Anti-HA immuno-staining reveals that 99.6% of HA-rTLP was located in the nucleus in all transfected cells (Fig. 6), indicating that the putative nuclear localization signals of rTLP protein seem to function in the cells.

DISCUSSION

We previously cloned the PER2 gene of rat (rPER2) and have made arrhythmic SCN-lesioned rats to monitor its circadian expression rhythms in peripheral tissues. The data suggest that the expression of rPER2 in the multiple tissues is under the control of SCN and rPER2 may be involved in the circadian rhythm of locomotor behavior in rats (15). In contrast to the *Drosophila* system, rTLP did not translocate rPER2 from cytoplasm to the nucleus (data not shown), which may be due to the fact that rTLP appears to lack the two PER interactive domains defined in dTIM (24).

Recently, mammalian homologues of dTIM were cloned (11, 24, 25). They have found that neither mTIM mRNA nor the protein are rhythmic in the SCN under a light/dark cycle. In this report, we have isolated a rat homologue of mTIM (rTLP) and elucidated the characteristics of rTLP. The rTLP shows 89% homology with mTIM and the mRNA expresses in all rat tissues from whole brain, lung, heart, liver, kidney, placenta and testis. The expression of rTLP mRNA was detected throughout the SCN, but it was not rhythmic in the SCN. These results are very similar with the characteristics of mTIM (26), but rhythmic mTIM expression in retina is not so evident in rats(data not shown). Recent *Drosophila* genome project indicates that m*tim* corresponds to the mammalian homologue of *Drosophila tim*-like sequence(AE003698, AE003699), but not to *Drosophila tim* itself. The second *timeless* gene in *Drosophila* (*tim* 2) shares greater sequence similarity with mammalian *tim* (27). Thus, we named the rat homologue of mouse *tim* as a rat *timeless*-like gene. Considering the facts that mTIM shares sequence similarity to TIM 2 in *Drosophila* (27) and m*tim*-knocked out mice did not show any significant effect on their circadian behavior (28), we suggest that mTIM is not a real homologue of dTIM.

Recombinant mTIM was mostly found in the nucleus and does not facilitate nuclear entry of mPER in mammalian cell lines (10). However, mTIM can inhibit CLOCK/BMAL-1-mediated transcriptional activation (10, 13, 14). Furthermore, Zylka *et al.* and Field *et al.*

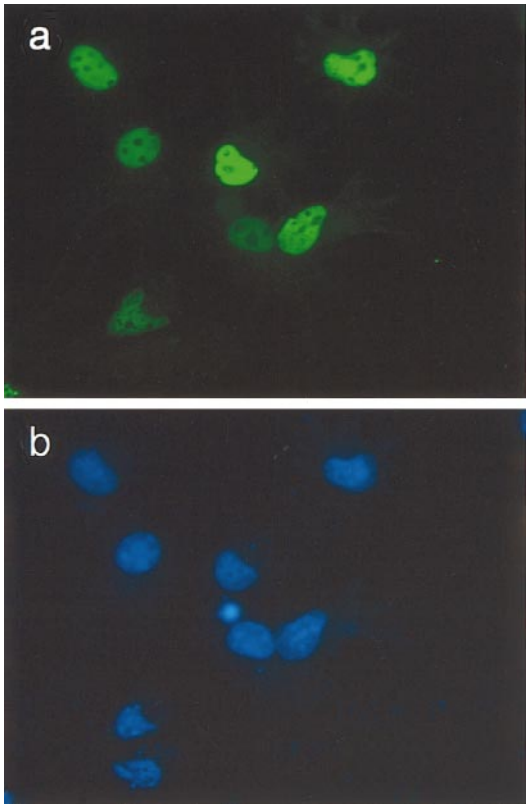

FIG. 6. Nuclear localization of rat TLP protein in mammalian cells. Cos-1 cells transfected with rTLP (a) or costained with DAPI (b).

reported that there was no interaction between mPER and mTIM (11, 29), while Takumi *et al.* showed mPER1 (amino acids 135–638), which included the PAS and CLD domain, were associated with mTIM (26). Therefore, the function of mTIM in the circadian loop is still controversial in mammals. In this paper, we have demonstrated the nuclear localization of singularly transfected rTLP in mammalian cell. In addition, rTLP did not translocate rPER2 from cytoplasm to nucleus (data not shown) like *Drosophila*. However, *in vitro* pull down assays showed that the PAS domain of rPER2 was associated with rTLP (Fig. 5). Hence, our present results suggest that rTLP probably function as the circadian molecule after rPER2 was translocated into the nucleus and the interaction of the PAS domain of rPER2 with rTLP may influence the molecular behavior of rTLP and rPER2 in the nucleus. Finally, our present results may provide a clue to elucidate the precise molecular mechanism between PERIOD and TIMELESS in the mammalian circadian loop.

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