Phosphorylation of clock protein PER1 regulates its circadian degradation in normal human fibroblasts

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Recent advances suggest that the molecular components of the circadian clock generate a self-sustaining transcriptional–translational feedback loop with a period of approx. 24 h. The precise expression profiles of human clock genes and their products have not been elucidated. We cloned human clock genes, including *per1*, *per2*, *per3*, *cry2* and *clock*, and evaluated their circadian mRNA expression profiles in WI-38 fibroblasts stimulated with serum. Transcripts of *hPer1*, *hPer2*, *hPer3*, *hBMAL1* and *hCry2* (where h is human) underwent circadian oscillation. Serum-stimulation also caused daily oscillations of hPER1 protein and the apparent molecular mass of hPER1 changed. Inhibitor studies indicated that the CKI (casein kinase I) family, including CKI ϵ and CKI δ , phosphorylated hPER1 and increased the apparent molecular mass of hPER1. The inhibition of hPER1 phosphory-

lation by CKI-7 [*N*-(2-aminoethyl)-5-chloro-isoquinoline-8-sulphonamide], a CKI inhibitor, disturbed hPER1 degradation, delayed the nuclear entry of hPER1 and allowed it to persist for longer in the nucleus. Furthermore, proteasome inhibitors specifically blocked hPER1 degradation. However leptomycin B, an inhibitor of nuclear export, did not alter the degradation state of hPER1 protein. These findings indicate that circadian hPER1 degradation through a proteasomal pathway can be regulated through phosphorylation by CKI, but not by subcellular localization.

Key words: casein kinase I ϵ , circadian rhythm, Period, phosphorylation, proteasomal degradation.

INTRODUCTION

The behaviour and physiology of most organisms is subject to circadian, 24-h rhythmicity. Negative-feedback loops in clock genes are thought to control circadian oscillators in all organisms from bacteria to mammals [1]. Genes that regulate the mammalian clock are involved in a negative autoregulatory feedback loop that underlies overt rhythm generation [2]. Oscillating molecules that control their own circadian expression seem to be very important for generating circadian rhythms.

The circadian expression of clock gene products such as PER and TIM in *Drosophila* is thought to be important for driving overt rhythms. Several studies have shown that the constitutive expression of *per* or *tim* restores locomotor rhythm and protein cycling of the null alleles of *per* and *tim* respectively [3]. Eliminating the oscillations of PER and TIM proteins by overexpression abrogates circadian rhythmicity [4]. These data indicate that the circadian rhythmic expression of PER and TIM proteins is more important than that of mRNA for maintaining locomotor rhythm in *Drosophila*.

Post-translational modification, including the phosphorylation and protein degradation of clock gene products, underlies the mechanism of circadian rhythm generation in *Drosophila*. The *double-time* (*dbt*) gene product phosphorylates PER and causes protein degradation [5,6]. *Drosophila* TIM (dTIM) is degraded by a photic entrainment cue and proteasome inhibitors

block tyrosine-phosphorylation-dependent TIM degradation *in vitro* [7]. Thus dTIM is degraded through the ubiquitin–proteasome pathway. The novel clock gene, *shaggy*/GSK-3 (glycogen synthase kinase-3) might also play a role in TIM phosphorylation [8] and *shaggy*-dependent TIM phosphorylation might increase PER–TIM heterodimerization or promote the nuclear translocation of PER–TIM complexes in wild-type *Drosophila*.

The phosphorylation and degradation mechanism should also be critical to regulating the generation of rhythm in mammals. Positional cloning has revealed that the *tau* locus (which shortens circadian rhythm) in hamsters is encoded by CKI (casein kinase I) ϵ [9], an orthologue of *dbt*. CKI ϵ phosphorylates PER1, PER2 and PER3, then renders them unstable [10]. Recent findings indicate that the human PER2 site phosphorylated by CKI ϵ is mutated in some individuals with familial advanced sleep-phase syndrome [11]. This condition affects ‘morning-type’ individuals with a 4-h advance in sleep, body temperature and melatonin rhythms. A recent study of the mouse liver has shown that mPER1 (mouse PER1) and mPER2, CLOCK and BMAL1 (brain and muscle Arnt-like protein 1) undergo circadian changes in terms of phosphorylation and abundance, and that these changes might function in maintenance of the rodent clock [12]. When overexpressed with CKI ϵ , mPER1 and mPER3 are phosphorylated and degraded through the ubiquitin–proteasome pathway, while phosphorylation in co-expressed PERs with function-loss

Abbreviations used: BAP, bacterial alkaline phosphatase; BMAL1, brain and muscle Arnt-like protein 1; CKI, casein kinase I; CKII, casein kinase II; CKI-7, *N*-(2-aminoethyl)-5-chloro-isoquinoline-8-sulphonamide; CRY, cryptochrome; *dbt*, *double-time*; DMEM-10, Dulbecco's modified Eagle's Medium supplemented with 10% (v/v) foetal bovine serum and penicillin/streptomycin; DRB, dichloro-1- β -D-ribofuranosylbenzimidazol; GST, glutathione S-transferase; LMB, leptomycin B; NES, nuclear export signal; NLS, nuclear localization signal; PAS, Per-Arnt-Sim; PER, PERIOD; hPER1, human PER1; mPER1, mouse PER1; poly(A)⁺, polyadenylated; RT, reverse transcriptase; dTIM, *Drosophila* TIM; TBB, 4,5,6,7-tetrabromo-2-azabenzimidazole.

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The nucleotide sequences reported have been submitted to the DDBJ, EMBL, GenBank[®] and GSDB Nucleotide Sequence Databases under the accession numbers AB088477 (*hPer1*), AB002345 (*hPer2*), AB047686 (*hPer3*), AB014558 (*hCry2*) and AB002332 (*hClock*).

CKI ϵ is inhibited, which protects the PER proteins from degradation [13]. However, the relationship between cycling of the mammalian clock protein and its post-translational modification in native systems remains obscure.

Some familial advanced and delayed sleep-phase syndromes can be attributed to mammalian *per* mutations in PER proteins, presumably CKI ϵ -binding domains [11,14]. Because a phosphorylation disorder might cause some sleep syndromes, a system with which to assay post-translational modifications of PER protein should be established.

To compare the molecular mechanisms of circadian clocks in divergent species, we cloned human clock genes and analysed their circadian expression in serum-shocked fibroblasts. We also developed a novel antiserum with which to examine the temporal expression of hPER1 (human PER1) protein. The results showed robust circadian profiles of hPER1 protein abundance, phosphorylation and degradation. We propose that phosphorylation by CKI ϵ and CKI δ , and degradation via the proteasome pathways of PER proteins is an important step towards understanding the human molecular clock.

MATERIALS AND METHODS

Cloning and sequencing clock genes

Complementary DNA clones for *hPer1*, *hPer2*, *hCry2* and *clock* genes were isolated during the Kazusa human cDNA project which aimed to accumulate information on the coding sequences of long cDNAs for unidentified human genes [15,16]. We used the nucleotide sequence of the *hPer2* gene as a query sequence for the *hPer3* gene in a similarity search against a library that included ESTs (expressed sequence tags) of more than 10 000 cDNA clones from size-fractionated cDNA libraries of the human brain [15]. A BLAST search by the Wisconsin Sequence Analysis Package™ (Version 8; Genetics Computer Group, Madison, WI, U.S.A.) revealed several clones with high sequence similarity to the ORF (open reading frame) of *hPer2*. These clones were classified with respect to two genes: one was identical with *hPer1*, and the other was a human orthologue of *mPer3* [17–19]. We sequenced DNA as described in [20].

Cell culture

Human lung diploid cells (WI-38; A.T.C.C. no. CCL 75; population doubling, 21) obtained from the American Type Culture Collection (Manassas, VA, U.S.A.) were maintained in DMEM-10 [Dulbecco's modified Eagle's Medium (Invitrogen) supplemented with 10% (v/v) foetal bovine serum and penicillin/streptomycin (Invitrogen)]. Cells were stimulated with serum by changing the medium to DMEM-50 [with 50% (v/v) horse serum] and then replacing that with serum-free DMEM-10 after 2 h. At the indicated times after serum-stimulation, cells were washed with PBS and total RNA was extracted with TRIzol® reagent (Invitrogen). Where indicated, cycloheximide was added to a final concentration of 10 µg/ml. The human immature myeloid cell line KG-1 (A.T.C.C. no. CCL 246) was maintained in Iscove's modified Dulbecco's medium supplemented with 20% (v/v) foetal bovine serum and the human epitheloid carcinoma cell line HeLa S3 (A.T.C.C. no. CCL 2.2) was cultured in F-12 medium (Invitrogen) containing 15% (v/v) calf serum.

RT (reverse transcriptase)-PCR

We examined the expression of clock genes in 14 human tissues and in three cell lines including serum-induced WI-38 cells by RT-PCR. Human tissue poly(A)⁺ (polyadenylated) RNAs were

obtained commercially (Clontech), whereas poly(A)⁺ RNAs of KG-1 and HeLa S3 cells were isolated by oligo(dT)-cellulose chromatography from cytoplasmic RNA prepared according to standard protocols [21]. The total RNAs of serum-shocked and control WI-38 cells were prepared using TRIzol® reagent. Contaminating DNA was removed from all RNAs, except for those of KG-1 and HeLa S3 cells, by digestion with DNase I (Roche). We synthesized cDNA templates for RT-PCR from RNAs [1 µg for poly(A)⁺ RNA and 4.5 µg for total RNA] using an excess of Superscript II reverse transcriptase (Invitrogen) and random hexamer primers. After synthesis of the first-strand cDNA, the remaining RNA in the reaction mixture was degraded with RNase A and RNase H, and the resulting cDNA templates were recovered by phenol extraction followed by ethanol precipitation. An aliquot of the cDNA template mixture [corresponding to 2 ng of the starting poly(A)⁺ RNA for human tissues, KG-1 and HeLa S3 cells, and 15 ng of the starting total RNA for WI-38 cells unless otherwise indicated] was amplified by PCR using 0.5 unit of LA Taq DNA polymerase (Takara Shuzo Co., Osaka, Japan) in 10 µl of total reaction mixture using a DNA Thermal Cycler PJ9600 (PerkinElmer). The cycling conditions were as follows: first denaturation at 95 °C for 2 min, followed by 30 cycles of 30 s of denaturation at 95 °C, 30 min of primer annealing at 55 °C and 1 min of polymerization at 72 °C. To confirm the PCR efficiency for individual genes, control PCR products were generated from serial dilutions of each cDNA clone (0.05 fg–0.5 pg in 10 µl of PCR mixture). The PCR products were resolved by electrophoresis on 2% (w/v) agarose gels and detected by staining with ethidium bromide. The PCR products were quantified using ELISA as described in [22]. Before evaluating products by ELISA, RT-PCR proceeded as described above, except in the presence of digoxigenin (DIG)-11-dUTP (DIG PCR labelling mix; Roche). The ELISA data were converted using the software package, SOFTmax PRO (Molecular Devices, Sunnyvale, CA, U.S.A.) to mRNA levels expressed as equivalent amounts (moles) of cDNA plasmid based on control curves generated from PCR products of known amounts of authentic plasmids. The primer sets were: *hPer1*, 5'-GAATC-AGATGTGGCTAGACC-3' and 3'-TGTCAGCAACTTTGTCC-AGGG-5'; *hPer2*, 5'-ACTGCCAAATCTTACTCTGC-3' and 3'-AGCAAGGCTCAACAAATCATC-5'; *hPer3*, 5'-CCTATT-GATTTCACCTTCC-3' and 3'-CAACAGCACTGGGTAAG-AAGC-5'; *hBMAL1*, 5'-CTCCAGGAGGCAAGAAGATTT-3' and 3'-CTACTTGATCCTTGCTGCTTG-5'; *hCry2*, 5'-CTCGG-AACAGTGCCTCAAATC-3' and 3'-AACACACCTTCCCAGC-AATAG-5'; and *G3PDH*, 5'-TCATCATCTCTGCCCTCTG-3' and 3'-TCCACCACCCTGTTGCTGTAG-5'.

Antisera

A polyclonal antiserum was raised in rabbits to GST (glutathione S-transferase)-hPER1 fragment fusion proteins. The coding sequences of hPER1 (amino acids 1187–1290) were amplified by PCR using *hPer1* cDNAs as templates, and then inserted into pGEX vectors (Amersham Bioscience). GST-fusion proteins were produced in *Escherichia coli* BL21 cells and purified by glutathione-Sepharose column chromatography according to the manufacturer's protocols (Amersham Bioscience). The purified proteins were dialysed extensively against PBS and then rabbits were immunized conventionally with 1.2 mg of each protein.

Western blotting

At the indicated time points, serum-stimulated cells were rinsed twice with PBS and proteins were extracted, lysed with SDS sample buffer and boiled for 5 min.

Figure 1 Alignment of the amino acid sequences from PER3 among human (Hu), mouse (Mu), quail (Qu) and zebrafish (Ze)

The sequences from mouse, quail and zebrafish were reported previously [17,25,26]. NES, CKI-binding domain, NLS and CRY-binding domain of PER3 from human, mouse, quail and zebrafish are aligned. Amino acid residue numbers of both ends of each sequence are shown at respective sides. Identical amino acids in more than three sequences are boxed in black and those in two sequences are shaded. Four human specific repeats consisting of 18 amino acids, are located near the C-terminus.

Western blotting proceeded as described in [23]. In brief, cell lysates in SDS sample buffer were separated on SDS/6% polyacrylamide gels and blotted on to nitrocellulose membranes (Schleicher & Schuell). Non-specific binding was blocked with 3% (w/v) non-fat dried milk, then the membranes were incubated with hPER1 primary antiserum (1:1000 in blocking solution), washed three times for 10 min each in PBST [PBS containing 0.5% (v/v) Tween 20], incubated with secondary antibodies (1:1000 in blocking solution) and washed again three times for 10 min each. Immunocomplexes were detected using the ECL[®] (enhanced chemiluminescence) immunoassay signal reagent (Amersham Bioscience).

Cell fractionation

At the indicated times after serum induction, cells were harvested from 90-mm-diameter dishes and resuspended in 0.5 ml of cold cell-fractionating buffer [20 mM Hepes (pH 7.4), 300 mM sucrose, 110 mM potassium acetate, 50 mM magnesium acetate, 5 mM sodium acetate, 1 mM EGTA and 2 mM DTT (dithiothreitol)] containing 100 µg/ml saponin. Cell suspensions were disrupted using a Dounce homogenizer on ice. The homogenates were clarified by centrifugation at 500 *g* for 10 min at 4 °C and the supernatants were recovered as the cytoplasmic fraction. The pellets (nuclear fractions) were rinsed twice with cold cell-fractionating buffer, and resuspended in 100 µl of cell-fractionating buffer. Each fraction (15 µl) was mixed with SDS sample buffer and immunoblotted against anti-hPER1 and anti-(pyruvate kinase) antibodies (Polysciences, Warrington, PA, U.S.A.).

Phosphatase digestion and inhibition of CKI activity, proteasome activity and nuclear export

Cells were lysed on ice 12 h after serum-stimulation. The lysate was dephosphorylated with 2 units of BAP (bacterial alkaline

phosphatase; Toyobo Biochemicals, Osaka, Japan) in 200 µl of a solution containing 1 mM MgCl₂ and 50 mM Tris/HCl (pH 8.0) at 37 °C for 1 h and Western-blotted as described above.

To determine the relationship between hPER1 phosphorylation and CKI, cells stimulated with serum were incubated with the casein kinase inhibitor, CKI-7 [*N*-(2-aminoethyl)-5-chloro-isoquinoline-8-sulphonamide] (Seikagaku-Kogyo, Tokyo, Japan). After serum-stimulation, the medium was replaced with fresh DMEM-10 and then CKI-7 was added to a final concentration of 400 µM [24]. Cells were incubated further, rinsed and then lysed for Western blotting as described above.

We inhibited proteasome activity by incorporating the proteasome inhibitor, MG-132, or lactacystin at a concentration of 20 µM each and then proceeded under the conditions described above.

Nuclear exporting activity was inhibited by adding either 10 ng/ml of LMB (leptomycin B) or vehicle to cells 2 h after serum stimulation. We included 100 µM genistein (Sigma) to block tyrosine kinase activity and inhibited CKII (casein kinase II) activity by incubation with 10 µM DRB (dichloro-1-β-D-ribofuranosylbenzimidazol; Biomol, Plymouth Meeting, PA, U.S.A.) or with 25 µM TBB (4,5,6,7-tetrabromo-2-azabenzimidazole; a gift from Dr L. A. Pinna and Dr F. Maggio) for the indicated periods. Thereafter the cell lysates were Western-blotted.

RESULTS

Serum-shock induced circadian expression of clock genes in human fibroblasts

We originally cloned the full-length human clock genes encoding *Per1* (KIAA0482), *Per2* (KIAA0347) *Per3* (KIAA1779), *Cry2* (KIAA0658) and *Clock* (KIAA0334) as part of the Kazusa human cDNA project. Predictions indicate that human *Per3* (*hPer3*) cDNA encodes 1210 amino acids. The sequence similarity of hPER3 to mouse, quail and zebrafish orthologues (mPER3, qPER3 and zPER3) is 77, 34 and 38%, respectively (Figure 1)

[17,25,26]. The sequence of hPER3 exhibits overall identity of 39% and 38% with hPER1 and hPER2 respectively [20,27]. Several regions are conserved in the three predicted human PER sequences (Figure 1). The PAS (Per-Arnt-Sim) domains consisting of PAS A (residues 126–175 of hPER3) and PAS B (residues 264–316 of hPER3) motifs, which are regions of protein–protein interaction with a protein containing PAS, are highly conserved. Figure 1 shows the amino acid sequence similarity among human, mouse, quail and zebrafish PER3 proteins around an NLS (nuclear localization signal; residues 744–752), a CKI-binding site (residues 630–643), an NES (nuclear export signal; residues 401–413) and a CRY (cryptochrome)-binding site (residues 1135–1188) predicted from the sequence similarity with mammalian PER1, PER2 and PER3. These motifs are also highly conserved among these PER3s [17,25,26]. In contrast with these conserved domains, human-specific repeats were located at residues 991–1064 of hPER3. The repetitive sequence in hPER3 has no similarity with known motifs and its function remains unknown.

We examined the distribution of clock gene transcripts in 14 tissues and in three cell lines using RT-PCR. Although steady-state levels of the transcripts differed among tissues, these genes were expressed in all tissues, including the heart, brain, placenta, lungs, liver, skeletal muscle, kidneys, pancreas, spleen, thymus, prostate, testes, ovaries and small intestine (results not shown). We detected all clock gene transcripts tested in cultured cells including the embryonic lung diploid fibroblast line WI-38, the immature myeloid cell line KG-1 and the epitheloid carcinoma cell line HeLa S3, although at different expression levels (results not shown). For example, less *hBMAL1* was expressed in KG-1 and HeLa S3 cells than in WI-38 cells. The expression levels of all clock genes were quite low in HeLa S3 cells. Such ubiquitous expression suggests that clock genes play important roles in the circadian rhythms of peripheral tissues.

Serum-shock induces the circadian oscillation of clock genes in several types of mammalian peripheral cells *in vitro* [28–30]. We examined whether serum induces the circadian expression of human clock genes in normal human diploid fibroblasts (WI-38 cells) using RT-PCR ELISA (see the Materials and methods section) to detect RNA levels of these genes. Since cultured WI-38 cells invariably undergo senescence after a finite number of doublings, we selected young cells. The data from RT-PCR and ELISA are expressed as moles of corresponding cDNA plasmids per 3 ng of total RNAs. The expression of *c-fos* transcripts was induced by over 20-fold at 1 h after adding serum addition as expected (Figure 2). Serum also stimulated the immediate expression of the *hPer1* and *hPer2* genes [28]. The mRNA levels of *hPer1* and *hPer2* genes reached a maximum at 1 and 4 h respectively, and minimal levels at 12 h for both the two *per* genes. Although *hPer3* was also expressed in a circadian fashion after 24 h, it was not immediate like the expression of *hPer1* and *hPer2*. The expression profile of *hCry2* was similar to that of *hPer3*. The peak expression of *hBMAL1* transcripts was a reciprocal fluctuation compared with that of *hPer* genes as it is *in vivo* [31]. On the other hand, the expression of *hClock* and *G3PDH* did not significantly differ (results not shown).

To confirm immediate-early induction of the *hPer1* and *hPer2* genes, we recorded *hPer* accumulation for up to 4 h after serum-shock in the presence of cycloheximide, an inhibitor of protein synthesis. Ongoing protein synthesis was not required for the immediate-early expression of *hPer1* and *hPer2* mRNAs in human fibroblasts as well as in rodent fibroblasts [28]. Moreover, the mRNA stability of *hPer1* and *hPer2* increased in cells incubated with cycloheximide. The regulatory profiles of various immediate-early genes, including *c-fos* and *c-myc*, are similar to those

Figure 2 Circadian expression profile and transient stimulation of human clock genes in serum-stimulated WI-38 cells

Expression of *hper1*, *hper2*, *hper3*, *hBMAL1*, *hCry2* and *c-fos* was analysed by RT-PCR. Quiescent WI-38 cells were stimulated by changing the medium to DMEM containing 50% horse serum for 2 h, then replacing this medium with DMEM-10. Total RNAs were prepared at various times (h) after exposure to high serum concentration. Complementary DNA templates corresponded to 15 ng of starting total. PCR products were quantified by ELISA. Results are means $\pm$ S.D. for two to six independent experiments. Relative RNA levels were determined from ratios of each template cDNA used in PCR. Data from RT-PCR ELISA are expressed as amounts (moles) of corresponding cDNA plasmids in 3 ng of total RNAs.

of *hPer1* and *hPer2* [32]. These findings suggest that *hPer1* and *hPer2* are products of immediate early genes.

Circadian oscillation and temporal change in apparent size of PERIOD proteins in WI-38 cells stimulated with serum

Figure 2 shows the circadian expression of three *per* genes in serum-stimulated WI-38. To understand whether the circadian expression of *per* genes leads to the expression of human PERIOD1 protein (hPER1), we raised antisera against the C-terminal portion of hPER1 as described in the Materials and methods section. Proteins extracted from serum-stimulated WI-38 were Western-blotted against anti-hPER serum. Figure 3(A) shows a specific immunoreactive band migrating at 183 kDa. The protein-accumulation profiles recorded after serum-shock showed that the abundance of hPER1 oscillated in serum-stimulated WI-38 (Figure 3A). The accumulation of hPER1 expression initially peaked at 6 h, then gradually fell during the next 20 h (Figure 3B). After 23 h, the levels of hPER1 were not as high as the first peak, but definitely increased once again to reach a second peak at 32 h (Figure 3B).

In addition to an oscillating amount of hPER1, the apparent size of hPER1 increased between 3 and 12 h from an apparent

Figure 3 Temporal change of hPER1 accumulation in serum-stimulated WI-38 cells

(A) WI-38 cells were grown to confluence then starved of serum for 24 h. Cells were washed and shifted to medium containing 50% calf serum. At various times (indicated above the gel in h) after serum-stimulation, cells were lysed and Western-blotted (hPER1 or actin) as described in the Materials and methods section. Lanes show 70 μ g of extracted proteins. (B) Signals obtained in Western blotting shown in (A) for hPER1 protein indicated as amounts relative to value at 0 h. (C) Cell lysates at 12 h were digested with BAP for 1 h (lane 2), then Western-blotted using hPER1 antiserum. WI-38 cells were stimulated and proteins were extracted with lysis buffer at 3 (lane 1) or 12 h (lanes 2 and 3). Upper and lower arrows indicate phosphorylated (204 kDa) and dephosphorylated (188 kDa) forms of hPER1 respectively.

molecular mass of 188 to 204 kDa (Figure 3A). This indicated that hPER1 undergoes significant post-translational modifications over time after serum stimulation. Edery et al. [33] reported that *Drosophila* PER protein in fruitfly head extracts undergoes daily oscillations in terms of apparent molecular mass as well as in abundance by phosphorylation [33]. Furthermore, mPERs in the liver are phosphorylated in a circadian manner [12]. To test whether the slowly migrating hPER1 in serum-stimulated cells is indeed caused by phosphorylation, we incubated cell lysates with BAP 12 h after serum stimulation. The mobility of BAP-treated hPER1 protein shifted to the level of mock-treated samples and was essentially indistinguishable from hPER1 that initially appeared at 3 h (Figure 3C). This indicated that most of the time-dependent size increases in hPER1 after serum-stimulation are due to phosphorylation.

The role of CKI in hPER1 phosphorylation and oscillation of its expression profile

CKI ϵ and CKI δ can phosphorylate overexpressed hPER1 and hPER2 *in vitro* and induce an increase in the apparent molecular size of PERs [10,34]. To understand the relationship between

Figure 4 CKI-7 inhibits temporal mobility shifts and oscillation of hPER1

(A) WI-38 cells were serum-stimulated for 2 h, then fresh medium alone (lanes 1 and 2) or that containing CKI-7 (final concentration, 400 μ M; lane 3) was added. Cell lysates were recovered with SDS sample buffer at 3 (lane 1) or 8 h (lanes 2 and 3) after serum-stimulation. Lysates were Western-blotted with hPER1 antiserum or anti-actin antibody. Upper and lower arrows indicate phosphorylated (204 kDa) and dephosphorylated (188 kDa) forms of hPER1 respectively. (B) Cells were stimulated with serum as described above, then hPER1 expression was examined in the presence (CKI-7) or absence (mock) of CKI-7. Cell lysates were recovered at the indicated times (in h) after serum stimulation.

CKIs and the altered molecular size caused by hPER1 phosphorylation after serum-stimulation, we examined the effect of the CKI inhibitor, CKI-7, which specifically inhibits both CKI ϵ and CKI δ [35]. We added CKI-7 after serum-stimulation to a final concentration of 400 μ M [24]. Phosphorylation was significantly inhibited as seen 12 h after serum-stimulation and the molecular size of hPER1 remained the same as it was at 3 h (Figure 4A). These results suggested that the CKI family, including CKI ϵ and CKI δ , can phosphorylate hPER1 and induce an electrophoretic mobility change. Camacho et al. [10] have indicated that other isoforms of the CKI family, such as CKI α and CKI γ cannot interact with hPER1 [10]. Considering these data, the upward mobility shift of hPER1 after serum-stimulation in human fibroblasts was partly due to phosphorylation by CKI ϵ and CKI δ .

The proper phosphorylation of dPER is essential for its degradation in *Drosophila*. The phosphorylation of PER is abnormal and the degradation of PER protein is delayed in the *Dbt* mutant, resulting in arrhythmic behaviour. *Dbt* is a *Drosophila* orthologue of mammalian CKI ϵ . Thus we studied whether or not the phosphorylation of hPER1 by CKI is related to the stability of hPER1 protein. WI-38 cells were stimulated with serum and were subsequently cultured in the presence of CKI-7. We then analysed hPER1 expression profiles for 48 h (Figure 4B). In the presence of CKI-7, hPER1 phosphorylation was inhibited for at least 36 h and hPER1 remained even 24 h after stimulation, when control hPER1 protein was degraded and barely detectable (Figure 4B, mock). We concluded that the inhibition of hPER1 phosphorylation by CKI-7 interfered with hPER1 degradation. These data suggest that phosphorylation by CKI ϵ and CKI δ is essential for the time-dependent degradation mechanism of

hPER1. Since incubation with CKI-7 for more than 40 h caused cell death, hPER1 was undetectable in cell lysates after 40 h (results not shown). CKI-7 could not totally inhibit hPER1 phosphorylation, because the apparent molecular mass increased slightly, even in the presence of the inhibitor. Kinases other than CKI ϵ and CKI δ might play a role in hPER1 phosphorylation after serum-stimulation. CKI-7 seems to suppress the protein synthesis of hPER1. To examine whether or not the suppression of hPER1 translation by CKI-7 inhibits the phosphorylation and degradation of hPER1 directly, CKI-7 was added 4 h after serum-stimulation when the initial acute induction of hPER1 had completed. Both the phosphorylation and degradation of hPER1 were suppressed (results not shown). Thus we concluded that the inhibited degradation in the presence of CKI-7 was not due to inhibiting the suppression of hPER1 translation.

In addition to serine and threonine residues, *Drosophila* TIM is phosphorylated on tyrosine residues, which leads to its degradation [7]. We Western-blotted phosphorylated hPER1 against the anti-phosphotyrosine antibody, 4G10 (Upstate Biotech) to determine whether or not tyrosine residues were phosphorylated. None of the phosphorylated hPER1 proteins reacted with the antibody (results not shown). Furthermore, the tyrosine kinase inhibitor, genistein ($< 100 \mu\text{M}$), could not block hPER1 degradation after hyperphosphorylation (results not shown). These data suggest that the tyrosine phosphorylation of hPER1 is not essential for its degradation.

Drosophila CKII phosphorylates PER and induces its nuclear entry [36,37]. The *Neurospora* clock protein, FREQUENCY (FRQ), is progressively phosphorylated over time and a FRQ-phosphorylating kinase has been identified as CKII [38]. To determine whether or not mammalian CKII also plays a role in the progressive phosphorylation of hPER1 in serum-stimulated cells, we incubated the cells with either $25 \mu\text{M}$ DRB or $25 \mu\text{M}$ TBB, potent CKII inhibitors [39,40]. However, hPER1 hyperphosphorylation was not prevented by these potent CKII-specific inhibitors (results not shown), suggesting that CKII does not function in mammalian clock systems as it does in other species.

Degradation of phosphorylated hPER1 through a ubiquitin–proteasome pathway

Several groups have reported that exogenously overexpressed mPER proteins are ubiquitinated and degraded via the ubiquitin–proteasome pathway [13,41]. To determine whether or not this pathway is involved in the native degradation system under our conditions, we added two universal proteasomal inhibitors of hPER1 oscillation after serum-stimulation. Figure 5(A) shows that hPER1 degradation was inhibited by MG-132, an inhibitor of the chymotryptic activity of the eukaryotic 26 S proteasome. Hyperphosphorylated hPER1 remained even 24 h after serum-stimulation in the presence of the inhibitor. The 20 S proteasome inhibitor, lactacystin ($20 \mu\text{M}$) also blocked hPER1 degradation (Figure 5B) as well as MG-132 (Figure 5A). These results indicate that endogenous hPER1 degradation proceeds via the ubiquitin–proteasome pathway in human fibroblasts stimulated with serum.

Effect of the phosphorylated state of hPER1 on subcellular localization

The phosphorylation of PER1 by CKI ϵ might modulate its nuclear localization. One report showed that hPER1 phosphorylation by CKI ϵ in HEK-293 cells could hide the NLS in PER1 and thus inhibit the nuclear entry of PER1 [42]. On the other hand, another report indicated that mPER1 phosphorylation by CKI ϵ promotes the nuclear localization in COS-7 cells [43]. Both of these results

Figure 5 Inhibition of hPER1 degradation with proteasome inhibitors

(A) WI-38 cells were stimulated with serum for 2 h, then incubated at 37°C in DMEM containing proteasome inhibitor, MG-132 (final concentration, $20 \mu\text{M}$). Control cells were incubated with DMSO alone (mock). At the indicated times (in h) after induction, cell lysates were Western-blotted. (B) WI-38 cells were stimulated with serum for 2 h, then incubated at 37°C in DMEM containing $20 \mu\text{M}$ lactacystin. Cell lysates recovered 20 h after stimulation were Western-blotted.

were generated from overexpression studies *in vitro*. Thus we examined the relationship between the phosphorylation state of hPER1 and its subcellular localization in native cell oscillation systems after serum-stimulation.

WI-38 cells were stimulated with serum, lysed with fractionating buffer and separated into nuclear and cytoplasmic fractions by centrifugation as described in the Materials and methods section. Figure 6(A) shows that hPER1 was present in both the nuclear and cytoplasmic fractions at three time points examined, while the amount and phosphorylation state changed with the time-course. Cytosolic marker protein, pyruvate kinase, was localized in cytosolic fraction. At 3 h after serum-stimulation, hPER1 was enriched in the cytosol fraction, whereas it localized mainly in the nuclear fraction at 6 h, when *per* transcription was suppressed (Figure 2). At 12 h, the amount of hPER1 in the nuclear fraction was decreased and the amount of hyperphosphorylated hPER1 was the same in both the cytosol and nuclear fractions. These results indicated that hPER1 can transfer into the nucleus for 6 h, and then is exported from the nucleus at around 12 h. Nuclear hPER1 was more phosphorylated (shifted higher) than that in the cytosol at 3 h after serum-stimulation. Furthermore, the phosphorylation level of nuclear hPER1 gradually increased over time, suggesting that phosphorylation was enhanced further after nuclear entry.

To confirm the relationship between nuclear localization and phosphorylation, we examined the subcellular localization of hPER1 after serum-shock in the presence of CKI-7 (Figure 6B). As described above, hPER1 phosphorylation was not completely inhibited even in the presence of CKI-7, but the inhibitor delayed the nuclear entry of hPER1 for over 4 h. At 24 h after serum-stimulation, not only hyperphosphorylation, but also nuclear

Figure 6 Subcellular localization and degradation of hPER1 in WI-38 cells after serum-stimulation

(A) Subcellular distribution of hPER1 at 3, 6 and 12 h after serum-stimulation. Cells were dispersed in fractionating buffer containing 100 μ g/ml saponin. Homogenates were separated by centrifugation at 500 g for 10 min. Supernatant (C, cytosolic fraction) and resuspended pellet (N, nuclear fraction) were Western-blotted and probed using anti-hPER1 or anti-(pyruvate kinase) (PK) antibodies. (B) Subcellular localization of hPER1 after serum-stimulation with or without CKI-7 (400 μ M). Cell lysates were fractionated, and then cytoplasmic (C) and nuclear (N) fractions were blotted and probed using anti-hPER1 or anti-PK antibodies. (C) Effect of LMB on hPER1 and p53 protein degradation after serum-stimulation. WI-38 cells were stimulated with 50% (v/v) horse serum in DMEM containing either LMB (back bar) or vehicle (mock, white bar). Relative hPER1 levels are indicated as percentages of value at 3 h after serum-stimulation. Relative p53 level is indicated as a percentage of the value in cells exposed to LMB 20 h after serum-stimulation.

hPER1 export, was inhibited (Figure 6B). Partially phosphorylated hPER1 was mainly separated in the nuclear fraction. Some initial cytoplasmic hPER1 phosphorylation might occur, which would lead to nuclear localization. Further phosphorylation by CKI in the nucleus seems to be essential for nuclear export.

Since hPER1 contains three NESs that function via the CRM1/Exportin 1 nuclear export system [44], we investigated whether or

not the export system influences hPER1 degradation. We incubated serum-stimulated WI-38 cells with LMB, a potent specific inhibitor of the CRM1/Exportin 1 nuclear export system, and recovered the cell lysate at indicated times after serum-stimulation. Figure 6(C) shows that hPER1 degradation could not be significantly blocked by LMB. On the other hand, LMB blocked p53 degradation, which is specifically degraded in cytoplasm after nuclear export (Figure 6C). These results indicated that hPER1 can be degraded on the proteasome in both nuclear and cytosolic compartments. Thus the degradation of hPER1 appeared to be controlled mainly by its phosphorylation state rather than by subcellular localization.

DISCUSSION

We cloned the human clock genes, *hPer1*, *hPer2*, *hPer3*, *hClock* and *hCry2*, and analysed their expression in peripheral tissues and cell lines. Functional structures, such as the NLS [23,29,42], residues phosphorylated by CKI ϵ [13,42], the NES [42] and the CRY-binding domain [23,41,45], are highly conserved in hPER1, hPER2 and hPER3. In addition to the conserved motifs, hPER3 contains a human-specific insertion at amino acid residues 991–1067. The function of this region is unknown.

The present study also demonstrated that circadian mRNA oscillations of not only *hPer1*, *hPer2*, and *hBMAL1*, but also *hPer3* and *hCry2*, in human fibroblasts were triggered by a high concentration of serum. The oscillating profiles of *per1* and *per2* mRNA in human WI-38 cells were similar to those in Rat-1 cells [28,46], NIH-3T3 [29], mouse embryonic fibroblasts [30], as well as human and rat vascular smooth muscle cells [47,48]. We could not detect hPer3 and hCry2 mRNA expression until 24 h after stimulation. This was distinct from hPer1 and hPer2 mRNAs, which were induced immediately after serum-stimulation. These results suggested that the expression mechanisms of *hper3* and *hCry2* differ from those of *Per1* and *Per2*. This is consistent with the notion that, whereas *mPer1* and *mPer2* are acutely induced by a light pulse in the rodent suprachiasmatic nucleus, *mPer3* and *mCry2* are not [18,49].

To characterize the post-translational modification and degradation of mammalian PER protein, we established an assay for hPER protein oscillation using specific antisera in human fibroblasts after serum-stimulation. The PER protein was induced 3–6 h after mRNA expression, and its abundance and electrophoretic mobility were altered (Figure 3). This was similar to the circadian profile of mPER1 in the liver [12]. We showed that the slow electrophoretic migration of hPER1 was due to initial sequential CKI phosphorylation events and to a CKI-independent phosphorylation mechanism (Figure 4). Recent reports indicate that hPER1 that is co-expressed with CKI ϵ and CKI δ in HEK-293 cells is sequentially phosphorylated and that the apparent molecular size of hPER1 increases [42]. These data indicate that the phosphorylation of hPER1 by CKI ϵ and CKI δ play an important role in the generation of circadian rhythm in humans.

The post-translational regulation of clock gene products is critical for maintaining the circadian oscillator [3]. Phosphorylation determines the cellular localization and stability of clock proteins in *Drosophila* [5,7,8,33]. Mammalian PER1, PER2 and PER3 are also phosphorylated and this state regulates their stability [13,34]. Keesler et al. [34] reported that the phosphorylation of hPER1 by CKI ϵ decreases hPER1 protein stability. We found that the CKI inhibitor, CKI-7, inhibited hPER1 phosphorylation and degradation. We could not exclude the possibility that hPER1 was phosphorylated by CKI α and CKI γ , because CKI-7 inhibits the activity of the entire CKI family. However, CKI ϵ and CKI δ can interact with and phosphorylate

hPERs, whereas CKI α and CKI γ cannot [34]. Thus we concluded that even during the oscillation of native PER protein, the phosphorylation of hPER1 by CKI ϵ and CKI δ controls hPER1 stability. CKI-7 could not completely inhibit hPER1 phosphorylation, suggesting that another kinase(s) is also involved in the control of PER phosphorylation and degradation. The tyrosine phosphorylation of dTIM is considered essential for degradation in *Drosophila* [7]. Our findings using a tyrosine kinase inhibitor suggested that the tyrosine phosphorylation of hPER1 is not essential for its degradation (results not shown).

The ubiquitin–proteasome pathway plays a key role in various cellular processes, including regulation of the cell cycle and transcription. This pathway is also the route of dTIM and dCRY degradation [7,50]. Our results showed that the proteasome inhibitors, MG-132 and lactacystin, inhibited the hPER1 degradation after its phosphorylation mainly by CKIs. Ubiquitin tagged with haemagglutinin is incorporated into overexpressed exogenous mPERs and is degraded on proteasomes [13,41]. Our data indicate that endogenous hPER1 is also degraded for protein cycling through the ubiquitin–proteasome pathway after serum-stimulation. The phosphorylation and proteasome degradation of circadian clock protein should play an important role in maintaining the circadian clock.

The relationship between the phosphorylation state of mammalian PERs and nuclear localization is controversial. Exogenous hPER1 overexpressed with CKI ϵ in HEK-293 cells localizes in the cytoplasm [42], whereas mPER1 co-expressed with CKI ϵ in COS-7 cells accumulates in the nucleus [43]. Thus the phosphorylation state might control the subcellular localization of PER1. The import and export timing of hPER1 seems to be important for clock maintenance [23]. We found that hPER1 is phosphorylated at various levels corresponding to the products of sequential CKI hyperphosphorylation. We therefore examined the subcellular localization in relation to the phosphorylation levels of hPER1 (Figure 6A). Human PER1 is probably phosphorylated in both the cytoplasm and the nucleus. The subcellular localization of hPER1 after CKI inhibition indicated that it can be phosphorylated to some extent in the cytoplasm, then sequential phosphorylation progresses after nuclear entry. Fully phosphorylated hPER1 in the cytoplasm 12 h after serum-stimulation might be transported from the nucleus via the nuclear exporting pathway (Figure 6A) [44]. Thus we concluded that complete hPER1 phosphorylation is not essential for nuclear entry.

Proteins destined for degradation are targeted to proteasome complexes that are localized in the cytoplasm and the nucleus [51]. The ubiquitin-mediated proteolysis of some proteins is regulated by their subcellular localization. For example, tumour suppressor p53 is specifically degraded in the cytoplasm [52,53], whereas the transcriptional repressor, Mat α 2 is degraded in the nucleus [54]. However, the nuclear export inhibitor, LMB, did not affect the degradation of hPER1, but blocked that of p53 (Figure 6C). We speculated that hPER1 is degraded at both nuclear and cytosolic proteasomes and that the process is regulated by phosphorylation rather than by subcellular localization.

Familial advanced [11] and delayed [14] sleep-phase syndrome is caused by disorders of the molecular circadian clock. To verify the molecular mechanism of human sleep disorders, an *in vitro* system is required with which to assay the human molecular clock. We developed a means of evaluating the post-translational modification of human clock molecules using normal diploid fibroblast WI-38 cells. Thus disorders in post-translational regulation systems can be examined using cultured skin fibroblasts derived from patients. Since PER protein does not undergo circadian oscillation in aged WI-38 cells that have exceeded 40 population doublings (K. Miyazaki, M. Mesaki and

N. Ishida, unpublished work), this could become a good model of insensitivity to circadian rhythms in the elderly. Further investigation of the post-translational regulation system in the human clock will help to clarify rhythm disorders and their associated molecular mechanisms.

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