Cloning and characterization of rat casein kinase 1ε

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Abstract **Genes differentially expressed in the subjective day and night in the rat suprachiasmatic nucleus (SCN) were surveyed by differential display. A gene homologous to human casein kinase 1ε (CK1ε) was isolated, which initially appeared to be expressed in the suprachiasmatic nucleus (SCN) in a circadian manner. We here describe the cDNA cloning of the rat CK1ε and characterization of the protein products. The rCK1ε is predominantly expressed in the brain including the SCN, binds and phosphorylates mPer1, mPer2, and mPer3 in vitro, and translocates mPer1 and mPer3, but not mPer2, to the cell nucleus depending on its kinase activity when coexpressed with these Per proteins in COS-7 cells.** © 2000 Federation of European Biochemical Societies. Published by Elsevier Science B.V. All rights reserved.

Key words: Circadian rhythm; Differential display; Suprachiasmatic nucleus; Per; Phosphorylation; Nuclear translocation

1. Introduction

Our understanding of the molecular mechanism of mammalian circadian rhythms has progressed considerably in recent years following the discovery of the *clock* gene [1] and mammalian homologs of *Drosophila* clock-related genes such as mPers and mTim [2–7]. In studies with mice: (1) mPer1, 2, and 3 were expressed in the suprachiasmatic nucleus (SCN) in a circadian rhythm pattern, and expression of both mPer1 and 2 was induced by light exposure [5,8]; (2) mPer proteins interacted with mCRY nuclear proteins and were translocated into the nucleus in cultured mammalian cells transformed with them [9]; (3) loss of the mPer2 function by deletion mutation eliminated circadian oscillation in wheel-running activity [10]; and (4) double knockout of the *cryptochrome* gene products mCry1 and mCry2 abolished the circadian rhythm of wheel-running activity [11–13]. Furthermore, in *Drosophila* the *double-time* (*dbt*) mutation eliminated the circadian rhythms of Per and Tim expression, and the protein responsible for the *dbt* mutation was shown to be a homolog of human casein kinase 1ε (hCK1ε) [14,15].

To clarify the circadian oscillation mechanism, we used the mRNA differential display method to search for genes differentially expressed in the subjective day and night in the rat SCN and isolated the gene *scop* [16] and another gene homol-ogous to human *casein kinase 1ε*. Here, we report on the cloning of rat *casein kinase ε* gene and characterization of the gene product (rCK1ε).

2. Materials and methods

2.1. Animals

Male Wistar strain rats weighing 250–350 g were housed individually in plastic cages in a room maintained at $24 \pm 1°C$ and illuminated for 12 h (0700–1900 h) by fluorescent light (about 80 lux). Food (type MF; Oriental Yeast Co.) and water were freely available. The animals were adapted to these conditions for at least 7 days prior to the experiments.

2.2. Screening of cDNA library and sequencing

In a previous study [16], a fragment of *ck1ε* cDNA was isolated by the differential display method. The fragment was radiolabeled with a Multiprime Labeling kit (Amersham Pharmacia Biotech UK Ltd., Buckinghamshire) and used as a probe to screen a rat hypothalamic cDNA library (Takara Biochemicals, Tokyo) according to standard laboratory protocols for molecular cloning. DNA sequencing was carried out using a DNA sequencing kit (Big Dye Terminator Cycle Sequencing Ready Reaction, PE Applied Biosystems) and a genetic analyzer (ABI 310, Perkin Elmer). cDNA fragments of mPer1, 2, and 3 were isolated as described previously [17,18].

2.3. Kinase-inactive mutant rCK1ε-1

To generate a kinase-inactive form of rCK1ε-1 (CK1-KN), lysine at amino acid residue (aa) 38 was changed to arginine by polymerase chain reaction (PCR).

2.4. Antibody

Anti-rCK1ε-1 antiserum was raised against a glutathione *S*-transferase (GST)-rCK1ε-1 fusion protein containing rCK1ε-1 from aa 294 to 416. To purify anti-GST-rCK1ε-1 antibody, a maltose binding protein (MBP)-rCK1ε-1 fusion protein containing the same part of the antigen was prepared. The GST- and MBP-rCK1ε-1 fusion proteins were produced in *Escherichia coli* using pGEX (Amersham Pharmacia Biotech) and pMAL (NEB) vectors, respectively. The MBP fusion protein was purified using amylose resin (NEB) according to the manufacturer's protocol. The GST fusion protein mixed with Freund's complete adjuvant was used to raise the antibody in rabbits. The resulting antiserum against GST-rCK1ε-1 was subjected to affinity purification with the MBP fusion protein coupled to HiTrap NHS-activated (Amersham Pharmacia Biotech).

2.5. Preparation of protein samples and immunoblot analysis

Rat organs were excised and snap-frozen in liquid nitrogen. Tissues were homogenized in 10 volumes of TNE buffer containing 20 mM Tris–HCl (pH 7.4), 1 mM EDTA, 0.15 M NaCl, 1% Nonidet P-40, 5% glycerol, 5 mM β-mercaptoethanol, 10 μg/ml aprotinin, 10 μg/ml leupeptin and 1 mM phenylmethylsulfonyl fluoride. The homogenates were centrifuged at $15\,000 \times g$ for 20 min at 4°C. The protein concentration of the supernatant (sup) fraction was determined by the Bradford method. For immunoprecipitation, sup fractions prepared from tissues or cells were incubated for 2 h at 4°C with anti-hemagglutinin (HA) antibody (Boehringer Mannheim) or anti-rCK1ε-1 antibody

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bound to protein G-Sepharose beads. The beads were then washed three times with TNE or RIPA lysis buffer and resuspended in SDS sample buffer. These samples were analyzed by the immunoblot method as follows. The samples were separated on 10% SDS–PAGE gels and transferred electrophoretically to nitrocellulose membranes. After the transfer, the membranes were stained with Ponceau S solution to ensure equal loading of proteins, and then blocked with 1% skim milk in Tris-buffered saline containing 0.1% Tween-20 (Tween-TBS) overnight at 4°C. After incubation with anti-rCK1ε-1 antibody (1:5000) or anti-HA antibody (1:2000), the membranes were incubated with horseradish peroxidase-conjugated anti-rabbit IgG (1:5000) or mouse IgG (1:2000) antiserum (Zymed). A chemiluminescence detection system (NEN Life Science Products) was used for visualization. When samples were treated with alkaline phosphatase in vitro, immunoprecipitated samples were subjected to dephosphorylation with calf intestine alkaline phosphatase (CIP; Takara Biochemicals) for 30 min at 37°C, washed twice with RIPA buffer, and resuspended in SDS sample buffer for the immunoblot analysis.

2.6. Immunohistochemistry

Rats were anesthetized with pentobarbital and their brains were perfused with 50 ml saline, followed by sequential perfusion with 500 ml of 4% paraformaldehyde in phosphate buffer (pH 7.2), and 250 ml of 1% glutaraldehyde (pH 2.5). The brains were post-fixed and dehydrated in 30% sucrose in phosphate buffer. Brain sections with a thickness of 20 μm were made using a cryostat and these were immunohistochemically stained using anti-rCK1ε-1 as described previously [19].

2.7. Cell cultures and transfection

Cultures of 293T and COS-7 cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum. For transfection experiments, cells were plated onto 100-mm culture dishes at a density of 10^4 cells/cm^2 and cultured for 2 days at 37°C under 5% CO_2. After trypsinization, the cells were plated onto 6-well plates and cultured overnight. When the culture reached 90–95% confluence, the cells were transfected with expression vectors carrying rck1ε-1 cDNA (pcDNA3-rck1ε-1) and HA-tagged mper cDNAs (pHM6-mper1, 2 and 3) with LipofectAmine 2000 (Gibco BRL) according to the manufacturer's protocol.

2.8. Cell staining

COS-7 cells were plated onto coverslips in 35-mm culture dishes at a density of 10^4 cells/cm^2 and cultured overnight. For cell staining, FuGENE 6 transfection reagent (Boehringer) was used according to the manufacturer's protocol. Cells were fixed by incubation with 4% paraformaldehyde for 15 min at room temperature, washed twice with phosphate-buffered saline (PBS) (pH 7.4), and permeabilized by incubation with 0.2% Triton X-100 in PBS for 10 min at room temperature. The fixed cells were incubated with 120 μl of the primary antibody solution (1:1000) for 2 h at room temperature in a 12-well dish, followed by incubation with 120 μl of a secondary antibody reagent (Fluorolink Cy3-labeled goat anti-mouse IgG 1:2000; Alexa 488 goat anti-rabbit IgG 1:200) for 30 min at room temperature. The stained coverslips were mounted on a slide glass and observed under a fluorescence microscope.

2.9. In vitro kinase assay

For the in vitro kinase assay of rCK1ε-1, hexahistidine (His)-tagged rCK1ε-1 (His-rCK1ε-1) or kinase-negative CK1-KN (His-CK1-KN) was produced in bacteria using the pTrcHis vector (Invitrogen), and partially purified with Ni-NTA resin (Qiagen). Prior to the reaction, the His-rCK1ε-1 was subjected to limited digestion with trypsin (15 min at 30°C) to eliminate autophosphorylation activity, which hampers the activity toward exogenous substrate. The kinase reaction was performed in a kinase buffer (50 mM Tris–HCl, pH 7.4, 10 mM $MgCl_2$, and 1 mM β-mercaptoethanol and 50 μM [γ-^{32}P]ATP) with immunopurified mPer proteins or bovine milk dephosphorylated α-casein (Sigma Chemicals) as substrates. The immunopurified mPer proteins were treated with iodoacetamide to kill contaminated kinases. After incubation for 10 min at 37°C, the reactions were stopped by the addition of SDS–PAGE sample buffer and analyzed by the immunoblot method and autoradiography using BAS 3000 (Fuji Film, Tokyo).

3. Results

3.1. Isolation of rCK1-ε cDNA clones

We first attempted to clone a full-length cDNA for rCK1-ε from a rat hypothalamic cDNA library. Four independent clones were obtained. Clone 1 was identical to the full-length cDNA for rat CK1δ [20]. Clone 2 contained an open reading frame of 1248 bp encoding a protein highly homologous to hCK1ε (99.3% amino acid identity) (Fig. 1a). Thus, clone 2 appeared to encode rCK1ε. Clone 3 encoded a protein identical to rCK1ε except that the C-terminal 10 amino acid residues of rCK1ε were replaced by 100 unrelated amino acids (Fig. 1a). The protein encoded by clone 4 was the same as rCK1ε from the initiation methionine up to aa 297, but the remaining C-terminal portion was dissimilar to that of rCK1ε (Fig. 1a). When the C-terminal sequence of clone 4 was compared with the genomic sequence of hCK1ε, the clone was found to encode a splicing variant having the seventh and eighth exons that are spliced out in hCK1ε. On the basis of these structural features, clones 2, 3, and 4 were designated rCK1ε-1, rCK1ε-2, and rCK1ε-3, respectively (Fig. 1b). rCK1ε-1 was selected for functional analysis because, as noted later, no kinase activities could be detected for rCK1ε-2 and rCK1ε-3.

3.2. Distribution of rCK1ε-1 protein

The tissue distribution of rCK1ε-1 protein was examined by immunoblot analysis using an antibody raised against the
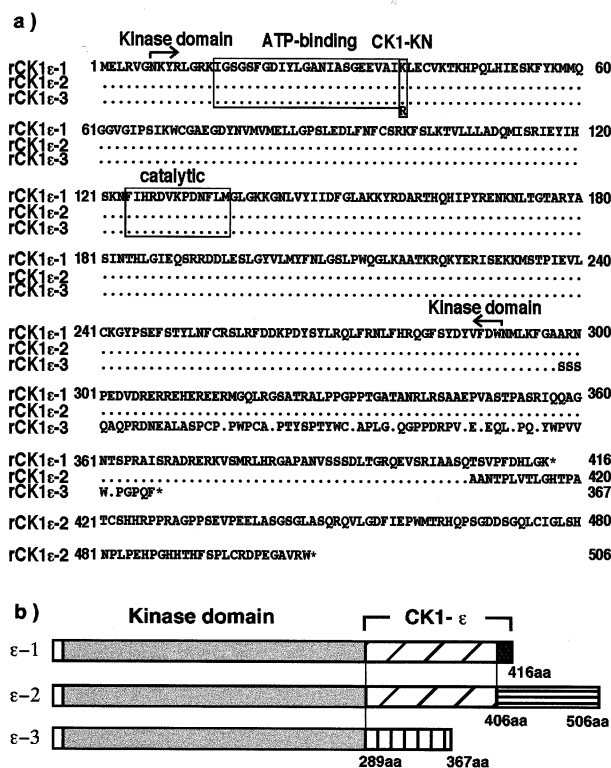

Fig. 1. Three rck1ε gene products. a: Alignment of amino acid sequences of rCK1ε-1, -2 and -3. The ATP binding site and serine/threonine kinase catalytic domain are boxed. The catalytic domain of rCK1ε is shown by arrows. b: Structures of three rCK1ε isoforms deduced from the cDNA sequences.
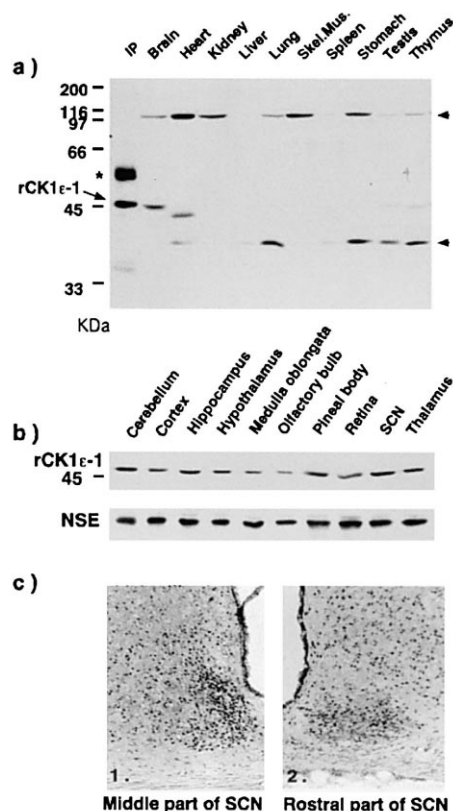
Fig. 2. Distribution of rCK1ε-1 protein. a: The tissue distribution of rCK1ε-1 protein was examined by immunoblot analysis using an anti-rCK1ε-1 antibody. In the first lane (IP), rCK1ε-1 was immunoprecipitated from the whole rat brain homogenate with anti-rCK1ε-1 antibody to show the size of rCK1ε-1. The arrow and asterisk respectively indicate rCK1ε-1-IR and rabbit IgG. The two arrowheads show non-specific immunoreactivities. b: Distribution of rCK1ε-1 protein in rat brain, pineal body and retina. The protein concentrations were normalized by immunoblot analysis using an antibody against NSE (neuron-specific enolase); the results are shown in the lower panel. c: Immunohistochemical staining of brain sections with antibody against rCK1ε-1. rCK1ε-1-IR in the middle (1) and rostral (2) parts of the SCN.

unique C-terminal sequence of rCK1ε-1. Immunoreactivity at the position corresponding to rCK1ε-1 (47.3 kDa) was predominantly detected in the brain (Fig. 2a). Weak immunoreactivity was detectable in the testis and thymus. Cross-reactivities to different sizes of proteins were detected in several tissues, but their relationship to rCK1ε-1 is unknown. The distribution of rCK1ε-1 protein in the adult rat brain, pineal body and retina was investigated by immunoblot analysis (Fig. 2b). The brain was divided into eight parts (cerebellum, cortex, hippocampus, hypothalamus, medulla oblongata, olfactory bulb, SCN, and thalamus). Expression of rCK1ε-1 was detected in every part of the brain and in the pineal body and retina in similar amounts. Localization of rCK1ε-1 protein was also examined immunohistochemically; rCK1ε-1 protein-like immunoreactivity (rCK1ε-1-IR) was observed in all regions of the brain (data not shown). When the antibody was preabsorbed by the antigen, rCK1ε-1-IR disappeared from all brain areas. In the SCN, rCK1ε-1-IR appeared to be concentrated in nuclei of neuron-like cells. Dense CK1ε-1-IR signals were obtained for the dorsomedial region of the middle part of the SCN (Fig. 2c-1) and the ventral and lateral region of the rostral part (Fig. 2c-2).

3.3. rCK1ε-1 phosphorylates and interacts with mPer proteins

To examine whether rCK1ε phosphorylates Per proteins, ectopic expression systems using COS-7 and 293T cells were employed. rCK1ε-1 and HA-tagged mPer1, 2, and 3 were transiently overexpressed in these cells and the phosphorylation state of the mPer proteins was initially assessed by the shift of electrophoretic mobility on an SDS–PAGE gel detected by the immunoblot method. The data obtained using COS-7 cells are shown in Fig. 3. The electrophoretic mobility of each mPer markedly decreased when the active form of rCK1ε-1 (Fig. 3, lanes 4, 7, and 10), but not the KN form (Fig. 3, lanes 5, 8, and 11), was coexpressed with mPer. When the mPer proteins were immunoprecipitated with anti-HA antibody, not only the active form but also the inactive form of rCK1ε-1 was coimmunoprecipitated with the mPer proteins (Fig. 3, lanes 4, 5, 7, 8, 10, and 11), although the mPer3 immunoprecipitate gave only a weak signal for rCK1ε-1 in COS-7 cells (Fig. 3, lanes 10 and 11). Conversely, when rCK1ε-1 was immunoprecipitated, mPer proteins were recovered in the immunoprecipitates with anti-rCK1ε-1 antibody, though apparently to a lesser extent in the case of mPer1 (Fig. 3). These findings indicated that rCK1ε-1 can interact directly or indirectly with the mPer proteins independent of their phosphorylation state, and that the most stable interaction occurs between rCK1ε-1 and mPer2. The results for 293T cells
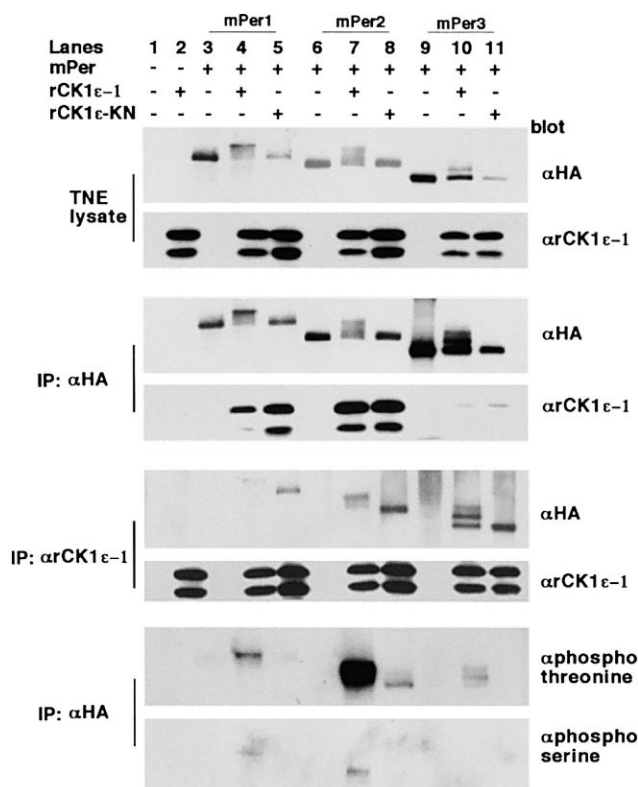

Fig. 3. rCK1ε-1 phosphorylates and interacts with mPer proteins in COS-7 cells. rCK1ε-1 and HA-mPer1, 2, and 3 were transiently overexpressed in COS-7 cells and the phosphorylation state of the mPer proteins was assessed by the shift in the electrophoretic mobility on an SDS–PAGE gel in immunoblot analysis. Samples (TNE lysate of COS-7 cells and its immunoprecipitates with anti-HA (IP: αHA) or anti-rCK1ε-1 (IP: αrCK1ε-1) antibody) were immunoblotted using anti-HA (αHA), anti-rCK1ε-1 (αrCK1ε-1), anti-phosphothreonine (αphosphothreonine) or anti-phosphoserine (αphosphoserine).
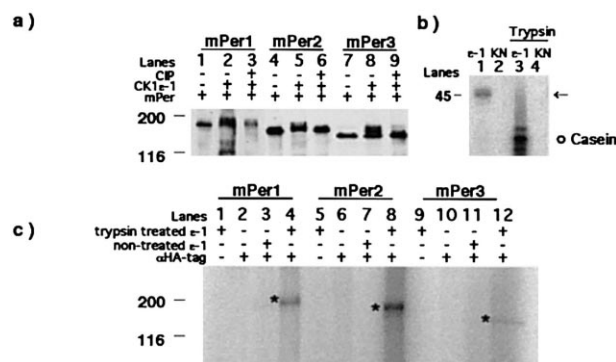
Fig. 4. Gel retardation assay and in vitro kinase assay. a: HA-tagged mPer1, 2 and 3 were immunoprecipitated with anti-HA antibody from COS-7 cells coexpressed with or without rCK1ε-1 and dephosphorylated by alkaline phosphatase (CIP) in vitro (lanes 3 and 6) and analyzed by the immunoblot method. The CIP treatment inhibited gel retardation of mPer proteins. b: Effect of trypsin treatment on casein (open circle) phosphorylation. Bacterially expressed His-tagged rCK1ε-1 (ε-1) and kinase-negative rCK1ε-1 (KN) were purified, treated with trypsin, incubated with dephosphorylated α-casein and [γ-^{32}P]ATP in vitro, and analyzed with the immunoblot method and autoradiography. The autophosphorylation of rCK1ε-1 is showed by an arrow. c: Effect of trypsin treatment on phosphorylation of mPer proteins. Bacterially expressed His-tagged rCK1ε-1 (ε-1) was purified, treated with trypsin, incubated with HA-mPer proteins immunoprecipitated with anti-HA antibody (αHA) and [γ-^{32}P]ATP in vitro, and analyzed by the immunoblot method and autoradiography.

were generally similar to those for COS-7 cells, though the affinity of the mPer proteins to interact with rCK1ε-1 in 293T cells seemed to be higher when the proteins were phosphorylated with the latter (data not shown). The phosphorylation of mPer proteins in COS-7 cells was directly detected by immunoblotting with anti-phosphothreonine and anti-phosphoserine antibodies (Fig. 3, bottom panels). The mPer proteins were found to be phosphorylated at both threonine and serine residues. The most prominent phosphorylation was observed at the threonine residues of mPer2. In addition, treatment of HA-mPer proteins, which were obtained from overexpressed COS-7 cells and immunoprecipitated with anti-rCK1ε-1 antibody, with alkaline phosphatase (CIP) eliminated the shifts of the mPer proteins (Fig. 4a), which suggests the retardation observed in the electrophoresis of the mPer proteins after coexpression of mPer and rCK1ε-1 (shown in Fig. 3) was due to their phosphorylation (Fig. 4a).

3.4. In vitro kinase assay of rCK1ε-1

To confirm the involvement of rCK1ε-1 in the phosphorylation of mPer proteins, in vitro kinase assays were performed using bacterially expressed and partially purified (with Ni-TNA resin) His-tagged rCK1ε-1. The CK1 family shares the conserved kinase domain and has a series of variable C-terminal tails. It is known that CK1ε has a 123-amino acid extension beyond the kinase domain that serves as a pseudosubstrate, inhibiting activity toward exogenous substrates [21]. Thus, prior to the assay, this His-tagged rCK1ε-1 was subjected to limited digestion with trypsin to eliminate the inhibitory C-terminal tail. Using rCK1ε-1 activated by trypsin, we
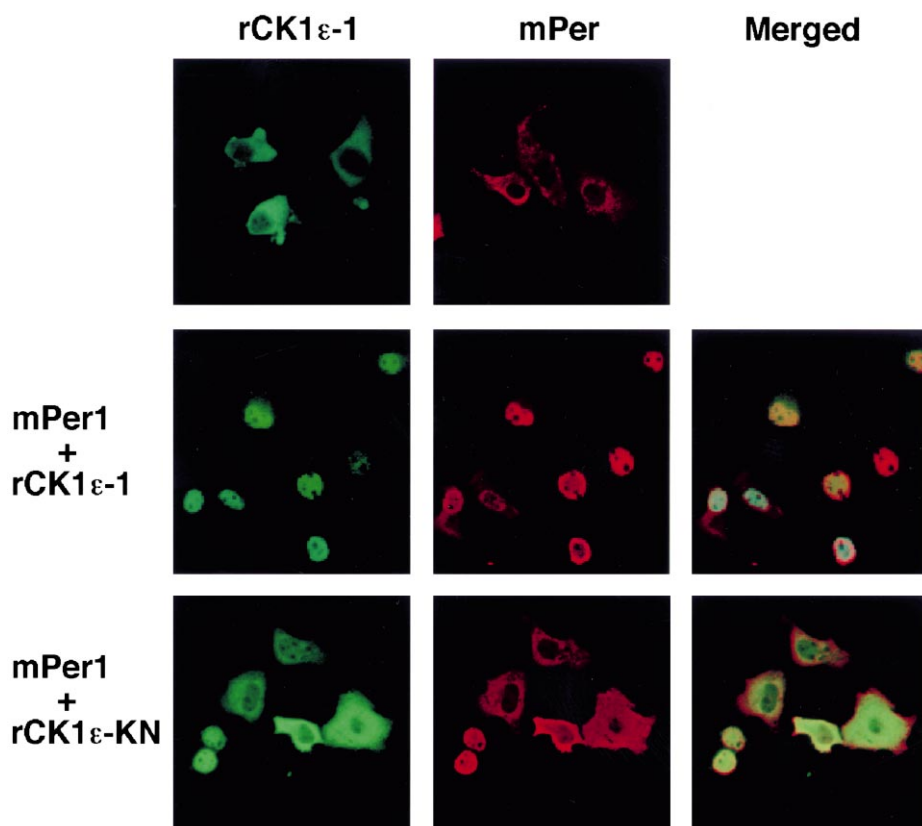

Fig. 5. Effects of coexpression of rCK1ε-1 or kinase-negative rCK1ε (rCK1εKN) with HA-mPer1 protein on the subcellular distribution of each protein studied by immunofluorescence cytochemistry using anti-rCK1ε-1 (green) or anti-HA (red) antibody in COS-7 cells.

Fig. 6. Effects of coexpression of rCK1ε-1 with HA-mPer2 or 3 proteins on the subcellular distribution of each protein studied by immunofluorescence cytochemistry using anti-rCK1ε-1 (green) or anti-HA (red) antibody in COS-7 cells.
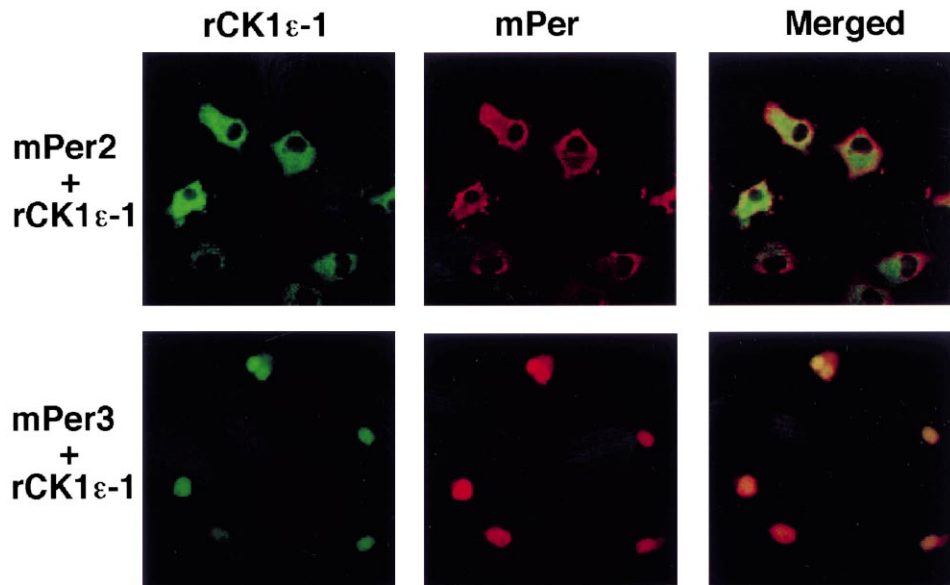
examined whether it phosphorylated mPer1, 2, and 3 in vitro. Before doing this experiment, we examined the effect of the trypsin treatment on the phosphorylation of α-casein. As seen in Fig. 4b, before the treatment autophosphorylation of rCK1ε-1, but not phosphorylation of α-casein, was observed (lane 1). However, after the treatment phosphorylation of α-casein was observed (Fig. 4b, lane 3). Furthermore, when His-CK1-KN was incubated, neither autophosphorylation nor the phosphorylation of α-casein was observed before and after the trypsin treatment (Fig. 4b, lanes 2 and 4). Then, we examined the phosphorylating activity of rCK1ε-1 on the HA-mPer proteins. The full-length mPer proteins expressed in COS-7 cells were immunoprecipitated with anti-HA antibody and used as substrates for the kinase assay. The results showed that each mPer was phosphorylated by rCK1ε-1 treated with trypsin, but not by non-treated rCK1ε-1 (Fig. 4c, lanes 4, 8, and 12). We repeated the last experiment three times, but could get only minute amounts of mPer proteins by immunoprecipitation using anti-HA antibody. Therefore, we did not succeed in determining the protein contents of mPers. Thus, we could not calculate the stoichiometry of phosphorylations. However, this stoichiometry study is important to give some clue to the answer to the question whether rCK1ε-1 can really phosphorylate mPer proteins in the clock neurons in the SCN. This must be clarified in future.

3.5. CK1ε-1 can translocate mPer proteins into the nucleus

Finally, the effect of coexpressing rCK1ε-1 with mPer proteins on the subcellular distribution of each protein was studied by immunofluorescence cytochemistry in COS-7 cells (Figs. 5 and 6). When rCK1ε-1 and mPer proteins were independently overexpressed, almost all the immunostaining of each protein was observed in the cytoplasm (Fig. 5), though a faint signal for mPer1 was occasionally recognizable in the nucleus. When mPer1 or mPer3 was coexpressed with rCK1ε-1, in both cases the majority of the immunostaining was found in the nucleus (Figs. 5 and 6). In contrast, coexpression of mPer2 with rCK1ε-1 did not affect the cytoplasmic

distribution of either protein (Fig. 6). Furthermore, coexpression of the mPers with CK1-KN could not translocate mPer1 (Fig. 5) or mPer3 (data not shown). In any event, it appears that the rCK1ε-1 and mPer proteins always exhibit the same localization pattern in the cells.

4. Discussion

4.1. Isolation of rCK1ε and its splicing variants

When rCK1ε was cloned from a rat hypothalamus cDNA library, at least three forms of rCK1ε were found to be potentially expressed in the rat brain. The expression of four alternatively spliced variants of CK1α has been reported in chicken tissues [22,23]. However, the existence of CK1ε splicing variants has not hitherto been reported. Rat CK1ε-1 shows 99.3% amino acid identity with hCK1ε, suggesting that it may be the dominant form of rCK1ε. The CK1ε isoforms found in this study contain an identical kinase domain and variable C-terminal tails. Since the kinase activity of hCK1ε is known to be autoregulated by the C-terminal tail, there are likely to be functional differences among the rCK1ε variants. We attempted to verify this by comparing their substrate specificities. However, we have so far been unable to detect any kinase activity for CK1ε-2 and CK1ε-3.

4.2. Expression of rCK1ε-1 protein in the rat brain

It has previously been reported that CK1ε is widely expressed in rat tissues, including the brain, testis, heart, liver, and spleen, and that it is localized in both nuclear and cytosolic fractions [24]. However, we observed that the expression of rCK1ε-1 was highly restricted to the brain and was localized preferentially in the nuclear fraction. A possible reason for the apparent discrepancy could be that the CK1ε detected in the previous work [24] might have been due to cross-reactivity of the antibody with a conserved region of the CK1ε family. It is noteworthy that in the brain sections examined in the present study, rCK1ε-1-IR was observed in the SCN, the master circadian oscillator.

4.3. rCK1ε-1 protein does not appear to oscillate in the SCN

When primers specific for CK1ε were used to detect differential expression by RT-PCR, a significant though subtle difference in expression between CT6 and CT18 was discerned (data not shown). In view of this, CK1ε was at first identified as a gene expressed in a circadian manner in the SCN. However, further analysis using a specific antibody against rCK1ε-1 failed to show a significant daily change in the expression of rCK1ε-1 protein under either LD or DD conditions (data not shown). This result was consistent with the absence of a daily rhythm in the expression of dbt in the *Drosophila* head [15]. Therefore, it is more likely that the expression of rCK1ε-1 does not necessarily oscillate in a circadian manner in mammals.

4.4. rCK1ε-1 phosphorylates and binds to mPer proteins

The transfection experiments revealed that rCK1ε-1 could be involved in the phosphorylation of mPer1, 2, and 3 in vivo (Fig. 3). Purified rCK1ε-1 was also capable of phosphorylating purified mPer proteins in vitro (Fig. 4). These findings strongly suggest that rCK1ε-1 directly phosphorylates mPer proteins in the cells. Moreover, interaction between rCK1ε-1 and the mPer proteins was clearly detected by the immunoprecipitation assay from the cotransfected cells (Fig. 3). Since the kinase-negative mutant of rCK1ε-1 used in this experiment was also able to associate with the mPer proteins to an extent similar to that of the active rCK1ε-1 in the case of COS-7 cells (Fig. 3), it seems that the interaction is not dependent on the phosphorylation of the mPer proteins. In the immunoprecipitation assay, the three mPer proteins exhibited some quantitative differences in their interaction efficiencies. Although a difference in protein stability in the cells may affect the efficiency, mPer2 appears to have the most stable interaction with rCK1ε-1. To further evaluate the interaction differences, competitive experiments among the mPer proteins need to be carried out.

4.5. Nuclear translocation of mPer through phosphorylation by rCK1ε-1

When overexpressed in COS-7 cells, mPer proteins were mainly distributed in the cytoplasm, though some nuclear localization of mPer1 was occasionally observed (Fig. 5). Co-expression with the active rCK1ε-1, but not with the kinase-inactive form, efficiently promoted the nuclear translocation of mPer1 and mPer3 (Figs. 5 and 6), suggesting that the phosphorylation of these proteins by rCK1ε-1 is required for their nuclear translocation. In the case of mPer2, however, the contribution of CK1ε-1 is not sufficient for nuclear translocation. Complex formation with another mPer might thus be necessary for mPer2 to be translocated to the nucleus. Whatever the case, it is clear that the rCK1ε-1 and mPer proteins are always colocalized in the cells, showing the intimate relationship between them.

It has been reported that mPer proteins interact with each other, and that mPer3 can serve as a carrier of mPer1 and mPer2 to bring them into the nucleus [9]. This nuclear entry is supposed to be critical for the generation of circadian signals in the SCN. However, the relationship among these mechanisms, including the rCK1ε-1-mediated mechanism presented in this paper, is currently unknown.

When overexpressed in COS-7 cells, rCK1ε-1 was diffusely distributed in the cytoplasm, which is apparently inconsistent with the nuclear localization of rCK1ε-1 observed in brain tissue. This discrepancy can, however, be explained by the fact that COS-7 cells lack factors required for the nuclear localization of rCK1ε-1, such as mPer1 and mPer2. In this regard, it can be hypothesized that mPer1 and mPer3 could serve as carriers of rCK1ε-1 into the nucleus. The classical functions of CK1ε include the regulation of DNA replication, DNA repair, and nuclear trafficking, all of which are related to nuclear functions [25–28]. It is therefore possible that mPer1 and/or mPer3 is involved in the mammalian circadian rhythm mechanism through regulating the function of rCK1ε-1.

4.6. rCK1ε-1 as a component of the mammalian circadian clock

In this study, we have presented a line of evidence supporting the proposition that rCK1ε-1 phosphorylates mPer proteins and promotes the nuclear translocation of at least mPer1 and mPer3. Currently, the basic system of the mammalian circadian clock is thought to involve a negative feedback loop comprised of the following steps: (1) activation of mPer and mCry expression by the CLOCK-BMAL1 complex through the E-boxes of the promoter regions of the *mper* and *mcry* genes; (2) accumulation of mPer and mCry proteins in the cytoplasm; (3) nuclear translocation of mPer and mCry proteins; and (4) inhibition of the CLOCK-BMAL1 function by mPer, mCry, or an mPer–mCry complex [9]. In this loop, rCK1ε-1 may be located upstream of the nuclear entry of mPer. To determine whether this is the case, loss-of-function and gain-of-function studies in the mammalian system will have to be conducted.

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