View metadata, citation and similar papers at core.ac.uk
brought to you by CORE
provided by Elsevier - Publisher Connector

Human casein kinase Iδ phosphorylation of human circadian clock proteins period 1 and 2

F. Camacho[a,b], M. Cilio[a,b], Y. Guo[a,b], D.M. Virshup[a,b], K. Patel[a,b], O. Khorkova[a,b], S. Styren[a,b], B. Morse[a,b], Z. Yao[a,b], G.A. Keesler[a,b,*]

[a] *CNS Molecular Biology and Genomics, Cell Biology and Neuropathology, and Immunology, Aventis Pharmaceuticals Inc., Bridgewater, NJ 08807, USA*
[b] *Department of Oncological Science and Department of Pediatrics, University of Utah, Salt Lake City, UT 84132, USA*

Received 20 November 2000; revised 17 December 2000; accepted 18 December 2000

First published online 15 January 2001

Edited by Shmuel Shaltiel

Abstract **Casein kinase Iε (CKIε), a central component of the circadian clock, interacts with and phosphorylates human period protein 1 (hPER1) [Keesler, G.A. et al. (2000) NeuroReport 5, 951–955]. A mutation in CKIε causes a shortened circadian period in Syrian Golden hamster. We have now extended our previous studies to show that human casein kinase Iδ (hCKIδ), the closest homologue to hCKIε, associates with and phosphorylates hPER1 and causes protein instability. Furthermore, we observed that both hCKIδ and hCKIε phosphorylated and caused protein instability of human period 2 protein (hPER2). Immunohistochemical staining of rat brains demonstrates that CKIδ protein is localized in the suprachiasmatic nuclei, the central location of the master clock. These results indicate that CKIδ may play a role similar to CKIε, suggesting that it may also be involved in regulating circadian rhythmicity by post-translation modification of mammalian clock proteins hPER1 and 2.** **© 2001 Federation of European Biochemical Societies. Published by Elsevier Science B.V. All rights reserved.**

Key words: Casein kinase; Period; Circadian rhythm; Phosphorylation

1. Introduction

Circadian rhythms are present in a diverse number of organisms including mammals, fungi, plants, insects, and cyanobacterium [2–4]. The control of biochemical, physiological, and behavioral processes is regulated by endogenous biological clocks whose components are relatively conserved among these organisms [5,6]. In mammals, the suprachiasmatic nucleus (SCN) is the site of the central pacemaker or 'gears' of the clock, and is responsible for resetting and controlling the clocks within the peripheral tissues [7–9]. One important component within the central clock is the period (PER) proteins whose function is critical in maintaining constant daily rhythms.

In mammals, three PER proteins have been identified: PER1, 2 and 3. All PER mRNAs and protein levels oscillate during the circadian day and are intimately involved in both the positive and negative regulation of the biological clock [10–12]. The function of PER3 is poorly understood; however, *mPer3* knockout mice have a subtle effect on circadian activity, and therefore have been suggested to be involved in the circadian controlled output pathways [13]. PER2 is the most well-characterized protein, and mPER2 mutant mice (*mPer2*^{brdm1}), lacking 87 residues at the carboxyl portion of the PAS dimerization domain, have a shortened circadian cycle in normal light–dark settings, but show arrhythmicity in complete darkness [14]. Shearman et al. [15] have shown that PER2 has a dual function in the regulation of the 'gears' of the central clock. In their study, PER2 binds to cryptochrome (CRY) proteins and translocates to the nucleus where CRY negatively regulates transcription driven by CLOCK and BMAL1 positive transcriptional complexes. Upon nuclear entry, PER2 initiates the positive arm of the clock by positively regulating BMAL1 transcription by a yet unidentified mechanism. PER1 is believed to be involved in the negative regulation of its own transcription in the feedback loop, but recent evidence points to it being involved in the input pathway [16]. In cell culture models, PER1 protein levels are also regulated post-translationally. PER1 protein phosphorylation leads to a decrease in cellular PER1 protein levels presumably through enhanced protein degradation rates [1,17].

To date, only one kinase has been discovered as a central clock component [18]. Young and colleagues first identified a kinase-like *Drosophila* protein, doubletime (DBT) that is thought to phosphorylate *Drosophila* PER [19,20]. Missense mutations in *dbt* result in an altered circadian rhythm. Null alleles of *dbt* result in hypophosphorylation of dPER and arrhythmia. The mammalian kinases most closely related to DBT are casein kinase I (CKI)ε and CKIδ. Both kinases have been shown to bind to mPER1, and several studies have shown that CKIε phosphorylates both mouse and human PER1 [19,20]. The consequence of PER1 phosphorylation by hCKIε includes both cytoplasmic retention and protein instability [17,21]. There has been no biochemical reason to choose between CKIε or CKIδ as a potential regulator in mammals until Lowery et al. [18] found that in the Syrian Golden hamster, semidominant mutations in CKIε (*tau*) caused a shortened circadian day in both heterozygous (22 h) and homozygous animals (20 h) [22]. In this instance, reduced levels of CKIε activity resulted in less PER phosphorylation with presumably higher levels of cytoplasmic PER protein leading to enhanced nuclear entry and altered circadian cycles.

Of the seven identified CKI isoforms, CKIδ is the closest homologue of CKIε. The kinase domains of both of these

*Corresponding author. Fax: (1)-908-231 4335.
E-mail: george.keesler@aventis.com

0014-5793/01/$20.00 © 2001 Federation of European Biochemical Societies. Published by Elsevier Science B.V. All rights reserved.
PII: S0014-5793(00)02434-0

proteins are 97% identical with only eight amino acid changes [23,24]. While the identification of mutant CKIε in the *tau* mutant hamster suggests CKIε is the important circadian regulator, an alternative hypothesis is that the *tau* mutation renders CKIε a dominant interfering kinase that may block the function of CKIε and CKIδ in the SCN. In this study, we set out to determine if hCKIδ was capable of playing a similar role as hCKIε in the phosphorylation of human clock proteins PER1 and 2.

2. Materials and methods

2.1. Plasmids

Mammalian expression vectors pCEP4-CKIε and pCEP4-CKIδ were constructed as described [24]. Full-length hPER1 kindly provided by Dr. Y. Sakaki [25] was ligated into plasmid vector pEYFP (Clonetech, Palo Alto, CA, USA) to create an in-frame fusion with the YFP-tag. Full-length open reading frame (ORF) hPER2 was cloned by PCR from a human brain cDNA library from Clonetech using the forward primer ATCTAGATCTAGAATGAATGGATACGCGGAATTTCCG and the reverse primer TCTGCTCGAGTCAAGGGGGATCCATTTTCGTCTT. The ORF encodes a 1246 amino acid protein. The DNA was subcloned into the pEYFP living color vector (Clonetech) creating a hPER2–N-terminal YFP fusion protein. All constructs were confirmed by DNA sequence analysis.

2.2. Transfection and radiolabeling of HEK 293T cells

Human embryonic kidney cells HEK 293T were cultured and transfected via lipofectamine plus (Gibco BRL Life Technologies, Rockville, MD, USA) according to the manufacturer's protocols. HEK 293T cells were radiolabeled 16 h post-transfection with 0.5 mCi/ml [^{35}S]methionine/cysteine (NEN, Boston, MA, USA) for 30 min in methionine and cysteine deficient media. Thereafter, cells were washed and lysates were prepared as described [1].

2.3. Immunoprecipitations and Western blot analysis

Lysates containing equal amounts of protein (100 mg total) were mixed with 5 μl of anti-YFP, anti-hematoxylin (HA), or anti-c-myc monoclonal antibody (mAb) and immunoprecipitated. Western blotting of proteins was performed using either anti-YFP at a 1:1000 dilution, anti-HA at 1:1000 dilution, or anti-c-myc at a 1:1000 dilution (Santa Cruz Biotech, Santa Cruz, CA, USA). Secondary antibodies (Gibco BRL Life Technologies) anti-rabbit horseradish peroxidase (HRP) were used at a 1:5000 dilution.

2.4. Immunohistochemistry

Male Sprague Dawley rats weighing 250–350 g were housed in plastic cages with free access to food and water, and a 12 h light–12 h dark cycle. Rats were anesthetized with chloral hydrate and were perfused through the heart with 200 ml saline, followed by 500 ml of 4% formaldehyde (phosphate-buffered saline (PBS) 0.01 M, pH 7.4). The brains were post-fixed overnight and cryoprotected in 30% sucrose in phosphate buffer prior to being frozen and sectioned to 30 μm in the coronal plane. Free-floating sections were incubated in 0.5% H_2O_2 for 30 min then washed in PBS (3×5 min) and blocked for 1 h in 10% mouse serum. The tissue was then incubated in anti-rat CKIδ antibody 1:500 dilution (R19 Santa Cruz Biotech) for 48 h at 4°C in buffer containing the primary antibody plus 2% mouse serum. Sections were rinsed in PBS, then incubated in biotin-labeled mouse anti-goat (1:1000 dilution) (Jackson Labs, Bar Harbor, ME, USA) for 1 h. The tissue was then incubated in avidin–HRP conjugate from the Vectastain Elite ABC kit (Vector Laboratories, Burlingame, CA, USA) according to the manufacturer's instructions. CKIδ immunoreactivity was visualized by incubating the avidin–HRP with H_2O_2 catalyzed 3,3′-diaminobenzidine-4HCL localization. Sections were mounted on gel-subbed slides and allowed to air dry prior to being dehydrated in a graded series of alcohols to xylene and then coverslipped. Selected sections were counter-stained with HA prior to being coverslipped. The tissue was evaluated on an Olympus AX-70 microscope and captured with a Sony STU-5 digital camera employing a Media Cybernetics Pro-Series 128 bit frame grabber board. Images were then transferred to a Dell Optiplex GX300 workstation utilizing Image ProPlus 4.0 image software.

3. Results

3.1. hCKIδ phosphorylates hPER1 and hPER2

To determine whether hCKIδ could phosphorylate hPER1, YFP-tagged hPER1 expression plasmid was co-transfected into HEK 293T cells with either hCKIδ or vector control. Co-transfection of hCKIδ with hPER1 resulted in a significant shift in molecular mass of hPER1 proteins as compared to hPER1 co-transfected with vector alone as demonstrated by Western analysis (Fig. 1, lanes 2 and 4). The change in molecular mass of hPER1 after co-transfection with hCKIδ is most likely due to a post-translation modification by phosphorylation for two reasons. The first is that co-transfected cells incubated with [^{32}P]ATP showed significant incorporation of radioactive label in the hPER proteins, and second samples treated with lambda phosphatase reduced the gel shift to control levels (data not shown).

We have previously found that hCKIε phosphorylates hPER1 in co-transfected HEK 293T cells (see Fig. 1, lane 3). We therefore sought to determine if both hCKIδ or hCKIε could phosphorylate another clock protein, hPER2. YFP-tagged hPER2 expression plasmid was co-transfected into HEK 293T cells with either vector control, hCKIδ or hCKIε. As shown in Fig. 1, cells co-transfected with hPER2 and hCKIδ or hCKIε show a shift molecular mass of hPER2 protein that is not apparent in the hPER2 singly transfected cells (Fig. 1, lanes 5–7), indicating that both kinases could phosphorylate hPER2.

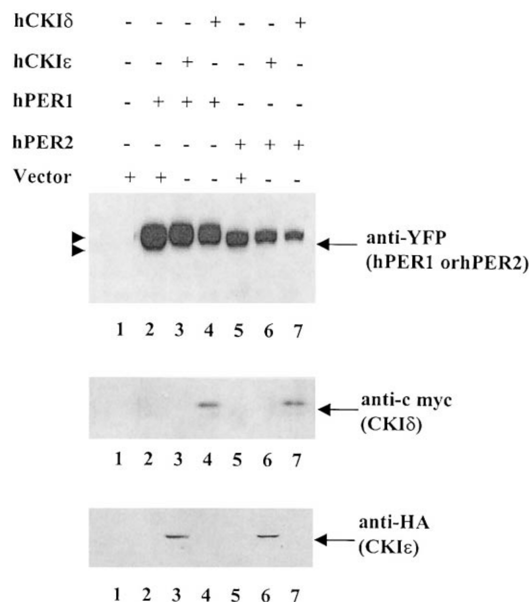

Fig. 1. Phosphorylation of hPER1 and hPER2 by recombinant hCKIδ or hCKIε in transfected cells, and Western blot analysis of hPER1, hPER2, hCKIδ, and hCKIε. HEK 293T cells were transfected with vector alone or with hPER1 or hPER2 (lanes 1, 2, and 5), hPER1 and hCKIδ (lane 4), hPER2 and hCKIδ (lane 7), or hPER1 and hCKIε (lane 3), or hPER2 and hCKIε (lane 6). At 16 h post-transfection, cells were harvested and lysates were prepared as described in Section 2. 20 mg of total protein from HEK 293T lysates was loaded onto a 3–8% gradient SDS–PAGE. Proteins were transferred to PVDF membranes and Western-blotted using the anti-YFP mAb (1:1000), or anti-c-myc (hCKIδ) mAb (1:1000), or anti-HA (hCKIε) mAb (1:1000).

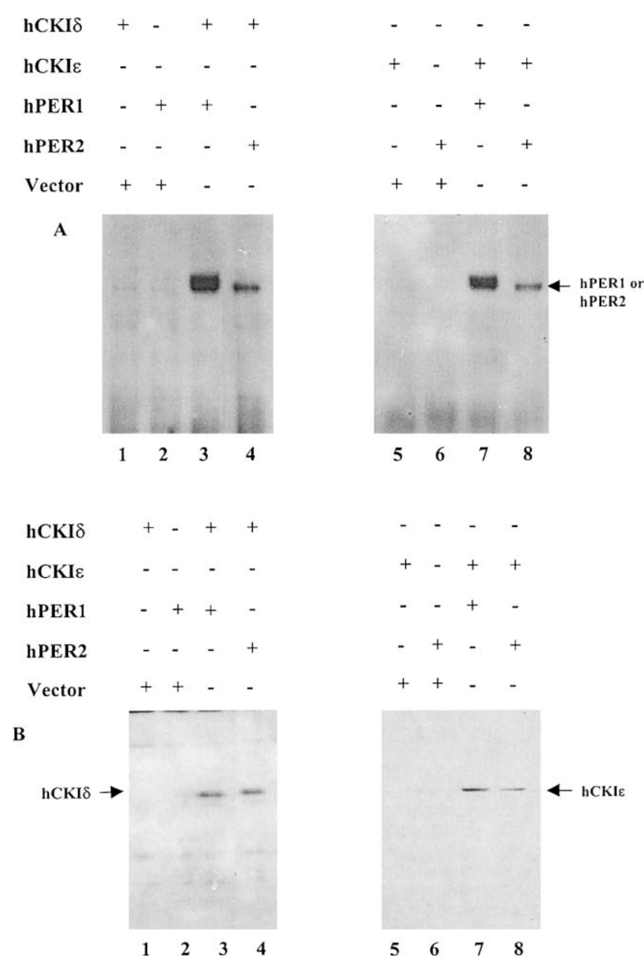

Fig. 2. Protein–protein interaction between hPER1, hPER2, and hCKIδ, or hCKIε. HEK 293T cells were transfected with vector and hCKIδ (lane 1), vector and hCKIε (lane 5), vector and hPER1 (lane 2), vector and hPER2 (lane 6) or hCKIδ and hPER1 (lane 3), hCKIδ and hPER2 (lane 4), hCKIε and hPER1 (lane 7), or hCKIε and hPER2 (lane 8). 16 h post-transfection, cells were harvested and lysates were prepared. Lysates were immunoprecipitated with either anti-c-myc mAb (upper panel, lanes 1–4), anti-HA mAb (upper panel, lanes 5–8), or with anti-YFP mAb (lower panels, lanes 1–8) and Western-blotted with anti-YFP mAb (upper panels, lanes 1–8), anti-c-myc mAb (lower panel, lanes 1–4), or anti-HA mAb (lower panel, lanes 5–8). All proteins were resolved on a 12% SDS–PAGE.

3.2. *hCKIδ physically associates with hPER1 and hPER2 in HEK 293T cells*

Because hCKIδ can phosphorylate hPER1 and hPER2 proteins, we next asked if hCKIδ associated or interacted with hPER1 and hPER2 in co-transfected cells. To determine this, we expressed c-myc-tagged hCKIδ, YFP-hPER1, YFP-hPER2, or vector control in HEK 293T cells. HA-tagged hCKIε expression plasmid was used as a positive control. Cells were lysed and either immunoprecipitated with anti-c-myc mAb (hCKIδ) or anti-HA mAb (hCKIε) and Western-blotted with either anti-YFP mAb, and in the reciprocal experiment immunoprecipitated with anti-YFP mAb (hPER) and Western-blotted with anti-c-myc or anti-HA mAb. As shown in Fig. 2, immunoprecipitating with anti-c-myc mAb and Western blotting with anti-YFP mAb revealed the appearance of hPER1 and 2 proteins (lanes 3 and 4; upper panels). Identical results were found for hCKIε by immunoprecipitating with anti-HA mAb (lanes 7 and 8; upper panels). Furthermore, the reciprocal experiment of immunoprecipitating with anti-YFP mAb followed by Western blotting with anti-c-myc mAb or anti-HA mAb shows hCKIδ and hCKIε protein (lanes 3, 4, 7, and 8; lower panels). These data indicate that hCKIδ interacts with both hPER1 and 2 in co-transfected cells.

3.3. *Phosphorylation of hPERs by hCKIδ leads to hPER protein destabilization*

It has been postulated that accumulation of PER protein could result in both a negative and positive effect on the central clock and that phosphorylation of PER might be a key post-translational event in regulating the PER protein levels. We therefore determined whether hCKIδ phosphorylation of hPER1 and hPER2 affected their protein stability. HEK 293T cells were co-transfected with expression plasmids encoding either hPER1 or hPER2 alone or hPER1 and hPER2 together and with or without hCKIδ, or vector control. HA-tagged hCKIε expression plasmid was used as a positive control. 16 h post-transfection, cells were pulse-labeled with [^{35}S]methionine and chased for the times indicated. As expected, cells co-transfected with hPER1 or hPER2 and vector showed a gradual minimal shift in electrophoretic mobility and the protein levels remained relatively constant throughout the time course (Fig. 3A,D, lanes 1–7). At later time points (16 and 24 h) both hPER1 and hPER2 showed a minimal shift in their molecular masses with the shift in hPER1 being more apparent. This is most likely due to phosphorylation by either endogenous CKI and/or as of yet unidentified additional kinase(s). However, in cells co-transfected with hCKIδ, hPER1 or hPER2 showed a rapid mobility shift as early as 4 h post-pulse, and reached a maximum between 8 and 16 h (Fig. 3B,E). Half-life estimates of hPER1 protein showed that in the vector control approximately 50% of the protein remained in the cells at the 24 h time point (Fig. 3A). Conversely, hPER1 protein levels after phosphorylation with hCKIδ revealed that only 50% of the protein remained after 8 h and less than 20% remained after 24 h and similar to our previously reported results with hCKIε (Fig. 3B,C) [1]. It is interesting to note that the hPER1 mobility shift was more pronounced than that of hPER2 suggesting that hPER1 is phosphorylated to a greater extent than hPER2 (Fig. 3B,E). It also appears that hPER2 is slightly more stable than hPER1 after phosphorylation by hCKIδ (Fig. 3B,E). The estimated half-life of hPER2 protein after phosphorylation with hCKIδ was similar to the vector control after 16 h in which approximately 50% of the total protein is present. The significant differences in hPER2 protein levels were only apparent at the 24 h time point in which 50% of the protein remains in the vector control and less than 25% of the protein remains after phosphorylation with hCKIδ (Fig. 3D,E). Similar results were found when hCKIε was co-transfected in replace of hCKIδ with either hPER1 or hPER2 (Fig. 3C,F).

PER1 and PER2 have been shown to form heterodimers when co-expressed. However, there appears to be no advantage with regard to hPER protein stability after phosphorylation by hCKIδ when both hPER1 and 2 are co-expressed. As shown in Fig. 3G,H, co-transfection of hPER1 and hPER2 in the presence of hCKIδ also led to degradation of the hPER proteins, and in particular hPER1, with an estimated half-life

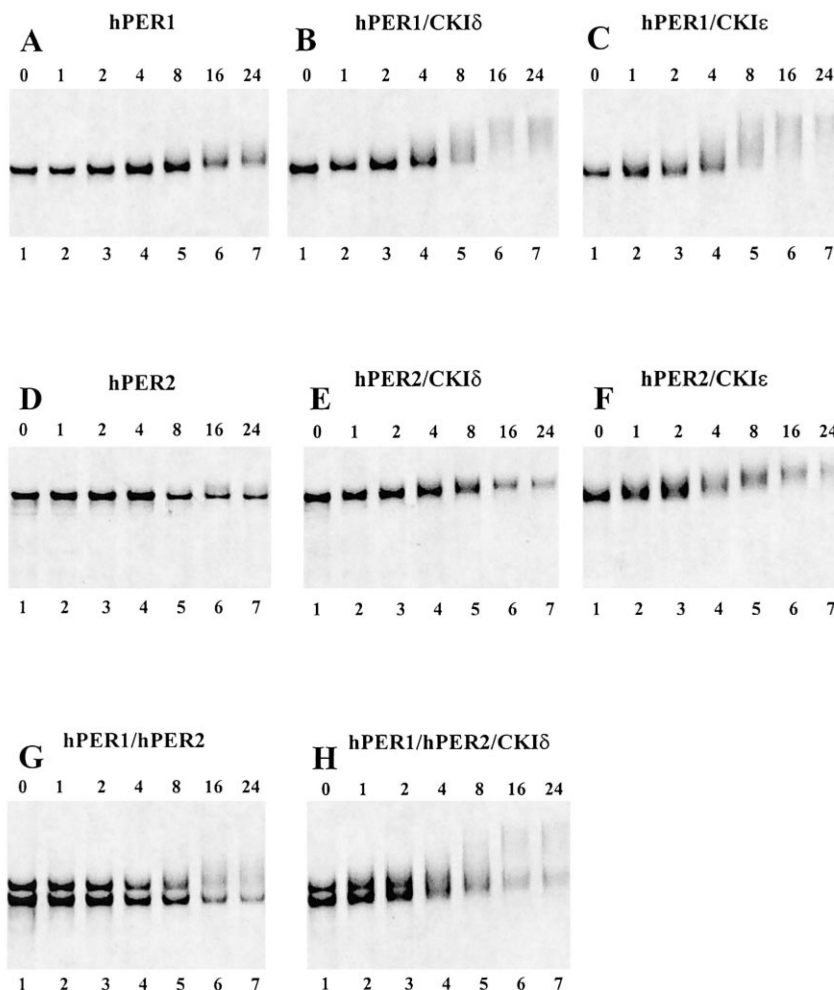

Fig. 3. Pulse-chase labeling of hPER1 and hPER2 co-transfected with hCKIδ, hCKIε, or vector. HEK 293T cells were co-transfected with either hPER1 and vector (A), hPER1 and hCKIδ (B), hPER1 and hCKIε (C), hPER2 and vector (D), hPER2 and hCKIδ (E), hPER2 and hCKIε (F), hPER1, hPER2 and vector (G), or hPER1, hPER2, and hCKIδ (H). 16 h post-transfection, HEK 293T cells were pulse-labeled with [^{35}S]methionine and cysteine (500 mCi/ml) for 30 min and then chased for the times (h) indicated at the top of each gel. Cells were lysed and immunoprecipitated with anti-YFP mAb and the hPER proteins were resolved on 8% SDS–PAGE.

similar to either hPER expressed singly in the presence of kinase (Fig. 3B,E,H).

3.4. *CKIδ protein is expressed in the SCN*

Given that the central clock is localized in the SCN, and CKIδ can phosphorylate and associate with the clock components hPER1 and 2, we determined whether CKIδ is expressed in SCN. To this end, we performed immunohistochemical studies on coronal sections of rat brain using a CKIδ specific polyclonal antibody. As shown in Fig. 4, examination of coronal sections containing the SCN demonstrated intense, localized immunoreactivity of CKIδ protein within this well-defined region as well as in both cortical and subcortical neurons (Fig. 4A). Higher magnification (Fig. 4A, inset) of this region showed that virtually all cells within the SCN stained positive for CKIδ protein. Examination of SCN at higher magnification (Fig. 4B) demonstrated that immunoreactivity was predominantly localized to nuclear profiles; although weak immunoreactivity could be observed in cytoplasm and to a lesser extent in neuronal processes. Based on the broad expression of CKIδ immunoreactivity observed in rat brain, we examined the localization of this protein in hippocampus (Fig. 4C). As in the SCN, CKIδ protein was localized predominantly to neuronal populations and was essentially absent in neuron free regions such as dentate gyrus molecular layer and corpus callosum. Immunoreactivity was most apparent in the ventral leaf of the dentate gyrus (VLDG) granule cell layer, although weak expression of CKIδ protein was also observed in the dorsal leaf of the dentate gyrus (DLDG) granule cell layer, subiculum and hippocampal pyramidal cell layer. Examination of neurons of the VLDG granule cell layer revealed that CKIδ protein was not present in nuclear profiles, but was instead localized to peri-nuclear cytoplasm (Fig. 4C, inset). This differential subcellular localization within neurons of the dentate gyrus and the SCN was consistently observed in all animals examined.

4. Discussion

4.1. *Human PER1 and PER2 are substrates for hCKIδ*

The role of CKI in the regulation of circadian rhythms is becoming increasingly apparent. Phosphorylation of PER

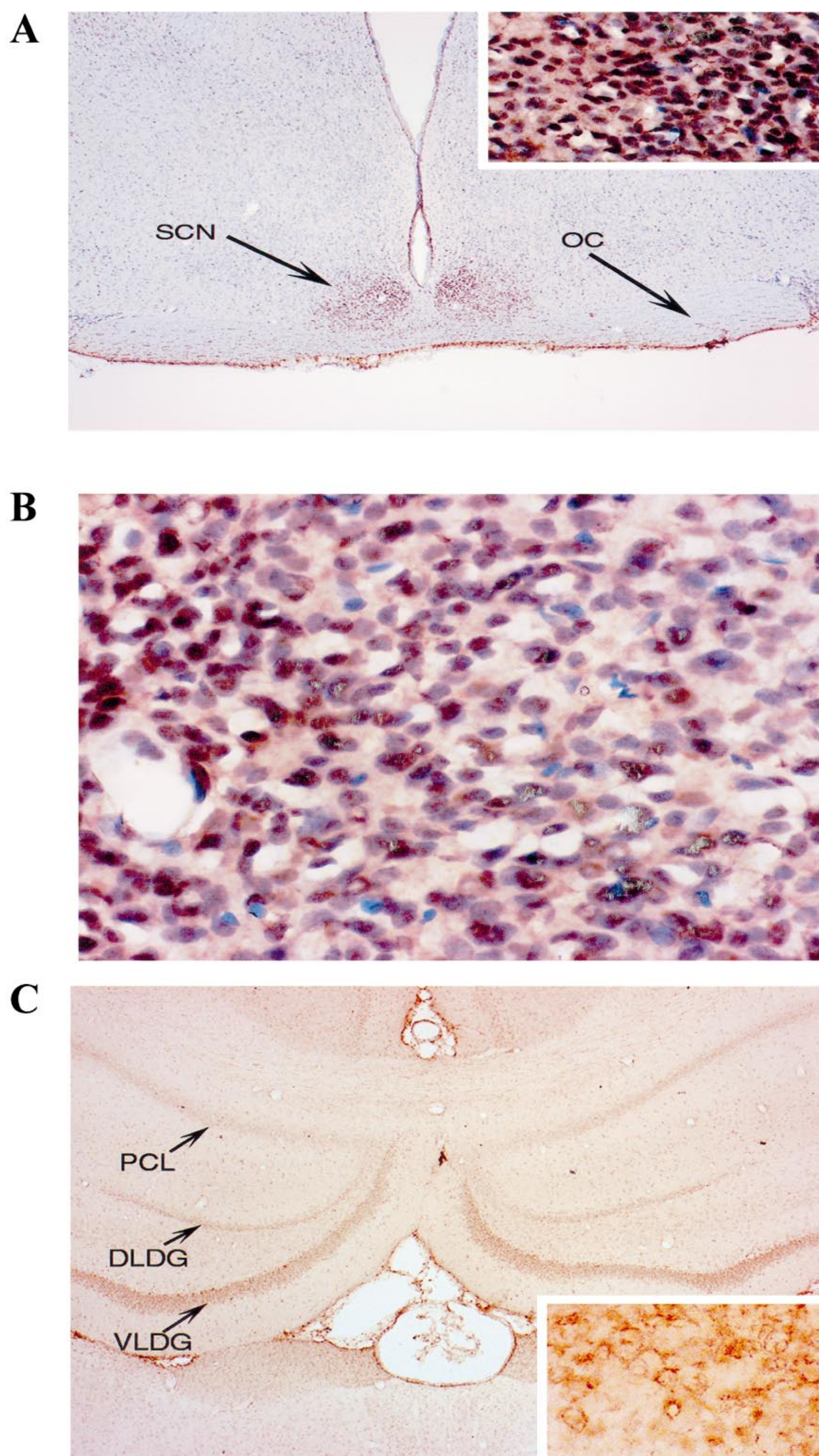

Fig. 4. Immunohistochemical staining for CKIδ in rat brain cross sectional samples. A: Rat brain coronal sections (15× magnification) containing the SCN showing positive staining CKIδ in the SCN and optic chiasm (OC), insert is 100× higher magnification of SCN cells. B: 100× magnification of rat brain coronal sections showing SCN neurons staining for CKIδ. Note the appearance of CKIδ nuclear localization within the SCN neurons. C: Rat brain cross sectional staining showing CKIδ immunoreactivity in dentate gyrus (VLDG, DLDG), and hippocampal pyramidal layers (PCL). Insert is 100× magnification showing staining in the peri-nuclear space of the neurons.

proteins by CKI(s) in the cell appears to be a key event in both the positive and negative regulation of the cycle [1,17,18,21]. As the circadian day progresses, PER protein accumulates and becomes phosphorylated and degraded; therefore, any perturbation of the phosphorylation event may alter the cellular levels of PER and change the homeostasis of the circadian cycle. A recent report by Lowery et al. [18] demonstrates this elegantly in the identification of the *tau*

mutant as a CKIε defect. Although CKIε is active in the *tau* mutant, it has less than 10% of wild-type activity suggesting that phosphorylation of PER proteins is perturbed leading to altered cellular PER protein levels and localization. In our own circadian mathematical modeling studies, it was determined that slight alterations of kinase activity resulting in decreased PER phosphorylation resulted in extreme changes (a shortening) in the circadian daily cycle [26].

Our previous findings demonstrated that hCKIε and hPER1 interact, hCKIε could phosphorylate hPER1, and that the consequence of hPER1 phosphorylation is destabilization of hPER1 protein [1]. This study furthers these observations, demonstrating that hCKIδ can interact with and phosphorylate hPER1 and hPER2. This is not unexpected due to the close homology between hCKIε and hCKIδ in the kinase domain. Perhaps the phosphorylation of PERs is a general signaling mechanism to target the proteins for degradation. Although Vielhaber et al. found that phosphorylation of mPER1 by hCKIε causes cytoplasmic retention in HEK 293T cells, we have found no evidence for that to be the case with hPER2 (unpublished observation), nor have we found that hPER phosphorylation alters hetero- or homodimer formation between the PERs.

4.2. *hCKIδ and hCKIε may have redundant roles in PER post-translational regulation*

If PER phosphorylation is a mechanism of regulation, and since PER protein levels oscillate during the day, then how does CKI become active at the appropriate time during the day and phosphorylate PER? hCKIε and hCKIδ are constitutively active kinases; therefore, a simple explanation is that PER phosphorylation occurs when intracellular protein levels reach a critical mass for enzyme substrate interaction. Another explanation is that hCKIε or hCKIδ mRNA levels may oscillate during the day similar to hPER1, 2 and 3 but are delayed by several hours. As PER protein levels begin to accumulate, CKI protein levels increase which results in the phosphorylation of PER. Although, Lowery et al. [18] found no evidence for CKIε oscillation and *dbt* mRNA levels do not fluctuate [19]. However, we are currently exploring this hypothesis.

Another question that remains is the possible redundant roles of CKIε and CKIδ in the phosphorylation of PERs. The data by Takahashi and colleagues [18] demonstrate that a hypomorphic allele of CKIε can alter circadian rhythm and those results may render CKIδ as having a minor role in circadian biology. An alternative hypothesis is that the *tau* mutant CKIε acts as a dominant negative kinase that binds to the PER proteins, fails to phosphorylate them, but also prevents other kinases, in particular CKIδ, from efficiently phosphorylating the bound PERs. In fact, experiments by our laboratory and Veilhaber et al. [17] have shown that hCKIε kinase negative mutants interact and associate with PER in co-transfected cells. Another possibility is that CKIε is the primary kinase in the SCN while CKIδ acts on PER in the periphery. However this may not be the case because of the intense CKIδ staining we observed in the rat SCN. Furthermore, CKIδ phosphorylation of PER might be just a consequence of the close homology in the kinase domain between the two proteins; however, we have not found evidence to suggest that other CKI isoforms efficiently phosphorylate PERs. In fact, in CKIα and CKIγ co-transfection experiments with the hPERs, we have found limited mobility shifts and $^{32}PO_4$ incorporation into PER1 and 2 (unpublished observation). Furthermore, we have not been able to demonstrate any interaction between CKIα and hPER1 or 2 in either a yeast two hybrid or co-immunoprecipitation assay [17].

4.3. *CKIδ as a component of the mammalian clock*

Our studies have shown that both hPER1 and hPER2 are substrates for the closely related CKI isoforms δ and ε. Not only do both kinases interact with hPER1 and 2, but they also both efficiently phosphorylate the proteins and cause a shift in the molecular mass of the protein. More importantly, phosphorylation of the PERs causes the proteins, in particular hPER1, to become unstable or degraded suggesting that phosphorylation is a key step in the regulation of cellular PER levels and in the regulation of the circadian cycle in mammals. Our data suggest that both hCKIδ and hCKIε are positioned to contribute to phosphorylation and destabilization of PER in the SCN. If in fact CKIδ is also a regulator of the central clock, then we would predict that expression of hypomorphic alleles of CKIδ in the SCN will also act as dominant negatives and result in the *tau* phenotype of period shortening.

Acknowledgements: The authors would like to thank Su-Fen Pu for her help with the HA staining.

References

[1] Keesler, G.A., Camacho, F., Guo, Y., Virshup, D., Mondadori, C. and Yao, Z. (2000) NeuroReport 5, 951–955.
[2] Johnson, C.H. and Golden, S.S. (1999) Annu. Rev. Microbiol. 53, 389–409.
[3] Young, M.W. (1998) Annu. Rev. Biochem. 67, 135–152.
[4] Dunlap, J.C. (1999) Cell 96, 271–290.
[5] Aschoff, J. (1981) Handbook of Behavioral Neurobiology: Biological Rhythms, Plenum, New York.
[6] Pittendrigh, C.S. (1993) Annu. Rev. Physiol. 55, 16–54.
[7] Liu, C., Weaver, D.R., Strogatz, S. and Reppert, S.M. (1997) Cell 91, 855–860.
[8] Herzog, E.D., Takahashi, J.S. and Block, G.D. (1998) Nat. Neurosci. 1, 708–713.
[9] Liu, C. and Reppert, S.M. (2000) Neuron 25, 123–128.
[10] Zylka, M.J., Shearman, L.P., Weaver, D.R. and Reppert, S.M. (1998) Neuron 20, 1103–1110.
[11] Albrecht, U., Sun, Z.S., Eichele, G. and Lee, C.C. (1997) Cell 91, 1055–1064.
[12] Shearman, L.P., Zylka, M.J., Weaver, D.R., Kolakowski, L.F. and Reppert, S.M. (1997) Neuron 19, 1261–1269.
[13] Shearman, L.P., Jin, X., Lee, C., Reppert, S.M. and Weaver, D.R. (2000) Mol. Cell. Biol. 17, 6269–6275.
[14] Zheng, B., Larkin, D.W., Albrecht, U., Sun, Z.S., Sage, M., Eichele, G., Lee, C.C. and Bradley, A. (1999) Nature 400, 167.
[15] Shearman, L.P., Sriram, S., Weaver, D.R., Maywood, E.S., Chaves, I., Zheng, B., Kume, K., Lee, C.C., van der Horst, G.T.J., Hastings, M.H. and Reppert, S.M. (2000) Science 288, 1013–1018.
[16] Hastings, M.H., Field, M.D., Maywood, E.S., Weaver, D.R. and Reppert, S.M. (1999) Proc. Natl. Acad. Sci. USA 26, 15211–15216.
[17] Vielhaber, E., Eide, E., Rivers, A., Gao, Z.H. and Virshup, D.M. (2000) Mol. Cell. Biol. 13, 4888–4899.
[18] Lowery, P.L., Shimomura, K., Antoch, M.P., Yamazaki, S., Zemenides, P.D., Ralph, M.R., Menaker, M. and Takahashi, J.S. (2000) Science 288, 483–491.
[19] Price, J.L., Blau, J., Rothenfluh, A., Abodeely, M., Kloss, B. and Young, M.W. (1998) Cell 94, 83–95.
[20] Kloss, B., Price, J.L., Saez, L., Blau, J., Rothenfluh, A., Wesley, C.S. and Young, M.W. (1998) Cell 94, 97–107.

[21] Takano, A., Shimizu, K., Kani, S., Buijs, R.M., Okada, M. and Nagai, K. (2000) FEBS Lett. 477, 106–112.
[22] Ralph, M.R. and Menaker, M. (1988) Science 241, 1225–1227.
[23] Graves, P.R., Haas, D.W., Hagedorn, C.H., Depaoli-Roach, A.A. and Roach, P.J. (1993) J. Biol. Chem. 268, 6394–6401.
[24] Fish, K.J., Cegielska, A., Getman, M.E., Landes, G.M. and Virshup, D.M. (1998) J. Biol. Chem. 270, 14875–14883.
[25] Tei, H., Okainura, H., Shigeyoshi, Y., Fukuhara, C., Ozawa, R., Hirose, M. and Sakaki, Y. (1997) Nature 389, 512–516.
[26] Camacho, F., Khorkova, O., Guo, Y., Metz, W., Tang, W., Morse, B., Dinerstein, R., Yao, Z. and Keesler, G.A. (2000) Society for Neuroscience Abstract, 469.16.