A Missense Variation in Human *Casein Kinase I Epsilon* Gene that Induces Functional Alteration and Shows an Inverse Association with Circadian Rhythm Sleep Disorders

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Recent studies have shown that functional variations in clock genes, which generate circadian rhythms through interactive positive/negative feedback loops, contribute to the development of circadian rhythm sleep disorders in humans. Another potential candidate for rhythm disorder susceptibility is casein kinase I epsilon (CKIε), which phosphorylates clock proteins and plays a pivotal role in the circadian clock. To determine whether variations in *CKIε* induce vulnerability to human circadian rhythm sleep disorders, such as delayed sleep phase syndrome (DSPS) and non-24-h sleep–wake syndrome (N-24), we analyzed all of the coding exons of the human *CKIε* gene. One of the variants identified encoded an amino-acid substitution S408N, eliminating one of the putative autophosphorylation sites in the carboxyl-terminal extension of CKIε. The N408 allele was less common in both DSPS ($p = 0.028$) and N-24 patients ($p = 0.035$) compared to controls. When DSPS and N-24 subjects were combined, based on an *a priori* prediction of a common mechanism underlying both DSPS and N-24, the inverse association between the N408 allele and rhythm disorders was highly significant ($p = 0.0067$, odds ratio $= 0.42$, 95% confidence interval: 0.22–0.79). *In vitro* kinase assay revealed that CKIε with the S408N variation was ~ 1.8-fold more active than wild-type CKIε. These results indicate that the N408 allele in *CKIε* plays a protective role in the development of DSPS and N-24 through alteration of the enzyme activity.
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INTRODUCTION

In mammals, including humans, circadian cycles of approximately 24 h are observed in behavior and physiology, including cycles of sleep, hormone secretion, and core body temperature. The master circadian pacemaker is localized in the hypothalamic suprachiasmatic nucleus (SCN). Clock genes, *Per1/2/3*, *Cry1/2*, *Bmal1*, and *CLOCK* are expressed in the SCN and produce a nearly 24 h cycle through interacting positive/negative feedback loops (Harmer *et al*, 2001; Reppert and Weaver, 2002). BMAL1 and CLOCK proteins bind to E-box elements and activate transcription of *Per* and *Cry* genes. As the PERs and CRYs are translated, they enter the nucleus and inhibit BMAL1/CLOCK-driven transcription in the negative feedback loop. The circadian pacemaker is synchronized (entrained) to the 24 h day, primarily by the environmental light/dark cycle.

Certain human sleep disorders, designated circadian rhythm sleep disorders, are attributed to the disruption of the circadian timing system (Weitzman *et al*, 1981; Campbell *et al*, 1999; Wijnen *et al*, 2002). Patients with circadian

rhythm sleep disorders, such as delayed sleep phase syndrome (DSPS), advanced sleep phase syndrome (ASPS), and non-24-h sleep–wake syndrome (N-24), fail to adjust their sleep/wake cycle to the daily schedule required for social life. Despite normal sleep architecture, sleep onset and offset are persistently delayed (DSPS) or advanced (ASPS) compared to the societal norm. N-24 patients suffer from daily delays of sleep onset and offset times, with the consequence of progressive cycling through the 24 h environmental day. The pathogenesis of DSPS and N-24 is not yet known, but several possible mechanisms have been proposed: reduced sensitivity of the oscillator to photic entrainment, a prolonged intrinsic period beyond the range of entrainment to 24 h day, and abnormal coupling of the sleep/wake cycle to the circadian rhythm (Weitzman *et al*, 1981; Campbell *et al*, 1999; Uchiyama *et al*, 2000). It is estimated that 0.13% (in Japan) (Yazaki *et al*, 1999), 0.17% (in Norway) (Schrader *et al*, 1993), and 0.7% (in USA) (Ando *et al*, 1995) of the general population suffer from DSPS, while the prevalence of N-24 is lower. Genetic factors reportedly confer predisposition to ASPS and DSPS (Ancoli-Israel *et al*, 2001; Jones *et al*, 1999; Reid *et al*, 2001).

Analysis of animals and humans with altered circadian rhythms demonstrated that casein kinase I epsilon (CKIε) (and presumably its most closely related homolog, CKIδ) plays a crucial role in regulating the circadian pacemaker (Eide and Virshup, 2001). CKIε (and CKI δ) phosphorylates PER proteins, leading to their destabilization and relocalization (Takano *et al*, 2000; Vielhaber *et al*, 2000; Keesler *et al*, 2000; Akashi *et al*, 2002; Camacho *et al*, 2001). CKIε/δ have long carboxyl-terminal (C-terminal) extensions, which can be autophosphorylated, with the consequence of autoinhibition of kinase activity (Graves and Roach, 1995; Cegielska *et al*, 1998). *Double-time* (*dbt*) gene, a Drosophila homolog of mammalian *CKIε*, was shown to alter or ablate circadian rhythm when functionally mutated (Price *et al*, 1998). In hamsters, a point mutation in *CKIε* that decreases kinase activity causes the semidominant short-period *tau* phenotype (Ralph and Menaker, 1988; Lowrey *et al*, 2000). A recent report showed that, in humans, familial ASPS can be induced by a *Per2* S662G mutation, which reduces CKIε-induced phosphorylation of the PER2 protein (Toh *et al*, 2001). We have reported that a *Per3* gene haplotype, in which one of the variations lies close to the CKIε target site and presumably alters PER3 protein phosphorylation, is significantly associated with DSPS (Ebisawa *et al*, 2001). These results suggest the possibility that human *CKIε* (h*CKIε*) gene may also be involved in susceptibility to circadian rhythm sleep disorders.

Accordingly, we set out to screen the complete coding region of the *CKIε* gene, as well as adjacent exon–intron boundaries for the presence of genetic variants in circadian rhythm sleep disorder patients and controls.

MATERIALS AND METHODS

Subjects

In all, 98 DSPS patients (60 males; 38 females; mean age: 27.1 ± 9.1 years) and 39 N-24 patients (29 males; 10 females; mean age: 26.9 ± 8.4 years) were recruited. Diagnosis was assigned by a trained psychiatrist according to the International Classification of Sleep Disorders (ICSD1990) criteria. All of the patients were unrelated, except for two sibling pairs, of which each consisted of a patient with DSPS and a patient with N-24. In a combined analysis of DSPS and N-24, two of the DSPS subjects with siblings of N-24 were excluded from the DSPS/N-24 group to avoid an increase in the Type I error rate. Neither of the sibling pairs carried the S408N variation. Another three patients with DSPS had relatives with probable DSPS, who were not involved in this study, and another patient with N-24 had a first-degree relative with severe insomnia. In all, 138 healthy subjects were recruited as controls (81 males; 57 females; mean age: 32.1 ± 8.6 years). Control individuals were free from sleep disorders or psychoses. All of the study subjects were sighted. In total, 59 DSPS patients, 36 N-24 patients, and 107 control subjects of the study population were reported previously (Iwase *et al*, 2002), while the others were newly recruited for this study. In order to minimize the effect of the population stratification, which may cause false results, all of the study subjects were Japanese and recruited in mainland Japan. The controls were geographically matched to the patients. Written informed consent was obtained from the subjects. The protocol was approved by the ethics committee of Saitama Medical School and the participating institutes.

Blood samples were drawn by venipuncture and genomic DNAs were prepared from leukocytes using QIAamp DNA Blood Maxi Kit or QIAGEN Blood & Cell Culture DNA Midi Kit (QIAGEN, Hilden, Germany).

DNA Analysis

Polymerase chain reaction/single-strand conformation polymorphism (PCR-SSCP) analysis was used to screen for variations in all coding exons of the *CKIε* gene. Fluorescein-labelled primers to amplify each of the coding exons and adjacent exon–intron junctions were derived from the genomic structure determined by alignment of the cDNA and genomic sequence of hCKIε (AB024597 and AL020993, respectively) (Table 1). PCR was performed in a total volume of 50 μl containing 100 ng DNA, 0.5 μM of each primer, $1 \times$ PCR buffer II, 0.2 mM dNTPs, 1.5 mM Mg^{2+}, and 1.25 U of AmpliTaq Gold DNA Polymerase. Conditions for PCR were preincubation at 95°C for 9 min to denature the DNA and to activate the polymerase, followed by 45 cycles at 95°C for 20 s, 63–68°C for 45 s, and 72°C for 1 min, with a subsequent final extension step at 72°C for 10 min.

SSCP electrophoresis was carried out on a denaturing gel in a DSQ-500S DNA sequencer (Shimadzu, Kyoto, Japan) basically as described (Ebisawa *et al*, 2001). Briefly, 1 μl of PCR products were mixed with 19 μl of formamide buffer (90% formamide, 5 mM EDTA, 10 mg/ml Blue dextran), heated at 80°C for 7 min, and 1.5 μl of the sample mixture was electrophoresed on a 0 or 5% glycerol SSCP Gel at 20°C, according to the manufacturer's protocol. Genomic DNAs in which variants were detected by SSCP were amplified using primers that encompass the SSCP-amplified region, and purified using QIAquick PCR Purification Kit (QIAGEN). Sequence reactions were performed on both strands using internal primers and the BigDye Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems, Foster City, CA, USA) according to the protocol of the

Table I Primers Used for SSCP Analysis of the Human *CKIε* Gene

Exon number	Primer name	Sequence (5′–3′)	Fragment size	Annealing temperature (°C)
I	IF	CCA CGT CGC TGA CCC TCA TGT TCC	234	68
	IR	GCC CCT GGA GCC ACA TTC TGA CTT C		
2	2F	CAC ACG CCA GAT CTC AGA AAT GCT TAG TGG	266	63
	2R	CTG TGC TCA TGG CTG CCC ACC G		
3	3F	CTG CCT GCC TCT GAC CCC TGA C	264	63
	3R	GGC AGG AGG CAG GGC TGG TAT C		
4	4F	CTG CCT GGC CCA GAG TGC TAG GCA AG	335	68
	4R	AGT GGC CCC GGG TGC ACA CTG C		
5	5F	CCC AGA GGA TGA GTT AGG GGC CTG AGT G	306	68
	5R	GCC TCA CCT TTC CCT TAG ACA GTG CCT C		
6	6F	GTG GCT AGG ACA GTG CTG GCT GCA G	310	68
	6R	CCA GCT CAC TCT GGC CCT CTG AGT C		
7	7F	CTG GCC TCT GGG GCT GAC TGG TG	271	68
	7R	CTG AAC CCA GCC CAC TGC CTG AGT C		
8	8F	GAC TCA GGC AGT GGG CTG GGT TCA G	267	63
	8R	CTC AGT TCT GAG GCC CAG AGG GAC TG		
9	9F	ATC GCC AGC GGC TAA GGG ACT TGA C	241	63
	9R	CCC ACC CCT CCA CAA CAC ATT GGT C		

F or R, in the primer names indicate the forward or reverse orientation of each primer.

manufacturer, and detected by an ABI PRISM 310 Genetic Analyzer (Applied Biosystems). One of the PCR-amplified fragments in which a deletion was detected by direct sequence analysis was cloned into pGEM-T Easy vector (Promega, Madison, WI, USA), and multiple isolates were sequenced on both strands. To determine the frequency of the S408N variant, all of the samples were amplified by PCR using 9F and 9R primers in Table 1 and subjected to either SSCP and/or denaturing high-performance liquid chromatography (DHPLC) analysis, followed by sequencing reactions as described above.

For DHPLC analysis, PCR products were denatured at 98°C for 30 s and 95°C for 7 min, followed by gradual reannealing from 95 to 15°C over 40 min. The crude PCR products (5–7 μl) were then injected into a DNASep column and separated through a 13.5–15.75% acetonitrile gradient at 61°C using a WAVE DNA Fragment Analysis System (Transgenomic, Omaha, NE, USA).

Purification of Recombinant Proteins

The partial cDNAs encoding mouse PER1 (mPER1) (amino acids 547–799), rat PER2 (rPER2) (486–793), and mouse PER3 (mPER3) (367–880) fragments, which correspond to the CKIε-binding regions (Takano, A *et al*, unpublished

observation), were subcloned into pGEX4T-3 or pGEX6P-1 vector (Pharmacia, Peapack, NJ, USA) for the production of glutathione-*S*-transferase (GST)-fused recombinant proteins. The partial fragments of PERs were used for *in vitro* kinase assay, because it is practically impossible to obtain enough amount of intact full-length PER proteins due to their instability when expressed in *Escherichia coli*. The PER fragments we used correspond to the CKIε-binding domains which contain the phosphorylation sites; therefore, they can be properly used for *in vitro* kinase assay of CKIεs to compare the kinase activity against PERs.

The S408N substitution was introduced into the rat CKIε (rCKIε) cDNA by site-directed mutagenesis using PCR, generating CKIε-S408N. The amino-acid sequence of rCKIε is identical to that of hCKIε, except for two amino acids. Neither of the two amino acids is a phosphoacceptor residue. The expression constructs encoding GST-fused wild-type rCKIε (GST-CKIε-WT) and CKIε-S408N (GST-CKIε-S408N) were prepared using pGEX4T-3 (for α-casein) or pGEX6P-1 (for GST-PERs) vector. *Escherichia coli* (*E. coli*) strain BL21 (DE3) was transformed with the expression plasmids and the fusion proteins expressed were purified with glutathione sepharose 4B (Pharmacia) according to the manufacturer's protocol. GST-CKIε proteins were easily degraded, therefore, for the use in kinetic analysis

against α-casein, the fusion proteins were further purified by immunoprecipitation with the specific antibody against the C-terminal end of rCKIε to remove the contamination of partially degraded recombinant rCKIε (Takano et al, 2000). To perform in vitro kinase assay using GST-fused PER fragments as substrates, GST tag was removed from GST-CKIε using PreScission protease (Amersham) to discriminate phosphorylated GST-PERs and autophosphorylated rCKIε on electrophoretic mobility.

In Vitro Kinase Assay and Kinetic Analysis

Kinase reactions were performed in buffer containing 45 mM Tris-HCl, pH 7.4, 9 mM MgCl$_2$, 0.9 mM β-mercaptoethanol, 40 μM ATP, 74 kBq of [γ-^{32}P], kinase and α-casein or GST-PER in a final volume of 20 μl. Approximately 40 ng of the immunoprecipitated GST-CKIε (for α-casein), 2 pmol of rCKIε (for GST-mPER1 and GST-rPER2), or 10 pmol of rCKIε (for GST-mPER3) was added to the reaction mixture. Varying concentrations of α-casein (0–100 μM), or 20 pmol of GST-mPER1, GST-rPER2, or GST-mPER3 protein, was used as a substrate. The amount of rCKIε, GST-CKIε, or GST-PER used in each reaction was confirmed by SDS-polyacrylamide gel electrophoresis (SDS-PAGE), followed by Coomassie brilliant blue staining using bovine serum albumin as a standard, revealing that the difference in the amount of rCKIε or GST-CKIε in each experiment was smaller than 7.3% of the wild type. The kinase reactions for α-casein were allowed to proceed at 37°C for 10 min, because the enzyme activity was linear with time for up to 20 min (data not shown). Reactions were terminated by addition of 20 μl SDS-PAGE sample buffer. A part of the reaction mixture was subjected to electrophoresis on 12% (for α-casein) or 7.5% (for GST-PERs) polyacrylamide gels, and [^{32}P] incorporation into the substrates was determined by a BAS-2000 image analyzer. When α-casein was used as a substrate, the data were presented as a double-reciprocal plot and V_{max} and K_m were obtained using computer software (Kaleida Graph, Abelbeck Software).

Statistical Analysis

Departure from Hardy–Weinberg equilibrium was tested using a χ^2 goodness-of-fit test. The allele and genotype frequencies were compared by means of Fisher's exact test. All p-values reported are two-tailed. Correction for multiple testing for the analyses in the previous studies was not performed since a considerable number of subjects were newly recruited for this study, which was conducted with a pre-established hypothesis (Perneger, 1998). Unpaired t-test was performed to compare the amounts of incorporated [^{32}P] into GST-PER by CKIε-WT and CKIε-S408N.

RESULTS

Using PCR-SSCP and subsequent sequencing of the PCR-amplified fragments, all of the coding exons and flanking exon–intron boundaries of the CKIε gene were screened for sequence variations. In an initial screen of 35 genomic DNA samples (17 of DSPS and 18 of N-24), three sequence variants were identified (Table 2). One single-nucleotide

Table 2 Sequence Variations Identified in the Human CKIε Gene

DNA polymorphism	Location	Amino-acid substitution
51C>T	Exon 1	None
77-63_77-60delGGCG	Intron 1	None
1223G>A	Exon 9	S408N
1263A>G	Exon 9 (3′-untranslated region)	None

Variations were named basically according to den Dunnen and Antonarakis (2001). Nucleotide numbers refer to the human CKIε cDNA sequence (AB024597) with the A of the ATG start codon denoted as 1. The S408N variation was submitted to DDBJ (http://www.ddbj.nig.ac.jp/, Accession no. AB080742).

Table 3 Frequency of the S408N Variant in Patients and Controls

		Allele frequency		
	n	N408 (%)	S408 (%)	p-value
Control	276	34 (12.3)	242 (87.7)	
DSPS	196	12 (6.1)	184 (93.9)	0.028[a]
N-24	78	3 (3.8)	75 (96.2)	0.035[b]
DSPS/N-24	270	15 (5.6)	255 (94.4)	0.0067[c]

[a]Odds ratio (OR) = 0.46, 95% confidence interval (CI): 0.23–0.92.
[b]OR = 0.28, 95% CI: 0.085–0.95.
[c]OR = 0.42, 95% CI: 0.22–0.79.

variation (51C>T) was located in exon 1, another variation (1223G>A) in exon 9, and one intronic deletion of 4 bp (77-63_77-60delGGCG) resided upstream of exon 2. The 1223G>A exonic variation predicted an amino-acid substitution, S408N. S408 is located in the C-terminal extension of the CKIε and is conserved in CKIεs of humans, hamsters, mice, rats, and Xenopus laevis, as well as in CKIδs of humans and rats. Previous studies demonstrated that the C-terminal extensions of mammalian CKIε (and CKIδ) can be autophosphorylated, inhibiting the kinase activity (Graves and Roach, 1995; Cegielska et al, 1998), and that S408 is one of the putative phosphoacceptor residues (Gietzen and Virshup, 1999). Therefore, the S408N variation is likely to eliminate one of the autophosphorylation sites, resulting in decreased autophosphorylation and increased enzyme activity. The 51C>T exonic variation resulted in synonymous substitution. Neither the 51C>T variation nor the intronic deletion (77-63_77-60delGGCG) appeared to affect known splice sites or to create better splice donor/acceptor consensus sequences, based on visual examination of the sequence context, so functional alterations appeared unlikely (Burset et al, 2000). Therefore, we focused on the S408N variation for further analysis.

The frequency of the S408N variation was analyzed in a total of 137 circadian rhythm sleep disorder patients and 138 control subjects. Allele and genotype distributions are shown in Tables 3 and 4. No significant deviation from Hardy–Weinberg equilibrium was detected for the variation either in patients or in controls. The distribution analysis resulted in the detection of an additional silent sequence variation (1263A>G) in the 3′-untranslated region of the

Table 4 Genotype Distribution of the S408N Variant in Patients and Controls

				Genotype		
	n	N/N (%)	N/S (%)	S/S (%)	N/N+N/S (%)	p-value
Control	138	4 (2.9)	26 (18.8)	108 (78.3)	30 (21.7)	
DSPS	98	1 (1.0)	10 (10.2)	87 (88.8)	11 (11.2)	0.038[a]
N-24	39	0 (0)	3 (7.7)	36 (92.3)	3 (7.7)	0.061[b]
DSPS/N-24	135	1 (0.7)	13 (9.6)	121 (89.6)	14 (10.3)	0.013[c]

The frequency of the N408-allele carrier is shown as (N/N+N/S). Odds ratio (OR) and 95% confidence interval (CI) are for (N/N+N/S) vs S/S.
[a]OR = 0.46, 95% CI: 0.22–0.96.
[b]OR = 0.3, 95% CI: 0.086–1.04.
[c]OR = 0.42, 95% CI: 0.21–0.83.
Two of the DSPS subjects, who had siblings with N-24, were excluded from the combined DSPS/N-24 group to avoid an increase in the Type I error rate. Neither of the sibling pairs carried the S408N variation.

CKIε gene, which was located 40 bp downstream of S408N polymorphic site (Table 2). One of the DSPS patients and two of the N-24 patients were heterozygous for the $1263A > G$ variation, while it was not detected in the control individuals. However, the frequency of the $1263A > G$ variation was too low to establish whether the variation affects the development of DSPS and N-24.

The N408 allele was significantly less frequent in DSPS ($p = 0.028$) and in N-24 ($p = 0.035$) than in control subjects (Table 3). The frequency of the N408-allele carrier was also significantly lower in DSPS subjects ($p = 0.038$) compared to controls, while the difference in carrier frequency between N-24 subjects and controls showed a similar tendency but did not come to statistical significance ($p = 0.061$) (Table 4). N-24 patients often suffer from DSPS during the course of the illness (Kamgar-Parsi et al, 1983; Oren and Wehr, 1992; McArthur et al, 1996), and reportedly share some of the physiological characteristics of DSPS, such as prolonged interval between natural wake time and the core body temperature trough (Uchiyama et al, 2000) or melatonin midpoint (Shibui et al, 1999; Uchiyama et al, 2002). These observations led to an a priori prediction that DSPS and N-24 are essentially the same disorder expressed with different degrees of severity (Weitzman et al, 1981; Campbell et al, 1999; Regestein and Monk, 1995). Indeed, when DSPS and N-24 subjects were combined, highly significant inverse associations were found between the N408 variant and DSPS/N-24 in both allele frequency ($p = 0.0067$, odds ratio (OR) = 0.42, 95% confidence interval (CI): 0.22–0.79) and carrier frequency ($p = 0.013$, OR = 0.42, 95% CI: 0.21–0.83), suggesting that the N408 allele protects against the development of DSPS/N-24. Our sample size had a 78% power to detect this effect of the S408N allele at a significance level of $p = 0.05$.

We next considered whether the S408N variation induces a functional alteration in CKIε, as expected from the location of the substitution. To determine whether the N408 variation in CKIε affects kinase activity in situ, phosphorylation of PER1 was assayed in transfected COS-7 cells by pulse-chase analysis. COS-7 cells were co-transfected with expression plasmids encoding mPer1 and either wild-type rCKIε or rCKIε with the S408N substitution. The transfected cells were pulse-labeled with [^{35}S]methionine for 1 h and chased for 0–6 h. After the chase period, cells were lysed and mPER1 protein expressed in COS-7 cells was immunopre-cipitated using anti-mPER1 antibody. The immunoprecipi-tates were electrophoresed, and [^{35}S]-labeled mPER1 was detected. rCKIε with the S408N substitution induced a more pronounced mobility shift and reduced mPER1 protein level at 6 h post-pulse, which was indistinguishable from the effects induced by wild-type rCKIε. These results indicate that, in situ, wild-type rCKIε and rCKIε with the S408N substitution induce similar levels of phosphorylation and subsequent instability of the mPER1 protein (data not shown).

Previous reports suggested that subsets of autophosphorylation sites in CKIε are dephosphorylated in HEK293 and NIH3T3 cells by endogenous phosphatases, thus activating CKIε activity (Gietzen and Virshup, 1999; Rivers et al, 1998). Therefore, it is possible that rCKIεs transfected into COS-7 cells are dephosphorylated, consequently masking the effect of the S408N substitution on kinase activity.

To test this hypothesis, we performed in vitro kinase assays of GST-CKIε with or without the S408N substitution, using α-casein as a substrate. Recombinant GST-CKIε proteins were expressed in E. coli, purified with glutathione sepharose 4B, and immunoprecipitated with the C-terminus-specific antibody for CKIε to remove partially degraded protein. The kinetic analysis was performed using varying substrate concentrations (0–100 μM). As expected, GST-CKIε-S408N exhibited higher kinase activity than GST-CKIε-WT (Figure 1a). The data were represented as a double-reciprocal plot (Figure 1b). GST-CKIε-S408N showed significantly increased V_{max} (181% of GST-CKIε-WT) and a slightly decreased apparent K_m (78% of GST-CKIε-WT) against casein. To investigate whether the S408N substitution in CKIε causes higher enzyme activity on endogenous clock components, in vitro kinase assays using GST-PERs as substrates were also performed. To distinguish the phosphorylated GST-PERs from autophosphorylated CKIεs, GST tags were removed from recombinant CKIεs. As shown in Figure 2, CKIε-S408N incorporated more [^{32}P] into PER1, PER2, and PER3 fragments, respectively, than CKIε-WT did.

DISCUSSION

CKIε is one of the seven isoforms of CKI, designated α, β, γ1–3, δ, and ε (Eide and Virshup, 2001). Activity of CKIε
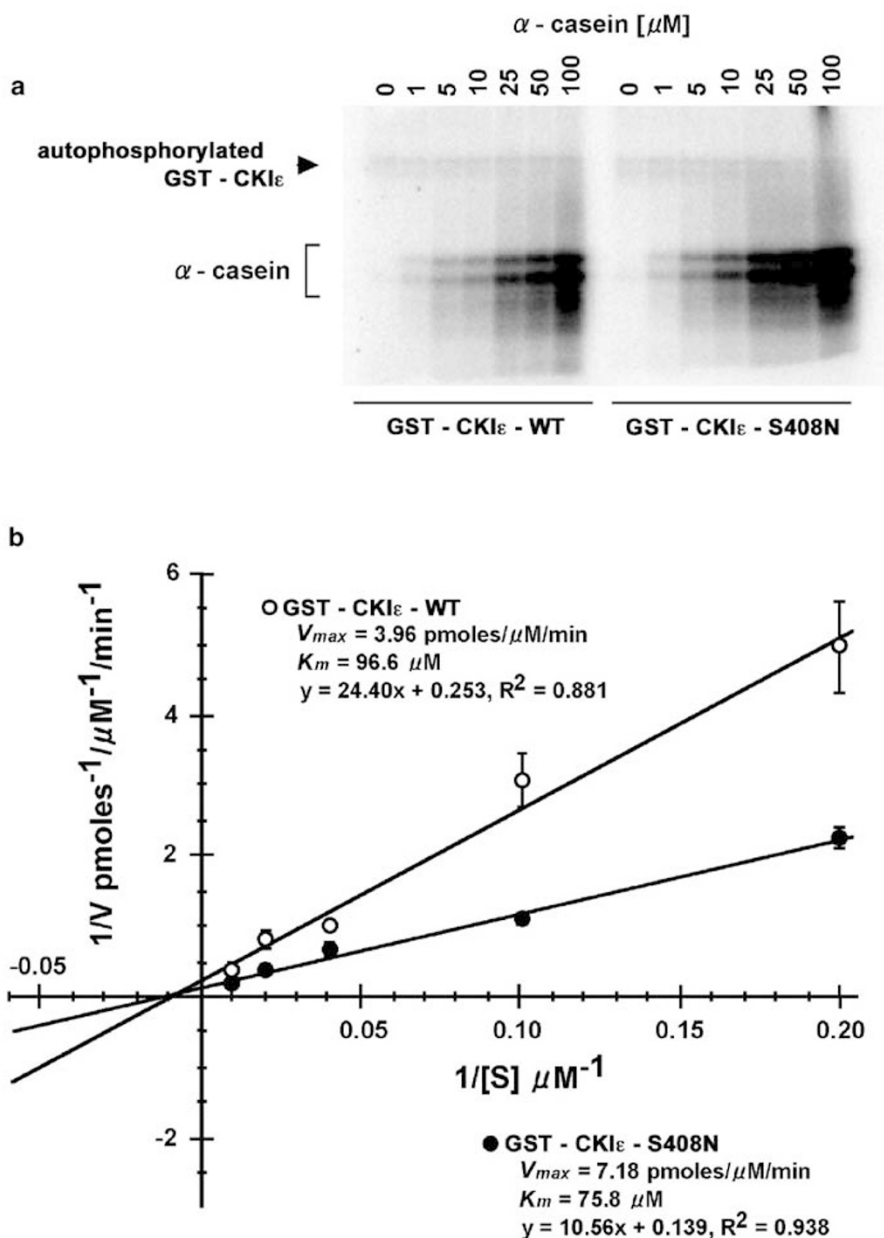
Figure 1 Kinetic analysis of recombinant GST-CKIε for α-casein. (a) Assays were performed with various concentrations of α-casein in the presence of GST-CKIε-WT or CKIε-S408N. Autophosphorylation of GST-CKIε-WT and CKIε-S408N that were only slightly visible in this figure were readily apparent when increased amounts of recombinant enzymes were used (data not shown). (b) Double-reciprocal plot of the data derived from the kinase assay performed with various concentrations of α-casein. Open and closed circles indicate the results for GST-CKIε-WT and CKIε-S408N, respectively. Calculated V_{max} and K_m of GST-CKIε-WT and CKIε-S408N are shown in the upper and the lower parts of the plot, respectively (means $\pm$ standard errors (SE) from three independent experiments).

(and the closely related CKIδ) is regulated in part by autophosphorylation of the C-terminal extension (Eide and Virshup, 2001; Graves and Roach, 1995; Cegielska et al, 1998). In vitro, CKIε is highly autophosphorylated, which inhibits enzyme activity (Gietzen and Virshup, 1999; Rivers et al, 1998). Both dephosphorylation by phosphatase treatment and removal of the C-terminal domain reactivate the kinase (Graves and Roach, 1995; Cegielska et al, 1998). In a site-directed mutagenesis study, eight amino acids in the C-terminal domain were identified as probable autophosphorylation sites, including serine-408 (Gietzen and Virshup, 1999). Therefore, the amino-acid change from

serine-408 to asparagine (S408N) in CKIε, which was found in this study, is likely to eliminate one of the autophosphorylation sites, and is expected to reactivate part, but not all, of the kinase activity. Indeed, in our in vitro kinase assay with α-casein, recombinant GST-CKIε with the S408N substitution purified from E. coli exhibited a moderate (1.8-fold) elevation of specific activity compared to that of wild-type GST-CKIε, while a previous study showed that a mutant CKIε, in which all of the putative autophosphorylation sites are disrupted, was eight-fold more active than wild-type CKIε (Gietzen and Virshup, 1999). The moderate elevation of CKIε activity by S408N substitution was
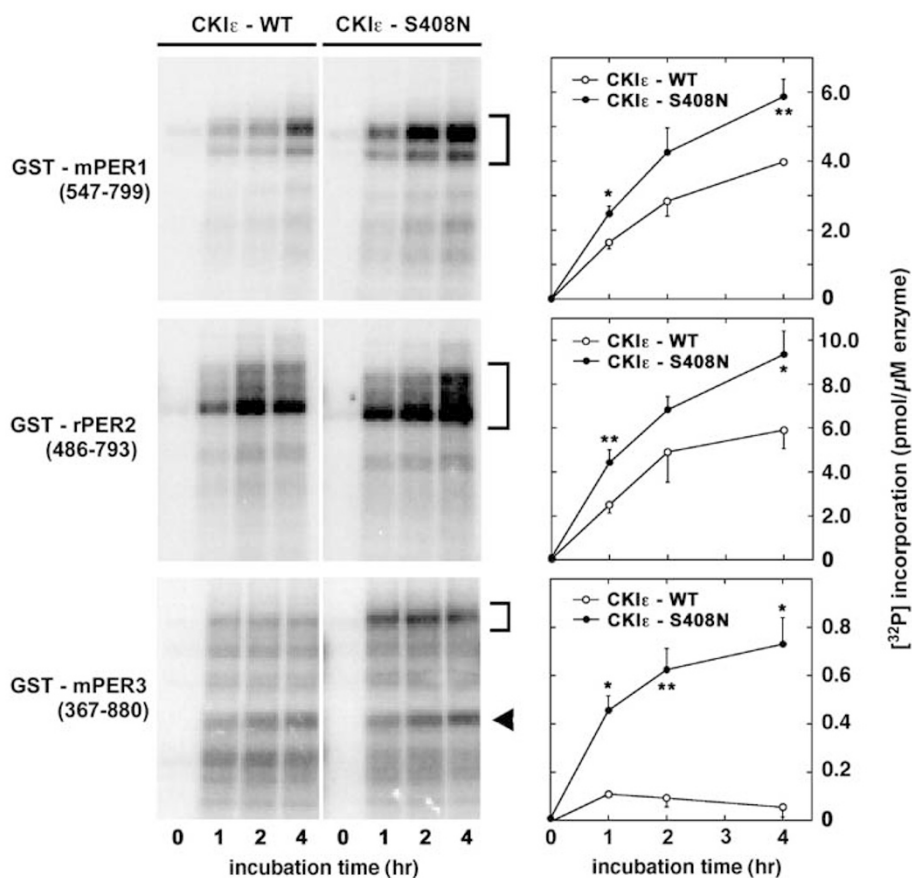
Figure 2 *In vitro* kinase assay of recombinant CKIε using GST-fused PER fragments as substrates. GST-mPER1 (amino acids 547–799) (top panels), GST-rPER2 (486–793) (middle panels), or GST-mPER3 (367–880) (bottom panels) fragment was incubated with recombinant CKIε-WT or CKIε-S408N for the indicated duration and analyzed by 7.5% polyacrylamide gels as described in Materials and methods. Representative autoradiograms are shown. Angle brackets indicate the phosphorylated GST-PER fragments. Arrowhead indicates autophosphorylated CKIε (left panels). Incorporated [^{32}P] was quantified and normalized to the total amount of kinase used (means $\pm$ SE from three to five independent experiments). Statistically significant differences in [^{32}P] incorporation induced by CKIε-WT and CKIε-S408N are shown by asterisks (*$p < 0.05$, **$p < 0.01$) (right panels).

identically observed in the *in vitro* kinase assay with each of the three subtypes of PER proteins, which are endogenous substrates for CKIε. It is intriguing that CKIε-S408N induced more phosphorylation of PER3 than CKIε-WT did, because we have previously reported that a *Per3* gene haplotype, which presumably alters PER3 protein phosphorylation, is significantly associated with DSPS (Ebisawa *et al*, 2001). However, it should be noted that we observed much less phosphorylation of PER3 compared with that of PER1 or PER2, which is consistent with the previous reports showing CKIε-induced phosphorylation of PER3 (Takano *et al*, 2000; Akashi *et al*, 2002) and unstable interaction of PER3 with CKIε in the absence of PER1 (Akashi *et al*, 2002; Lee *et al*, 2004). We could not find any elevation of enzyme activity in pulse-chase analysis *in situ*, presumably because of dephosphorylation by endogenous phosphatases as described in 'Results', or because the analysis was insufficiently sensitive to detect a moderate difference of activity.

The *tau* mutation in hamster *CKIε* decreases kinase activity by as much as eight-fold (Lowrey *et al*, 2000), whereas the S408N variant in h*CKIε* results in only 1.8-fold change (an increase) in the activity. This difference might explain the reason why the N408 allele of h*CKIε* induces a

significant but modest effect ($\sim$2-fold reduction in the risk to develop DSPS/N-24), compared with the *tau* mutation in hamster *CKIε*, which causes a semidominant short-period phenotype (Ralph and Menaker, 1988).

Studies in flies and mammals suggest that CKIε binds to and phosphorylates PER proteins, leading to instability and intracellular relocalization of the PERs (Takano *et al*, 2000; Vielhaber *et al*, 2000; Keesler *et al*, 2000; Akashi *et al*, 2002). Mutant *CKIε* in the Syrian Golden hamster is deficient in PER phosphorylation (Lowrey *et al*, 2000). *Per2* S662G mutation in a reported familial ASPS cause hypophosphorylation by CKIε (Toh *et al*, 2001). In both cases, the PER protein(s) seems to undergo delayed degradation and accelerated accumulation, leading to hastened nuclear entry and shortened circadian period. In contrast, in flies with dbt^L or dbt^{ar} (long-period alleles of *dbt*, the Drosophila homolog of *CKIε*), it is likely that delayed phosphorylation and increased nuclear stability of PER protein slow the rate of PER elimination from the nucleus and lengthen circadian rhythm (Price *et al*, 1998; Rothenfluh *et al*, 2000). Therefore, hypophosphorylation of PER protein appears to cause different phenotypes depending on the subcellular localization of the stabilized PERs. hCKIε with an S408N substitution appears more active than wild type only when

the protein is autophosphorylated. A recent study suggests that the autophosphorylation level of CKIε, in neuroblastoma N2a cells, is dynamically regulated through transient dephosphorylation and subsequent phosphorylation, thus regulating the kinase activity (Liu et al, 2002). Additionally, in clock-relevant cells, CKIε intracellular localization is under circadian control (Lee et al, 2001); therefore, it is possible that a dynamic autophosphorylation/dephosphorylation cycle could differentially regulate CKIε activity at different subcellular locations in pacemaker cells. The S408N variation of hCKIε might alter circadian rhythmicity through increased phosphorylation and decreased stability of PER protein; the expected phenotypic consequences, however, would differ depending on the levels of CKIε autophosphorylation in each subcellular location. It will be of interest to investigate the autophosphorylation status of CKIε-S408N and to clarify its functional role in circadian clock machinery.

Although a significant inverse association was observed between the N408 variant and DSPS/N-24, 10.3% of the patients carried the N408 allele, indicating that DSPS/N-24 is genetically heterogeneous and multiple genes affect susceptibility to the development of DSPS/N-24.

The 1263A>G variation in the 3'-untranslated region of hCKIε was detected only in three of the rhythm disorder subjects, but not in controls. A larger sample size will be necessary to ascertain its relevance to DSPS/N-24.

Owing to the potential role of CKIε in the circadian rhythm, all of the coding exons in hCKIε gene were screened for variations in circadian rhythm sleep disorder patients and controls. We found a missense variation S408N, for the first time, which eliminates one of the putative autophosphorylation sites in hCKIε and confers 1.8-fold higher enzyme activity in vitro. There was a significant difference in the frequency of N408 allele between controls and DSPS or N-24, respectively, with an excess of N408 allele in controls. When considering the whole sample of circadian rhythm sleep disorders (DSPS/N-24), we found a highly significant inverse association between N408 allele and DSPS/N-24 ($p = 0.0067$, OR = 0.42, 95% CI: 0.22–0.79). These results indicate that the N408 allele of the hCKIε gene is a marker for decreased risk of DSPS/N-24. S408N variation would also be useful to investigate other disorders related to disturbed circadian rhythm or interindividual differences of circadian rhythmicity in apparently normal subjects (Johansson et al, 2003). Our results will yield a new insight into the mechanism of DSPS/N-24 and raise a question in the role of CKIε autophosphorylation on mammalian clock functioning.

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