学 位 論 文
Doctor's Thesis

The Study on Activation Mechanism of mPer1 Promoter by
Calcium/Calmodulin-Dependent Protein Kinase II.
(カルシウム/カルモデュリン依存性プロテインキナーゼ II による
mPer1 プロモーターの活性化機構に関する研究)

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1. Summary

Recent our studies suggest that CaM kinase II was involved in light-induced phase delays and induction of Per1 and Per2 genes in the hamster suprachiasmatic nucleus (SCN)(Yokota et al., 2001). I here focused on intracellular mechanisms of the CaM kinase II-induced mPer1 gene expression. Immunoblotting and immunohistochemical analyses with isoform specific antibodies against different isoforms of CaM kinase II and CaM kinase IV showed abundant expression of the δ isoform of CaM kinase II without significant expression of CaM kinase IV in the lateral ventral part of the rat SCN. I next defined CaM kinase II-responsive region on the mPer1 promoter using luciferase reporter gene assay. Transfection of the constitutively active CaM kinase IIδ largely increased the mPer1 promoter activity in NG108-15 cells and slightly but significantly in NB2A and C6 glioma cells. Similarly, transfection of a constitutively active MEKK, an upstream kinase of MAPK largely increased the promoter activity in NB2A cells. Deletion and mutation analyses of the mPer1 promoter revealed that 5'-GAGGGG-3' sequence motif near by exon 1B, in which several zinc finger proteins seem to bind, was essential for the CaM kinase II-induced activation of the mPer1 promoter. These results suggest that CaM kinase IIδ but not CaM kinase IV plays an essential role for mPer1 expression through 5'-GAGGGG-3' motif on the mPer1 promoter.

2. List of Published Paper

1) Involvement of Calcium/Calmodulin-Dependent Protein Kinase II in The Induction of mPer1.

Kazumi Nomura, Yusuke Takeuchi, Shun Yamaguchi, Hitoshi Okamura, and Kohji Fukunaga

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3. Acknowledgements

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4. Abbreviations and Acronyms

CaM kinase II $=Ca^{2+}$/calmodulin-dependent protein kinase II

CaM kinase IV$=Ca^{2+}$/calmodulin-dependent protein kinase IV

CREB=cAMP-responsive element binding protein

DTT= 1,4-ditio-D,L-treitol

NG108-15=neuroblastoma×glioma hybrid cells

PBS=phosphate-buffered saline

TBS=Tris-buffered saline

KN-93 = 2-[*N*-(2-hidroxyethyl)-*N*-(4-methoxybenzenesulfonyl)]-amino-*N*-(4-chlorocinnamyl)-*N*-methylbenzylamine

NMDA = *N*-methyl-D-aspartate

PAGE = polyacrylamide gel electrophoresis

SDS = sodium dodecyl sulfate

5. Background and Purpose

5.1. Circadian Rhythm

Most organisms, from prokaryotes to eukaryotes, have their own biological rhythms. Especially those having periods of approximately 24 hours are called circadian rhythms. The original periods are synchronized to a 24-hr day/ night cycle by several environmental cues such as light, temperature, and endogenous bioactive substances. Mammalian circadian rhythms are governed by a biological clock located in the suprachiasmatic nucleus (SCN) of the hypothalamus and the output of a circadian oscillation appears as locomotive activity, hormonal secretion, sleep-wake cycle, and many other physiological functions. Recent studies advance molecular mechanisms of the rhythmic expression of clock genes, thereby generating mammalian circadian rhythms. The hetero-complex of Clock/BMAL1, both basic helix-loop-helix (bHLH)-PAS proteins, activate the transcription of three mammalian period genes (Per1, Per2 and Per3) and two *cryptochrome* genes (Cry1 and Cry2) by binding to the E-box on their promoters. Thereafter, the heteromultimeric complexes repress transcription of Per genes by attenuating the activation potential of the Clock/BMAL1, forming a negative feed back loop.

5.2. Expression of mPer1 and Light Exposure

Expression of mPer1 in the SCN is robustly rhythmic for a number of cycles in constant darkness with a dependable peak in the subjective morning suggesting the operation of a day-phase clock in the SCN. Light exposure during subjected night induces rapid mPer1 mRNA expression, thereby resetting the biological clock in the SCN (Sherman et al., 1997; Takumi et al., 1998; Shigeyoshi et al., 1997). However, the signaling cascade involved in mPer1 gene expression following photic stimulation is not fully understood. The photic stimulation strongly synchronizes the endogenous

circadian rhythm by conveying the photic signals from retina to the SCN via the retinohyothalamic tract (RHT). Released glutamate from the RHT activates NMDA receptors (Ebling 1996; Ding et al., 1997), thereby activating Ca^{2+}-dependent phosphorylation of cAMP responsive element binding protein (CREB). The cAMP responsive element (CRE) on the mPer gene promoter may play an important role in the light-induced Per gene expression. However any precise elements interacting with CREB on the mPer gene promoter are not identified. Yokota et al. recently found that Ca^{2+}/calmodulin-dependent protein kinases (CaM kinases) but not mitogen-activated protein kinase (MAPK) was required for the light-induced Per1 gene expression (Yokota et al., 2001). The results were consistent with the previous observation suggesting a calmodulin-induced phase delay in the rat SCN (Fukushima et al., 1997). In addition, CaM kinase II was abundantly detected in the rat SCN (Fukushima et al., 1997). Fukunaga et al. also reported that CaM kinase II activation following induction of long-term potentiation (LTP) was generated by glutamate receptor stimulation in the rat SCN (Fukunaga et al., 2002). In the report, high frequency stimulation of the optic nerve led an activation of CaM kinase II in the VIP neurons in the SCN. Taken together, we hypothesized that CaM kinase II signaling pathway is involved in the *Per* gene expression in the SCN and in turn the light-induced phase delays in the SCN.

5.3. Ca^{2+}/Calmodulin-Dependent Protein Kinases

The information encoded in transient Ca^{2+} signals is deciphered by various intracellular Ca^{2+} binding proteins that convert the signals into a variety of biochemical changes. One of the key proteins that transduce Ca^{2+} signals in response to increase in intracellular Ca^{2+} is calmodulin (CaM) (Crivici and Ikura 1995; Nelson and Chazin 1998). It is well known that CaM is the central protein mediator of intracellular Ca^{2+} signaling. Following an increase in intracellular Ca^{2+}, Ca^{2+} binds to CaM to alter its conformation, and the resulting Ca^{2+}/CaM complex activates a large array of different target proteins

and enzymes. Among the enzymes, one of the primary effectors of CaM is a family of serine/threonine protein kinases called Ca^{2+}/CaM-dependent protein kinases (CaM kinases) (reviewed by Hanson and Schulman 1992). This family includes kinases such as phosphorylase kinase, myosin-light chain kinase (MLCK), and CaM kinases I, II, and IV. These kinases are grouped according to whether they are dedicated kinases having a single substrate (phosphorylase kinase and MLCK) or whether they have several substrates (CaM kinase I, II and IV) (Picciotto *et al.* 1996; Soderling 2000; Fukunaga and Miyamoto 2000; Corcoran and Means 2001). CaM kinases I, II and IV are known to be abundant in the nervous system and thought to regulate a broad spectrum of neuronal processes ranging from the regulation of enzyme activities for short-term modulation to gene expression for long-term changes .

5.4. CaM Kinase II

Ca^{2+}/calmodulin-dependent protein kinase II (CaM kinase II) is highly expressed in neuronal and nonneuronal tissues and can regulate many calcium-mediated functions such as neurotransmitter and hormone release (Matsumoto et al., 1999; Tabuchi et al., 2000) and synthesis, receptor functions (McGlade-McCulloh et al., 1993), neuronal plasticity (Fukunaga and Miyamoto 2000) and gene expression (Hanson and Schulman, 1992, Takeuchi et al., 2000 and 2002b). Four subunits, termed α, β, γ and δ are encoded by distinct genes in eukaryotes (Shulman and Hanson 1993). These subunits comprise large holoenzymes (~600 kDa) of 8-12 subunits with their carboxyl-terminal association domains. Immunoelectron microscopy of CaM kinase II revealed that CaM kinase II forms a "flower"-shaped homopolymer with a central core consisted with association domains, indicated by binding of the antibody against the α subunit. Various isoforms of each subunit, which are different in the variable domain between the regulatory and the association domains, have been identified in all subunits such as α (Lin et al., 1987). The isoforms from four types of CaM kinase II subunits, α, β, γ, and

δ, have been identified in the brain (Colbran et al., 1990; Schulman et al., 1993). Each isoform shows different regional distribution in the brain and different intracellular localization in cultured neuronal cells when they were overexpressed (Brocke et al., 1995; Srinvasan et al., 1994; Takeuchi et al., 1999 and 2000; Tobimatsu et al., 1989). In this study, I tried to define which type of CaM kinase II isoform is expressed in the SCN and which element on the mPer1 promoter is responsible for the activation of its expression by CaM kinase II.

6. Methods

6.1. Materials

Monoclonal antibodies to the α subunit (CBα-2) and the β subunit (CBβ-1) of CaM kinase II were obtained from Life Technology (Tokyo Japan); anti-CaM kinase IIγ antibody [CaM kinase IIγ (C-18)] was obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA); the polyclonal antibody specific for the δ1-δ4 isoforms of CaM kinase II (anti-CaM kinase IIδ1-δ4 antibody) was prepared by immunizing rabbits with a synthesized peptide corresponding to the common carboxyl-terminal end of the isoforms (Takeuchi et al., 1999). The polyclonal antibody to the CaM kinase IV (anti-CaM kinase IV antibody) was prepared by immunizing rabbits with a synthesized peptide corresponding to the common carboxyl-terminal end (Kasahara et al., 1999). Other chemicals used were of analytical grade.

6.2. Immunoblot Analysis

The SCN were isolated from 5 rats and homogenized with 250 μl ice-cold solution (0.32 M sucrose, 10 mM HEPES-NaOH, pH 7.5, 2 mM EDTA, 2 mM EGTA, 15 mM $Na_4P_2O_4$, 150 mM β-glycerophosphate, 25 mM NaF, 0.1 mM Leupeptin, 0.1 mM Pepstatin, 0.1 mM Trypsin inhibitor, 0.1 mM DTT and 1% TritonX-100). The homogenate was treated with 1% SDS in the final concentration and boiled for 2 min at 100 °C according to the methods of Laemmli (1970). Proteins separated by SDS-polyacrylamide (10%) gels were transferred to PVDF membranes (Millipore Corporation, Bedford, MA). The blots were incubated at room temperature for 1hr with 4 % skim milk in TBS (20mM Tris-HCl, pH 7.5, 0.15M NaCl), and then incubated at 4 °C overnight with each primary antibody in the blocking solution. CaM kinase IIα, β were detected by anti-mouse IgG antibody. CaM kinase IIγ were detected by anti-goat IgG antibody. CaM kinase IIδ and CaM

kinase IV were detected by anti-rabbit antibody. The blots were visualized by an enhanced chemiluminescence detection system (Amersham Pharmacia Biotech, UK).

6.3. Immnostaining

The SCN slices were washed with phosphate-buffered saline (PBS) and treated with 0.3% bovine serum albumin (BSA) in PBS (blocking solution) for 1hr. Slices were incubated overnight with the antibodies against CaM kinase II γ, δ or CaM kinase IV at a dilution of 1 : 500 in the blocking solution. After the first antibody treatments, slices were washed with PBS and then incubated with the second antibody, fluorescein isothiocyanate (FITC)-conjugated anti-rabbit IgG antibody for the anti-CaM kinase IIδ antibody or anti-CaM kinase IV antibody, the rhodamine-conjugated anti-goat IgG for the anti-CaM kinase IIγ antibody. Immunostaining was examined under a confocal laser scanning light microscope (CLSM) (Olympus, Tokyo Japan).

6.4. Plasmid Construction

The pCAGGSneo expression vector was kindly provided by Prof. J. Miyazaki (Osaka University, Japan). The pFC-MEKK and pFC-PKA were purchased from STRATEGENE (La Jolla, CA, USA). The cDNA for CaM kinase IIδ isoform was prepared and inserted into the *Eco*RI site of pCAGGSneo under control of the chicken β-actin promoter (Takeuchi et al., 1999 and 2000). The constitutively active mutant of CaM kinase IIδ1 was obtained by replacing Thr 287 of the δ1 isoform with Asp as described (Takeuchi et al., 2002b). To obtain a constitutively active mutant of CaM kinase IV, the fragments spanning from +1 to +313 of CaM kinase IV were obtained using PCR with a sense primer (5'-ATCTCGAGATGCTCAAAGTCACGGTGCCCT- 3') and an antisense primer (5'-TACTCGAGTTAGTACTCTGGCAGAATAGCA-3') of mouse

cDNA. The fragment was digested with *Xho*I and inserted into the *Xho*I site of pCAGGSneo. The fragments were digested with *Eco*RI and inserted into the *Eco*RI site of pCAGGSneo. The mPer1 complete reporter vector (mPer1 comp) and the mPer1 1A reporter vector (mPer1 1A) were prepared as described by Yamaguchi et al.(2000). The mPer1 truncated promoter fragments [-1848/-21 (mPer1 -1848), -1813/-21 (mPer1 -1813), -1735/-21 (mPer1 -1735), -1721/-21 (mPer1 -1721), -1706/-21 (mPer1 -1706), -1678/-21 (mPer1 exon 1B)] were obtained by PCR using a sense primer and an antisense primer (5'-ATCTCGAGGAC GAAACAGGGAA-3') with the whole fragment containing mPer1 promoter region (-7227 to -21) as shown in Table 1. The mutant promoter fragments (mutated at GAGGGG, shown as mPer1 -1813M) were obtained by connecting the PCR fragments using two set of primers [mPer1 -1813 sense primer/mPer1 -1813M antisense primer and mPer1 -1813M sense primer/antisense primer (5'-ATCTCGAGGACG AAACAGGGAA-3')]. The truncated promoter fragments digested with *Nhe*I and *Xho*I were subcloned into the pGL3-basic encloning vector and sequenced. The fragments cording C/EBPβ were obtained by PCR using a sense primer (5'-ATGAATTCATGCACCGCCTGCTGGCCTGGGAC-3') and an antisense primer (5'-ATGAATTCCTAGCAGTGGCCCGCCGAGGCCAG-3') from mouse cDNA after amplification using outside primers coding 5'or 3' flanking region of C/EBPβ. The fragments were digested with *Eco*RI and inserted into the *Eco*RI site of pCAGGSneo. We examined the expression and activity of C/EBPβ in NG108-15 cells. SP-1 expression vector was made by Takeuchi et al. (2002a).

Table 1. *Oligonucleotide sequences and nucleotide positions of PCR primers*

Primer	Oligonucleotide sequence	
mPer1 –1848	5'-TTAGCTAGCATGTGTGTGACAC -3'	Sense
mPer1 –1813	5'-TTAGCTAGCAGTAGCCAATCAG -3'	Sense
mPer1 -1735	5'-TTAGCTAGCGAGGGGCAGGGC -3'	Sense
mPer1 –1721	5'-TTAGCTAGCGGCATTATGCAAC -3'	Sense
mPer1 –1706	5'-TTAGCTAGCGCCTCCCAGCCTC -3'	Sense
mPer1 –1813M	5'-TTAGCTAGCGAATTCCAGGGCCTGGCATTA -3'	Sense
	5'-CTGGAATTCATTGGCCTTCTGC-3'	Antisense
mPer1 Exon1B	5'-TTAGCTAGCTGCGGGCCGAAAC-3'	Sense
mPer1 XhoI 3'	5'-ATCTCGA GGACGAAACAGGGAA- 3'	Antisense

6.5. Cell Culture and Transfection

NG108-15 cells were grown in DMEM containing additions, as described (Takeuchi et al., 2002a), NB2A cells were grown in MEM containing 10% fetal calf serum, 10mM glutamine and 7.5% $NaHCO_3$, and C6 glioma cells were grown in DMEM containing 10% fetal calf serum. Cells were cultured in a humidified incubator with a 5% CO_2 atmosphere at 37°C. NG108-15, NB2A and C6 glioma cells (3×10^5 cells/dish) were plated in a 35-mm dish and cultured in the standard medium for 24 h. These cells were transfected with the mPer1, CRE or SRE reporter vectors (0.5μg of each DNA), 1.0μg of each kinase expression vectors (PKA, MEKK, CaM kinase IIδ, CaM kinase IV) or mock vector with pRL-TK (0.1μg of DNA; Promega Corp.) which contains *Renilla* luciferase under the herpes simplex virus thymidine kinase promoter using 4.8 μl of FuGENE transfection regent (Roche Applied Science) in 1.5 ml of serum-free medium for 6 h. The culture medium was changed to the standard medium and the cells were further cultured for 48 h. After the cells were treated with chemicals for each experiment, the activities of firefly luciferase and *Renilla* luciferase were measured by Dual-Luciferase Reporter Assay System (Promega, corp.) with luminometor (TD-20/20, Promega, corp.) according to the manufacture's protocol. The ratio of luminescence signal by firefly luciferase to that by *Renilla* luciferase was measured. To define the

CaM kinase II δ-sensitive element on the mPer1 promoter, the cells were co-transfected with each mPer1 reporter vector (0.9μg of DNA) and the constitutively active CaM kinase IIδ1 (0.5μg of DNA) with pRL-TK (0.1μg of DNA; Promega Corp.). For the assay using C/EBPβ and SP-1, the cells were co-transfected with each mPer1 -1813 reporter vector (0.9μg of DNA), CaM kinase II δ1 (0.5μg of DNA), C/EBPβ or SP-1 (0.1μg of DNA) and pRL-TK (0.1μg of DNA; Promega Corp.).

To confirm activity of the constitutively active mutant of CaM kinas IIδ1, Ca^{2+}/CaM-dependent (total activity) and Ca^{2+}/CaM –independent activities were measured using cell extracts obtained from the transfected NG108-15 cells as described (Fukunaga et al., 1993). Briefly, cells were homogenized at 4 °C by sonication in a 0.1 % Triton X-100, 50 mM HEPES, pH 7.4, 4 mM EGTA, 10 mM EDTA, 15 mM $Na_4P_2O_7$, 100 mM β-glycerophosphate, 25 mM NaF, 1 mM dithiothreitol, 0.1 mM leupeptin, 75 μM pepstatin A and 0.1 mg/ml aprotinin. The insoluble material was removed by centrifugation at 15, 000 x *g* for 5 min. The assay medium contained (for 25 μl): HEPES (50 mM), [γ-^{32}P]ATP (0.1 mM), syntide-2 (40 μM), Mg acetate (10 mM), bovine serum albumin (1 mg/ml), PKI-tide (5 μM) and PKC_{19-36} (2 μM) to block cAMP-dependent protein kinase and protein kinase C, respectively. The assay was carried out either in the presence of EGTA (1 mM), to measure the Ca^{2+}/CaM-independent activity, or $CaCl_2$ (1 mM) and calmodulin (3 μM), to measure the total activity.

7. Results

7.1. Expression Pattern of CaM Kinase II Isoforms and CaM Kinase IV in SCN

To difine which type of CaM kinase II is involved in light-induced mPer1 expression, immunoblot analysis using extracts from rat SCN was carried out with CaM kinase IIα, β, γ and δ antibodies. Immunoreactive bands with anti-CaM kinase IIδ and anti-CaM kinase IIγ antibodies were detected, whereas those with anti-CaM kinase IIα and CaM kinase IV antibodies were faint. No apparent band with anti-CaM kinase IIβ was detected in the SCN (Fig. 1).

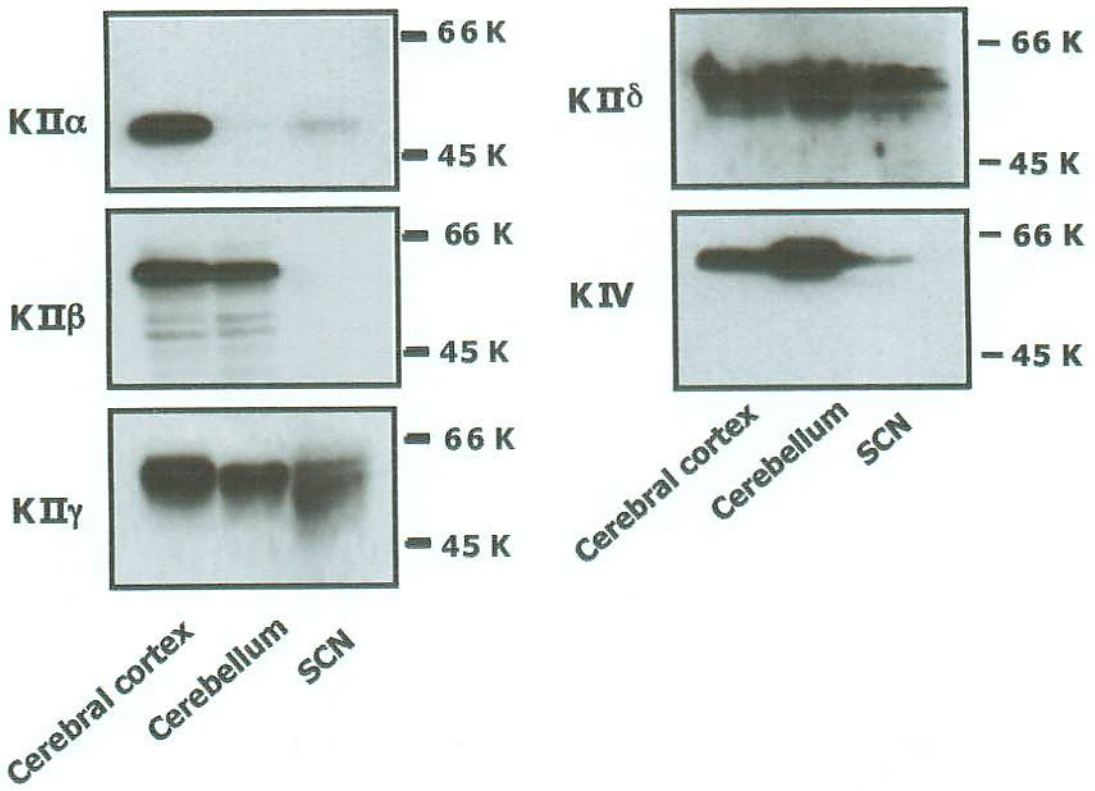

Fig. 1. Immunoblot analysis of various subunits of CaM Kinase II, or CaM Kinase IV in the rat SCN

Extracts from the cerebrum, cerebellum and SCN from the rat were separated on SDS-PAGE and transferred to PVDF membranes. The subunits of CaM kinase II and CaM kinase IV were detected with the subunit specific antibodies for each subunit of CaM kinase II and CaM kinase IV (see MATERIALS AND METHODS). The positions of the molecular makers with 45 and 66 K were shown in the right side in each panel.

In addition, immunohistochemical studies revealed that the CaM kinase IIδ subunit was highly expressed in the neurons of the rat SCN (Fig. 2a, b). This is in contrast with weak immunoreactivities for anti-CaM kinase IIγ and CaM kinase IV antibodies in the SCN (Fig. 2c-f). The weak immunoreactivity with anti-CaM kinase IIγ antibody was observed along the optic nerve (Fig. 2c,d).

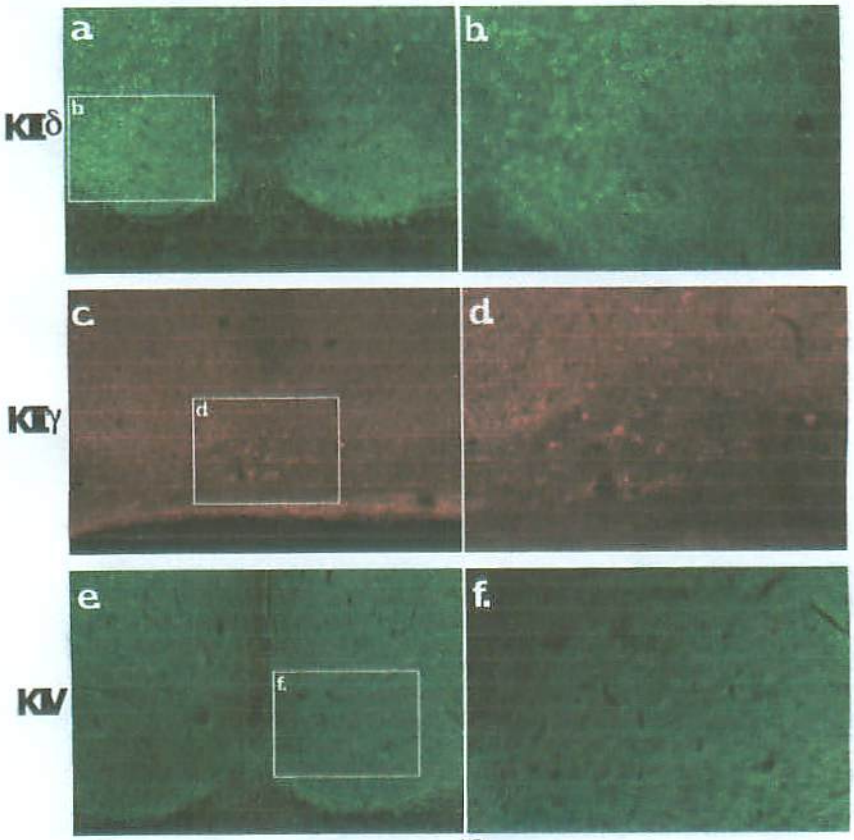

Fig. 2. Confocal laser scanning light microscope (CLSM) images in the rat SCN stained with the anti-CaM Kinase II γ, δ antibodies and anti-CaM Kinase IV antibody.

Rat SCN slices were examined by immunofluorescence microscopy after staining with anti-CaM kinase IIδ, CaM kinase IIγ or CaM kinase IV antibody. **a. b.** The SCN slices were immunostained with the anti-CaM kinase IIδ antibody followed by FITC-conjugated anti-rabbit IgG antibody. **c. d.** The SCN slices were immunostained with the anti-CaM kinase IIγ antibody followed by rhodamine-conjugated anti-goat IgG antibody. **e. f.** The SCN slices were immunostained with the anti-CaM kinase IV antibody followed by FITC-conjugated anti-rabbit IgG antibody.

7.2. Activation of mPer1 Promoter by CaM Kinase IIδ1

I next examined wherther CaM kinase IIδ was involved in the mPer1 expression. To confirm the activity of the constitutively-active mutant of CaM kinase IIδ1, I measured the CaM kinase II activity using extracts from the transfected cells. As shown in Fig. 3a, the total activity of CaM kinase II increased significantly in the transfected cells with CaM kinase IIδ1 and constitutively-active CaM kinase IIδ1, as compared to those with mock vector. The activities of constitutively-active MEKK, PKA, and CaM kinase IV were confirmed also, with stimulatory effects of the SRE promoter for MEKK and the CRE promoter for the three protein kinases in NG108-15 cells (Fig. 3b). Therefore, the effects of four types of constitutively-active protein kinases, including CaM kinase IIδ1, CaM kinase IV, MEKK and PKA, on mPer1 promoter activity were examined in NG108-15, NB2A and C6 glioma cells using a luciferase reporter assay system (Fig. 3c). Overexpression of MEKK and CaM kinase IIδ1 resulted in increases in the mPer1 promoter activity to different extents among the cells (Fig. 3c).

Overexpression of CaM kinase IIδ1 had the highest effect (three-fold increase) on the mPer1 promoter activity in NG108-15 cells. By contrast, overexpression of MEKK greatly increased the promoter activity by 2.5- and 8-fold in the C6 and NB2A cells, respectively. Overexpression of PKA enhanced slightly the promoter activity only in NG108-15 cells (Fig. 3c).

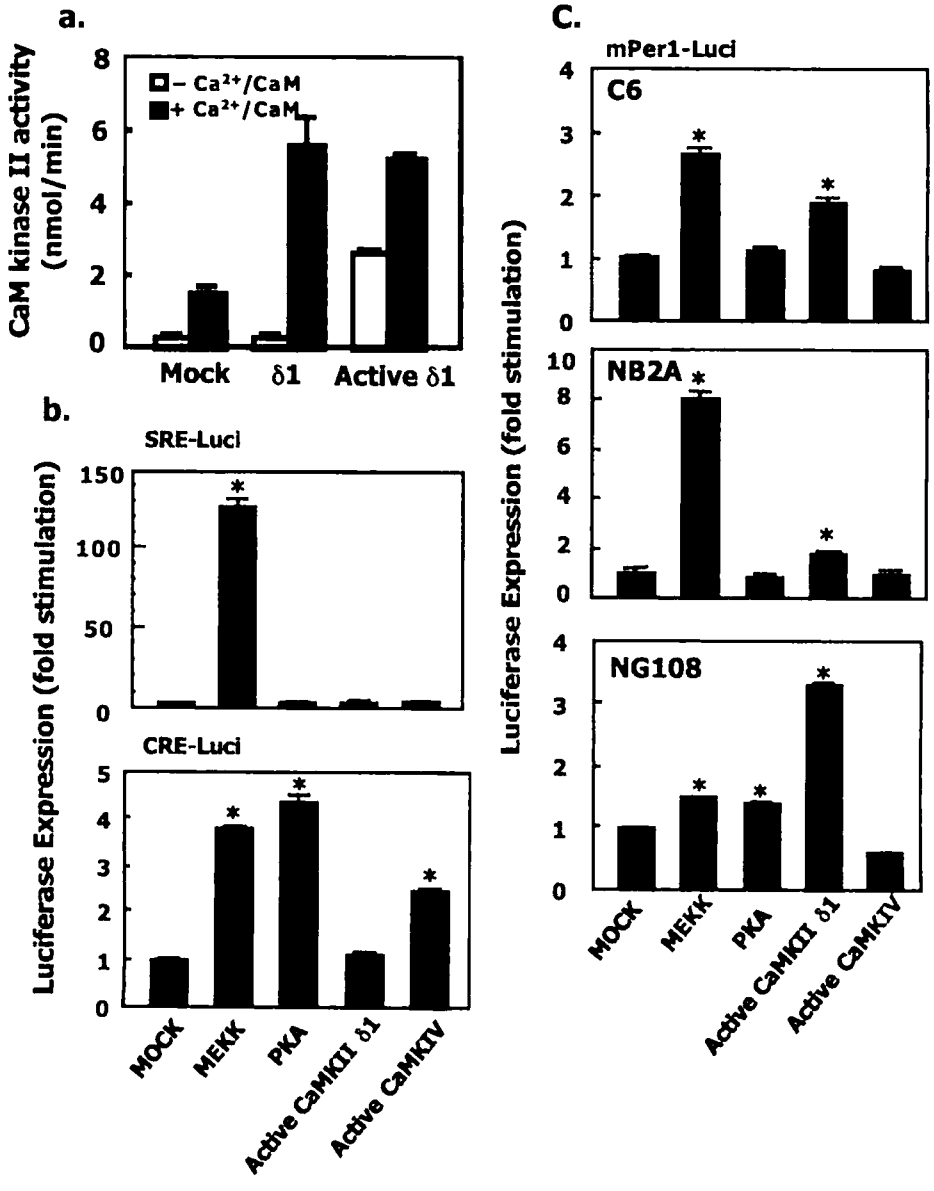

Fig.3. Effects of overexpression of constitutively active protein kinases on activities of SRE or CRE containing promoter and mPer1 promoter

a. NG108-15 cell were transfected with control plasmid (Mock), CaM kinase IIδ1, and the active mutant of CaM kinase IIδ1. The extracts obtained from the transfected cells were subjected to CaM kinase II activity assay in the presence or absence of Ca^{2+}/CaM. **b.** NG-108-15 cells were co-transfected with SRE or CRE containing vectors and pRL-TK together with the empty vector (Mock) or individual constitutively- active kinase expression vectors. Luciferase activities were determined as described in Materials and Methods and expressed relative to that of Mock-transfected cells. **c.** C6, NB2A and NG108-15 cell were co-transfected with mPer1 complete promoter vector and pRL-TK together with individual constitutively-active kinase expression vectors. The data are represented as the mean ± S.D. for three independent experiments. $*p < 0.01$ compared with the Mock.

7.3. CaM Kinase II Responsive Region in mPer1 Promoter

To identify CaM kinase II-responsive regions in the mPer1 promoter, I assessed the responsiveness to CaM kinase IIδ1 using truncated promoter constructs. Schematic representations of the truncated mPer1 promoter constructs and their luciferase activities in NG108-15 cells are shown in Fig. 4a. The luciferase activity of mPer1-1848 construct was lost largely, but that of mPer1-1813 construct restored by 80% of the complete promoter. The luciferase activities of mPer1-1721 and mPer1-1706 then decreased gradually. The marked decrease of the luciferase activity of mPer1-1848 suggested the presence of a negatively regulatory sequence in the region from -1848 to -1813 that was masked in the case of complete promoter of mPer1. Significant

promoter activities of mPer1-exon 1B and mPer1-exon 1A constructs were not observed, as shown in Fig. 4a. NG108-15 cells were then co-transfected with each of these luciferase constructs plus CaM kinase IIδ1 expression vector, and relative luciferase activities in CaM kinase IIδ1-trunsfected cells were compared to that in mock-transected cells (Fig. 4b). The mPer1-1848 and mPer1-1813 constructs showed essentially the same stimulatory effects of CaM kinase IIδ1 on their promoter activation as the full-length promter (mPer1 comp.). Whereas mPer1-1721, mPer1-1706 and mPer1-exon1B constructs largely lost the stimulatory effects by CaM kinase IIδ1, and the effects were not observed in the mPer1A construct. These results showed clearly that the region from -1813 to -1721 was essential for CaM kinase IIδ1 responsiveness.

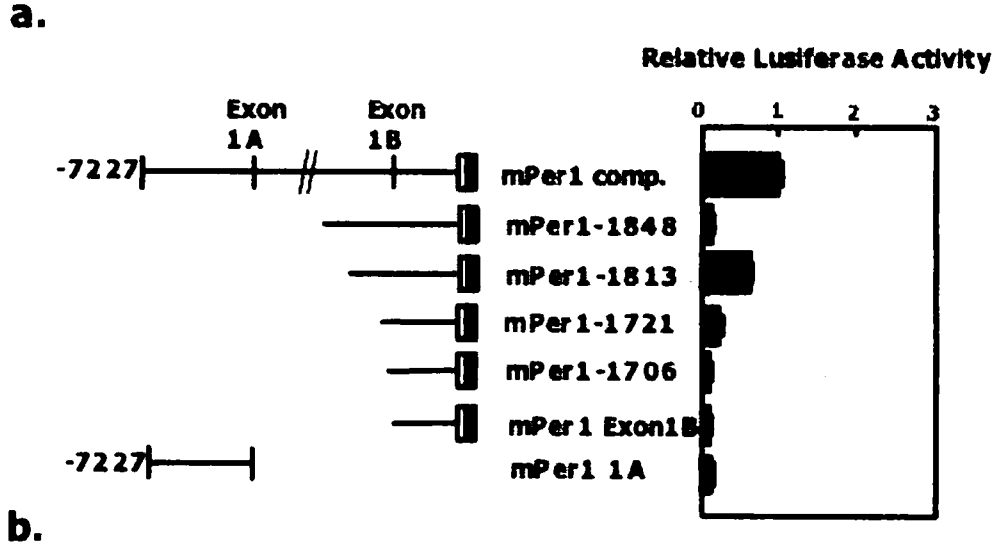

Fig.4. Relative luciferase activities in the mPer1 promoter constructs and CaM kinase II responsiveness

a. Schematic structures of the mPer1 promoter constructs and their relative luciferase activities in NG108-15 cells. NG108-15 cells were co-transfected with each reporter vectors and pRL-TK. Luciferase activities relative to mPer1 complete vector (mPer1 comp.) are expressed in each column. **b.** Relative luciferase activities in NG108-15 cells co-transfected with indicated promoter constructs together with or without constitutively active CaM kinase IIδ vector. The data are presented as the mean ± S.D. for three independent experiments. *$p<0.01$ versus without CaM kinase IIδ.

7.4. Effects of C/EBPβ and SP-1 on mPer1 Promoter Activity

Because the CaM kinase II responsive region contains two CCAAT and two GC-box elements, which are responsive to C/EBPβ and SP-1, respectively, we investigated whether C/EBPβ and SP-1 were involved in CaM kinase II-induced potentiation of mPer1 promoter activity by co-transfecting C/EBPβ and SP-1 into NG108-15 cells. Among the C/EBP family members, C/EBPβ is a possible transcription factor responsible for CaM kinase II (Yukawa et al., 1998). Transfection of C/EBPβ had no effects either on the basal promoter activity or on the CaM kinase II-induced promoter activity using the mPer1-1813 construct (Fig. 5). In contrast, SP-1 caused a significant decrease in the mPer1-1813 construct promoter activity, and co-transfection of CaM kinase IIδ1 totally abolished the inhibition (Fig. 5).

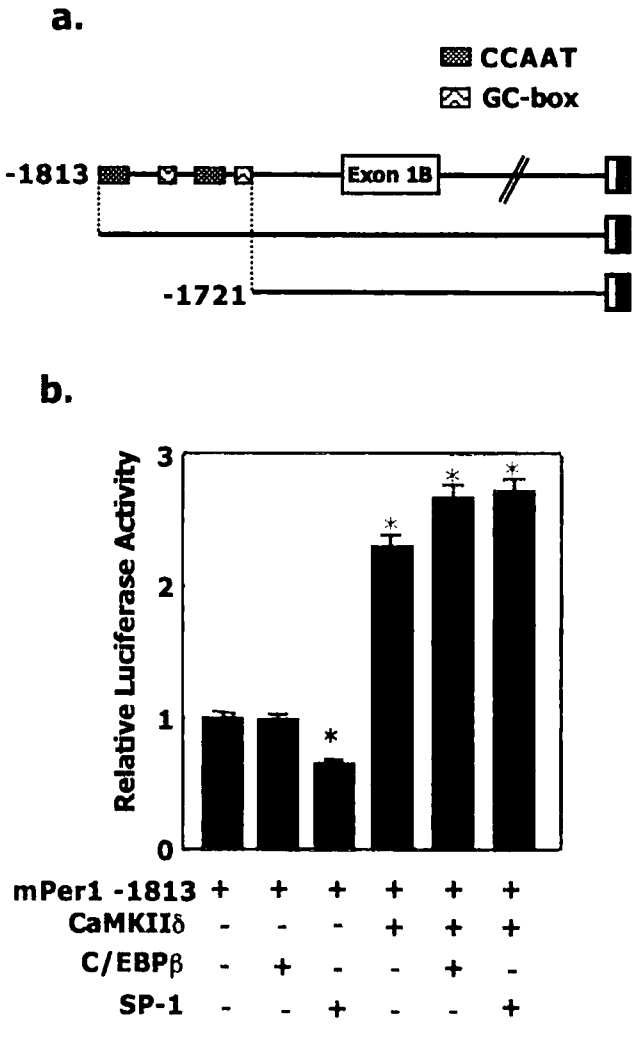

Fig.5. Effects of co-expression of C/EBPβ and SP-1 with CaM kinase IIδ on the mPer1 promoter activity

Schematic structures of mPer1 promoter and CCAAT and GC-box elements around -1813 to -1721 region. **b.** Effect of C/EBPβ or SP-1 on mPer1 promoter. NG108-15 cells were co-transfected with mPer1 -1813 constructs together with C/EBPβ or SP-1 vectors in the presence or absence

of CaM kinase IIδ. The luciferase activities are relative to that of mPer1-1813 vector alone and presented as the mean ± S.D. for three independent experiments.

7.5. Identification of CaM Kinase II-Responsive Regions

To identify the CaM kinase II-responsive regions, additional truncated and mutated promoter constructs were used for the luciferase promoter assay as shown in Fig. 6. Schematic representation of these mPer1 promoter constructs and their luciferase activities in NG108-15 cells are shown in Fig. 6A. Consistent with the result in Fig. 5, elimination of two CCAAT and one GC-box in the mPer1 -1735 construct showed essentially the same stimulatory effect of CaM kinase II on the promoter activity as shown in Fig. 6a. The result indicated that CaM kinase IIδ regulated the mPer1 promoter via the 14 bp from -1735 to -1720. I then searched about the 14 bp (5'-GAGGGGCAGGGGCCT-3') as binding regions some zinc finger proteins. Interestingly , mutation of 5'-GAGGGG-3' to 5'-GAATTC-3' at the position -1735 to -1730 totally abolished the CaM kinase IIδ-responsiveness in the mPer1 promoter activity. This results indicated that CaM kinase IIδ regulated mPer1 expression via the 5'-GAGGGG-3' element on the mPer1 promoter region.

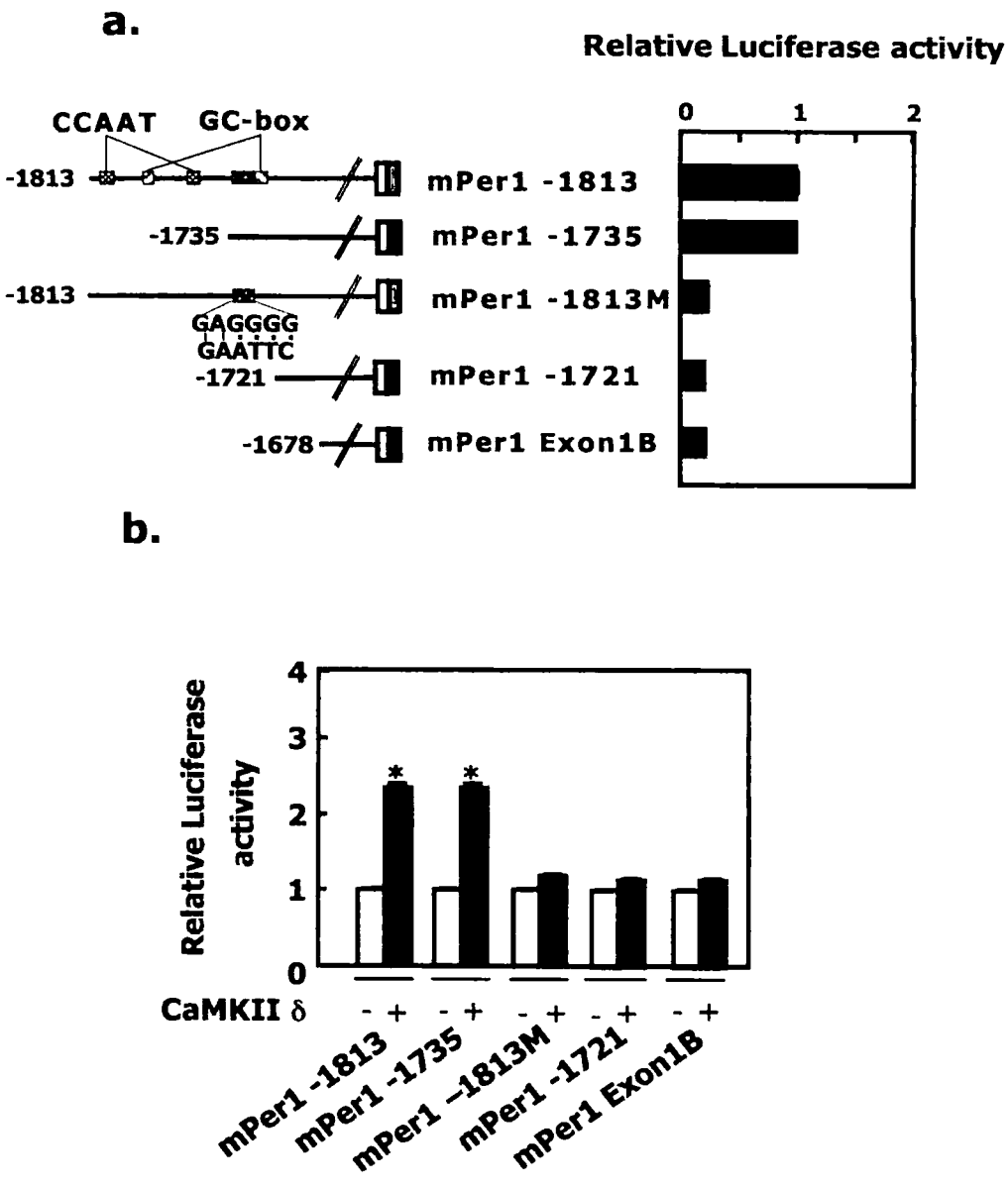

Fig.6. Deletional and mutational analyses of CaM kinase II-responsive regions of the mPer1 promoter.

a. The number of each constructs indicate positions relative to the transcription start site (+1). mPer1 -1813M had mutation on the indicated region. Luciferase activity of the individual promoter constructs was presented relative to that of mPer1-1813 promoter. Luciferase activities were presented as the mean ± S.D. for three independent experiments. b. CaM kinase II-responsiveness in each construct. Relative luciferase activities are relative to that without CaM kinase IIδ transfection and presented as the mean ± S.D. for three independent experiments.

8. Discussion

The light-induced Per1 and Per2 expression in the hamster SCN was attenuated by CaM kinase II inhibitor, KN-93, but not by MAPK pathway inhibitors such as PD98059 and U0126 (Yokota et al., 2001). Similarly, KN-93 but not PD98059 or U0126 blocked the phase delays induced by light exposure at CT 13.5 (Yokota et al., 2001). These data supported the involvement of CaM kinase II in the Per1 gene expression following light exposure. In this study, I first demonstrated that CaM kinase IIδ is predominantly expressed in neurons of the rat SCN, which are in contrast with the predominant expression of CaM kinase IIα and β in the forebrain such as cortex and hippocampus. The second important observation is that the possible CaM kinase II-responsive element was defined in the upstream of the DBP binding site near exon 1B. In support of these results, overexpression of a constitutively active CaM kinase IIδ in the C6, NB2A and NG108-15 cells stimulated the promoter of mPer1. I here used the longest genomic DNA fragment (mPer1 -7.2 kbp) that spanned the preceding region of exon 1A to the untranslated region of exon 2 (Yamaguchi et al., 2000). The longest promoter construct had the highest transcriptional activity. Travnickova-Bendova et al (2002) reported that essential role of cAMP-responsive element binding site (CRE) in the cAMP and MAPK pathways in the mPer1 expression. The promoter construct of mPer1 used in the previous work contained 1.8 kbp from the putative transcription start site in exon 1B. Using the promoter region, they clearly demonstrated that the combined stimulation with PKA and MAPK pathways largely enhanced the mPer1 promoter activity in a CREB-dependent manner. Consistent with their results, I observed the significant increase in the promoter activity by overexpression of the constitutively active MEKK in all of the neural cell lines used. However, the constitutively-active PKA had a weak stimulatory effect only in NG108-15 cells. Overexpression of constitutively-active CaM kinase IV failed to stimulate the mPer1 promoter. Like

MAPK and PKA, CaM kinase IV has been known to phosphorylate CREB, thereby activating CRE-dependent transcription factor. Indeed, I here confirmed that overexpression of CaM kinase IV activates CRE containing promoter activity (Fig. 3a). The reason for lack of CaM kinase IV-induced induction of the mPer1 is not clear in the present study. The observation of the increased promoter activity by CaM kinase IIδ was in contrast with having no effect of the Ca^{2+} in the mPer1 promoter activity in the previous work (Travnickova-Bendova et al., 2002). The precise reasons for the difference between the previous and our results are not clear at present. The diversity of the effects of constitutively active MEKK, PKA and CaM kinase IIδ was observed in each cell line. The lack of downstream targets for each protein kinase in each cell type was possibly responsible for the different effects by each protein kinase on the mPer1 promoter in different cell lines. Furthermore, a low level of expression of CaM kinase II was expected in the human choriocarcinoma JEG3 cells used in the previous work compared with neuronal cell lines used here. The difference in the promoter constructs between the previous and our works also possibly accounts for the difference in Ca^{2+}-responsiveness in the mPer1 promoter activity. I also observed a decreased promoter activity in the shorter promoter construct (mPer1-1848), suggesting the presence of negative regulators that do not work in the longest mPer1 promoter. Taken together, the increased mPer1 promoter activity by CaM kinase II was consistent with the previous observation, in which activation of CaM kinase II in the SCN was involved in the photic stimulation-induced Per1 expression in the hamster SCN.

In the present study, I tried to define the intracellular signaling underlying the CaM kinase II-induced mPer1 expression. Deletional and mutational analyses of the mPer1 promoter demonstrated that the adjacent upstream region of exon 1B is more important for the promoter activity than that of exon 1A regarding the CaM kinase II-responsiveness and that 5'-GAGGGG-3' motif at the position -1735 to -1721 on mPer1 promoter is essential for its responsiveness. The site is putative motif for SP-1 like

proteins that bind to GA or GC boxes. Indeed, CaM kinase II and IV activities are reported to be involved in the SP-1 signaling pathway controlling cell cycle progression in the retinoblastoma (Shom et al., 1999). Unexpectively, the co-transfection of SP-1 with active CaM kinase II did not enhance the mPer1 promoter activity in NG108-15 cells (Fig. 5b). However, it was noticed that overexpression of SP-1 alone caused a significant decrease in the promoter activity and co-expression with CaM kinase II relieved the inhibition by SP-1. Taken together, removal of the negative regulator such as SP-1 by CaM kinase II partly accounts for the upregulation of mPer1 gene by the enzyme. Transcriptional regulation by SP-1 is regulated by its induction, ratio of SP-1/SP-3, phosphorylation (Hagen et al 1994). Thus further extensive studies are required to define the SP-1 function in the mPer1 promoter activity. Similarly, C/EBPβ, a member of C/EBP family, which is phosphorylated and activated by CaM kinase II (Wegner et al., 1992; Yano et al., 1996) failed to stimulate the mPer1 promoter with CaM kinase II in NG108-15 cells. The mutation analysis of the CCAAT site of mPer -1813 promoter construct had no effect on the CaM kinase II-induced activation (data not shown). Previous study reported several zinc finger transcription factors such as MAZ and MZF family members, which bind to G-box (Fujimoto et al., 2000; Song et al., 2001). However, there is little information about expression of these transcription factors in the brain and large family members of the transcription factors are reported. Further extensive studies are needed to identify downstream targets for CaM kinase II regarding mPer1 induction in the SCN. Yamaguchi et al. recently reported that DBP activated the mPer1 promoter containing a DBP-binding site located at -1718 to -1709, where is adjacent downstream of the GAGGG motif, suggesting that possible protein-protein interaction with DBP is responsible for the CaM kinase II responsiveness.

In conclusion, I define CaM kinase II-responsive element in the mPer1 promoter, which is possibly involved in the light-induced phase shift. Among CaM kinase II isoforms, CaM kinase IIδ is abundant in the rat SCN and may have characteristic

functions in the phase shift. However, diversity of the effects of various constitutively-active kinases in the various cell types suggested that I should focus on the issue how the signaling contributes in the SCN. Furthermore, the molecular mechanisms in the mPer1 expression are possibly different between the phase delay and advance in the photic entrainment. In addition, further studies will need to define transcription factors or signaling complexes as downstream target for CaM kinase II in the SCN. Since synergistic action in both MAPK and PKA pathways was demonstrated in the CREB-dependent mPer1 expression (Travnickova-Bendova et al., 2002), I will next study cross-talk between CaM kinase II and MAPK/PKA signaling for the mPer1 expression in the SCN.

9. References

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