SCOP, a novel gene product expressed in a circadian manner in rat suprachiasmatic nucleus

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Abstract To elucidate the mechanism of the circadian rhythm, genes differentially expressed during subjective day and night in the rat suprachiasmatic nucleus (SCN), a circadian oscillator in mammals, were surveyed by a differential display method. We isolated a novel gene, scop (SCN circadian oscillatory protein), that was expressed in a circadian manner in the SCN. SCOP protein is predominantly expressed in the brain and has domains including a pleckstrin homology domain, leucine-rich repeats, a protein phosphatase 2C-like domain and a glutamine-rich region. The structural feature of SCOP protein suggests its role in the intracellular signaling in the SCN.

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Key words: Circadian rhythm; Differential display; Suprachiasmatic nucleus; Leucine-rich repeat; Protein phosphatase 2C; Rat brain

1. Introduction

Almost all living organisms show circadian rhythms in their behaviors and physiological phenomena [1,2]. These circadian rhythms are driven by an endogenous oscillator with a period of about 24 h, and are synchronized to exactly 24 h by environmental time cues on the earth such as the light-dark cycle. In mammals, a circadian oscillator is known to be located in the hypothalamic suprachiasmatic nucleus (SCN) [1,2]. It has been shown that the circadian rhythm in *Drosophila* requires the interaction of Period (Per) and Timeless (Tim) proteins [3–5], their heterodimer's nuclear translocation [6,7] and the inhibitory regulation of per and tim transcriptions through an autoregulatory feedback loop mechanism mediated by other clock-related proteins, Jrk (*Drosophila* homolog of Clock) and Cycle (*Drosophila* homolog of BMAL-1) [8]. Progress has been made in cloning some mammalian homologs for *Drosophila* clock components to elucidate molecular components of mammalian clocks [9–16]. Therefore, a framework of the mechanism for the generation of the circadian oscillation seems to be established. However, the precise mechanism has not been fully clarified yet.

In order to find genes that might be involved in the mechanism of the circadian rhythm in mammals, we tried to identify the genes whose mRNA expressions were different during subjective day and night in the rat SCN. To this end, we employed a mRNA differential display method, which is based on reverse transcription (RT) and arbitrarily primed polymerase chain reaction (PCR), and identified a molecule (we termed this protein SCOP for SCN circadian oscillatory protein) that oscillates in the SCN under a constant dim light condition. The characterization of this molecule is described.

2. Materials and methods

2.1. Animals

Male Wistar strain rats, weighing 250–350 g, were first housed individually in plastic cages in a room maintained at 24 ± 1°C and illuminated for 12 h (7.00–19.00 h) by fluorescent light (about 80 lux). Then, they were transferred to a room constantly illuminated with a dim (about 0.2 lux) red light (DD) throughout the whole day. Food (type MF; Oriental Yeast Co.) and water were freely available. These rats were adapted to this condition for at least 7 days before the experiments.

2.2. Collection of the tissue of the SCN

For SCN samplings under the DD condition, the subjective circadian time of each animal was determined by monitoring its locomotor activity pattern. Animals were killed by decapitation at various circadian times of the day, and their brains were quickly removed and frozen in dry ice. Coronal brain sections with a thickness of 700 μm including the SCN were made using a cryostat, and bilateral SCNs were punched out from the brain section using a 20-gauge needle. Tissues outside the SCN within the hypothalamus which located dorsolaterally to the SCN were similarly obtained.

2.3. mRNA differential display method

Total RNA was isolated from the SCN tissues of five rats sampled at circadian times (CT) 6 (the middle of the subjective light period) and CT 18 (the middle of the subjective dark period) using the RNeasy total RNA kit (Qiagen), and reverse-transcribed with an oligo dT primer according to the protocol for SuperScript II (Gibco BRL). In brief, denatured total RNA was incubated in a reaction mixture (20 μl) consisting of 50 mM Tris-HCl (pH 8.3), 71 mM KCl, 3 mM $MgCl_2$, 10 mM dithiothreitol, 0.5 mM dNTP and SuperScript II (200 units) at 42°C for 50 min, and the reaction was terminated by heating at 70°C for 15 min. To remove RNA complementary to the cDNA, the reaction mixture was incubated with 2 units of RNase H (Gibco BRL) at 37°C for 20 min. One milliliter of the first strand cDNA mixture (10-fold diluted) was used for arbitrarily primed PCR (AP-PCR). AP-PCR was performed in a supplied Ex-Taq buffer (Takara) containing 0.5 mM of arbitrary primers, 0.2 mM of cold dNTP, 0.18 mCi/66 nmol/l of [α-^{32}P]dCTP, and 2.5 U/ml Taq DNA polymerase (Ex-Taq, Takara). PCR conditions were 95°C for 5 min, 75°C for several minutes for hot start, 45°C for 2 min and 72°C for 2 min for 1 cycle, and then 95°C for 1 min, 45°C for 1 min, 72°C for 1 min for 40 cycles. A final elongation cycle was performed at 72°C for 5 min. A 6 ml aliquot of each PCR sample was mixed with 4 ml denaturing loading buffer and heated at 78°C for 3 min, and then resolved on a 6% denaturing polyacrylamide gel for DNA sequence. PCR products were detected by autoradiography.

2.4. Subcloning of PCR products

Bands selected from the autoradiograms were excised from the dried gel and the DNA was eluted by soaking in 50 ml TE buffer (10 mM Tris-HCl, pH 7.6, 1 mM EDTA) at 4°C for 2 days. One milliliter of eluted cDNA solution was subjected to the secondary PCR using the same primer set and PCR conditions except that [α-^{32}P]dCTP was omitted. The PCR products were treated with re-

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striction enzyme (*Bam*HI, Takara), and subcloned into pBluescript II SK$^-$ vector.

2.5. Screening of cDNA library and sequencing

The cDNA fragment of SCOP was radiolabeled with multiprime labeling kit (Amersham), and used as a probe to screen the rat hypothalamic cDNA library (Takara) and the human fetal brain cDNA library (Clontech) using standard laboratory protocols of molecular cloning. DNA sequencing was carried out using the DNA Sequencing kit (Big Dye Terminator Cycle Sequencing Ready Reaction, PE Applied Biosystems) and ABI 310 genetic analyzer (Perkin Elmer).

2.6. Competitive RT-PCR analysis

Total RNA was isolated from the SCN of each five rats killed at CT 0, 6, 12 and 18 using RNeasy total RNA kit (Qiagen). Reverse transcription was performed as described for AP-PCR. A competitor for the scop gene was made by deleting 680 bp from the fragment (nt 1281–2293). A control competitor for glyceraldehyde 3-phosphate dehydrogenase (G3PDH) was constructed by the PCR-based overlap extension method reported previously [17,18]. The competitor for G3PDH was 145 bp shorter than the original insert. The nucleotide sequences of the primers for the scop gene and its competitor were (forward) 5′-TCCGAGGAACTAGAAGCTGA-3′ and (reverse) 5′-GCAAGACACATTCAGCTCTG-3′, and for G3PDH and its competitor they were (forward) 5′-ACCACAGTCCATGCCATCAC-3′ and (reverse) 5′-TCCACCACCCTGTTGCTGTA-3′. PCR was carried out in a supplied Ex-Taq buffer (Takara) containing 0.25 mM primers, 0.2 mM cold dNTP, 2.5 U/ml Taq DNA polymerase (Ex Taq, Takara), 0.68×10^{-5} nM competitors. PCR was hot-started under the conditions: 95°C for 30 s, 75°C for several minutes for hot start, 60°C for 30 s and 68°C for 1 min for 1 cycle, and then 95°C for 10 s, 60°C for 10 s, 68°C for 1 min for 29 cycles. A final elongation cycle was performed at 68°C for 5 min. For quantitative analysis, aliquots of each PCR reaction were electrophoresed on 6% polyacrylamide gels and then stained with ethidium bromide.

2.7. Expression of fusion proteins and antibody production

Anti-SCOP antiserum was raised against glutathione *S*-transferase (GST)-SCOP fusion proteins containing the amino acids 735–1027 (GST-EC), 1029–1241 (GST-CB) and 1490–1696 (GST-C) of SCOP (Fig. 2). For purification of anti-EC and anti-CB antibodies, maltose binding protein (MBP)-SCOP fusion proteins containing the same parts of antigen were prepared. The GST and MBP fusion proteins were produced in *Escherichia coli* using pGEX (Pharmacia) and pMAL (NEB) vectors, respectively. The GST fusion proteins mixed with Freund's complete adjuvant were injected to rabbits. The resulting antisera against GST-EC and -CB proteins (αEC and αCB) were subjected to affinity purification using the MBP fusion proteins coupled to Affigel-10 (Bio-Rad). The antibody against GST-C protein (αC) was purified using the GST fusion protein coupled to Affigel-10.

2.8. Preparation of protein samples and immunoblot analysis

Tissues except the SCN were homogenized in 8 volumes of TNE buffer containing 20 mM Tris-HCl (pH 7.4), 1 mM EDTA, 0.15 M NaCl, 1% Nonidet P-40, 5% glycerol, 5 mM β-mercaptoethanol, 10 mg/ml aprotinin, 10 mg/ml leupeptin and 1 mM PMSF. The homogenates were centrifuged at $15\,000\times g$ for 20 min at 4°C. For subcellular fractionation, the brain tissue was homogenized in ice-cold 10 volumes of 10 mM Tris-HCl (pH 7.4) containing 0.32 M sucrose, and the homogenate was centrifuged at $1000\times g$ for 15 min (the first centrifugation). The precipitate (ppt) was resuspended in the same solution and centrifuged under the same conditions as above. The ppt was designated the P1 fraction (nuclear fraction). The supernatant (sup) was then combined with the sup from the first centrifugation and centrifuged at $10\,000\times g$ for 20 min. The ppt obtained was designated the P2 fraction (mitochondrial fraction). This sup was further centrifuged at $100\,000\times g$ for 60 min to separate the ppt, the P3 fraction (microsomal fraction), and the sup, the S fraction (cytosolic fraction). All fractions were adjusted to the same volume with the homogenization buffer. These protein samples were separated by 6% SDS-PAGE gels and transferred electrophoretically to a nitrocellulose membrane. After probing with anti-SCOP antibodies (1:400), immunoreactivities were visualized with alkaline phosphatase-conjugated anti-rabbit IgG (1:3000) using NBT and BCIP as substrates.

The SCNs and the regions outside the SCN in the hypothalamus punched out from five rats were directly homogenized in 100 μl of SDS sample buffer. To determine the protein concentration, these samples were separated by SDS-PAGE and stained with Coomassie brilliant blue. Immunoblot analysis was performed under the same conditions as above except that the visualization was performed by the chemiluminescence detection system (NEN).

3. Results

3.1. Isolation of differentially expressed mRNAs in the SCN

In order to screen genes that are differentially expressed in the SCN between subjective circadian day and night, total RNAs were prepared from the SCN tissue punched out from brains of rats which were killed at CT 6 and CT 18. The autoradiograms of the RNA fingerprints showed that most of the amplified fragments had similar radioactivities at the two circadian times, when primer sets A–D were used (Fig. 1). When primer E was used, however, there was a dramatic change in the radioactivity of the fingerprints between CT 6 and CT 18. Fourteen different bands were picked up and reamplified by PCR with the same primer. These products were then subcloned, sequenced and reexamined their expressions by RT-PCR using specific primers for each

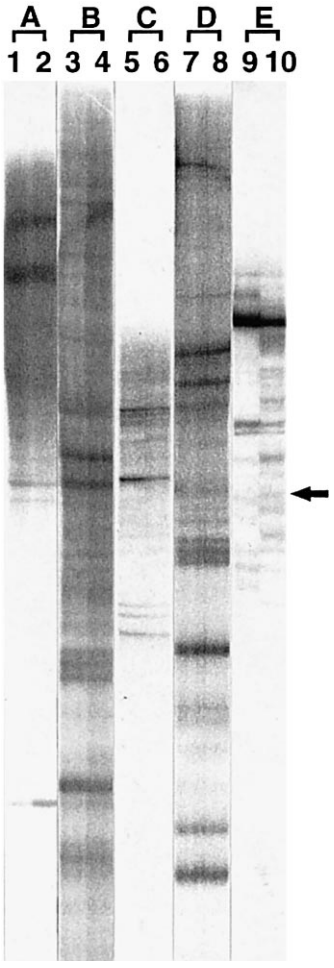

Fig. 1. Differential display of mRNA in the rat SCN. Total RNAs were prepared from the rat SCN at CT 6 and CT 18, and subjected to differential display using five sets of primers (A–E). Autoradiograms of the RNA fingerprints are shown. Lanes 1, 3, 5, 7 and 9 indicate cDNAs sampled at CT 6, lanes 2, 4, 6, 8 and 10 at CT 18. The arrow indicates the position of SCOP cDNA.

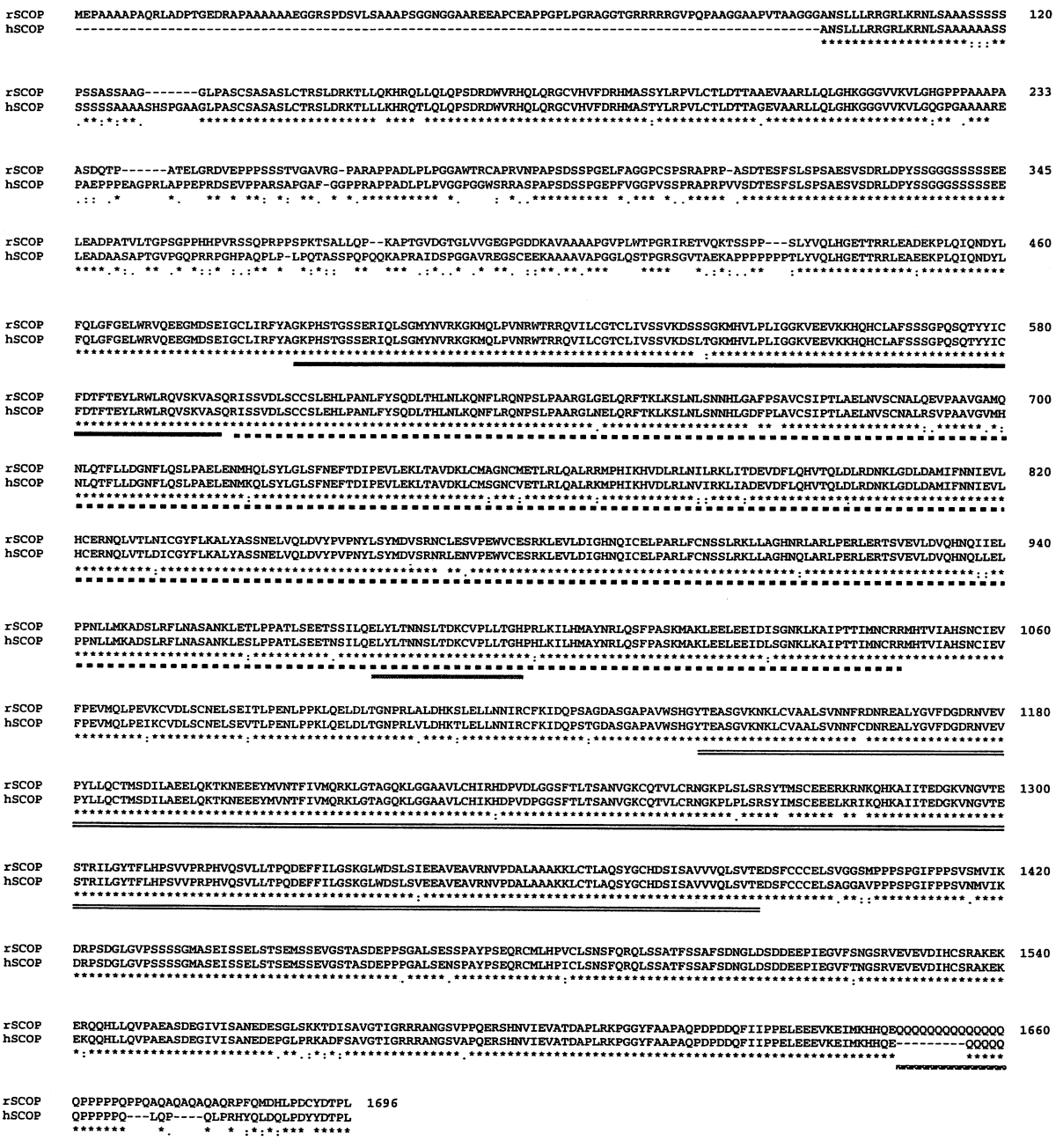

Fig. 2. Alignment of amino acid sequences of rat and human SCOP. The PH domain (black line), the LRRs (dotted line), the leucine zipper (gray line), the PP2C-like domain (double solid lines) and the Q-rich domain (meshed line) are underlined. Amino acid identity and similarity between rat SCOP (rSCOP) and human SCOP (hSCOP) are indicated by asterisks and dots below the hSCOP sequence, respectively. The nucleotide sequence data reported in this paper will appear in the DDBJ/EMBL/GenBank nucleotide sequence databases with the accession number AB023624.

clone. Consequently, only one clone (termed SCOP) was confirmed to be expressed differentially (CT 6 < CT 18) in the SCN.

Thus we focused on SCOP. We cloned a full-length cDNA for the rat SCOP from the hypothalamic cDNA library and a partial cDNA, lacking about 90 amino acids in its amino-terminus, for a human counterpart from the fetal brain cDNA library. The nucleotide sequence of the rat SCOP cDNA suggested that the clone encodes a novel protein consisting of 1696 amino acids (Fig. 2). The open reading frame was confirmed by in vitro translation and immunoblot analyses as described below. A similarity search for the deduced amino acid sequence revealed that SCOP is a novel protein that has multiple functional domains including a pleckstrin homology (PH) domain, leucine-rich repeats (LRRs), a leucine zipper (LZip), a protein phosphatase 2C (PP2C)-like domain and a glutamine-rich (Q-rich) region (Fig. 3A. The LRRs of rat SCOP contain 18 repeats of consensus motif and show the highest identity (28%) to the LRRs of the Ras-binding protein SUR-8 (Fig. 3B). The PP2C-like domain of SCOP is similar to not only PP2C catalytic domains of yeast (28% identity) and *Paramecium* (26%), but also PP2C-like domains of adenylate cyclases of yeast (26%) and smut fungus (30%) (Fig. 3C). The amino acid sequences in these

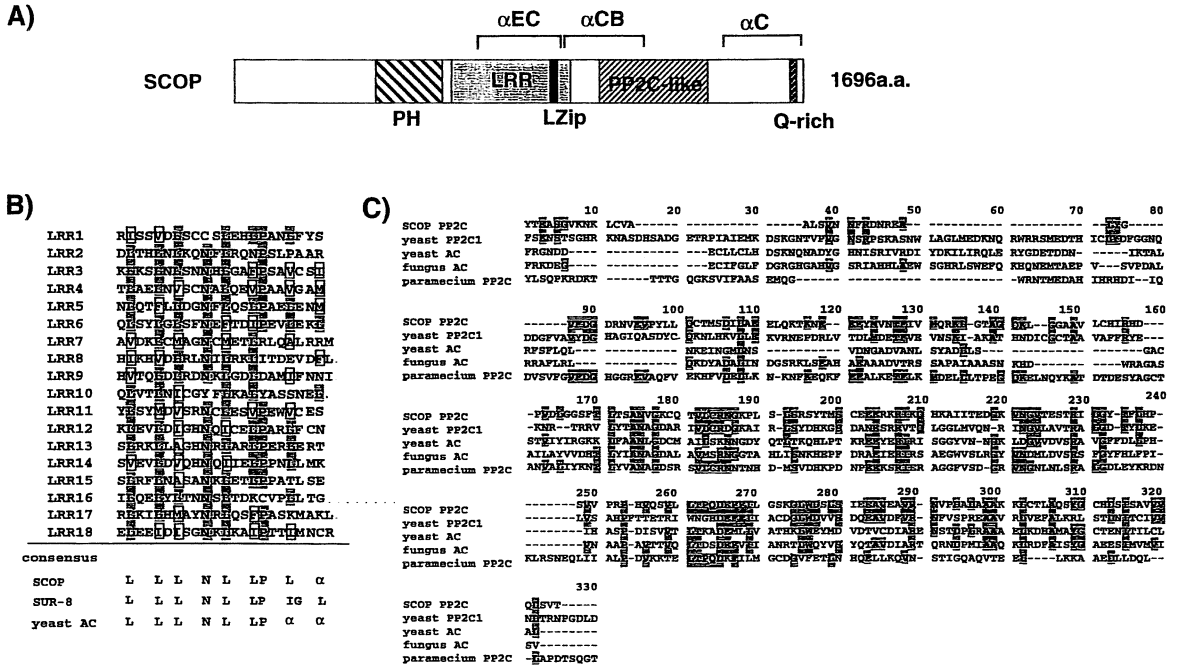

Fig. 3. The domain structures of SCOP. A: Positions of PH, LRR, LZip, PP2C-like and Q-rich domains in the rat SCOP protein are shown. The portions of SCOP protein to which antibodies were raised are indicated with upper bars (αEC, αCB and αC). B: Alignment of SCOP LRRs and the consensus of rat SCOP, human SUR-8 and yeast adenylate cyclase (yeast AC) is shown. Residues conserved in the majority of LRRs are shaded in black. Allowable substitutions for leucines within the LRRs are boxed. C: Comparison of the amino acid sequence of the PP2C-like domain of SCOP with the sequences of PP2C1 of *Schizosaccharomyces pombe* (yeast PP2C1), adenylate cyclase of *Saccharomyces cerevisiae* (yeast AC), adenylate cyclase of smut fungus (fungus AC), and PP2C of *Paramecium tetraurelia* (paramecium PP2C). Conserved amino acids are shaded in black.

domains were highly conserved across rat and human, implicating their crucial roles throughout evolution (Fig. 2).

3.2. Circadian changes in expressions of SCOP mRNA

The expression of SCOP mRNA in the SCN and a portion of the hypothalamus outside the SCN under the DD condition was semi-quantified by competitive RT-PCR (Fig. 4). To normalize the amount of mRNA in each sample, we used the detection levels of G3PDH. The expression of SCOP mRNA in the SCN was detected at the highest level at CT 24, gradually decreased through CT 6, and reached the lowest level at CT 12 under the DD condition. In the part of the hypothalamus outside the SCN, however, there was no change in the expression of SCOP mRNA.

3.3. Expression of SCOP protein

In order to examine the expression of SCOP protein, we raised three kinds of anti-SCOP antibodies that recognize amino acid sequences 735–1027 (αEC), 1029–1241 (αCB) and 1490–1696 (αC) of SCOP (Fig. 3A). Immunoblot analysis demonstrated that in vitro translated product of the SCOP cDNA was detected by these three antibodies, and that the molecular size of the product (183 kDa) was the same as that of the endogenous protein seen in the brain lysate (data not shown), confirming that the antibodies indeed recognize the SCOP protein.

The changes in the SCOP protein in the SCN were examined by immunoblot analysis using an anti-SCOP antibody (αEC) (Fig. 5). The immunoreactivity at the position corresponding to 183 kDa SCOP protein was readily detected in the SCN, and the level of the immunoreactive protein in the SCN increased during subjective night from CT 12 to CT 21 under the DD condition. In the hypothalamic part outside the SCN, the level of the immunoreactivity for SCOP protein was unchanged, consistent with the pattern of its mRNA expression.

The tissue distribution of SCOP protein was examined by immunoblot analysis using αEC. Immunoreactivity corresponding to 183 kDa SCOP protein was detected only in

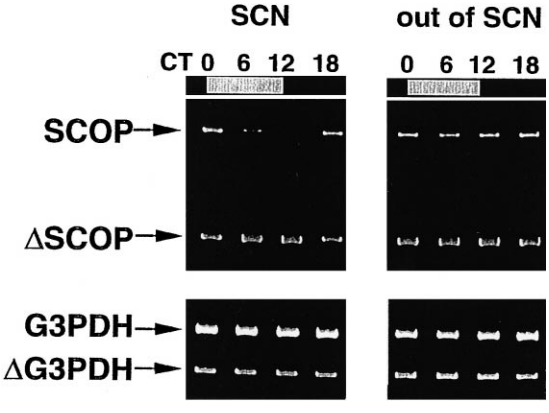

Fig. 4. Competitive RT-PCR using specific primers for SCOP and G3PDH. Samplings of the brain tissues were taken at CT 0, 6, 12 and 18 under constant dark (DD) condition, and total RNA was prepared for the RT-PCR. Ethidium bromide stainings of the PCR products are shown. ΔSCOP, a product of SCOP competitor; ΔG3PDH, G3PDH competitor. The expressions of G3PDH mRNA did not show daily changes in either the SCN or the part of the hypothalamus outside the SCN.

tissues of the central nervous system, cerebrum and cerebellum (Fig. 6A). In the testis, a marked immunoreactivity migrating slightly faster than the 183 kDa SCOP was detected by αEC. The distribution of SCOP protein in the nervous system was further analyzed by immunoblotting. As shown in Fig. 6B, immunoreactivity for SCOP protein was detected in various brain areas, but was relatively enriched in the hippocampus and the cerebellum. The intracellular localization of SCOP protein was examined by subcellular fractionation, followed by immunoblot analysis (Fig. 6C). Comparable immunoreactivities for SCOP protein were detected in the nuclear (P1), mitochondrial (P2) and cytosolic (S) fractions, potentially representing heterogeneous localization of SCOP in the cells. Immunoblot analysis for primary cultured rat brain neurons and astrocytes demonstrated that the SCOP immunoreactivity was highly concentrated in neurons (data not shown), suggesting specific functions in neurons.

4. Discussion

Current understanding of the mechanism of the circadian rhythm in mammals has progressed due to discoveries of mammalian homologs for Per and Tim and other related molecules [9–16]. Although mechanisms underlying circadian rhythms are believed to be conserved between flies and mammals, there is no direct evidence that the *Drosophila* system can indeed be applied to mammals. Moreover, the output mechanism downstream of the endogenous oscillation loop in the SCN is thoroughly unknown. In this study, we isolated a new gene, scop, that is expressed in a circadian manner in the SCN. By competitive RT-PCR and immunoblot analyses, it was found that the SCOP mRNA and protein in the SCN were high at dawn (CT 0 or CT 24) and low at dusk (CT 12) under the DD condition (Figs. 4 and 5). In contrast, there was no significant change in the level of SCOP mRNA outside the SCN under the DD condition. These findings suggest that the expression of SCOP protein is related to the generation or transmission of endogenous circadian oscillation in the SCN. The oscillation of the SCOP protein was also detected in the retina (data not shown), another possible circadian oscillator in mammals [19,20]. Considering these facts, it seems likely that SCOP protein is a candidate for a part of the clock machinery in the SCN, although its function remains to be elucidated.

From the deduced amino acid sequence, it was revealed that SCOP protein has characteristic domains. The PH domain is

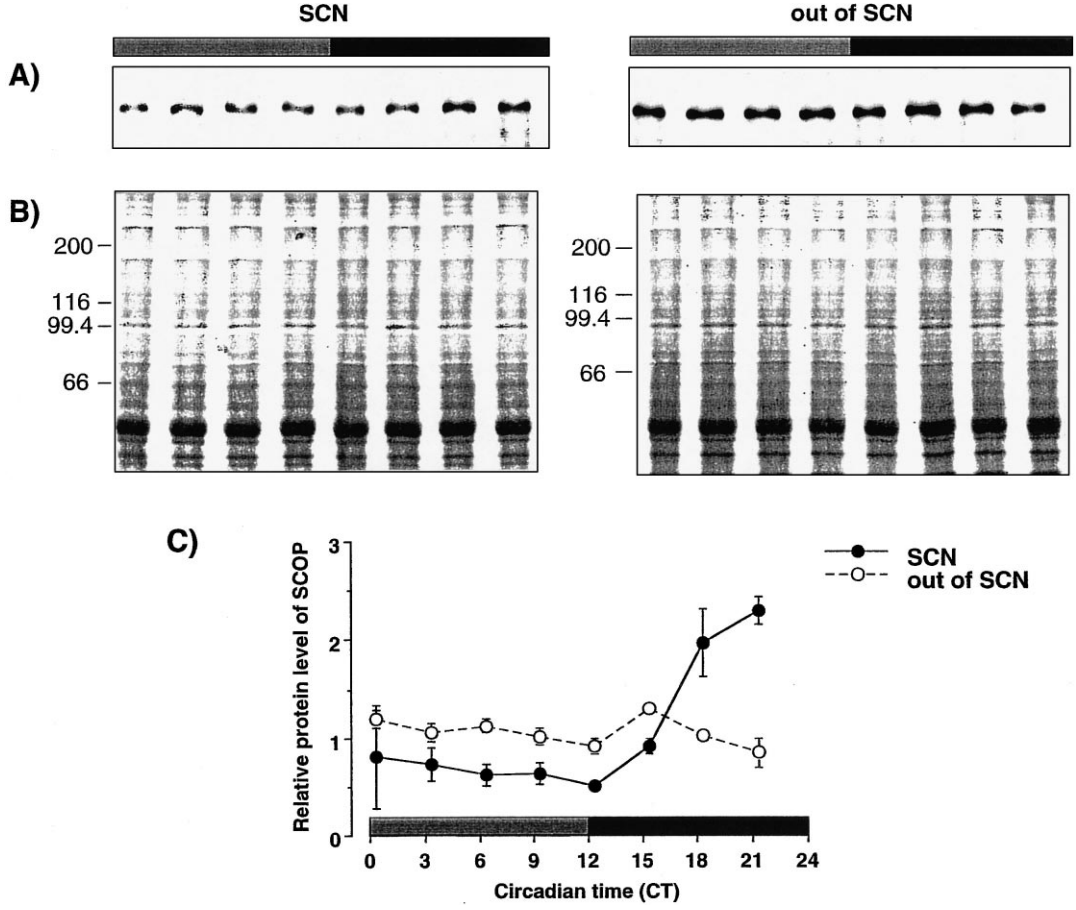

Fig. 5. Immunoblot analysis for the expression of SCOP protein in the SCN. Protein extracts (10 μg protein per lane) were prepared at different CTs from the SCN and the part of the hypothalamus outside the SCN of 6 week old male rats, and analyzed for the expression of SCOP protein by Western blotting with anti-SCOP antibody (αEC). A: Each case of Western blots done twice under DD condition is shown. The lanes from left to right show the data sampled at CT 0, 3, 6, 9, 12, 15, 18 and 21, respectively. B: Coomassie brilliant blue staining of the proteins resolved on the SDS-PAGE gel, indicating that the same amount of protein was applied to each lane. Numbers shown on the left side of the gels indicate positions of molecular size marker proteins in kDa. C: Quantification of the relative levels of the immunoreactivities for SCOP protein determined by a densitometer. The times of sampling are indicated on the abscissa. Data are expressed as means ± S.E.M.

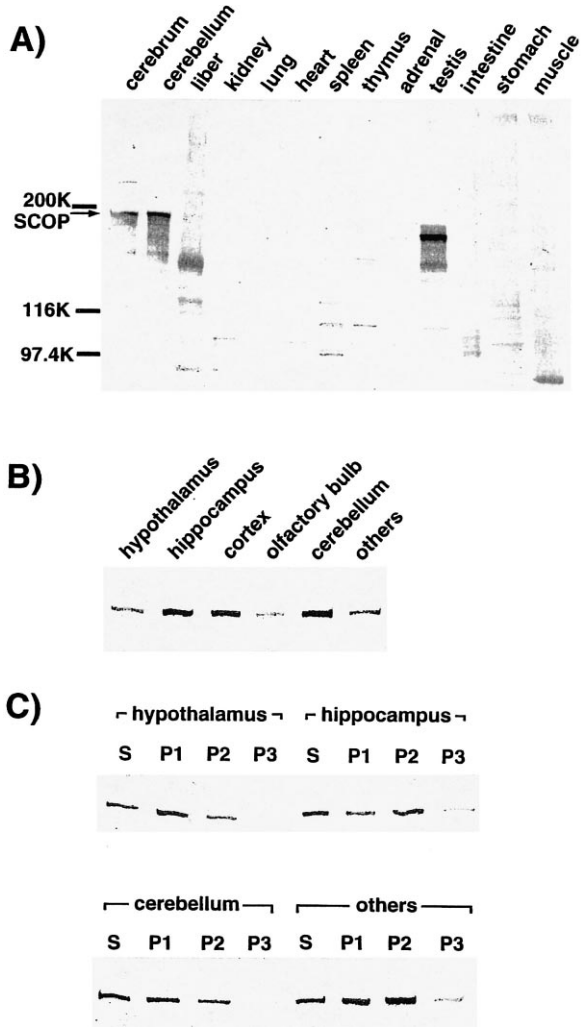

Fig. 6. Immunoblot analysis for the distribution of SCOP protein. A: Protein extracts were prepared from various tissues of adult male rat, and aliquots (10 μg protein per lane) were subjected to immunoblot analysis with αEC. Marked immunoreactivity was observed only in the brain (cerebrum and cerebellum) and testis. B: The rat brain was dissected into six parts (hypothalamus, hippocampus, cortex, olfactory bulb, cerebellum and others) and whole cell lysates were prepared. 20 μg protein of each sample was separated by SDS-PAGE, followed by immunoblot analysis with αEC. C: The tissues of hypothalamus, hippocampus, cerebellum and the others were subcellular fractionated into P1 (nuclear fraction), P2 (mitochondrial fraction), P3 (microsomal fraction) and S (cytosolic fraction) by sequential centrifugation. The volume of each fraction was normalized and an aliquot (10 μl) was separated by SDS-PAGE, followed by immunoblot analysis with αEC.

known to be included in some signaling molecules such as phospholipid-dependent protein kinase, Akt [21,22]. The interaction of the PH domain with phosphatidylinositol is known to be crucial for intracellular signal transmission mediated by PI3 kinase [23]. This raises the possibility that SCOP is involved in the intracellular signaling pathway through interaction with a membrane component. On the other hand, SCOP has LZip and a Q-rich region which have functional implications in the nucleus. From these structural features, it is hypothesized that SCOP's function is different in the membrane compartment and in the nucleus, or is a mediator of a signal from the membrane to the nucleus. In fact, the subcellular fractionation of the rat brain demonstrated that SCOP protein was recovered in either the membrane, cytoplasmic or the nuclear fractions. Further analysis must be done to elucidate the function of SCOP.

Both LRRs and PP2C-like domains are known to be conserved in adenylate cyclase of yeast. LRRs are also found in a variety of proteins with diverse biological functions and are proposed to mediate protein-protein interactions. Yeast adenylate cyclase contains 26 LRRs [24] that are required for binding to and activation by Ras during vegetative yeast growth [25,26]. Recent findings suggest that SOC-2 [27] and SUR-8 [28] having LRR domains homologous to yeast adenylate cyclase LRR bind to Ras and regulate Ras-mediated signaling. Thus, it is possible that SCOP is involved in the Ras-mediated signaling through the interaction between the SCOP LRR and Ras. The PP2C-like domain of SCOP is similar to not only the catalytic domains of PP2C of yeast and *Paramecium*, but also the PP2C-like domains of adenylate cyclases of several species. However, there is no report describing the phosphatase activity in the PP2C-like domain of adenylate cyclase. Likewise, the phosphatase activity of SCOP has not yet been detected in vitro in our laboratory (data not shown). There may be a specific activator or specific substrates for SCOP in vivo, although the possibility cannot be excluded that the PP2C-like domain has other functions than that of phosphatase. To clarify the function of these domains of SCOP, interacting proteins to the domains in vivo should be examined.

Here we described the identification of a novel protein, SCOP, that oscillated in a circadian manner in the SCN. Whether SCOP is indeed a part of the circadian clock, and if so, how SCOP is involved in the circadian oscillators, and what the actual function of the SCOP molecule is in the neural cells must be examined in the near future.

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