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Molecular Signals of Mammalian Circadian Clock

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INTRODUCTION

The feature of the circadian system is the prevalence of the oscillation at the levels of genes reflects at cells, tissues, and system levels. Oscillation starts at the intracellular molecular core feedback loop of clock genes. The oscillation starts first at the transcription of two main oscillators, *mPer1* and *mPer2*. The bHLH-PAS proteins (CLOCK and BMAL1) bind and initiate the transcription of *mPer* genes which are translated in the cytoplasm to mPER1 and mPER2 proteins. These proteins translocate into the nucleus, and form negative complex that comprises mCRY1, mCRY2, mPER1, mPER2, mPER3 and mTIM, which suppresses the transcription of the *mPer1* and *mPer2* genes by binding to the positive factors (CLOCK/BMAL1).

If the concentration of negative factors determines the time for the shut off of the transcription, the question remains what mechanisms determine the concentration of clock proteins. The mPERs are made in the cytoplasm, translocate into the nucleus, and form a negative complex comprised of mCRY1, mCRY2, mPER1, mPER2, mPER3 and mTIM that suppresses the transcription of the *mPer1* and *mPer2* genes by binding to the positive factors. Phosphorylation of PER1 and PER2 by casein kinase Iε (CKIε) is crucial for determining the circadian period length. Experiments leading to this conclusion and others point strongly to the importance of posttranscriptional and posttranslational regulatory mechanisms in the cell clock. Furthermore, there are growing evidences suggesting that clock proteins are regulated dynamically in both spatial (nuclear and cytoplasm) and temporal (production and degradation) dimensions. The main clock oscillatory protein mPER2 usually shuttles between the cytoplasm and the nucleus and is easily degraded by ubiquitination and the proteasome pathway. Ubiquitination of mPER proteins is

inhibited by the presence of mCRY proteins. Since mCRY protein can also be ubiquitinated when mPER proteins are absent, the mPER/mCRY dimer is stabilized, suppresses *mPer1* and *mPer2* transcription, and shuts off mPER translocation. Since it is speculated that the transcription level of *mPer* genes is understandable as the concentration of mPER/mCRY dimer in the nucleus, re-starting *mPer* transcription will depend on the nuclear export of the mPER proteins. The decrease of mPER in the nucleus by the CRM1/Exportin1 nuclear export machinery causes destabilization of mCRY, and the decrease of mCRY allows *mPer1* and *mPer2* gene transcription to restart.

In *Drosophila per*, the main oscillatory gene in the fly, the PER protein is known to show rhythm without accompanying the rhythm at its transcription level. In mammals, we recently found that mPER2 protein accumulation in the peripheral cells showed clear circadian oscillation even in the presence of constitutive *mPer2* mRNA expression by using the fibroblast cell lines in which expression of *mPer2* is controlled through tetracycline-regulatable promoter. This finding suggests that post-transcriptional regulation plays an important role in generating the core clock oscillation in mammals as in *Drosophila*. Since the mutation of *Drosophila slimb*, an F-box protein constituting ubiquitin ligase, shows the constant accumulation of PER protein and behavioral arrhythmicity, ubiquitin-proteasome mediated degradation will be involved in this process. The involvement of ubiquitin ligase to circadian clock oscillatory machinery will be evolutionally conserved since FWD1, an F-box protein, negatively regulates the ubiquitination of *Neurospora* FRQ, which is the main oscillatory component of circadian feedback loop in this species.

METHODS AND RESULTS

To reveal the role of ubiquitin-proteasome system in mammals, we examined the expression of ubiquitin-related substances in the SCN by in situ hybridization. The animals used for the in situ hybridization were male Balb/c mice at 6 weeks age. They were housed under standard 12h:12h light-dark (LD) cycles, and the expression of ubiquitin-related enzymes at ZT4 (ZT stands for Zeitgeber time in a LD cycle; ZT0 is lights-on and ZT12 is lights-off and thus ZT4 means 4 hours after the light onset). ^{33}P-UTP (New England Nuclear, Boston, MA) labeled antisense probe to UchL1, valosin-containing protein (VCP) and β-TRCP were made with a standard protocol for cRNA synthesis. Mice were deeply anesthetized with ether, and intracardially perfused with 10 ml of autoclaved ice cold saline, followed by a fixative containing 4% paraformaldehyde in 0.1 M phosphate buffer (PB) (pH7.4). For the animals housed in darkness, anesthesia was performed under safe dark red light. The brains were removed, postfixed in the same fixative for 24 h at 4°C and placed in 0.1 M PB containing 20% sucrose for 48 h. These brains were frozen using dry ice and stored at -80°C until use. Mouse brain sections were made 40 μm in thickness by a cryostat. In situ hybridization histochemistry was performed as described previously. Briefly, tissue sections were processed with 1 μg/ml proteinase K and 0.25% acetic anhydride. The sections were then incubated in the hybridization buffer (60% formamide, 10% dextran sulphate, 10 mM Tris–HCl, pH 8.0, 1 μM EDTA, pH 8.0, 0.6 M NaCl, 0.2% N-laurylsarcosine, 500 mg/ml transfer RNA, 1×Denhardt's and 0.25% sodium dodecyl sulphate) containing the ^{33}P-UTP-labeled antisense cRNA probes for 16 h at 60°C. After hybridization, these sections were rinsed in 2×SSC/50% formamide for 45 min at 60°C, and rinsed in 2×SSC/50%

formamide for 15 min at 60°C and the sections were treated with a solution containing 10 μg/ml RNase A for 30 min at 37°C. After rinsing, sections for free floating in situ hybridization were mounted onto gelatin-coated microscope slides, air-dried, and dehydrated.

β-TRCP, a mammalian homologue of *Drosophila slimb*, was only slightly expressed in the master clock in the suprachiasmatic nucleus (SCN). This suggests that this F-box protein seems not to be involved in the oscillation of the master clock in the SCN. Thus, the involvement of F-box protein on mammalian circadian system waits for further future analysis. Contrary to β-TRCP, in the SCN, we found the high levels of expression of UchL1, a main member of deubiquitinating enzyme in the brain. UchL1 recycles ubiquitin from ubiquitin-protein complexes or polyubiquitin chains by cleaving the amide linkage neighboring the C-terminal glycine of ubiquitin (19). We also found a high level of expression of molecular chaperone VCP in the SCN. More broader and deeper analyses are needed for elucidating the involvement of ubiquitin-proteasome on mammalian circadian system.

DISCUSSION AND CONCLUSION

In the SCN, clock genes keep timing with a clear circadian rhythmicity *in vitro* as *in vivo*. We found that molecular oscillatory components and their oscillatory mechanism of central clocks and peripheral clocks are mostly identical. In the SCN, the clock gene oscillation generated by the core loop in each SCN neuron produces the robust spike rhythms at each cell level. Thousands of multi-phased, clock oscillating cells synchronize and produce a stable and robust rhythm, which is transmitted to the peripheral tissues. Since peripheral tissue cultures *en bloc* show the circadian rhythm of clock genes, there must also be synchronizing ability among cells in peripheral tissues. It is thought that mammalian clock system displays a complex hierarchical structure of cell clocks at various levels headed by the oscillating SCN cell clocks at the top.

The unique feature of circadian biology is that the gene transcription occurring in the SCN, and reflects the behavioral and physiological rhythms. This means that the clock gene oscillation generated by the core loop in each SCN neuron is coupled and amplified, and spread into the whole brain and to all those peripheral organs including liver through oscillation conducting systems. In the peripheral organs, arriving clock signals entrain the cell clocks, and the intracellular oscillating molecular loop coordinates the timing of the expression of a variety of genes with specific cellular function. Gene array studies have demonstrated that hundreds of genes are controlled by circadian clock with its tissue specificity. From the core oscillatory loop to these clock controlled genes (ccgs), two routes are commonly used. The first is by E-box (CACGTG, CACGTT), and the second is by D-box (RTTAYGTAAY: R, purine; Y, pyrimidine).

The proteins are key players of cellular function. To perform effective cellular functions, things must be organized in ensemble in time dimension as well as in space dimension. Intracellular clock oscillating loop may be worth existing for controlling cellular events into proper and adequate time organization. TIME is the key word for clock genes to perform non-clock cellular functions.

神戸大学大学院医学系研究科（博士課程）

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（要旨は１，０００字～２，０００字程度）

地球上のほとんど全ての生物は、約24時間周期の行動や生理の変動を来し、これを概日リズム（circadian rhythm）と言う。医療の面からも、臨床症状、薬物動態・薬物効果にも日内変動があり、時間を考慮した時間医学が今注目を集めている。ショウジョウバエと同様、哺乳類でも、時計遺伝子が転写・翻訳後産生された時計蛋白質が、自分自身の転写制御を抑制するというコア・フィードバックループによるリズム発振分子機構が想定されている。まず、発振の中心となる振動子は、*Per1* と *Per2* の２つの遺伝子である。*Per1, Per2* の転写は、bHLH-PAS 蛋白である CLOCK と BMAL1 のヘテロダイマーが *Per1, Per2* のプロモーターの E-box にポジティブ因子として結合して促進される。続いて、*Per1*、*Per2* の転写によって産生された *Per1* RNA, *Per2* RNA から、PER1 蛋白質、PER2 蛋白質ができる。これが、細胞質から核の中へ入って、ポジティブ因子の転写を押さえるネガティブ因子になる。これには、リン酸化、ユビキチン化などによる蛋白質の修飾が関与している。

最近、時計蛋白質がユビキチン・プロテアゾーム系により分解されるメカニズムが明らかになりつつある。ショウジョウバエにおいては、*slimb* 変異体が恒常暗条件では行動の周期性が損なわれることが報告された。*slimb* 蛋白質は、リン酸化蛋白質を分解系へと向かわせる SCF 型 E3 ユビキチンリガーゼ複合体を構成する F-box/WD40 ファミリーに属する。恒常暗条件のもとでは *slimb* 変異体では高度にリン酸化された時計蛋白質が蓄積しており、時計蛋白質の周期的な分解が障害されていた。アカパンカビにおいても F-box/WD40 蛋白質の FWD1 ミュータントで蛋白質 FRQ が蓄積する。体内時計の発振の分子機構は進化的にも保存されているが、残念ながら、現在まで哺乳類においては SLIMB や FWD1 に相当するユビキチンリガーゼ複合体を形成する蛋白質は同定されていない。しかし、ヒトの線維芽細胞の細胞株で MG-132 や lactacystin の添加による PER1、PER2 の蓄積するなど、さまざまな証拠がユビキチン・プロテアソーム系の関与を示唆している。

この中で今回、ユビキチン・プロテアソーム系と同期して、分子シャペロンとして働く valocin containint peptide (VCP)に着目し、脳内発現を検索した。最も発現が高いのは、梨状葉、ブローカの対角帯核、視交叉上核、弓状核、視床下部背内側核、背側縫線核、小脳皮質、孤束核である。このうち、最も高い濃度が認められるリズムセンターの視交叉上核での日内変動を検索したが、概日リズムは認められなかった。視交叉上核において高い発現を示す VCP のターゲットはわかっていないが、PER、CRY などの時計蛋白質がターゲットである可能性はある。今回の検索の結果、VCP は時計被制御遺伝子 clock controlled gene でないことはあきらかであるので、恒常的に発現することでシャペロンの役割を果たすと考えられる。

概日リズムの重要な特質に、遺伝子の転写リズムがほぼ完璧な形で行動リズムに反映している事がある。具体的に述べると、リズムセンターである視交叉上核の各細胞におけるごく少数の遺伝子で構成されるコア・ループによる遺伝子レベルの時計発振が、視交叉上核という神経核レベルで同期・増幅され、全脳に伝播し、ついには行動・ホルモン分泌な

ど個体レベルの概日リズムを生み出す。発振は視交叉上核の各細胞であるが、振動の増幅・伝達機構は脳機能の効率性・特殊性が生かされていると言える。遺伝子から行動まで同じ現象を追うことができる事は生体リズムの重要な特質で、同じレベルの追求が可能な脳や生体機能はほとんど無いと言ってよい。今後、細胞内の遺伝子振動がいかにしてトータルとしての脳・生体機能である行動にまで至るかを解析する有力な系として、概日リズムの研究は用いられて行くであろう。

本研究は、哺乳類の時計発振機構を検索しVCPの脳内局在を明らかにした初めての論文で、重要な価値ある業績であると認められる。よって本研究者は、博士（医学）の学位を得る資格があると認める。