Circadian Rhythm

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Control of Circadian Phase by an Artificial Zinc Finger Transcription Regulator**

Miki Imanishi, Atsushi Nakamura, Masao Doi, Shiroh Futaki, and Hitoshi Okamura**

An internal circadian clock (from the Latin "*circa*" meaning "about" and "*dien*" meaning "day") has been found across kingdoms of life, a testimony that circadian rhythms are a basic feature of life on earth. Physiologically relevant circadian time originates from clock genes interlocked in transcription–translation feedback loops, which machinery can be found in most cells throughout the body.[1] The *cis*-regulatory elements of clock genes *Per* and *Cry* play pivotal roles in the autonomous rhythmic transcription and the entrainment of the clock to the external environment: E-box for the generation of rhythm,[2] Ca^{II}/cAMP response element (CRE),[3] and glucocorticoid responsive element (GRE)[4] for its entrainment (Figure 1 a). The GRE is especially interesting since it is the target of glucocorticoid hormones, potent regulators synchronizing peripheral clocks, potentially in the whole body.[4,5] Although the core machinery of oscillation and entrainment of the clock has been described in detail to date, along with the astonishing discovery that these gene transcription rhythms reflect behavioral rhythms,[1] so far no efforts seeking to adjust circadian time through direct action on the core-clock components have been reported.

Recently, C2H2-type zinc-finger-based artificial DNA binding proteins have seen tremendous development to specifically and efficiently manipulate the target genes.[6] In the present study, we succeeded in changing the phase of the clock, and hence to elicit its entrainment, by using an artificial ligand-inducible zinc finger transcriptional regulator specifically targeted to the GRE on the *Per1* promoter (Figure 1 a). Our results demonstrate the feasibility of using engineered zinc finger transcription factor to directly and specifically adjust the phase of the circadian clock.

[*] Dr. M. Imanishi, Dr. A. Nakamura, Prof. S. Futaki
Institute for Chemical Research, Kyoto University
Uji, Kyoto 611-0011 (Japan)
E-mail: imiki@scl.kyoto-u.ac.jp
Dr. M. Doi, Prof. H. Okamura
Graduate School of Pharmaceutical Sciences, Kyoto University
Sakyo-ku, Kyoto 606-8501 (Japan)
E-mail: okamura@pharm.kyoto-u.ac.jp

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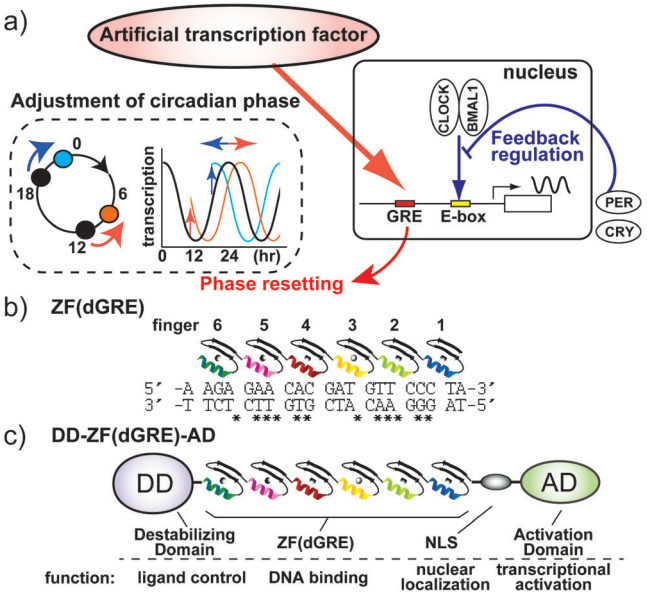

Figure 1. Schematic representation of artificial zinc finger proteins. a) Feedback regulation of clock genes through E-box and adjustment of circadian phases through GRE (solid square). In response to external stimuli, phase advances or delays are observed (dotted rectangle, blue and red, respectively). DD-ZF(dGRE)-AD targets a specific GRE on *mPer1* promoter. b) ZF(dGRE) and the target DNA. * = consensus GRE. c) Design of an artificial transcription factor, DD-ZF(dGRE)-AD. NLS = nuclear localization signal.

Mouse *Period1* (*mPer1*) promoter contains two possible GREs that locate distally (-3566, dGRE) and proximally (-1221, pGRE) to the transcription start site. Since mutation analyses indicated that dGRE is functionally active,[7] we created an artificial transcriptional regulator specifically recognizing dGRE. Here, we designed a six-zinc-finger protein, ZF(dGRE), which recognizes the dGRE (12 bp) together with six DNA base pairs flanking it in order to selectively bind to *mPer1* dGRE but not other GREs, by using the modular assembly of zinc finger units binding to specific DNA triplet sequences[8] (Figure 1 b and Table S1 in the Supporting Information).

The electrophoretic mobility shift assay demonstrated that the ZF(dGRE) binds to dGRE with over 20-fold higher affinity than to pGRE (the dissociation constant (K_d) values of dGRE and pGRE are (5 ± 0.36) and (115 ± 12) nM, respectively), which shares 15 identical bases among 18 with dGRE (Figure 2 a). Since ZF(dGRE) did not show any significant binding affinity to other GREs in dexamethasone-responding genes,[9] we can conclude that ZF(dGRE) is very selective to the *mPer1* dGRE. The binding specificity of ZF(dGRE) to the *mPer1* dGRE sequence is further sup-

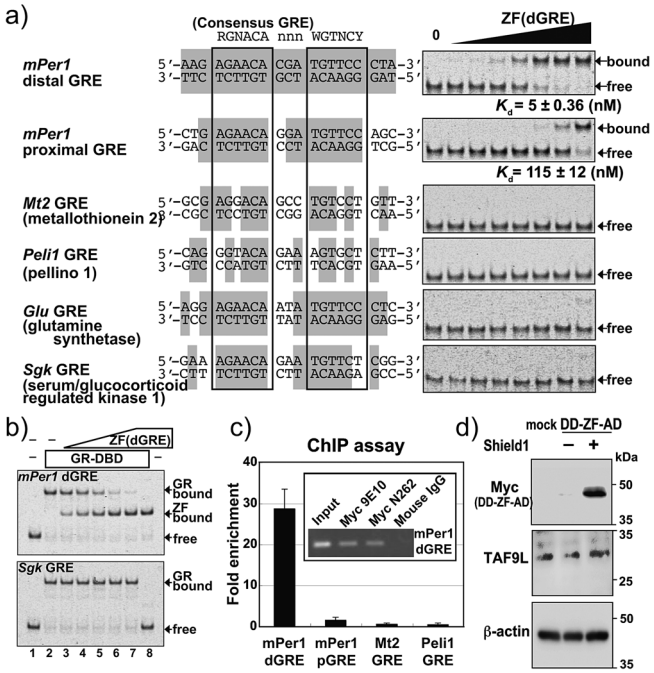

Figure 2. Specific DNA binding of ZF(dGRE) to mouse *Period1* distal GRE. a) Electrophoretic mobility shift assay of ZF(dGRE) (0.05–250 nM) to various GREs. The shaded bases are identical to those in the *mPer1* dGRE target. The K_d values are mean ± standard deviation (s.d., nM; $n=3$). b) Competitive DNA binding assays of GR-DBD and ZF(dGRE) for *mPer1* dGRE (top) and *Sgk* GRE (bottom). Lane 1, protein free; lanes 2–7, 0–164 nM ZF(dGRE) and 200 nM GR-DBD; lane 8, 164 nM ZF(dGRE). c) ChIP assays for a myc-epitope-tagged ZF(dGRE)-AD expressed in NIH3T3 cells. The inset shows a representative result of PCR amplification of immunoprecipitated samples by the indicated antibodies using *mPer1* dGRE primer. The data are mean ± s.d. ($n=3$). d) Nuclear accumulation of DD-ZF(dGRE)-AD after ligand (Shield1) addition. At 48 h after ligand addition, the nuclear extracts of mock and pCMV/6myc-DD-ZF(dGRE)-AD transfected cells were analyzed by Western blotting. The antibody for TAF9L was used as a control for the nuclear fraction. pCMV = plasmid containing the cytomegalovirus promoter.

ported by selective competition of ZF(dGRE) with the DNA binding domain of glucocorticoid receptor (GR-DBD) (Figure 2b). Although the application of ZF(dGRE) dose-dependently decreases GR-DBD binding to *mPer1* dGRE, the same treatment did not affect the GR-DBD binding to the GRE of *serum/glucocorticoid-inducible protein kinase 1* (*Sgk*).

For biological analysis in cell culture, we constructed an artificial zinc finger transcriptional regulator named ZF(dGRE)-AD, which consisted of a myc-epitope tag at the N terminus linked with the ZF(dGRE) as a DNA binding domain, followed by a nuclear localization signal (NLS), and a VP16-based transcriptional activation domain (AD) at the C terminus. First, we checked the specificity of this protein to genomic *mPer1* dGRE by a chromatin immunoprecipitation (ChIP) assay. The NIH3T3 cell line was used since this cell line is well known for having a core-clock system similar to that of the suprachiasmatic nucleus (SCN), the site of the master clock in mammals.[10] In this cell line, it was demonstrated that dexamethasone can reset the phase of the clock in cultured cells.[4,11] As shown in Figure 2c and Figure S1 in the Supporting Information, the DNA fragment containing *mPer1* dGRE was precipitated readily with myc-tagged ZF(dGRE)-AD, whereas co-precipitation of other GREs was much less abundant. These data indicate that the DNA binding activity and sequence specificity of ZF(dGRE) are functionally preserved even when its DNA target site (that is, *mPer1* dGRE) is chromosomally structured within the cells.

The activity of many clock gene proteins is limited to a precise time window during the circadian cycle.[1] To control the time specificity of our zinc finger construct, we added the FK506 binding protein (FKBP)-based ligand-controllable destabilizing domain (DD)[12] to ZF(dGRE)-AD (Figure 1c). The DD-fused proteins are rapidly and constitutively degraded by the proteasome, but the addition of the synthetic ligand Shield1 that binds to DD protects DD-fused proteins from degradation, thereby markedly extending the lifetime of the fusion proteins.[12] Indeed, after the transfection of an expression vector of DD-ZF(dGRE)-AD into NIH3T3 cells, accumulation of the engineered protein was only detected in the nucleus after the application of Shield1 (Figure 2d). This demonstrates that the DD–Shield1 system can successfully control the cellular expression of DD-ZF(dGRE)-AD. Nuclear-accumulated DD-ZF(dGRE)-AD binding to *mPer1* dGRE specifically activates the transcription of *mPer1*, since the application of Shield1 increased the *mPer1* mRNA without altering *mSgk* or *mBmal1* mRNA levels (see Figure S2 in the Supporting Information).

Next we sought to characterize the effect of our engineered DD-ZF(dGRE)-AD protein on clock gene expression rhythms in NIH3T3 cells by using a real-time reporter assay (see Figure S3 in the Supporting Information). To directly measure the effect of our protein on the *mPer1* promoter, we co-transfected an *mPer1* promoter-driven reporter (*mPer1-Luc*) containing the zinc-finger-targeting dGRE region with an expression vector of DD-ZF(dGRE)-AD (see Figure S4a in the Supporting Information). The application of Shield1 changed the rhythm of *mPer1-Luc* luminescence, although Shield1 itself without co-transfected DD-ZF(dGRE)-AD had no significant influence.

The circadian oscillator is thought to be composed of clock genes interlocked in an autoregulatory transcription–(post)translation feedback loop (see Figure S3 in the Supporting Information). Therefore, we further examined the effect of the evoked *mPer1* expression rhythm on the expression of other clock genes not under the direct control of the target GRE of DD-ZF(dGRE)-AD. For this, we selected *Bmal1* since its expression is regulated by the interlocked transcription feedback loop of the circadian clock (see Figure S3).[13] Shield1 treatment changes the *mBmal1-Luc* rhythm, although Shield1 itself does not (see Figure S4b in the Supporting Information). Importantly, the expression rhythm of *mBmal1-Luc* was completely antiphasic to that of *mPer1-Luc*, which is the expected phase relationship between the two genes, as previously observed in vitro and in vivo.[10,13,14] These findings strongly suggest that the ligand-mediated accumulation of the nuclear DD-ZF(dGRE)-AD level not only has a direct influence on *mPer1*

transcription, but also has the ability to change the rhythm of the core-clock oscillation as a whole.

Preexisting clock synchronizers such as glucocorticoids are known to shift the clock in a phase-dependent manner.[4,11] For examining its phase dependency precisely, we first prepared NIH3T3 cells stably transfected with DD-ZF-(dGRE)-AD. After transfecting these cells with an *mPeriod2* promoter-luciferase reporter in which luciferase is destabilized by a PEST sequence (*mPer2-dLuc*), we examined the phase-dependent effect of our artificial zinc finger transcription regulator. In this system, a clear rhythm of luciferase activity is obtained after a single medium change. Addition of Shield1 at 1 h after the second peak of the luciferase rhythm (Figure 3a, left, arrow) yielded a (1.13 ± 0.38) h (mean ± s.d.; $n = 9$) phase delay compared with vehicle (ethanol)-treated cells (Figure 3a; see also Figure S5 in the Supporting Information). In contrast, when Shield1 was applied before the second peak (1 h after the trough on the first day), a (0.87 ± 0.21) h (mean ± s.d.; $n = 11$) phase advance was observed (Figure 3b; see also Figure S5). In addition, no significant phase shifts were induced by Shield1 in wild-type NIH3T3 cells without DD-ZF(dGRE)-AD (Figure 3, right, gray bars (ZF(−)), and Figure S6 in the Supporting Information). These findings clearly demonstrate that accumulation of DD-ZF(dGRE)-AD actually induces phase-dependent phase shifts of core-clock oscillations.

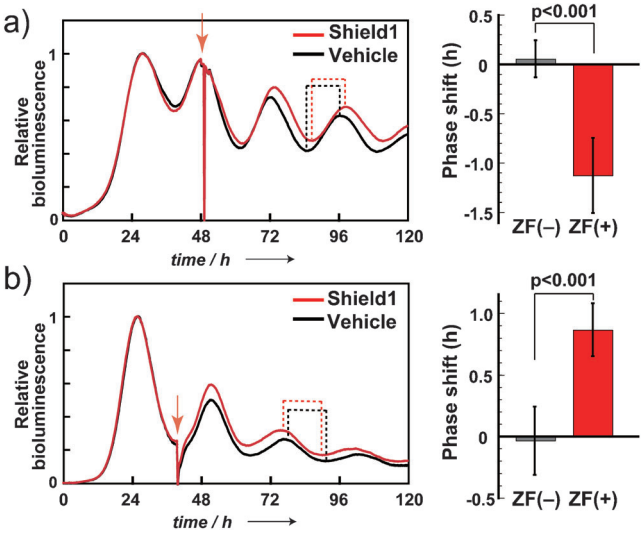

Figure 3. Phase-dependent phase shift of the DD-ZF(dGRE)-AD-expressing cells by addition of the Shield1 ligand. Left: The change of *mPer2-dLuc* luminescence was monitored by the time-dependent application of Shield1 on NIH3T3 cells stably expressing DD-ZF-(dGRE)-AD. At time 0, the *mPer2-dLuc* reporter plasmid was transfected and bioluminescence monitoring was started. Arrows indicate the time of application of Shield1 or vehicle into the medium at 1 h after the peak (a) or trough (b). The large clefts on the curve at the time of the arrows were noises resulting from treatments of ligand application. The dotted lines indicate the peak and trough after the addition of Shield1 (red) or vehicle (black). Phase delay (a) and advance (b) were observed. Right: Quantification of the phase shifts of the wild-type (gray, not expressing DD-ZF(dGRE)-AD; ZF(−)) and the DD-ZF(dGRE)-AD stable transfectant cells (red, ZF(+)). Phase-advance experiments (a) were performed 9 times, and phase-delay experiments (b) 11 times. Representative data are shown on the left side, and the cumulative calculated values are shown on the right [mean ± s.d. (h) ; $n = 9$ for (a), $n = 11$ for (b)]. The changes between Shield1 and vehicle treatments are statistically significant [Student *t*-test: $p < 0.001$ ($n = 9$) for the phase-advance experiment (a); $p < 0.001$ ($n = 11$) for the phase-delay experiment (b)].

Adjustment of cellular circadian rhythms has so far been limited to nonspecific chemical stimulations such as serum, dexamethasone, and forskolin.[4,11,15] Photoperturbation of the cellular clock has also been reported by expressing melanopsin receptors on the cell surface.[16] However, these stimulations can activate multiple genes, thereby inducing extraordinarily large phase shifts. There has been no synthetic agent available that can directly and specifically target *cis* elements on the promoter of the clock gene and thereby modulate the phases of the core-clock machinery. In this study, we have successfully engineered an inducible zinc finger transcriptional regulator specifically targeted to the dGRE of the clock gene *mPer1*. Activation of this synthetic protein can phase-shift the cellular clock either forward or backward.

It is believed that circadian rhythms are generated at an autoregulatory transcription–(post)translation feedback loop composed of a dozen clock genes, and circadian time is determined at the level of transcription.[1] However, it has remained unknown which gene is targetable for the modulation of the phase (or time) of the cellular clocks. Thus, our results suggest that *mPer1* is a state variable in the generation of circadian rhythms, and support the role of *mPer1* in the SCN on the light-induced behavioral phase shift.[17] Our present artificial transcription factor specific to *Per1* promoter not only changes the transcription of this gene, but also changes the oscillatory clock machinery as a whole. To our knowledge, this is the first report of an artificially designed protein that can externally control the cellular clock at the genomic level. Further improvements of this system, such as achieving tissue and organ specificity for readjustment of local circadian rhythms, will help the understanding of clock systems at the fundamental level and provide new avenues for the therapy of compromised circadian rhythms and associated pathologies.[18] Since our new zinc finger protein is selective and biologically effective, the present results will stimulate the creation of target-specific agents towards its clinical use, notably to avoid many side effects of nonspecific drugs such as dexamethasone or adrenocortical hormone derivatives.

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