# CKI$\varepsilon$/$\delta$-dependent phosphorylation is a temperature-insensitive, period-determining process in the mammalian circadian clock

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**A striking feature of the circadian clock is its flexible yet robust response to various environmental conditions. To analyze the biochemical processes underlying this flexible-yet-robust characteristic, we examined the effects of 1,260 pharmacologically active compounds in mouse and human clock cell lines. Compounds that markedly (>10 s.d.) lengthened the period in both cell lines, also lengthened it in central clock tissues and peripheral clock cells. Most compounds inhibited casein kinase I$\varepsilon$ (CKI$\varepsilon$) or CKI$\delta$ phosphorylation of the PER2 protein. Manipulation of CKI$\varepsilon$/$\delta$-dependent phosphorylation by these compounds lengthened the period of the mammalian clock from circadian (24 h) to circabidian (48 h), revealing its high sensitivity to chemical perturbation. The degradation rate of PER2, which is regulated by CKI$\varepsilon$/$\delta$-dependent phosphorylation, was temperature-insensitive in living clock cells, yet sensitive to chemical perturbations. This temperature-insensitivity was preserved in the CKI$\varepsilon$/$\delta$-dependent phosphorylation of a synthetic peptide in vitro. Thus, CKI$\varepsilon$/$\delta$-dependent phosphorylation is likely a temperature-insensitive period-determining process in the mammalian circadian clock.**

chemical biological approach | temperature compensation

The circadian clock is a molecular mechanism underlying endogenous, self-sustained oscillations with a period of ≈24 h, manifested in diverse physiological and metabolic processes (1–3). The most striking feature of circadian clock is its flexible yet robust response to various environmental conditions. For example, circadian periodicity varies with light intensity (4–6) while remaining robust over a wide range of temperatures ("temperature compensation") (1, 3, 7–9). This flexible-yet-robust characteristic is evolutionarily conserved in organisms ranging from photosynthetic bacteria to warm-blooded mammals (3, 10–12), and has interested researchers from a broad range of disciplines. However, despite many genetic and molecular studies (13–22), the detailed biochemical mechanism underlying this characteristic remains poorly elucidated (3).

The simplest explanation for this flexible-yet-robust property is that the key period-determining reactions are insensitive to temperature but responsive to other environmental conditions. Indeed, Pittendrigh proposed the existence of a temperature-insensitive component in the clock system in 1954 (7), and in 1968, he and his colleagues demonstrated that both the wave form and the period of circadian oscillations are invariant with temperature (23). However, the idea of a temperature-insensitive biochemical reaction is counterintuitive, as elementary chemical processes are highly temperature-sensitive. One exception is the cyanobacterial clock, in which temperature-insensitive enzymatic reactions are observed (24, 25). However, the cyanobacterial clock is quite distinct from other clock systems, and this biochemical mechanism has not been demonstrated in other clocks.

Recently, a chemical-biological approach was proposed to help elucidate the basic processes underlying circadian clocks (26), and high-throughput screening of a large chemical compound library was performed (27). In this report, to analyze systematically the fundamental processes involved in determining the period length of mammalian clocks, we tested 1,260 pharmacologically active compounds for their effect on period length in mouse and human clock cell lines, and found 10 compounds that most markedly lengthened the period of both clock cell lines affected both the central and peripheral circadian clocks. Most compounds inhibited CKI$\varepsilon$ or CKI$\delta$ activity, suggesting that CKI$\varepsilon$/$\delta$-dependent phosphorylation is an important period-determining process in the mammalian circadian clock. Surprisingly, the degradation rate of endogenous PER2, which is regulated by CKI$\varepsilon$-dependent phosphorylation (28) and probably by CKI$\delta$-dependent phosphorylation, was temperature-insensitive in the living clock cells, and the temperature-insensitivity was preserved even for the in vitro CKI$\varepsilon$/$\delta$-dependent phosphorylation of a synthetic peptide derived from PER2. These results suggest that this period-determining process is flexible in response to chemical perturbation yet robust in the face of temperature perturbations. Based on these findings, we propose that CKI$\varepsilon$/$\delta$-dependent phosphorylation is a temperature-insensitive period-determining process in the mammalian circadian clock.

## Results

**Ten Compounds Markedly Lengthened Period Length.** We examined 1,260 pharmacologically active chemical compounds from the

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LOPAC[1280] library for their effect on circadian period length in mammalian clock cell lines, NIH 3T3-*mPer2-Luc* and U2OS-*hPer2-Luc* (Table S1 and Table S2 and *SI Text*). We identified 10, all with period-lengthening effects, that substantially altered the period in both cell lines ($> +10$ s.d. or $< -10$ s.d., ≈2.0–3.0 h) (Fig. S1 *A* and *D* and Tables S3 and S4). These compounds, labeled "potent," also lengthened the period of primary cultures of mouse embryonic fibroblasts (MEFs, as peripheral clock cells) (Fig. S1 *B* and *E*) and slice cultures of suprachiasmatic nucleus (SCN; as central clock tissue) (Fig. S1 *C* and *E*). These results supported the idea that the potent compounds alter canonical clock processes.

**Potent Compounds Inhibit CKIε/δ Enzymatic Activity.** Most of the proposed targets of the 10 potent compounds on the list in Table S3 have not been identified as components of the mammalian circadian clock, although one of the 10 is an inhibitor of CK2, recently found as a component of the mammalian circadian clock (29, 30). The potent protein kinase inhibitors SB202190, SP600125, and roscovitine were previously reported to inhibit CKI in addition to their primary targets (31, 32). Since even specific protein kinase inhibitors also usually affect other kinases (31, 33), we hypothesized that the seven protein kinase inhibitors that we identified suppress CKI activity in addition to their primary targets.

To examine this hypothesis, we performed the siRNA knockdown of CKI genes, as well as the genes related to the 10 potent compounds (Fig. 1 *A–C* and Fig. S2 *B–G*). The knockdown of *CKIε* or *CKIδ* exhibited the greatest period-lengthening effects (Fig. 1*B*), more than 28 h in CT, whereas knockdown of other genes showed no or only a slight effect (Fig. S2 *B–G*). Moreover, the combinatorial knockdown of *CKIε* and *CKIδ* additively lengthened the period of circadian oscillations to over 30 h in CT (Fig. 1*C*), supporting the idea that CKIε/δ play one of the most important roles in the period-determination processes of mammalian circadian clocks. These results suggest that at least some of our compounds were not acting via their primary target, but through inhibition of CKIε/δ.

The inhibitory effect of the potent compounds on CKIε/δ activity was confirmed by the in vitro CKIε or CKIδ kinase assay. Importantly, nine of the 10 compounds [i.e., not 17α-hydroxyprogesterone (17-OHP)] strongly inhibited the catalytic domain of wild-type CKIε, lacking the C-terminal regulatory domain (ΔCKIε) as effectively as or better than IC261, a specific inhibitor of CKI (Fig. 1*D*). SP600125 and TG003, which dramatically extended the circadian period and potently inhibited ΔCKIε activity, strongly inhibited both activity of the ΔCKIε and catalytic domain of CKIδ (ΔCKIδ), with lower $IC_{50}$ ($< 0.55$ μM) than the $IC_{50}$ of IC261 (about 4 μM), whereas 17-OHP did not inhibit both of ΔCKIε and ΔCKIδ (Fig. 1*E*). Since double knockdown of the *CKIε* and *CKIδ* genes using siRNA had additive effects on period lengthening (Fig. 1*C*), and these chemical compounds, except 17-OHP, inhibited CKIε and CKIδ similarly, they probably acted on both CKIs to affect the period length of circadian oscillations.

We also examined the effect of potent compounds on the stability of the mPER2 protein, which was regulated by CKIε (28) (and probably by CKIδ) in non-clock cell, 293T cells. Fig. S3 *A* and *B* show that the two putative CKI inhibitors significantly enhanced the stability and slowed the degradation rate of LUC::mPER2 ($P < 0.01$, one-way ANOVA), whereas 17-OHP did not significantly affect LUC::mPER2 stability ($P = 0.18$, one-way ANOVA). These results were supported by immunoblot experiments (Fig. S4). This degradation rate of overexpressed LUC::mPER2 was not affected without the co-overexpression of CKIε(*tau*) (Fig. S3*C*; $P = 0.193$ for SP600125 and $P = 0.728$ for TG003; two-way ANOVA), presumably because relative expression levels of LUC::mPER2 in 293T cells compared with CKIε/δ were much higher that in MEFs. We used CKIε(*tau*) for these assays because the degradation rate with co-overexpression of CKIε(*tau*) was faster than that of CKIε(wt), which enable us to measure the PER2 degradation rate precisely. We next confirmed that the stability of LUC-fused mPER1 protein (LUC::mPER1), the closest relative of mPER2, was also decreased with co-expression of CKIε(*tau*) as well as LUC::mPER2, whereas LUC-fused mBMAL1 (LUC::mBMAL1) and native LUC were not (Fig. S5). These results suggest that the inhibitory effect of the potent compounds on CKIε/δ activity is the primary mechanism by which they lengthened the period of clock cells. In contrast, 17-OHP apparently functions via a different mechanism.

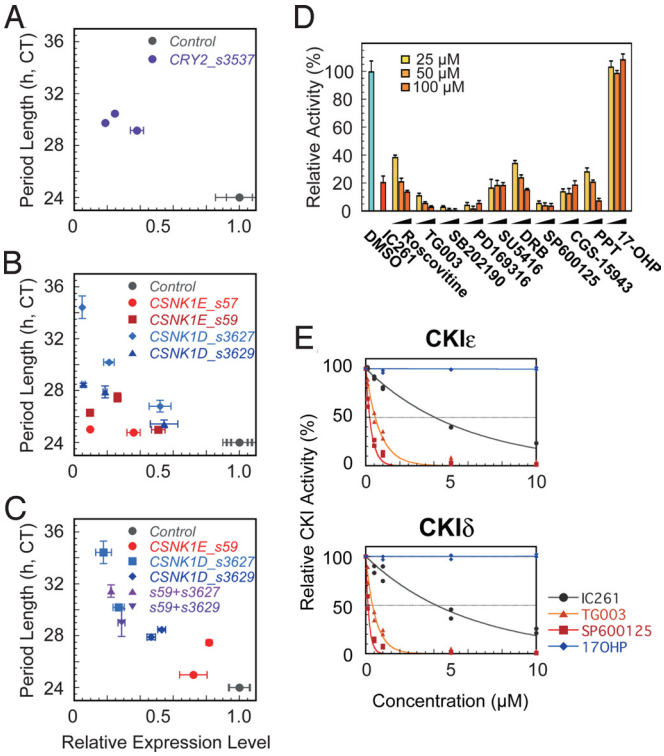



**Fig. 1.** Effects of knockdowns of *CSNK1*s on the period length and effect of potent compounds on the CKIε/δ activity in vitro. (*A–C*) Graphs indicate relationship between gene knockdown effects and period length in U2OS-*hPer2-Luc* cells. The x-axis indicates the expression level of genes relative to the samples transfected with control siRNA. The y-axis indicates the period length, described in circadian time (CT), with the control samples assigned as 24 h. Each symbol represents the mean ± SEM of independent experiments ($n \geq 3$). (*A*) The effect of *CRY2* knockdown as a positive control. (*B*) Gene knockdowns of CKIε/δ. *CSNK1E*, CKIε; *CSNK1D*, CKIδ. (*C*) Effect of the double knockdown of *CSNK1E* and *CSNK1D*. The gene expression level represents the total amount of CKIδ and CKIε. (*D*) ΔCKIε phosphorylation activity for a synthetic mPER2 peptide in the presence of chemical compounds [25, 50, or 100 μM for the 10 compounds, 100 μM for IC261] was measured using a modified IMAP assay with 100 μM ATP. The average results for each condition are shown as the relative activity compared to the control condition (DMSO). Error bars denote 1 s.d. (*E*) Dose-dependent effects of SP600125 and TG003 on ΔCKIε/δ phosphorylation activity. ΔCKIε/δ phosphorylation activity was measured in the presence of compounds at the indicated concentrations. Each symbol indicates the activity relative to the control condition in two independent experiments. The lines are approximate functions using the equation: $y = 100e^{-ax}$. The $IC_{50}$s for CKIε calculated from the equations were 4.0, 0.55, and 0.22 μM for IC261, TG003, and SP600125, respectively, and for CKIδ were 4.1, 0.40, and 0.13 μM.

**Flexibility and Robustness of a Period Determination Process.** To explore how important this process is for determining the period

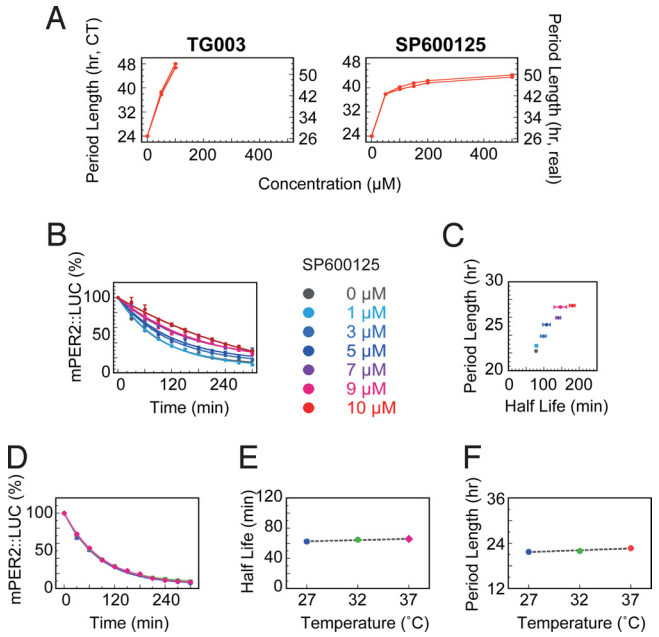



**Fig. 2.** Flexibility and robustness of a period-determination process of the mammalian circadian clock. (*A*) Dose-dependent effects of SP600125 and TG003 on period length in U2OS-*hPer2-Luc* cells. The period length is indicated both in real-time (right axis) and in circadian time (left axis). For circadian time, the average period length in two independent control experiments was assigned as 24.0 h. The two lines in each graph correspond to two independent experiments. Each value represents the mean ± SEM. At the concentrations without data points, the cells behaved arrhythmically. (*B* and *C*) Dose-dependent effect of SP600125 on the period length and degradation rate of mPER2::LUC in *mPer2*$^{Luc}$ MEFs. A pair of plates with cultured *mPer2*$^{Luc}$ MEFs, to which 0 to 10 μM SP600125 was applied, were prepared. One was used to measure mPER2::LUC decay and the other to determine the period. (*B*) Decay of mPer2::LUC bioluminescence in *mPer2*$^{Luc}$ MEFs. The degradation of mPer2::LUC protein was monitored after the administration of CHX to MEFs. The time-course data of each sample were normalized to approximate functions in which time point 0 was 100%. Each value represents the mean ± SEM. of the normalized data. The lines represent approximated curves in which y = 100 at time = 0 and y = 50 at the averaged half-life time. The colors in order from gray to blue to red represent the concentration of SP600125 with 0.25% DMSO ($n$ = 6). (*C*) Correlation between the period length and degradation rate of mPER::LUC in *mPer2*$^{Luc}$ MEFs with the administration of SP600125. Each value represents the mean ± SEM ($n$ = 6). (*D*) Temperature dependency of decay of the mPER2::LUC bioluminescence in *mPer2*$^{Luc}$ MEFs. The degradation of mPER2::LUC protein was monitored after the addition of CHX to MEFs. The time-course data of each sample were normalized to an approximate function in which time point 0 was 100%. Each value represents the mean ± SEM of the normalized data. The lines represent an approximated curve in which y = 100 at time = 0 and y = 50 at the averaged half-life time. The blue dots and line indicate the data at 27 °C; green, 32 °C; and magenta, 37 °C ($n$ = 23). (*E*) Temperature compensation in the half-lives of the mPer2::LUC protein. The graph indicates the mean ± SEM. The gray broken line indicates the approximated line described by the equation: y = 53.69 + 0.333x, and the $Q_{10}$ value between 27 and 37 °C calculated from the equation is 0.950. (*F*) Temperature compensation in the period length of *mPer2*$^{Luc}$ MEFs. The graph indicates the mean ± SEM. The gray broken line indicates the approximated line described by the equation: y = 19.02 + 0.097x, and the $Q_{10}$ value between 27 and 37 °C calculated from the equation is 0.957.

length of the mammalian circadian clock, we examined the effects of these two compounds on the circadian period length at higher concentrations (50–500 μM). Interestingly, the higher concentrations approximately doubled the period length (to 53.98 and 52.50 h at 100 μM for TG003, and 48.63 and 49.74 h at 500 μM for SP600125) relative to the control U2OS-*hPer2-Luc* cells (26.89 and 27.02 h) (Fig. 2*A*). Therefore, these results suggest that a period-determining process mediated by CKIε/δ-dependent phosphorylation is remarkably flexible to chemical perturbation, since a single compound lengthened the period from circadian (≈24 h) to almost circabidian (≈48 h).

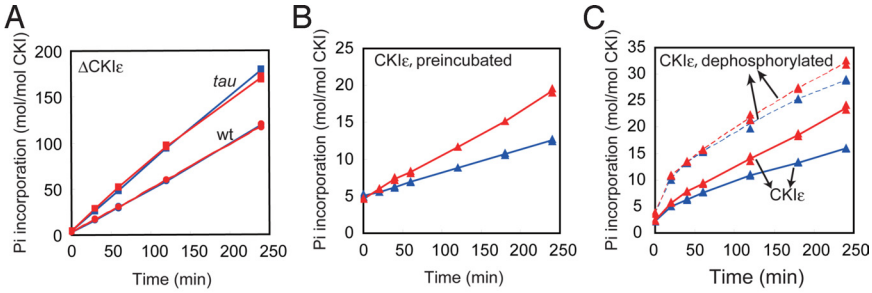
To further confirm this flexibility, we next investigated the sensitivity of this process to chemical perturbation in living clock cells by using *mPer2*$^{Luc}$ MEFs. We observed that the period length of the circadian oscillation in MEFs correlated well with the mPER2::LUC stability under the administration of a potent compound (Fig. 2 *B* and *C*). Given this strong correlation, we concluded that CKIε/δ activity on the PER2 protein is one of the most important period-determining processes in the mammalian circadian oscillator.

If important period-determination processes were highly sensitive to temperature, it would be very difficult to maintain the temperature compensation over the entire circadian period. Thus, we next investigated the temperature dependency of this process in living clock cells by using *mPer2*$^{Luc}$ MEFs. We found that the degradation rate of mPER2::LUC and the period length were completely temperature-insensitive in the MEFs (Fig. 2 *D*–*F*). The observed temperature-insensitivity seemed to reflect a molecular property of the endogenous mPER2 protein, because the degradation rate of LUC is sensitive to temperature in mammalian cells (Fig. 4 *A* and *B*). These results imply that the period-determination process, which was sensitive to chemical perturbation, was remarkably robust against physical perturbation such as temperature differences.

**CKIε/δ Activity Is Insensitive to Temperature.** To examine the biochemical foundation underlying the observed temperature-insensitivity, we analyzed the phosphorylation activity of CKIε and CKIδ in vitro. To facilitate this analysis, we designed a synthetic peptide substrate derived from the putative βTrCP-binding region of mouse PER2 (referred to here as βTrCP-peptide) (Fig. S6*A*), because this region is important for phosphorylation-dependent degradation of PER2 in the mammalian clock system (34). We first used just the catalytic domain of wild-type CKIε, lacking the C-terminal regulatory domain [ΔCKIε(wt)] to prevent potential confusion that could result from the autophosphorylation of this regulatory domain and the subsequent repression of CKIε kinase activity. This use of the catalytic domain was also justified by evidence that CKIε is kept in a dephosphorylated, active state in vivo (35).

Under this experimental setup, we successfully recapitulated, at least partially, the enhanced phosphorylation seen with a ΔCKIε *tau* mutation [ΔCKIε(*tau*)] (36) in comparison with ΔCKIε(wt) at 35 °C (Fig. 3*A*, red). Consistent with our other findings, ΔCKIε(wt) and ΔCKIε(*tau*) phosphorylated the peptide substrate at similar rates whether at 25 °C (Fig. 3*A*, blue) or 35 °C, indicating a strong temperature-insensitivity ($Q_{10}$ = 1.0) [Fig. 3*D*, ΔCKIε(wt) and ΔCKIε(*tau*)]. Similar temperature-insensitivity were also observed by using catalytic domain of wild-type CKIδ lacking the C-terminal regulatory domain [ΔCKIδ(wt)] ($Q_{10}$ = 1.2) [Fig. 3*D*, ΔCKIδ].

The temperature-insensitivity of the CKIε reaction depended substantially on the phosphorylation state of CKIε itself, because the $Q_{10}$ of the autophosphorylated full-length construct (preincubated with ATP) was greater than for the isolated catalytic domain (Fig. 3 *B* and *D*, CKIε, preincubated). We also confirmed an earlier report that preincubating full-length CKIε with ATP repressed its enzymatic activity (≈15-fold and 8-fold at 25 ° and 35 °C, respectively) (37); this was probably due to the autophosphorylation of the C-terminal regulatory domain (Fig. S6 *B* and *C*). The effects of autophosphorylation on enzymatic activity were also recapitulated by a mutant in which the eight autophosphorylated serine residues in the C-terminal regulatory domain (37) were replaced with glutamate [Fig. 3*D*, CKIε(D8)]. Using this autophosphorylation mutant, we observed both a slightly enhanced temperature sensitivity [$Q_{10}$ = 1.3, an increase




D

| Substrate | Enzyme | Phosphorylation activity (mol Pi/mol CKI/min) 25°C | 35°C | $Q_{10}$ |
|---|---|---|---|---|
| βTrCP-peptide | ΔCKIε(wt) | 0.52±0.057 | 0.53±0.066 | 1.0 |
| | ΔCKIε(*tau*) | 0.93±0.18 | 0.94±0.18 | 1.0 |
| | ΔCKIε(wt), preincubated | 0.34±0.043 | 0.37±0.038 | 1.1 |
| | CKIε(wt), preincubated | 0.031±0.0011 | 0.057±0.0024 | 1.8 |
| | CKIε(D8) | 0.077±0.0057 | 0.098±0.0074 | 1.3 |
| | ΔCKIδ | 0.65±0.13 | 0.76±0.17 | 1.2 |
| | CKIδ, preincubated | 0.060±0.0037 | 0.084±0.0068 | 1.4 |
| CK1tide | ΔCKIε(wt) | 79±2.8 | 111±8.7 | 1.4 |
| | CKIε(wt), preincubated | 14±0.54 | 28±0.81 | 2.0 |
| casein | ΔCKIε(wt) | 29±2.0 | 46±3.0 | 1.6 |
| | CKIε(wt), preincubated | 1.4±0.043 | 3.3±0.032 | 2.4 |

**Fig. 3.** Temperature insensitivity of the CKIε/δ phosphorylation activity. (*A*) Temperature dependency of the ΔCKIε(wt) (circles) and ΔCKIε (*tau*) (squares) phosphorylation activity for the βTrCP-peptide substrate. Assays were performed at 25 °C (blue) and 35 °C (red). (*B*) Phosphorylation activity of full-length CKIε(wt) autophosphorylated by preincubation with ATP. Assays were performed at 25 °C (blue) and 35 °C (red). (*C*) Temperature dependency of the full-length CKIε phosphorylation activity for the βTrCP-peptide substrate. Phosphorylation activity of full-length CKIε(wt) (CKIε, solid line), and CKIε(wt) dephosphorylated with λ protein phosphatase (CKIε, dephosphorylated) (broken line). Assays were performed at 25 °C (blue) and 35 °C (red). (*D*) Summary for temperature dependency of CKIε/δ enzymatic activities.

of 0.3 compared with ΔCKIε(wt)] and a repression of enzymatic activity (≈6-fold and ≈5-fold at 25 ° and 35 °C, respectively). We confirmed these effects on the time-course of phosphorylation by full-length CKIε that was not preincubated with ATP: As the incubation time increased (and probably as autophosphorylation increased), the enzymatic activity slowed (slopes of the curves decreased), and the temperature sensitivity (i.e., the difference in slope between 25 ° and 35 °C) increased (Fig. 3*C*, CKIε). We further confirmed these effects on the time course of phosphorylation using dephosphorylated full-length CKIε without preincubation (Fig. S6 *B* and *C*) and found that the enzymatic activity slowed substantially and the temperature sensitivity increased between the first hour and the next 3 h (Fig. 3*C*, CKIε, dephosphorylated). The effect of autophosphorylation on the temperature dependency was more prominent with CKIε than with the closely related CKIδ; the repression of enzymatic activity was, however, also observed for CKIδ (Fig. 3*D*, ΔCKIδ and CKIδ, preincubation).

PER2 phosphorylation is a complicated process involving multiple phosphorylation sites (38). To explore the temperature dependency of CKIε with another substrate, we designed a synthetic peptide substrate derived from the region near the functional amino acids in PER2, which is responsible for familial advanced sleep phase syndrome (FASPS) (referred to here as FASPS-peptide) (Fig. S6*A*). We chose this site because a serine at position 662 of human PER2 (amino acid 659 of mPER2) was identified as a mutation for FASPS (39). We found that the phosphorylation of FASPS-peptide by ΔCKIε(wt) was also temperature-insensitive, with a $Q_{10}$ = 1.2, whereas the phosphorylation by ΔCKIε(*tau*) was more temperature-sensitive ($Q_{10}$ = 1.6) (Fig. S6*D*). These results with the FASPS- and βTrCP-peptides suggest that CKI-dependent phosphorylation can be temperature-insensitive, depending on both the substrate and the state of the enzyme.

Since CKIε/δ is involved in a variety of other biological processes, we next investigated the temperature dependency of its activity for two clock-unrelated substrates: casein, a conventional protein substrate, and CK1tide, a commercially available peptide substrate for CKIε/δ, which was a peptide substrate phosphorylated at two residues N-terminal of CKIε/δ phosphorylation site. Enhanced temperature sensitivity was observed for these substrates ($Q_{10}$ = 1.6 for casein and 1.4 for CK1tide) (Fig. 3*D*), although it was still less than expected for canonical enzymatic reactions ($Q_{10}$ = 2–3). As with βTrCP-peptide, enhanced temperature sensitivity due to autophosphorylation was also observed for these substrates ($Q_{10}$ = 2.4 for casein, an increase of 0.8; and $Q_{10}$ = 2.0 for CK1tide, an increase of 0.6) (Fig. 3*D*), confirming that the temperature insensitivity depends substantially on the substrate as well as on the state of the enzyme (see also *SI Text*).

Although we demonstrated that the enzymatic activity of CKIε was temperature-insensitive for two synthetic peptide substrates (βTrCP-peptide and FASPS-peptide) derived from mPER2 as described above, it was still unclear if this temperature insensitivity would hold for the full-length mPER2 protein, which may have multiple phosphorylation sites outside the putative βTrCP-binding region and FASPS site. We therefore examined the degradation rate of mPER2::LUC, which was overexpressed in mouse clock cells (NIH 3T3) and compared it to that of LUC. As expected, the degradation rate of LUC was highly sensitive to temperature between 27 ° and 32 °C ($Q_5$ = 2.08 and 2.10, extrapolated $Q_{10}$ = 4.33 and 4.41, without or with ΔCKIε, respectively; Fig. 4 *A*, *B*, and *E*). The $Q_5$ represent the relative change of a physical property as a consequence of increasing the temperature by 5 °C. On the other hand, the degradation rate of mPER2::LUC was less sensitive to temperature ($Q_5$ = 1.51, extrapolated $Q_{10}$ = 2.28; Fig. 4 *C* and *E*). Importantly, enhancing the enzymatic reaction between mPER2 and CKIε by additionally expressing the catalytic domain of CKIε (ΔCKIε) in NIH 3T3 cells recapitulated the temperature insensitivity of the mPER2::LUC degradation rate ($Q_5$ = 1.22, extrapolated $Q_{10}$ = 1.49; Fig. 4 *D* and *E*). Based on these results together with our finding in *mPer2*$^{Luc}$ MEFs (Fig. 2 *D*–*F*), we concluded that CKIε/δ-dependent phosphorylation of the

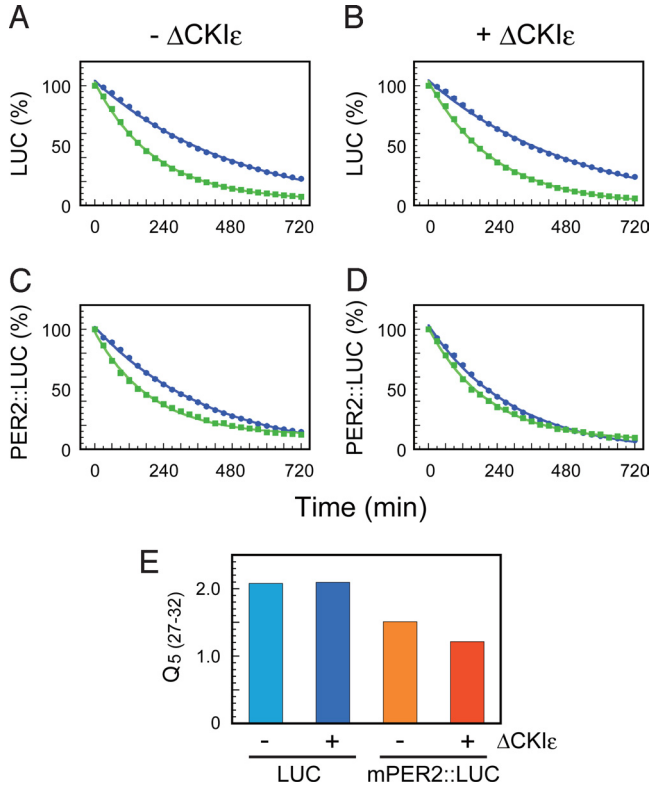



**Fig. 4.** Expression of full-length mPER2 and a catalytic domain of CKIε in NIH 3T3 cells recapitulates the temperature-insensitive reaction. (*A–D*) Temperature dependency of decay of LUC (*A* and *B*) or mPer2::LUC (*C* and *D*) bioluminescence in NIH 3T3 cells. NIH 3T3 cells, transfected with reporter vector (pMU2-*Luc* or pMU2-*mPer2*::*Luc*) and an expression vector for ΔCKIε (*B* and *D*) or empty vector (*A* and *C*), were used. The degradation of LUC or mPer2::LUC was monitored after the administration of CHX to cells. The time-course data of each sample were normalized to approximate functions in which time point 0 was 100%. Each value represents the mean ± SEM of the normalized data. The lines represent an approximated curve in which y = 100 at time = 0 and y = 50 at the averaged half-life time. Blue dots and line indicate the data at 27 °C; and green, 32 °C ($n$ = 6–11). (*E*) $Q_5$ values (the ratio between 27 and 32 °C) of the degradation rate of LUC or mPER2::LUC with or without co-expression of ΔCKIε. The half-lives of LUC or mPER2::LUC in each sample were calculated as described in the *SI Materials and Methods*.

mPER2 protein, which can determine the period length of circadian oscillations in clock cells, is likely temperature-insensitive.

## Discussion

**A Period-Determining and Temperature-Insensitive Process.** We discovered a highly flexible period-lengthening response in the mammalian clock: A human clock cell's circadian period could be stretched into a circabidian period (Fig. 2 *A* and *E*) through chemical manipulation, probably of the CKI activity, by SP600125 and TG003. We found that the knock-down of *CSNK1E* and *CSNK1D* had strong period-lengthening effects, but that of other kinases had much weaker effects, if any (Fig. 1 *A–C* and Fig. S2 *B–G*). Although we cannot completely exclude the possibility that our identified compounds inhibit other enzymes involved in mammalian circadian oscillations, we reason, as a first-order approximation, that these compounds lengthen the mammalian circadian oscillation primarily by inhibiting the CKIε and CKIδ enzymatic activity (see also *SI Text*).

We also found that this chemically flexible period-determination process is highly robust against temperature differences, because the degradation of endogenous mPER2, which is regulated by CKIε- or CKIδ-dependent phosphorylation, was temperature-insensitive in the living clock cells (Fig. 2 *D* and *F*). We also found that temperature insensitivity was preserved even in the CKIε/δ-dependent phosphorylation of a synthetic peptide derived from mPER2 in vitro (Fig. 3). Moreover, the expression of exogenous full-length mPER2 protein and the catalytic domain of CKIε in NIH 3T3 cells recapitulated the temperature-insensitive reaction (Fig. 4). Based on these experimental data, we propose that CKIε/δ-dependent phosphorylation is likely a temperature-insensitive period-determining process in the mammalian circadian clock. In particular, we note that this enzymatic reaction is a period-accelerating reaction in mammalian circadian clocks, because enhancing this reaction by genetic mutation leads to shortening of the circadian period (36, 40), and because pharmacological inhibition of this reaction led to lengthening of the circadian period of human clock cells from a circadian (≈24 h) to almost circabidian (≈48 h) period.

**A Period-Accelerating and Temperature-Insensitive Reaction Is Consistent with a General Theory of Temperature Compensation.** The observed period-accelerating and temperature-insensitive reaction is consistent with the previously proposed general theory for the temperature compensation of circadian clocks (41). In 1957, Sweeney and Hastings proposed that the temperature insensitivity of the primary period-determining reaction might be achieved through a balance between multiple, counteracting chemical reactions (8). This balance idea, which was mathematically formulated and generalized by Ruoff in 1992 into the balance theory (41), describes a system in which there is a balance between period-accelerating and period-decelerating reactions that yields the temperature-compensated circadian oscillation. In detail, if the period-accelerating and period-decelerating reactions were equally sensitive to temperature, the circadian oscillation would get faster with an increase in temperature. To reconcile this issue, the balance theory proposes that the period-accelerating reaction(s) are more temperature-insensitive than the period-decelerating reaction(s), and, hence, these two sets of enzymatic reactions are balanced to maintain a constant circadian oscillation. In an extreme and ideal case, the balance theory predicts that the period-accelerating reaction would be temperature-insensitive (41), which was suggested by this study.

**A Period-Accelerating and Temperature-Insensitive Reaction Is an Evolutionarily Conserved Design Principle from Cyanobacteria to Mammals.** This observed temperature insensitivity of an enzymatic reaction is the second example of such a characteristic in a circadian system. A similar temperature insensitivity was recently observed in the evolutionarily distinct cyanobacterial clock system ($Q_{10}$ = ≈1.3 for autophosphorylation of the cyanobacteria clock protein KaiC) (24). This remarkable similarity between two very different systems implies that temperature-insensitive enzymatic reactions might represent an evolutionarily conserved design principle. We do not insist here that all individual period-determining reactions in the mammalian circadian clock are temperature-insensitive, as is observed in the cyanobacteria clock. Rather, we propose that there are some temperature-insensitive period-determining enzymatic reactions in the mammalian circadian clock, which is consistent with the balance theory. Since biochemical reactions have been believed to be highly sensitive to temperature differences for a long time, our results on the existence of temperature-insensitive reaction in vitro and in cellulo, with possible implications for temperature compensation of circadian clocks, suggest the surprising capability of CKIε/δ-dependent phosphorylation. Therefore, remaining challenges are to obtain the atomic resolution model of this temperature-insensitive reaction, as well as to

demonstrate the physiological relevance of this temperature-insensitive reaction in circadian temperature compensation. Our in vitro temperature-insensitive reaction system, consisting of a short peptide, $\beta$TrCP-peptide, and a monomeric enzyme, CKI$\varepsilon$ or CKI$\delta$, would be an ideal experimental platform, which will provide atomic and molecular information required to test the physiological relevance of this temperature-insensitive reaction in circadian temperature compensation.

## Materials and Methods

**Analysis of the Effects of Compounds on Period Length in Cultured Cells.** NIH 3T3-*mPer2-Luc* cells and U2OS-*hPer2-Luc* cells were cultured on 24-well (PerkinElmer), 96-well, or 384-well (Falcon) culture plates in medium supplemented with 200 $\mu$M luciferin (Promega) and a chemical compound with stimulation with 10 nM forskolin (Nacalai Tesque). The time-course bioluminescence data were analyzed as reported previously (42).

**Analysis of the Temperature Sensitivity of mPER2 Degradation in the *mPer2$^{Luc}$* MEFs.** The *mPer2$^{Luc}$* MEFs cultures were maintained on 24-well culture plate at 27, 32, or 37 °C for 4 days, and the bioluminescence from the cells was monitored by the PMT-Tron system until the oscillations of the bioluminescence from mPER2::LUC triggered by the temperature change were diminished. The cells were then treated with 400 $\mu$g/mL CHX. The bioluminescence was recorded every 30 min for 5 h by the PMT-Tron system. The time course of the bioluminescence in each well was normalized and analyzed as described for the degradation rate of the LUC::mPER2 protein in 293T cells (*SI Text*).

**Measurement of CKI$\varepsilon$ and CKI$\delta$ Enzymatic Activity.** CKI activity was measured by the IMAP assay using the IMAP Screening Express kit (Molecular Devices), according to the manufacturer's protocol. For the P81 phosphocellulose paper assay, the full-length CKI$\varepsilon$ and CKI$\delta$, the catalytic domain of CKI$\varepsilon$, CKI$\delta$, and the tau-mutant of CKI$\varepsilon$ [$\Delta$CKI$\varepsilon$, $\Delta$CKI$\delta$, $\Delta$CKI$\varepsilon$ (*tau*)], and a mutant in which the eight autophosphorylated serine residues in the C-terminal regulatory domain were replaced with glutamate [CKI$\varepsilon$(D8)] purified from bacterial lysate were used as the enzymes in this assay.

**Analysis of Temperature Sensitivity of mPER2 or LUC Degradation in NIH 3T3 Cells.** NIH 3T3 cells were transfected with reporter vector (pMU2-*mPer2*::*Luc* or pMU2-*Luc*) and pMU2-$\Delta$*CKI*$\varepsilon$(wt) or empty pMU2 using FuGene6. The transfected cells were harvested by trypsin and plated on 24-well culture plates 24 h after transfection, and the degradation of mPER2::LUC or LUC was measured as described above for *mPer2$^{Luc}$* MEFs.

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