Sequential and Compartment-specific Phosphorylation Controls the Life Cycle of the Circadian CLOCK Protein*

Hsiu-Cheng Hung, Christian Maurer, Daniela Zorn, Wai-Ling Chang, and Frank Weber

From the Biochemistry Center Heidelberg (BZH), University of Heidelberg, Heidelberg 69120, Germany

The circadian clock facilitates a temporal coordination of most homeostatic activities and their synchronization with the environmental cycles of day and night. The core oscillating activity of the circadian clock is formed by a heterodimer of the transcription factors CLOCK (CLK) and CYCLE (CYC). Post-translational regulation of CLK/CYC has previously been shown to be crucial for clock function and accurate timing of circadian transcription. Here we report that a sequential and compartment-specific phosphorylation of the Drosophila CLK protein assigns specific localization and activity patterns. Total and nuclear amounts of CLK protein were found to oscillate over the course of a day in circadian neurons. Detailed analysis of the cellular distribution and phosphorylation of CLK revealed that newly synthesized CLK is hypophosphorylated in the cytoplasm prior to nuclear import. In the nucleus, CLK is converted into an intermediate phosphorylation state that correlates with trans-activation of circadian transcription. Hyperphosphorylation and degradation are promoted by nuclear export of the CLK protein. Surprisingly, CLK localized to discrete nuclear foci in cell culture as well as in circadian neurons of the larval brain. These subnuclear sites likely contain a storage form of the transcription factor, while homogeneously distributed nuclear CLK appears to be the transcriptionally active form. These results show that sequential post-translational modifications and subcellular distribution regulate the activity of the CLK protein, indicating a core post-translational timing mechanism of the circadian clock.

The circadian clock provides a molecular mechanism that orchestrates behavior and physiology in a temporal fashion and synchronizes homeostatic functions with the environmental cycles of day and night (1–3). The central oscillating activity of the Drosophila and mammalian circadian clock is formed by the heterodimeric complex of the transcription factors CLOCK (CLK) and CYCLE (CYC) that ultimately controls genome-wide transcription and activity states of key regulatory components (4–7). Importantly, the circadian oscillator is synchronized by environmental cycles, primarily light/dark and temperature cycles (8, 9), but keeps time on its own in the absence of environmental cues, therefore representing a true molecular clock.

Despite the crucial role of CLK/CYC for the circadian orchestration of physiology in Drosophila and mammals, little is known about their post-translational regulation (2, 10, 11). Transcript levels of Clk reveal robust circadian oscillations in Drosophila, due to rhythmic binding of the activator PAR-DOMAIN PROTEIN 1 and the repressor VRILLE to V/P-elements in the Clk-promoter (12–14). Rhythmic Clk transcription appears however not essential for self-sustained molecular oscillations, because expression of CLK from a per-promoter in anti-phase to its endogenous rhythm was found to support normal clock function (15). The post-translational regulation of CLK/CYC is however crucial for constitution of a circadian oscillator. Two CLK/CYC-activated genes period (per) and timeless (tim) feed back onto CLK/CYC activity, by binding and inhibition of CLK/CYC (16). Nucleocyttoplasmic shuttling of TIM (17) allows a temporal control of nuclear import and inhibitor activity of PER (18–21). PER-mediated inhibition of CLK/CYC (22, 23) involves the recruitment of the casein kinase 1ε homolog DOUBLE-TIME, which contributes to phosphorylation-induced inhibition and degradation of the CLK protein (24, 25). Activation of CLK/CYC is affected by calcium/calmodulin-dependent kinase II and mitogen-activated protein kinase, which both phosphorylate CLK in vitro (26). Phosphorylation of CLK may also control recruitment of the transcription co-activator CREB-binding protein to the CLK/CYC complex, which is essential for trans-activation by CLK/CYC (27, 28). These studies suggest a fundamental role of post-translational modifications for the temporal regulation of CLK/CYC-dependent transcription.

Here we show that sequential and compartment-specific phosphorylation controls the life cycle of the CLK protein. Newly synthesized CLK accumulates in the cytoplasm in a hypophosphorylated state. Subsequent nuclear translocation converts the CLK protein into an intermediately phosphorylated form. Interestingly, we found that CLK is stored in subnuclear compartments, and homogeneously distributed CLK protein represents a transcriptionally active species. Hyperphosphorylation as well as degradation of CLK are promoted by nuclear export of the transcription factor. The data reveal specific localization and activity patterns for the phosphorylation states of the CLK protein that oscillate over the course of a day (29).
EXPERIMENTAL PROCEDURES

Expression Constructs—CLK proteins were expressed from a pAc5.1/V5-HisA vector (Invitrogen) either without any tag (pAc-CLK) for Western blot analysis and transcription activation assays as described in a previous study (26, 27), or as fusion proteins with a N-terminal DsRed1 red fluorescent protein tag (pDsRed-CLK) for fluorescence microscopy (30). Mutations and deletions were inserted into these constructs with the QuikChange II XL system (Stratagene, La Jolla, CA) according to the instructions by the manufacturer and using the following primers: CLKNLs (substitution of Pro-484 to Leu, Lys-485 to Gln, and Lys-487 to Glu; fwd, CCA CAG GAA TAT CGC TGG AGG CCC AAC AGT GC; rev, GCA CTT TCG TTC GCC CTC GAC GAG TAT TCC TGG GG), CLKANES (deletion of 25 amino acids from Gln-836 to Leu-860; fwd, GGC TCG Glu, and Lys-487 to Glu; fwd, CCA CAG GAA TAT CGC TCG DYPRYFQS) that compensates for the change in protein size toward the terminal 25-amino acid tag (MDYKDDDDKDYKDDDDK-DYPYFQS) which compensates for the change in protein size caused by the NES deletion.

Fluorescence Microscopy—For cellular localization studies, DsRed-CLK constructs were expressed in Drosophila S2 cells or S2R+ cells as specified in the figure legends after transient transfection with Lipofectin (Invitrogen) as instructed by the manufacturer. 5 × 10^5 cells were transfected with 1 or 2 μg of plasmid DNA and 10 μl of Lipofectin, as described previously (16, 22, 26). Cells were cultured in poly-l-lysine (Sigma)-coated cover slide chambers (Nalgene, Rochester, NY). Fluorescence microscopy was performed using an Axiostar 200M (Zeiss, Jena, Germany) fluorescence microscope or a Nikon C1si-CLEM confocal microscope (Fig. 6) after staining of nuclei with Hoechst 33342 (Invitrogen). At least 60 transfected cells from at least two independent experiments were analyzed per construct. Images were collected with a laser wavelength of 563 nm for DsRed-CLK and 352 nm for Hoechst 33342-stained nuclei.

Immunohistochemistry—Third instar larvae were raised and collected at time points as indicated in the figure in cycles of 12-h light and 12-h darkness. Larvae were dissected and brains were fixed in 4% formaldehyde in PEM buffer (100 mM Pipes, pH 6.9, 1 mM EGTA, 2 mM magnesium sulfate) for 2 h, blocked with 10% normal goat serum (NGS) in PT buffer (phosphate-buffered saline plus 0.3% Triton X-100) for 2 h at room temperature and subsequently incubated for 48 h at 4 °C in 50 μl of primary antiserum solution containing 1:500-diluted rabbit anti-CLK (27) and 1:200-diluted guinea pig anti-PAP (31) in PT buffer with 10% NGS. The rabbit anti-CLK antibody was incubated with CLKNLs protein extract to remove unspecific interactions prior to the application in immunohistochemistry. Brains were rinsed with PT buffer and PT buffer plus 5% NGS for 20 min each and then incubated at room temperature for 2 h in secondary antiserum solution containing 1:200-diluted TRITC-conjugated donkey anti-guinea pig (Jackson Immuno Research, West Grove, PA) and 1:200-diluted Cy2-conjugated goat anti-rabbit (Jackson ImmunoResearch) in PT buffer with 10% NGS. After incubation, brains were rinsed with PT buffer and PT buffer plus 5% NGS for 20 min each and mounted in the mounting medium (50 mM Tris-Cl, pH 8, 90% glycerol, 2.5% DABCO (Sigma)).

Confocal Microscopy and Quantification of Staining Intensity—Optical sections of larval lateral neurons (LNs) were imaged on a Nikon C1si-CLEM confocal microscope. For each Zeitgeber Time (ZT), LNs from at least 10 brain hemispheres were scanned. For each LN sample, PAP staining was used to identify and select an optical section, which was then scanned for CLK immunoreactivity. A 0.31-μm Z-stack of images was taken per LN with single laser at 488 nm, 543 nm, and with both lasers together. Images were imported to NIH Image 1.34S, and the localization of neuronal PAP and CLK staining was determined. Total pixel intensity of CLK staining was measured for the whole cell or nucleus only and the means ± S.E. from all brains at a particular ZT are reported in the graphs.

Transcription Activity Assays—Transcriptional activation assays in Drosophila S2 and S2R+ cells were performed as described previously (16, 22, 26). In brief, cells from 500 μl of culture (10^6 cells/ml) were transfected after 12-h incubation at 25 °C with 200 μl of serum-free Schneider’s insect medium (Sigma) containing 1.5% Lipofectin (Invitrogen), 25 ng of pRL-copia, 10 ng of pGL3-(4-per-E-box)hs::luc+, 200 ng of pHT control vector, and 0.25 ng of pAc-CLK construct. Identical controls were performed for each condition without pAc-CLK. 200 μl of 20% fetal bovine serum (Invitrogen) in Schneider’s insect medium was added 4–6 h after transfection. After 40–48 h luciferase activities were determined using the dual-luciferase reporter assay system (Promega) according to the instructions by the manufacturer. Firefly luciferase activity was normalized toward Renilla luciferase activity to control for transfection efficiency and lysate concentration (16, 26).

Subcellular Fractionation—w1118 flies were entrained during eclosion in LD cycles of 12-h light and 12-h darkness. Flies, 1–7 days old, were harvested in LD 2 h after lights were on (ZT 2), and 2 h after lights were off (ZT 14). 400–500 μl of fly heads were subjected to subcellular fractionation according to a protocol that was adapted from a previous study (32). Fly heads were gently homogenized in liquid nitrogen and transferred to 800 μl of buffer A (1 M sorbitol, 7% Ficoll, 20% glycerol, 50 mM Tris, pH 7.5, 5 mM magnesium acetate, 5 mM EGTA, 3 mM CaCl_2, 3 mM dithiothreitol, 1× complete protease inhibitor (Roche Applied Science)). Coarse debris was filtered by passing the homogenate through a nylon mesh. Subsequently, Nonidet P-40 was added to a final concentration of 0.1%, and the mixture was passed through a 20-gauge needle. 1200 μl of iced-cold buffer B (10% glycerol, 25 mM Tris, pH 7.5, 5 mM magnesium acetate, 5 mM EGTA, 1× complete protease inhibitor (Roche Applied Science)) were added during gentle mixing. The mixture was carefully added on top of 300 μl of buffer AB (at a ratio of 1:1.7) and centrifuged at 700 × g for 3 min. 120 μl of the supernatant was collected as total protein fraction, and the rest was layered on top of a sucrose step-gradient (1 M sucrose, 10% glycerol, 25 mM Tris, pH 7.5, 5 mM magnesium acetate, 1 mM dithiothreitol, 1× complete protease inhibitor (Roche Applied Science)). After centrifugation at 9000 × g for 8 min, the nuclear pellet was resuspended in 50 μl of SDS-loading buffer. The supernatant was collected as cytosolic fraction, and proteins were precipitated with trichloroacetic acid (Sigma) and...
Sequential Post-translational Regulation of CLOCK

For phosphatase treatment, CLK proteins were similarly expressed in S2R+ cells. Cells were lysed in 4× SDS loading buffer and incubated at 95 °C, and lysates were subjected to SDS-PAGE on 6% gels. CLK protein was analyzed by Western blot using an antibody raised in rabbits against the C terminus of CLK (27).

Western Blot Analysis of Phosphorylation States and Degradation Kinetics—CLK proteins were analyzed from fly head extracts or after expression in S2 or S2R+ cells that were transiently transfected as described for fluorescence microscopy with 2 µg of pAc-Clk constructs. After 40–48 h cells were harvested and lysed in 4× SDS loading buffer (240 mM Tris/HCl, pH 6.8, 8% SDS, 20% glycerol) and boiled at 95 °C, and lysates were subjected to SDS-PAGE on 6% gels. CLK protein was analyzed by Western blot using an antibody raised in rabbits against the C terminus of CLK (27).

To determine degradation kinetics, S2 cells (2 ml of 10⁶ cells/ml) were transfected as described for fluorescence microscopy with 2 µg of pAc-Clk expression constructs. One day after transfection, cells were split in 24-well plates and incubated for a further 24 h. Subsequently, new protein synthesis was inhibited by the addition of cycloheximide at a final concentration of 260 µM. Cells were harvested over a time course after the addition of cycloheximide, and CLK protein levels were determined by Western blot analysis on 6% SDS-PAGE. CLK protein levels were quantified for different time points by densitometry and normalized toward an unspecified control band, which was detected by the anti-CLK antibody. Amounts of CLK proteins are shown relative to the amount of CLK prior to the addition of cycloheximide (time point 0, set to 100).

RESULTS

Circadian Oscillations on the CLK Protein Level—We first investigated the cellular distribution of the CLK protein in circadian neurons of the larval brain (Fig. 1A). We found that the major CLK protein localizes inside the nucleus (33). Interestingly, nuclear and total amounts of CLK revealed robust oscillations over the course of a day, with a maximum in the morning and trough levels in the evening (Fig. 1, A and B). These oscillations correlated with the circadian profile of CLK transcription that reaches a maximum toward the end of the night (16). In addition, phosphorylation states of the CLK protein oscillate in abundance over the course of a day (Fig. 1C) in accord with previous reports (25, 29, 33). Hypo- and hyperphosphorylated forms of CLK were mainly observed during late night and in the morning, whereas intermediate phosphorylation states were the predominant species in the evening, when CLK/CYC is transcriptionally active.

Compartment-specific Phosphorylation of CLK—To investigate the cellular localization of CLK in more detail, we expressed CLK with an N-terminal red fluorescent protein (DsRed) fusion tag in Drosophila Schneider 2 (S2) cells and analyzed the localization by fluorescence microscopy. The Drosophila CLK sequence reveals two consensus nuclear localization signals (NLSs) (Fig. 2A). The first consensus NLS is located in the N terminus at the beginning of the basic helix-loop-helix (bHLH) DNA-binding domain. Although this region is conserved in mammalian CLK orthologs, mutagenesis of this site did not affect the subcellular localization of the CLK protein, indicating that the consensus NLS in the bHLH domain is not involved in nuclear translocation (data not shown). A second consensus NLS located C-terminal of the PAS domains is unique to the Drosophila CLK protein. Rendering this site non-functional by mutagenesis (Fig. 2A) caused a cytoplasmic localization of the CLKNLS mutant protein in S2 cells (Fig. 2B). These results show that the consensus NLS located C-terminal of the PAS domains is a functional nuclear translocation site and essential for nuclear import of the CLK protein. Interestingly, nuclear import-deficient CLKNLS localized largely in cytoplasmic speckles (Fig. 2B). These speckles were also observed for non-tagged CLKNLS mutants, indicating that the localization pattern is not due to the fluorescent protein fusion tag (data not shown). Similarly, PER/TIM complexes were found to localize in cytoplasmic foci shortly before they translocate into the nucleus.
The nuclear import inefficient mutant of CLK may therefore be stalled in pre-import cytoplasmic foci. Cellular fractionation experiments of mammalian CLK from mouse liver (34) revealed a hypophosphorylation of cytoplasmic mCLK, whereas nuclear mCLK was hyperphosphorylated. We therefore analyzed the phosphorylation state of nuclear and cytoplasmic CLK proteins (Fig. 2, B and C) in Drosophila S2 cells. Different hypo-, intermediate, and hyperphosphorylated forms of CLK could be resolved by one-dimensional gel electrophoresis. Phosphatase treatment converted these phosphorylated forms into two bands with increased electrophoretic mobility that likely represent a non- and a hypophosphorylated species (supplemental Fig. S1). Wild-type CLK showed predominantly hyperphosphorylated, low electrophoretic mobility forms (Fig. 2C). In contrast, cytoplasmic CLK^{NLS} accumulated mainly in a hypophosphorylated state, indicating that newly synthesized CLK protein is converted to a low phosphorylated species in the cytoplasm, whereas hyperphosphorylation requires nuclear import (Fig. 2C).

Sequential and Compartment-specific Phosphorylation of the CLK Protein—The nuclear localization of wild-type CLK in S2 cells (Fig. 2) mimics the predominant nuclear localization of CLK in circadian neurons of the larval brain (Fig. 1). When we investigated the cellular localization of CLK in S2R + cells, we made the surprising observation that CLK showed a strong cytoplasmic localization in most cells, in addition to nuclear staining (Fig. 3A). Co-expression of CYC facilitated, however, an efficient nuclear localization of CLK in S2R + cells, suggesting that CYC promotes the nuclear localization of CLK. Such a role of CYC is consistent with the finding of reduced endogenous cyc levels in S2R + cells that reached only 50% of the transcript levels found in S2 cells, as determined by quantitative real-time PCR. In addition, pharmacological inhibition of CRM1-dependent nuclear export by leptomycin B significantly increased the amount of nuclear CLK, suggesting that rapid nuclear export causes an inefficient nuclear localization of CLK in S2R + cells (Fig. 3A). Interestingly, deletion of a C-terminal consensus nuclear export signal (NES) resulted in a complete nuclear localization of the mutant CLK^{ANES} protein in most cells, indicating that this sequence is important for nuclear export of CLK (Fig. 3A). The CLK^{ANES} mutant activated transcription of a luciferase reporter gene in S2R + cells similar as wild type (Fig. 3B) demonstrating that deletion of the NES did not compromise CLK function and allowed a native fold of the mutant protein. The results are consistent with a nucleocytoplasmic shuttling of the CLK protein, which, in the absence of CYC, results in a cytoplasmic localization of CLK, due to inefficient nuclear retention.

When the phosphorylation states of cytoplasmic and nuclear CLK proteins were investigated after expression in S2R + cells, we observed that cytoplasmic CLK^{NLS/ANES} was hypophosphorylated (Fig. 3C and data not shown), whereas wild-type CLK showed predominantly hyperphosphorylated forms (Fig. 3C). Interestingly, the nuclear export-deficient CLK^{ANES} protein accumulated in an intermediate and a hyperphosphorylated state. The phosphorylation level of CLK^{ANES} was increased compared with nuclear import-deficient CLK^{NLS/ANES}, indicating that nuclear translocation is important for hyperphosphorylation of CLK. On the other hand, CLK^{ANES} showed a stronger accumulation in the intermediate phosphorylation state compared with shuttling wild-type CLK (Fig. 3C), which was mainly hyperphosphorylated. These findings suggest that nuclear export promotes phosphorylation of the CLK protein and further phosphorylation events may take place in the cytoplasm after nuclear export. Because the CLK^{ANES} construct carries a 25-amino acid deletion of the NES, we validated these findings using a CLK^{ANES} construct that carries a 25-amino acid tag (25tag-CLK^{ANES}) (supplemental Fig. S2). No significant differences were however observed in the phosphorylation patterns of CLK^{ANES} and 25tag-CLK^{ANES} (supplemental Fig. S2A). Similarly, 25tag-CLK^{ANES} accumulated in a phosphorylation
Sequential Post-translational Regulation of CLOCK

A

FIGURE 3. Sequential and compartment-specific phosphorylation during nucleo-cytoplasmic shuttling of CLK. A, fluorescence microscopy of DsRed-CLK localization in S2R+ cells either in the absence (first row) or presence (second row) of coexpressed CYC or after addition of the nuclear export inhibitor leptomycin B (LMB) (third row). Experiments were conducted as in Fig. 2. The first row shows the localization of a DsRed-CLKNES mutant that carries a deletion of the C-terminal consensus NES sequence. Insets show a quantification of the percentage of S2R+ cells that show either significant cytoplasmic (C/N) or only nuclear (N) localization of DsRed-CLK proteins. B, CLK/CYC-dependent luciferase reporter gene expression in S2R+ cells for wild-type CLK, CLK NES, and in the absence of a CLK expression construct (control). Mean luciferase activity ± S.E. from at least four independent experiments is shown as the percentage of the wild-type control set to 100. C, analysis of the phosphorylation states of shuttling wild-type CLK, nuclear CLK NES, and cytoplasmic CLK NLS/NES (please see also supplemental Fig. S2). Brackets indicate hypo-, intermediate, and hyperphosphorylated forms of CLK. Representative Western blots from at least three independent experiments are shown.

state that was intermediate compared with mainly hyperphosphorylated wild-type CLK and hypophosphorylated cytoplasmic CLK NES (supplemental Fig. S2B).

The above observations reveal a sequential and compartment-specific phosphorylation of the CLK protein, which starts with hypophosphorylation in the cytoplasm prior to nuclear import, followed by further phosphorylation in the nucleus toward an intermediate phosphorylation state, and finally proceeds to hyperphosphorylation, which is promoted by nuclear export. The observations are consistent with a transcriptionally active low or moderately phosphorylated form of nuclear CLK that is the major phosphorylation species in the evening of a circadian cycle, when CLK/CYC activity is at its peak (Fig. 1) (24, 29). During late night and early morning hypophosphorylated CLK can be observed (Fig. 1) (29). The hypophosphorylated protein likely represents newly synthesized CLK that enters the nucleus, whereas the hyperphosphorylated form represents CLK protein from the previous circadian cycle that is exported and targeted to degradation.

Compartment-specific CLK Phosphorylation in Vivo—To analyze a compartment-specific phosphorylation of CLK in vivo, we performed a nucleocytoplasmic fractionation of fly heads that were collected in the morning 2 h after lights were on (ZT 2), and in the evening 2 h after lights were off (ZT 14) (Fig. 4A). In the morning, when CLK/CYC-dependent transcription is at its minimum, we found hyperphosphorylated CLK in the nucleus and in the cytoplasm, which likely represents CLK protein from the previous circadian cycle that is targeted to degradation (Fig. 4A). A weak band of hypophosphorylated CLK could be detected in the cytoplasm, consistent with newly synthesized CLK protein. During early night, a strong band of intermediately phosphorylated CLK could be observed inside the nucleus, which correlates with maximal transcriptional activity of CLK/CYC (Fig. 4A). In addition, hyperphosphorylated CLK is present in the nucleus and in the cytoplasm. Interestingly, in the evening nuclear CLK protein showed a similar phosphorylation pattern as the CLK NES construct in cell culture. These findings support the observations in cell culture, indicating that hypophosphorylated cytoplasmic CLK protein is converted to an intermediately phosphorylated species after translocation into the nucleus. The accumulation of intermediately phosphorylated nuclear CLK correlates with CLK/CYC-activated transcription in the evening, which is consistent with a transcriptional activity of intermediately phosphorylated CLK. Significant amounts of intermediately phosphorylated CLK were only observed inside the nucleus (Fig. 4A). In contrast, hyperphosphorylated CLK is present in the nucleus as well as in the cytoplasm, which is consistent with a nuclear export of the CLK protein after transcriptional inhibition by PER-mediated hyperphosphorylation (24, 25, 35). Because in cell culture, nuclear CLK NES revealed a stronger accumulation in the intermediate phosphorylation state, whereas shuttling wild-type CLK accumulated mainly in a hyperphosphorylated form, nuclear export likely enhances the hyperphosphorylation of the CLK protein.

Nuclear Export Promotes Turnover of the CLK Protein—The above results suggested that nuclear export plays a role in the regulation of the CLK life cycle. We therefore analyzed compartment-specific degradation kinetics of the CLK protein in cell culture, to address a role of nuclear export for turnover of CLK (Fig. 4B). Degradation kinetics were analyzed by cycloheximide chase experiments for cytoplasmic CLK NLS, nuclear CLK NES, and shuttling wild-type CLK. We found that cytoplasmic and shuttling CLK proteins were degraded with similar...
kinetics, suggesting that phosphorylation events, which trigger CLK degradation, occur in the cytoplasm. CLK^NLS was significantly more stable, demonstrating that nuclear localization stabilizes the CLK protein and nuclear export is important for turnover of CLK.

CLK Is Stored in Nuclear Foci—An interesting observation from cell culture was the finding that CLK localized to nuclear foci (Fig. 5A). To investigate whether this localization pattern is relevant in vivo, we performed a detailed analysis of endogenous CLK localization in wild-type larval brains by immunohistochemistry (Fig. 5). Similar to the observations in cell culture we found that endogenous CLK localizes in nuclear foci of lateral neurons (Fig. 5B), demonstrating that the nuclear localization pattern is not an artifact of CLK expression in cell culture but occurs under normal physiological conditions in vivo. The size of the nuclear foci is similar to the size of secretory vesicles to which the circadian neuropeptide PDF localizes.

A detailed investigation of CLK localization in S2 cells using confocal microscopy confirmed the localization of wild-type CLK in nuclear foci (Fig. 6), whereas the CLK^NLS mutant localized mainly to the cytoplasm (see also Fig. 2). Surprisingly, a
weak nuclear staining could however be observed for the NLS mutant (Fig. 6A). In contrast to wild-type CLK, the small amount of nuclear CLK^{NLS} revealed a homogeneous distribution. When cells that expressed different levels of wild-type CLK were compared, those cells that expressed high levels of CLK showed an accumulation of CLK in nuclear foci, whereas cells that expressed lower levels of CLK revealed a more homogeneous nuclear staining, similar to the small amount of nuclear CLK^{NLS} (Fig. 6A). These results suggested that the amount of nuclear CLK affects the subnuclear distribution.

We next asked whether the CLK protein present in nuclear foci or the homogeneously distributed CLK represents the transcriptionally active species. Because CLK^{NLS} showed a weak but homogeneous distribution in the nucleus, we analyzed the transcriptional activity of this mutant in cell culture. Surprisingly, CLK^{NLS} activated the expression of a luciferase reporter gene almost as efficient as wild-type CLK (Fig. 6B). Because only small amounts of CLK^{NLS} enter the nucleus and show a homogeneous nuclear distribution (Figs. 2 and 5A), the results suggest that homogeneously distributed nuclear CLK is the transcriptionally active species.

**DISCUSSION**

The heterodimeric complex of the transcription factors CLK and CYC forms the core oscillating activity of the circadian clock in *Drosophila* and mammals, ultimately controlling genome wide transcription and activity states of key regulatory factors in a temporal fashion. The circadian oscillation of CLK/CYC activity thereby allows a temporal orchestration of homeostatic functions and their synchronization with the environmental cycles of day and night. Although precise timing of CLK/CYC activation and inhibition appears to be crucial for clock function and accurate timing of circadian transcription (36), little is known about the molecular mechanisms that facilitate these regulatory events. CYC is constitutively expressed in *Drosophila*, whereas CLK transcription and protein phosphorylation reveal robust oscillations over the course of a day (16, 29). A number of studies indicated an important role of post-translational regulation of the CLK protein for clock function and accurate timing of circadian transcription (15, 24, 25, 29, 37). Because many transcription factors are regulated at the level of their cellular distribution, we investigated the contribution of nucleocytoplasmic transport and compartment-specific phosphorylation to the control of CLK/CYC activity.

We found that CLK shuttles between the nucleus and the cytoplasm and interacts with the heterodimerization partner CYC promotes an efficient nuclear localization. Similar nucleocytoplasmic shuttling has been observed for the mammalian CYC homolog BMAL1, which promotes the nuclear localization of mCLK (38–40). Because CLK is the limiting factor for circadian transcription (41), CYC levels are likely sufficient to facilitate the nuclear localization of CLK in neurons throughout a circadian cycle (Fig. 1). Nucleocytoplasmic shuttling of CLK suggests a regulatory role of cellular transport for trans-activation by CLK/CYC. Such regulation is supported by oscillating total and nuclear amounts of CLK protein in circadian neurons of the larval brain (Fig. 1) and by compartment-specific phosphorylation states of the CLK protein (Figs. 2–4).

Cytoplasmic CLK is hypophosphorylated, while phosphorylation increases after nuclear import (Figs. 3 and 4), similar to the situation in mammals (30, 34). The initial phosphorylation of newly synthesized CLK is likely crucial for nuclear import, because we identified consensus phosphorylation sites that prevent nuclear import after mutagenesis to the constitutively unphosphorylated form (data not shown). Upon nuclear import, the CLK protein was found to be further phosphorylated (Fig. 3). Similarly, a weak band of hypophosphorylated CLK was observed in cytoplasmic fractions of fly head extracts during morning, while in the evening intermediate and hyperphosphorylated forms of CLK accumulated in the nucleus *in vivo* (Fig. 4). These findings reveal a temporal and compartment-specific sequence of phosphorylation events prior to, and after nuclear translocation of the CLK protein.

Phosphorylation of CLK in the nucleus is likely linked to activation and inhibition of CLK/CYC-dependent transcription. The enhanced intermediate phosphorylation state of nuclear CLK^{ANES} in cell culture (Fig. 3), together with the accumulation of an intermediately phosphorylated form of nuclear CLK during the evening of a circadian cycle *in vivo* (Fig. 4), correlates with CLK/CYC-dependent transcription, indicating that an intermediately phosphorylated form of CLK is transcriptionally active. Recently, an inhibitory PER-dependent hyperphosphorylation of chromatin-bound CLK by the casein kinase 1ε homolog DOUBLE-TIME has been shown (24, 35). PER-mediated hyperphosphorylation is thought to trigger inhibition and degradation of the CLK protein (24, 25, 35). Interestingly, hyperphosphorylated forms of CLK are present in nuclear and cytoplasmic fractions of fly head extracts, while the intermediate phosphorylation form is mainly nuclear (Fig. 4). This finding suggests that hyperphosphorylated CLK is exported from the nucleus after transcriptional inhibition. Similarly, export-deficient nuclear CLK^{ANES} revealed a reduced hyperphosphorylation compared with shuttling wild-type CLK, suggesting that nuclear export promotes the hyperphosphorylation of CLK. These observations in cell culture and *in vivo* indicate that nuclear export is an integral step of the CLK life cycle. This conclusion is supported by the finding that nuclear CLK protein is more stable than cytoplasmic and shuttling wild-type CLK. Nuclear export therefore promotes degradation of the CLK protein. Because wild-type CLK and a cytoplasmic CLK mutant reveal similar degradation kinetics, the modifications that trigger CLK degradation, such as phosphorylation and/or ubiquitination, likely occur in the cytoplasm.

In conclusion, these results uncover a sequential and compartment-specific phosphorylation of the CLK protein, which assigns specific localization and activity patterns to the phosphorylation states of CLK that are found to oscillate over the course of a day (Fig. 7). Importantly, the subcellular and, as discussed later, subnuclear distribution together with the sequential and compartment-specific phosphorylation of CLK provides time of day specific modification states of the CLK protein throughout the circadian cycle (Fig. 7). In the morning, when mRNA levels and new protein synthesis of CLK are high, newly synthesized, hypophosphorylated forms of CLK that enter the nucleus can be observed, together with hyperphosphorylated CLK from the previous
circadian cycle that is targeted to degradation after nuclear export (Figs. 1 and 4) (29). In the evening, when CLK/CYC transcriptional activity reaches its peak, intermediate phosphorylated forms of CLK are most abundant (Figs. 1 and 4) (24, 25, 29). These findings identify a temporal and compartment-specific sequence of phosphorylation events that allow a precise post-translational regulation of the CLK life cycle and likely provide a core post-translational interval-timer of the circadian clock.

Interestingly, localization of CLK in nuclear foci was not only observed in cell culture, but also under endogenous conditions in neurons of wild-type larval brains (Fig. 5). These foci likely contain a storage form of the CLK protein, because the heterodimerization partner CYC revealed a homogeneous nuclear distribution (30, 42). In addition, cells that express low levels of CLK/CYC and further phosphorylation may activate the transcriptional activity, which requires recruitment of the co-activator CREB-binding protein (CBP) (27). Further PER-mediated phosphorylation inhibits CLK/CYC activity (24) and possibly triggers nuclear export that promotes hyperphosphorylation and degradation of the CLK protein.

In conclusion, our results show a sequential and compartment specific phosphorylation of the CLK protein that is accompanied by nucleocytoplasmic transport and specific subnuclear distribution (Fig. 7). The specific phosphorylation patterns observed prior to nuclear import, inside the nucleus and after transit through the nucleus assign specific cellular localization and activity patterns to the different phosphorylated species of the CLK protein that oscillate over the course of a circadian cycle. These findings suggest a post-translational timing mechanism of the circadian clock that is based on a temporally controlled sequential phosphorylation and specific subcellular localization of the circadian transcription factor CLK.

Acknowledgments—We thank Natalie Mena, Tatjana Wüst, and Axel Diernfellner for technical assistance, Ulrike Engel and Christian Ackermann from the Nikon Imaging Center at the University of Heidelberg for help with imaging, and Michael Brunner for helpful discussions.

REFERENCES

1. Yu, W., and Hardin, P. E. (2006) J. Cell Sci. 119, 4793–4795
2. Weber, F. (2009) Naturwissenschaften 96, 321–337
3. Hung, H. C., Kay, S. A., and Weber, F. (2009) J. Biol. Rhythms 24, 183–189
4. McDonald, M. J., and Rosbash, M. (2001) Cell 107, 567–578
5. Claridge-Chang, A., Wijnen, H., Naef, F., Boothroyd, C., Rajewsky, N., and Young, M. W. (2001) Neuron 32, 657–671
6. Akhtar, R. A., Reddy, A. B., Maywood, E. S., Clayton, J. D., King, V. M., Smith, A. G., Giant, T. W., Hastings, M. H., and Kiyasut, C. P. (2002) Curr. Biol. 12, 540–550
7. Panda, S., Antoch, M. P., Miller, B. H., Su, A. L., Schook, A. B., Straume, M., Schultz, P. G., Kay, S. A., Takahashi, J. S., and Hogenesch, J. B. (2002) Cell 109, 307–320
8. Stanewsky, R., Kaneko, M., Emery, P., Beretta, B., Wager-Smith, K., Kay, S. A., Rosbash, M., and Hall, J. C. (1998) Cell 95, 681–692
9. Glaser, F. T., and Stanewsky, R. (2005) Curr. Biol. 15, 1352–1363
10. Gallego, M., and Virshup, D. M. (2007) Nat. Rev. Mol. Cell Biol. 8, 139–148
11. Bae, K., and Edery, I. (2006) J. Biochem. 140, 609–617
12. Cyran, S. A., Buchsbaum, A. M., Reddy, K. L., Lin, M. C., Glossop, N. R., Hardin, P. E., Young, M. W., Storti, R. V., and Blau, J. (2003) Cell 112, 329–341
13. Glossop, N. R., Hou, J. H., Zheng, H., Ng, F. S., Dudek, S. M., and Hardin, P. E. (2003) Neuron 37, 29–461
14. Blau, J., and Young, M. W. (1999) Cell 99, 661–671
15. Kim, E. Y., Bae, K., Ng, F. S., Glossop, N. R., Hardin, P. E., and Edery, I. (2002) Neuron 34, 69–81
16. Darlington, T. K., Wager-Smith, K., Ceriani, M. F., Staknis, D., Gekakis, N., Steeves, T. D., Weitz, C. J., Takahashi, J. S., and Kay, S. A. (1998) Science 280, 1599–1603
17. Ashmore, L. J., Sathyanarayanan, S., Silvestre, D. W., Emerson, M. M., Schotland, P., and Sehgal, A. (2003) J. Neurosci. 23, 7810–7819
18. Sathyanarayanan, S., Zheng, X., Xiao, R., and Sehgal, A. (2004) Cell 116, 603–615
19. Fang, Y., Sathyanarayanan, S., and Sehgal, A. (2007) Genes Dev. 21, 1506–1518
20. Saez, L., and Young, M. W. (1996) Neuron 17, 911–920
21. Meyer, P., Saez, L., and Young, M. W. (2006) Science 311, 226–229
22. Weber, F., and Kay, S. A. (2003) FEBS Lett. 555, 341–345

Sequential Post-translational Regulation of CLOCK
Sequential Post-translational Regulation of CLOCK

23. Rothenfluh, A., Young, M. W., and Saez, L. (2000) Neuron 26, 505–514
24. Yu, W., Zheng, H., Hou, J. H., Dauwalder, B., and Hardin, P. E. (2006) Genes Dev. 20, 723–733
25. Kim, E. Y., and Edery, I. (2006) Proc. Natl. Acad. Sci. U.S.A. 103, 6178–6183
26. Weber, F., Hung, H. C., Maurer, C., and Kay, S. A. (2006) J. Neurochem. 98, 248–257
27. Hung, H. C., Maurer, C., Kay, S. A., and Weber, F. (2007) J. Biol. Chem. 282, 31349–31357
28. Etchegaray, J. P., Lee, C., Wade, P. A., and Reppert, S. M. (2003) Nature 421, 177–182
29. Lee, C., Bae, K., and Edery, I. (1998) Neuron 21, 857–867
30. Maurer, C., Hung, H. C., and Weber, F. (2009) FEBS Lett 583, 1561–1566
31. Renn, S. C., Park, J. H., Rosbash, M., Hall, J. C., and Taghert, P. H. (1999) Cell 99, 791–802
32. Luo, C., Loros, J. J., and Dunlap, J. C. (1998) EMBO J. 17, 1228–1235
33. Hou, J. H., Yu, W., Dudek, S. M., and Hardin, P. E. (2006) J. Biol. Rhythms 21, 93–103
34. Lee, C., Etchegaray, J. P., Cagampang, F. R., Loudon, A. S., and Reppert, S. M. (2001) Cell 107, 855–867
35. Yu, W., Zheng, H., Price, J. L., and Hardin, P. E. (2009) Mol. Cell. Biol. 29, 1452–1458
36. Kadener, S., Menet, J. S., Schoer, R., and Rosbash, M. (2008) PLoS Biol. 6, e119
37. Taylor, P., and Hardin, P. E. (2008) Mol. Cell. Biol. 28, 4642–4652
38. Kwon, I., Lee, J., Chang, S. H., Jung, N. C., Lee, B. J., Son, G. H., Kim, K., and Lee, K. H. (2006) Mol. Cell. Biol. 26, 7318–7330
39. Tamaru, T., Isojima, Y., van der Horst, G. T., Takei, K., Nagai, K., and Takamatsu, K. (2003) Genes Cells 8, 973–983
40. Kondratov, R. V., Chernov, M. V., Kondratova, A. A., Gorbacheva, V. Y., Gudkov, A. V., and Antoch, M. P. (2003) Genes Dev. 17, 1921–1932
41. Bae, K., Lee, C., Hardin, P. E., and Edery, I. (2000) J. Neurosci. 20, 1746–1753
42. Lim, C., Lee, J., Choi, C., Kim, J., Doh, E., and Choe, J. (2007) Mol. Cell. Biol. 27, 4876–4890
43. Lee, J., Lee, Y., Lee, M. J., Park, E., Kang, S. H., Chung, C. H., Lee, K. H., and Kim, K. (2008) Mol. Cell. Biol. 28, 6056–6065
44. Cardone, L., Hirayama, J., Giordano, F., Tamaru, T., Palvimo, J. J., and Sassone-Corsi, P. (2005) Science 309, 1390–1394
45. Heun, P. (2007) Curr. Opin. Cell Biol. 19, 350–355
46. Seeler, J. S., and Dejean, A. (2003) Nat. Rev. Mol. Cell Biol. 4, 690–699