Identification of mPer1 Phosphorylation Sites Responsible for the Nuclear Entry*

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Atsuko Takano‡, Yasushi Isojima, and Katsuya Nagai
From the Division of Protein Metabolism, Institute for Protein Research, Osaka University, 3-2 Yamada-Oka, Suita, Osaka 565-0871, Japan

Casein kinase 1 epsilon (CK1ε) is an essential component of the circadian clock in mammals and Drosophila. The phosphorylation of Period (Per) proteins by CK1ε is believed to be implicated in their subcellular localization and degradation, but the precise mechanism by which CK1ε affects Per proteins has not been determined. In this study, three putative CK1ε phosphorylation motif clusters in mouse Per1 (mPer1) were identified, and the phosphorylation status of serine and threonine residues in these clusters was examined. Phosphorylation of residues within a region defined by amino acids 653–663 and in particular of Ser-661 and Ser-663, was identified as responsible for the nuclear translocation of mPer1. Furthermore, phosphorylation of these residues may influence the nuclear translocation of a clock protein complex containing mPer1. These findings indicate that mPer1 phosphorylation is a critical aspect of the circadian clock mechanism.

The metabolism and behavior of organisms are influenced by endogenous circadian rhythms. Recently it has been revealed that a positive-negative transcriptional feedback mechanism may be responsible for the generation of the circadian clock signal in various species (1–4). Experiments with cultured cells and knockout and transgenic mice have shown that the Clock, Bma1, Per2, and Cryptochrome (Cry)1 and 2 proteins are essential for generating circadian rhythms in mammals (5–8).

In this context, it has been suggested that the phosphorylation of clock-related proteins such as Per1 has an important role in regulating the circadian clock. The Drosophila Period protein (dPer) and mammalian Per proteins (Per1–3) have been reported to undergo temporal shifts in electrophoretic mobility due to phosphorylation (9, 10). Drosophila Double-time (Dbt) protein, a homologue of mammalian CK1ε, was suggested to be a putative kinase for the dPer protein (11). Mutant alleles of dbt that confer shortened or lengthened circadian behavioral rhythms, or that abolish circadian rhythms altogether, have been identified (12). These alterations are correlated with changes in the phosphorylation and stability of dPer, indicating that Dbt is a necessary component of the circadian clock mechanism (12). Similar roles for mammalian Dbt have also been suggested by recent findings. The Syrian hamster Tau mutation confers free running periods of 22 h for heterozygotes and 20 h for homozygotes. The tau locus was cloned and found to encode the hamster homologue of CK1ε. The Tau mutation is a single nucleotide change that causes an arginine-to-cysteine substitution at amino acid (aa) residue 178, which is highly conserved in other members of the CK1 family. The mutant protein can still associate with mPer proteins, but its kinase activity is much lower than that of wild-type CK1ε (13). Furthermore, hPer2 was shown to be implicated in a human disorder, familial advanced sleep-phase syndrome (FASPS), an autosomal dominant circadian rhythm disorder characterized by a 4-h phase advance in the daily sleep-wake cycle (14). FASPS is caused by a serine-to-glycine substitution affecting aa 662 of hPer2, which is located within a domain thought to be bound and phosphorylated by CK1ε. The hPer2 mutant was shown to be less phosphorylated by CK1ε than the wild type hPer2 in vitro. In addition, CK1ε phosphorylates not only Per proteins but also Cry1 and BMAL1 (15). Collectively, these data suggest that CK1ε is a component of the mechanism that regulates circadian rhythms.

In experiments with COS-7 cells, we previously found evidence that CK1ε interacts with the mouse Per proteins mPer1, mPer2, and mPer3 and that it causes mPer1 and mPer3, but not mPer2, to enter the nucleus in a phosphorylation-dependent manner (16). In contrast, it was reported that coexpression of mPer1 and CK1ε in HEK293 cells causes mPer1 to be transferred to the cytoplasm from the nucleus, which was explained as being due to the masking of the mPer1 nuclear localization signal (NLS) by phosphorylation of the protein (17). Although these data suggested that CK1ε is important in generating circadian rhythms in mammals, the precise molecular effect of CK1ε on mPer proteins remained unclear.

In this work, we identify putative CK1ε-dependent phosphorylation sites in mPer1 that are responsible for its entry into the nucleus. Our results suggest that one of three clusters of these sites is involved in controlling the subcellular localization of mPer1 and thus is implicated in the circadian clock mechanism.

EXPERIMENTAL PROCEDURES

In Vitro Kinase Assay—Recombinant rat CK1ε (rCK1ε) was used for in vitro kinase assays. cDNA encoding the full-length protein or the catalytic domain (aa 1–297) of rCK1ε was subcloned into the vectors pTrcHisB (Invitrogen) or pET21T-3 (Qiagen) to produce hexahistidine-tagged rCK1ε (His-rCK1ε) or GST-fused rCK1ε (GST-rCK1ε), respectively. These expression vectors were transformed into the BL21(DE3) Escherichia coli expression system (Stratagene). His-rCK1ε was purified with nickel-nitrilotriacetic acid resin (Qiagen) and GST-rCK1ε with glutathione-Sepharose 4B (Amersham Biosciences), according to the manufacturers’ protocol. Prior to use in kinase assays, recombinant His-rCK1ε was subjected to limited digestion with trypsin (15 min at 30 °C) to eliminate the COOH terminus and to reduce autophosphorylation.
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**RESULTS**

Identification of CK1-dependent Phosphorylation Sites in mPer1—Previous reports by others and from our laboratory indicated that CK1e phosphorylates mPer proteins and affects their subcellular localization and turnover (16, 17, 19, 20). To clarify the function of CK1e in the circadian clock mechanism, we identified mPer sites phosphorylated by CK1e by performing in vitro kinase assays using a series of GST-mPer1 fragments as substrates for rCK1e (Fig. 1A). We first found that rCK1e phosphorylated a GST-mPer1 fragment containing aa 547–799 (Fig. 1A, lane 6). To further define the phosphorylated region of mPer1, four smaller GST-tagged fragments covering aa 548–799 were constructed (Fig. 1A, lanes 8–11). Three of these fragments (aa 627–667, aa 667–742, and aa 742–799) were phosphorylated by rCK1e (Fig. 1A, lanes 9–11), but the fourth (aa 547–628) was not (Fig. 1A, lane 8). This result suggests that mPer1 contains at least three CK1e phosphorylation sites, within the region from aa 628–799.

The sites of CK1-mediated phosphorylation in mPer2 and mPer3 were also examined. rCK1e was found to phosphorylate only a GST-tagged mPer2 fragment containing aa 486–753 and a GST-tagged mPer3 fragment containing aa 387–880 (data not shown).

Next, we searched for the CK1e-phosphorylation consensus motif (Ser/Thr-X-X-Ser/Thr or Asp-Asp-Asp-X-Ser/Thr) (21) within the CK1e-targeted regions of the mPer proteins. For mPer1, two consensus motif clusters were found, from aa 653 to 663 (ST653–663) and aa 714 to 726 (ST714–726), as well as an isolated single motif from aa 784 to 787 (ST784–787) (Fig. 1B). The ST714–726 region, which is conserved among all mPer proteins, has been suggested to be relevant for their ubiquitination (20). Moreover, a mutation in the corresponding region of hPer2 was reported to cause familial advanced sleep-phase syndrome (FASPS) (14). ST784–787 is conserved in mPer3 but not mPer2, and ST653–663 is not found in mPer2 or mPer3. To clarify whether these putative motifs are indeed phosphorylated and, if so, whether they are responsible for the phosphorylation-dependent nuclear export of mPer proteins, we constructed several HA-mPer1 mutants. In three of these, the serine and threonine residues in a single consensus motif cluster were replaced by alanine (ST653–663A, S714–726A, and S784A). An additional fourth derivative all three consensus motif clusters were mutated (S/ST653–784A). Another replacement mutant with alterations of the motif cluster from aa 902 to 916 (ST902–916), which has been reported to be a region phosphorylated by CK1e (17), was also constructed (S/ST902–916A) (Fig. 1B).

Identification of Phosphorylation Sites Necessary for the Nuclear Entry of mPer1—To determine how many CK1e-dependent phosphorylation sites are present in mPer1, the phosphorylation status of each mPer1 mutant in the presence of rCK1e was examined. COS-7 cells were transfected with vectors expressing HA-tagged full-length or mutant mPer1 derivatives, along with wild type rCK1e, a kinase-inactive rCK1e mutant (rCK1e-KN) in which the lysine residue at aa 38 was replaced by arginine or an empty control vector. The levels of phosphorylation of the mPer1 proteins were initially assessed by a shift in electrophoretic mobility on SDS-PAGE gels, as detected by immunoblot analysis. Wild type mPer1 and the mPer1 mutant S784A underwent an obvious shift in mobility in the presence of intact rCK1e (Fig. 2A, lanes 1–3 and 10–12). However, the S/ST653–663A mutant showed a lower mobility shift than did the wild type protein (Fig. 2A, lanes 4–6), and the S/ST714–726A mutant exhibited a striking electrophoretic mobility even in the absence of rCK1e protein (Fig. 2A, lane 7–9). The S/ST653–784A mutant, in which all serine and threonine residues of the three consensus motif clusters were replaced, was still phos-
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Fig. 1. A. in vitro kinase assay to identify mPer1 regions phosphorylated by rCK1e. GST-mPer1 fragments were incubated with buffer and 40 ng of trypsinized His-rCK1e for 10 min. The substrates used in the assay are as follows: full-length HA-mPer1 without (lane 1) or with (lane 2) His-rCK1e. GST-mPer1 fragments corresponding to amino acid residues (aa) 1–130 (lane 3), aa 22–495 (lane 4), aa 351–547 (lane 5), aa 547–799 (lane 6), aa 799–1324 (lane 7), aa 547–627 (lane 8), aa 627–667 (lane 9), aa 667–742 (lane 10) and aa 742–799 (lane 11). Left panel: autoradiography with [γ-32P]ATP; right panel: CBB staining. Molecular masses are indicated in kilodaltons, and the positions of phosphorylated substrates are indicated with arrowheads. B. alignment of the amino acid sequences of mPer1, -2, and -3 with respect to the rCK1e-phosphorylated region of mPer1. The mPer1 diagram shows the positions of rCK1e target areas. A and B indicate PAS-A and PAS-B domains; NES, nuclear export signal; NLS, nuclear localization signal. Note that the phosphorylated fragment is aa 628–799, between the PAS-B domain and the NLS, and aa 902–920, boxed. The Ser or Thr residues found in the CK1e phosphorylation motif are represented in bold, and conserved amino acids are indicated by gray boxes. The positions of putative phosphorylation motif clusters are boxed. Alanine mutation sites are shown above the sequences.

phorylated (Fig. 2A, lanes 13–15). Each mPer1 mutant could be coimmunoprecipitated with rCK1e. These results suggest that there are multiple CK1e-dependent phosphorylation sites in mPer1 and that each phosphorylation site differentially influences the phosphorylation status of mPer1.

Our previous study showed that the coexpression of rCK1e causes mPer1 and mPer3, but not mPer2, to enter the nucleus in COS-7 cells (16). To identify the Ser/Thr residues responsible for mediating the phosphorylation-dependent nuclear entry of mPer1, HA-tagged wild type or mutant mPer1 proteins were coexpressed with rCK1e in COS-7 cells, and their subcellular localizations were determined by immunocytochemistry (Fig. 2, B and C). When HA-mPer1 or mutant proteins were overexpressed alone, they localized mainly in the cytoplasm and were observed in the nucleus in less than one-third of the cells (Fig. 2B, panels a, c, e, g, and i). When wild type HA-mPer1 was coexpressed with rCK1e, most of the protein was found in the nucleus (Fig. 2B, panel b). This difference in the subcellular localization of HA-mPer1 is likely due to its phosphorylation by rCK1e, as indicated by its cytoplasmic localization when it was coexpressed with the kinase-inactive version of rCK1e (Fig. 2C). Interestingly, coexpression of HA-mPer1 S/T[653–663]A or S/T[714–726]A with rCK1e did not result in the nuclear localization of these proteins (Fig. 2B, panels d and f). HA-mPer1 S/T[653–663]A coexpressed with the empty vector or with kinase-inactive rCK1e-KN did not appear in the nucleus, but approximately one-third of the HA-mPer1 S/T[714–726]A protein was observed in the nucleus when it was coexpressed with the vector or rCK1e-KN. These data indicate that the rCK1e-dependent phosphorylation motif clusters ST653–663 and S714–726 of mPer1 are important for its nuclear entry and that the ST653–663 region is of special importance, because the HA-mPer1 S/T[653–663]A mutant did not localize to the nucleus even when it was coexpressed with empty vector or the kinase-inactive rCK1e-KN. In contrast, HA-mPer1 S/T[784]A and S/T[902–916]A entered the nucleus in a manner dependent on phosphorylation by rCK1e, but the proportion of these mutant proteins in the nucleus was significantly lower compared with wild type mPer1 (Fig. 2B). For HEK 293 cells, which express considerable amounts of endogenous CK1e, it was previously reported that overexpressed mPer1 was found in the nucleus but that in the presence of exogenous CK1e it was exported to the cytoplasm (17). Therefore, we examined the subcellular localization of mPer1 and mutant derivatives in HEK 293 cells by methods similar to those used for COS-7 cells. Surprisingly, the HA-mPer1 S/T[653–663]A protein was observed only in the cytoplasm irrespective of the presence of rCK1e (data not shown). Another mutant, HA-mPer1 S/T[714–726]A, was distributed in a pattern similar to that of wild type mPer1. These observations strongly suggest that the mPer1 ST653–663 motif cluster is essential for the nuclear entry of mPer1 mediated by CK1e-dependent phosphorylation, because the mPer1 S/T[653–663]A protein is found only in the cytoplasm, irrespective of host cell type or the presence of rCK1e.

A Chimeric mPer2 Protein Is Transferred into the Nucleus by CK1e—In our previous study (16), we showed that coexpression with CK1e caused mPer1 and mPer3, but not mPer2, to enter the nucleus in COS-7 cells. Moreover, the mPer1 ST653–663
The Cytoplasmic Localization of the mPer1 S[661–663]A Protein Is Not Inhibited by Leptomycin B—The mPer2 protein was shown to shuttle between the cytoplasm and nucleus (23), and the absence of rCK1 is an apparent explanation for this difference. Thus, to confirm the nuclear import function of the ST653–663 motif cluster, we constructed a chimeric mPer2 mutant in which aa 611–620 were replaced by aa 652–667 of mPer1 (mPer2 [mPer1 652–667]). Furthermore, we constructed another HA-mPer2 mutant by replacing the alanine residue at position 723 with serine (mPer2 A[723]S), because this site corresponds to Ser-784 of mPer1, which is conserved in mPer3. As expected, the HA-mPer2 [mPer1 652–667] protein entered the nucleus when coexpressed with rCK1 in COS-7 cells, but the HA-mPer2 A[723]S protein did not (Fig. 3, A, panels c–f). These data indicate that the ST653–663 motif cluster of mPer1 is essential for its phosphorylation-dependent entry into the nucleus. Both of these mPer2 mutants exhibited a shift in electrophoretic mobility similar to that of wild type mPer2 when coexpressed with rCK1 (data not shown). Either the potential change in phosphorylation was beyond the sensitivity of the assay, or residues 653–663 may have an NLS function. These two possibilities should be addressed.

Ser-661 and Ser-663 May Be Essential for the CK1ε-dependent Nuclear Translocation of mPer1—To identify essential phosphorylation sites in the ST653–663 motif cluster that mediate the nuclear entry of mPer1, mutant proteins in which each serine or threonine residue of the cluster was replaced by alanine were constructed and expressed in COS-7 cells (Fig. 4, A and B). Only two mPer1 mutants, S[661]A and S[663]A, showed marked and significant decreases in the frequency of nuclear localization when coexpressed with rCK1 (37.8% for S[661]A and 14.0% for S[663]A (Fig. 4A, panels i and j)), and the other mPer1 mutants were observed mainly in the nucleus. A mPer1 mutant in which both Ser-661 and Ser-663 were replaced by alanine (S[661,663]A) localized only in the cytoplasm, like mPer1 S[653–663]A (Fig. 4A, panels b and k).

Next, we constructed a mPer1 mutant in which both Ser-661 and Ser-663 were replaced by aspartic acid (S[661,663]D) and expressed it in COS-7 cells (Fig. 4C). Overexpressed S[661,663]D mPer1 was found in the nucleus even in the absence of rCK1 (Fig. 4C, panel c). These findings suggest that Ser-661 and Ser-663 are sufficient for the phosphorylation-dependent nuclear entry of mPer1 when it is coexpressed with rCK1.

Ser-661 and Ser-663 are part of the Ser-661–Ser-663 Aspartic and Serine motif (mPer2 A[723]S) and are conserved in mPer2 (Fig. 1C). Thus, to confirm the nuclear import function of the ST653–663 motif cluster, we constructed a chimeric mPer2 mutant in which aa 611–620 were replaced by aa 652–667 of mPer1 (mPer2 [mPer1 652–667]). Furthermore, we constructed another HA-mPer2 mutant by replacing the alanine residue at position 723 with serine (mPer2 A[723]S), because this site corresponds to Ser-784 of mPer1, which is conserved in mPer3. As expected, the HA-mPer2 [mPer1 652–667] protein entered the nucleus when coexpressed with rCK1 in COS-7 cells, but the HA-mPer2 A[723]S protein did not (Fig. 3, A, panels c–f). These data indicate that the ST653–663 motif cluster of mPer1 is essential for its phosphorylation-dependent entry into the nucleus. Both of these mPer2 mutants exhibited a shift in electrophoretic mobility similar to that of wild type mPer2 when coexpressed with rCK1 (data not shown). Either the potential change in phosphorylation was beyond the sensitivity of the assay, or residues 653–663 may have an NLS function. These two possibilities should be addressed.

Ser-661 and Ser-663 May Be Essential for the CK1ε-dependent Nuclear Translocation of mPer1—To identify essential phosphorylation sites in the ST653–663 motif cluster that mediate the nuclear entry of mPer1, mutant proteins in which each serine or threonine residue of the cluster was replaced by alanine were constructed and expressed in COS-7 cells (Fig. 4, A and B). Only two mPer1 mutants, S[661]A and S[663]A, showed marked and significant decreases in the frequency of nuclear localization when coexpressed with rCK1 (37.8% for S[661]A and 14.0% for S[663]A (Fig. 4A, panels i and j)), and the other mPer1 mutants were observed mainly in the nucleus. A mPer1 mutant in which both Ser-661 and Ser-663 were replaced by alanine (S[661,663]A) localized only in the cytoplasm, like mPer1 S[653–663]A (Fig. 4A, panels b and k).

Next, we constructed a mPer1 mutant in which both Ser-661 and Ser-663 were replaced by aspartic acid (S[661,663]D) and expressed it in COS-7 cells (Fig. 4C). Overexpressed S[661,663]D mPer1 was found in the nucleus even in the absence of rCK1 (Fig. 4C, panel c). These findings suggest that Ser-661 and Ser-663 are sufficient for the phosphorylation-dependent nuclear entry of mPer1 when it is coexpressed with rCK1.
suggesting that the subcellular localization of mPer proteins is determined by the balance between nuclear entry and export. A question that arises in this respect is whether the phosphorylation-dependent nuclear localization of mPer1 is caused mainly by the stimulation of nuclear import or by the inhibition of nuclear export. Therefore, we examined the effect of the nuclear export inhibitor Leptomycin B (LMB) on the subcellular distribution of mPer1 and mutant derivatives. At least three independent trials, in which 30–100 cells were counted, were performed for each experiment. As described above, wild type mPer1 was observed in the cytoplasm when it was expressed alone in COS-7 cells, and it was observed in the nucleus when coexpressed with rCK1ε (Fig. 5A, panels a and e). Treatment with LMB resulted in the nuclear localization of wild type mPer1 irrespective of the presence of rCK1ε (Fig. 5A, panels b and f). This result suggests that mPer1 as well as mPer2 shuttles between the cytoplasm and nucleus. In contrast, the mPer1 S[661,663]A mutant was observed only in the cytoplasm irrespective of the presence of rCK1ε and treatment with LMB (Fig. 5A, panels d and h). Thus we suggest that Ser-661 and Ser-663 are necessary for the nuclear import of mPer1.

Two mPer1 Serine Residues, Ser-661 and Ser-663, Are Phosphorylation Sites for CK1ε—In vitro kinase assays were performed to confirm whether the S/T653–663 putative phosphorylation motif cluster of mPer1, especially residues Ser-661 and Ser-663, is phosphorylated by rCK1ε. GST-mPer1 fragments corresponding to aa 547–799 of wild type mPer1 and of the mPer1 S/T[653–663]A and mPer1 S[661,663]A mutant proteins were constructed and used as substrates for GST-rCK1ε. Each GST-mPer1 fragment was phosphorylated by GST-rCK1ε in vitro (Fig. 6A, lanes 5–7). However, the GST-mPer1 S[661,663]A and S/T[653–663]A fragments were phosphorylated to lesser extents than was the wild type GST-mPer1 fragment (Fig. 6A, lanes 6 and 7). A quantitative analysis of phosphorylation showed that the wild type GST-mPer1 fragment incorporated 8–9 pmol of Pι per pmol of protein (Fig. 6, B and C), whereas the mPer1 mutants S[661,663]A and S/T[653–663]A incorporated 5–6 and 3–4 pmol of Pι per pmol of protein, respectively (Fig. 6, B and C). In light of these observations, we suggest that two mPer1 serine residues, Ser-661 and Ser-663, are true CK1ε phosphorylation sites.

The Nuclear Entry of the Clock-related Protein Complex Requires the CK1ε-dependent Phosphorylation of mPer1—It has been suggested that Cry1 and Cry2 are essential for the generation of the circadian rhythm in mice (7) and that they form complexes with Per proteins that enter the nuclei of clock
Identification of mPer1 Phosphorylation Sites

In this report, we first examined sites in the mPer1 protein that are phosphorylated by rCK1e. The region of mPer1 that is targeted by rCK1e is located between the PAS domain and the putative NLS (Fig. 1B). The rCK1e target areas of mPer2 and mPer3 are also positioned between PAS domains and putative NLs. The rCK1e binding site of mPer1 was reported to contain phosphorylated residues (aa 593–815 in mPer1 and aa 554–763 in mPer2) (14, 17). A recent report suggested that the CK1e binding domain was essential for the functions of mPer1 and mPer2 in the circadian clock mechanism (23). We also examined the CK1e binding site of mPer1 and found that a derivative lacking aa 742–799 did not interact with CK1e (data not shown). Taken together, these results indicate that the mPer1 area from aa 628 to 799, between the PAS domain and putative NLs, is important for binding to and phosphorylation by CK1e.

Three putative CK1e phosphorylation consensus motif clusters (ST653–663, ST714–726, and ST784–787) were identified in the mPer1 target area (Fig. 1C). The ST[653–784]A mutant, in which all serine and threonine residues in these three clusters were replaced by alanine, still underwent phosphorylation (Fig. 2A), indicating that there are other unidentified phosphorylation sites in mPer1. Interestingly, putative motif clusters are not found in the Drosophila Per (dPer) protein. In addition, Dbt interacts directly with an amino-terminal region of dPer and not with carboxyl-terminal residues beyond the PAS domain (11). These data suggest that the role played by CK1e-phosphorylated mPer proteins might differ from that of dPer.

In the transcriptional feedback loop model, the delay between the synthesis of clock proteins and the inhibition of transcription is thought to be necessary for maintaining circadian oscillations. In Drosophila, dPer and Timeless (dTIm) first accumulate in the cytoplasm and enter the nucleus in a time-dependent manner after forming a complex (9). In this study, we examined the sites of phosphorylation responsible for the CK1e-dependent nuclear translocation of mPer1. We found that the phosphorylation of serine and threonine residues within a region defined by aa 653–663 is likely responsible for the nuclear translocation of mPer1 with cotransfection assay to COS-7 cells and HEK293 cells. Furthermore, Ser-661 and Ser-663 are phosphorylation sites particularly important for the nuclear entry of mPer1 (Fig. 4). The significance of Ser-661 and Ser-663 in the phosphorylation-dependent nuclear translocation of mPer1 was also confirmed with a chimeric mPer2 mutant (mPer2 [mPer1 652–667]), in which the corresponding region was substituted with the mPer1 region that contains the ST653–663 motif cluster. The mPer1 652–667 sequence contains no known NLS motif. We found that the chimeric mutant mPer2 [mPer1 652–667] acquired the ability to be translocated into the nucleus by rCK1e (Fig. 3).

These two serine residues were confirmed as rCK1e-dependent phosphorylation sites by an in vitro kinase assay (Fig. 6). The incorporation of phosphates mediated by rCK1e was ~8 pmol per pmol of protein for a wild type mPer1 fragment and ~6 pmol per pmol of protein for the S[661,663]A fragment.
for each experiment. The subcellular localization of mPer1 or mutant derivatives. At least three inhibitor LMB (right panels) for 6h.

kinase assays using GST-wild type (lanes 2 and 5), GST-S[661,663]A (lanes 3 and 6), or GST-S[653–663]A (lanes 4 and 7) mPer1 (aa 547–799) fragments as substrates. These substrates were incubated with the GST-rCK1e fragment (aa 1–673) (lane 1), and radioactivity was analyzed by SDS-PAGE. The incubation time was 2 h, and 300 ng of the substrates was used. Upper panel: CBB staining; lower panels: autoradiography with γ-32P]ATP. Molecular masses are displayed in kilodaltons, and the positions of phosphorylated substrates are indicated with arrows. B and C, quantitative analysis of time-dependent phosphorylation changes. Wild type (WT) GST-mPer1 or GST-mPer1 mutants were incubated with GST-rCK1e for various lengths of time. The incorporation of 32P into mPer1 protein was quantified by an image analyzer and plotted against incubation time.

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Fig. 7. Subcellular localization of the clock-related protein complex depends on Ser-661 and Ser-663 of mPer1. A, representative examples of the subcellular distribution patterns of wild type mPer1 (panel b), S[661,663]A (panels c and h) in COS-7 cells coexpressed with CFP-rCK1e (panels a, d, and g) and HA-mCry1 (panels c, f, and i). The distribution of mPer1 or mutant derivatives is represented in green (middle panels), HA-tagged protein in red (lower panels), and CFP-fused protein in blue (upper panels). Transfected cells were untreated (left and middle panels) or treated with the nuclear export inhibitor LMB (right panels) for 6 h. B, quantitative analysis of the subcellular localization of mPer1 or mutant derivatives. At least three independent trials, in which 30–100 cells were counted, were performed for each experiment.

Because the incorporation of phosphate into the ST[653–663]A fragment was lower (by about 3–4 pmol per pmol of protein), we suppose that there must be two other phosphorylation sites recognized by rCK1e in the mPer1 ST[653–663] motif cluster. In an attempt to directly identify phosphorylated residues, we also performed a MALDI-TOF/MS analysis of phosphorylated mPer1 fragment but did not observe peptide peaks corresponding to the motif cluster ST[653–663].

Recently, Per, CRY, Clock, BMAL1, and CK1e were reported to be phosphorylated, to form multimeric complexes, and to enter the nucleus (10). These phenomena appear to be critical for the transcriptional feedback mechanism responsible for circadian rhythms, and they support a model for the regulated phosphorylation-dependant nuclear entry of Per proteins. Another report (25) has proposed that mPer2 shuttles between the cytoplasm and nucleus and that this translocation is related to the stability of mPer2, suggesting a different role for the subcellular localization of Per proteins. The NLS and NES motifs in mPer2 are thought to be necessary for translocation. Overexpressed mPer1 localized mainly in the cytoplasm of COS-7 cells but exhibited nuclear localization in cells treated with LMB (Fig. 5). This result implies that mPer1, like mPer2, is also transported through the nuclear membrane. The mutant S[661,663]A protein remains in the cytoplasm irrespective of the presence of LMB (Fig. 5, A and B). Thus, the phosphorylation-dependent nuclear translocation of mPer1 mediated via the serine 661 and 663 residues is likely to be based on nuclear import mechanisms. Thus the phosphorylation of serine 661 and 663 by CK1e may direct mPer1 to the nucleus, and, in contrast, the ST902–916 cluster may direct it to the cytoplasm. mPer1 has also putative NLS and NES motifs, but they are not near the ST653–663 motif cluster. Thus we speculate that the phosphorylation of serine 661 and 663 by CK1e changes the conformation of mPer1 and influences the masking of NLS and NES, but this remains to be demonstrated at the molecular level.

The S[714–726]A mutant was also observed in the cytoplasm of COS-7 cells when coexpressed with rCK1e (Fig. 2, B and C). However, a mutant protein in which only Ser-714 was replaced with alanine (S[714]A) exhibited a distribution similar to that of wild type mPer1 (data not shown). Furthermore, in cells treated with LMB, the S[714]A mutant was observed in the nucleus (data not shown). Ser-714 may be the most important residue in the S[714–726] motif cluster. Because phosphorylation of this residue by CK1e has been suggested to initiate a cascade of phosphorylation throughout the cluster (24). These results suggest that Ser-714 and the S[714–726] motif cluster are not essential for the nuclear entry of mPer1. However, the S[714–726]A mutant exhibited mobility shifts independent of the presence of rCK1e. This finding has led to more detailed work now in progress.

In this study, we also examined the roles of the Ser-661 and Ser-663 residues of mPer1 with respect to the clock protein complex, including mCry1 and rCK1e (Fig. 7). mPer1 or rCK1e alone tended to localize to the cytoplasm, but mCry1 localized to the nucleus. mCry proteins may exert more influence in specifying the subcellular localization of the mPer-mCry1 complex than do mPer proteins, because this complex localized mainly in the nucleus. But surprisingly, the mPer1 S[661,663]A mutant caused a complex containing mCry1 to be
detected in the cytoplasm. Our results suggest that mPer1, as well as the mCry proteins, has an important role in the nuclear translocation of complexes containing clock proteins. It is thought that mPer proteins interact with mCry proteins and CK1ε in cytoplasm and that these proteins then enter the nucleus. Accordingly, we propose that a conformational change in mPer1 dependent on CK1ε-induced phosphorylation triggers the nuclear translocation of clock protein complexes. In HEK293 cells, the mPer1 S(661,663)A mutant is also observed in the cytoplasm when coexpressed with mCry1 (data not shown). This result suggests that the phosphorylation of mPer1 influences the localization of mCry and that Ser-661 and Ser-663 of mPer1 have important roles in determining the subcellular localization of the clock protein complex.

The results of this work suggest that there are multiple CK1ε-dependent phosphorylation sites in mPer1 (Fig. 2A), which prompts a question concerning their regulation. CK1ε effectively phosphorlates Ser/Thr residues on the carboxy-terminal side of the Ser/Thr-X-Ser/Thr motif, but only when the Ser/Thr residue at the amino-terminal side of the motif is already phosphorylated. This raises the possibilities that other kinases may work as a "switch" to control CK1ε-dependent phosphorylation and that each motif is differently regulated by this mechanism. In Drosophila, CK2α and Shaggy, a glycogen synthase kinase-3 orthologue, are suggested to contribute to the mechanism of the circadian clock (26, 27). In mammals, CK1ε is thought to cooperate with these kinases in the Wnt signaling pathway (22). Taken together, other kinases, including CK2 and glycogen synthase kinase-3, may cooperatively work with CK1ε to regulate the subcellular localization and stability of mPer proteins. Their roles await future investigation.

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Atsuko Takano, Yasushi Isojima and Katsuya Nagai

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