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Casein kinase I in the mammalian circadian clock

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Abstract

The circadian clock is characterized by daily fluctuations in gene expression, protein abundance and posttranslational modification of regulatory proteins. The *Drosophila* PERIOD (dPER) protein is phosphorylated by the serine/threonine protein kinase, DOUBLETIME (DBT). Similarly, the murine PERIOD proteins, mPER1 and mPER2, are phosphorylated by Casein kinase I epsilon (CKIε), the mammalian homolog of DBT. CKIε also phosphorylates and partially activates the transcription factor BMAL1. Given the variety of potential targets for CKIε and other cellular kinases, the precise role of phosphorylation is likely to be a complex one. Biochemical analysis of these and other circadian regulatory proteins has proven to be a fruitful approach in determining how they function within the context of the molecular clockworks.

I. Introduction

In terms of overall mechanism, molecular clocks share considerable similarity between species. The murine clock is essentially a negative feedback loop in which the heteromeric transcription factor CLOCK/BMAL1 drives transcription of its negative regulators *Per* (*mPer1* and *mPer2*) and *Cryptochrome* (*mCry1* and *mCry2*). The net effect is a daily cycle in which transcript and protein abundance fluctuates (extensively reviewed in Dunlap (1999) and Reppert and Weaver (2002)). A striking feature of the clock is that many of these proteins are phosphorylated at some point during the cycle. Phosphorylation of clock proteins may in fact be an indispensable feature for maintaining circadian rhythmicity. The *Drosophila* PERIOD (dPER) protein, as well as mammalian mPER1 and mPER2 are rhythmically phosphorylated throughout the day (Ederly et al. 1994; Lee et al. 2001). The transcriptional activator BMAL1 is phosphorylated as well (Eide et al. 2002). The consequences of protein phosphorylation in circadian regulation include alterations in activity, subcellular localization, protein-protein interactions, and protein stability. The first protein kinase shown to regulate the circadian clock is Casein kinase I epsilon (CKIε). Several alleles of the homologous *doubletime* (*dbt*) gene that conferred aberrant period length were identified in a mutagenesis screen designed to identify novel clock genes (Kloss et al. 1998; Price et al. 1998). *In vitro* and tissue culture studies examining the effect of CKIε on clock proteins such as mPER2 complement genetic and whole animal studies, allowing insights into molecular mechanisms of circadian timing. Here we discuss purification of CKIε and mPER2, as well as assays allowing analysis of CKIε function in extracts and *in vivo*.

II. Bacterial Expression and Purification of an active form of CKIε

The carboxyl-terminus autoregulatory domain of CKIε and CKIδ potently autoinhibits the kinase activity after un-opposed autophosphorylation (Graves and Roach 1995; Gietzen and Virshup 1999). It is therefore critical when performing *in vitro* experiments to use a form of the kinase that does not autophosphorylate. Generally, we use bacterial expression to produce a truncated form of CKIε with a stop codon after residue 319, a form with good *in vitro* activity

that does not auto-inhibit. Experience has shown that PCR amplification and site directed mutagenesis of the CKI ϵ gene, is much more efficient when DMSO (4% final concentration) is added to the reactions. Alternatively, CKI ϵ can be activated by dephosphorylation (although this can complicate additional assays), or by proteolytic removal of the carboxyl-terminus.

The yield of soluble active CKI ϵ using bacterial expression is markedly enhanced by induction at room temperature (20–25°C) for 6 hours or more, as opposed to short induction periods at 37°C.

1. Purification of Casein Kinase I

CKI ϵ (Δ 319) was cloned by PCR into pET-32 Xa/LIC vector (Novagen) which contains a 105 amino acid thioredoxin tag upstream of the inserted gene, as well as His and S-tags. Other constructs without the thioredoxin tag have similarly worked well in our experience.

-Transform competent BL21(DE3) *E. coli* with the CKI ϵ (Δ 319) expression plasmid. Then inoculate 10 ml of LB medium supplemented with ampicillin (100 μ g/ml final concentration) with a single colony from a freshly streaked plate.

-The next day, start a new culture by adding the overnight culture (diluted 1:100) and incubate at 37°C with vigorous shaking until the OD600 reaches 0.5–0.7.

-Cool the culture to room temperature by brief immersion in cold water. Next add IPTG (0.1 mM final concentration) to the culture, followed by incubation at 28°C for 5–7 hours. Induction conditions, including IPTG concentration, temperature and time should be determined by a series of pilot experiments beforehand.

-Collect the bacteria by centrifugation for 10 min at 4°C.

-Freeze the pellet at –80°C for at least 15 minutes and then resuspend in CellLytic B bacterial cell lysis extraction reagent (Sigma #B3553) supplemented with lysozyme (0.5 μ g/ml), 1 mM PMSF, 10 mM MgCl₂ and Dnase I (10 μ g/ml) and incubate on ice for 30 minutes.

-Sonicate for three 30 sec pulses using output power 20% to shear genomic DNA. Cell debris and insoluble recombinant kinase are removed by centrifugation at 30,000 X g in a JA-17 rotor at 4°C for 30 min. This centrifugation step can be repeated once more if necessary.

-Dialyze the supernatant against cation exchange column buffer (20 mM HEPES pH 7.5, 10 mM NaCl, 0.02% NP-40, 1 mM EDTA, 1 mM DTT, 10% sucrose) overnight with several buffer changes.

-A cation exchange column is used as a first purification step to reduce contaminants in the final purification step, taking advantage of the basic isoelectric point of Trx-His-CKI ϵ (j319) (pI~10). The dialyzed supernatant from 2 L of bacterial culture is loaded on a 50 ml bed volume S-Sepharose column pre-equilibrated with column buffer.

-Wash the column with 10 column volumes of column buffer.

-Step-elute the bound proteins in a volume of about 200 ml using column buffer supplemented with 300 mM salt.

-Dialyzed the S-sepharose eluate against Ni-NTA column buffer (50 mM HEPES pH8.0, 300 mM NaCl, 10 mM imidazole, pH 7.9). After dialysis, adjust the pH of the column buffer to 7.9.

- Load the dialyzed protein solution by gravity flow onto a pre-equilibrated Ni-NTA column (20 ml bed volume) with column buffer (50 mM HEPES, 300 mM NaCl, 10 mM imidazole, pH 7.9).
- Wash with 10 column volumes of column buffer containing 20 mM imidazole.
- Bound proteins are eluted with elution buffer (50 mM HEPES, 300 mM NaCl, 300 mM imidazole, pH 7.0) in 5ml volume.
- Concentrate and buffer-exchange the Ni-NTA column eluate using ultrafiltration (Millipore, Amicon Ultra centrifugal filter Cat.# UFC801024) into CKI storage buffer (20mM HEPES (pH 7.5), 25 mM NaCl, 1 mM EDTA, 1 mM DTT, 0.02% NP-40, 10% sucrose).
- The protein is stable and can be stored at -80°C . The protein retains full activity for several freeze-thaw cycles.

2. Purification of MBP-mPER2(450–763)

Attempts to produce full length mPER1 and mPER2 using several bacterial expression systems or a baculovirus expression system were hampered by minimal solubility of the recombinant protein. However expression of a fragment of mPER2 in *E. coli* as a maltose-binding protein fusion protein (MBP-PER2(450–763)) was readily accomplished.

The procedure is similar to the CKI purification except the cells were grown in LB with 0.2% glucose and lysed in 30 mM HEPES (pH 7.5), 150 mM NaCl, 2 mM DTT, 2 mM EDTA, 0.1% NP-40. Overall, the protocol recommended by New England Biolabs was followed with a slight modification: Binding of the MBP fusion protein to amylose beads was carried out for 2 hrs (in batch) in column buffer (20 mM HEPES pH 7.5, 200 mM NaCl, 1 mM EDTA, 1 mM DTT) and washes were also performed in batch with 10 bead volumes of column buffer. The bound MBP fusion protein was eluted with column buffer supplemented with 100 mM maltose, and then dialyzed against protein storage buffer (20 mM HEPES(pH 8.0), 25 mM NaCl) overnight with 2–3 buffer changes.

Figure 1A shows the purified proteins, and figure 1B illustrates the stoichiometry of mPER2 phosphorylation *in vitro*.

III. Examination of mPER2 stability in tissue culture cells

Several effects of phosphorylation on circadian rhythm proteins have been described including regulation of nucleocytoplasmic shuttling and protein stability (Eide and Virshup 2001). Examination of protein localization and nuclear export is described elsewhere (Vielhaber et al. 2000; Vielhaber et al. 2001). Several approaches to examining the effect of phosphorylation on protein stability are available. The mPER2 stability assays utilize transiently expressed protein in cultured mammalian cells. As circadian rhythms have been observed in multiple tissue culture lines, it is likely that relevant biological pathways can be explored using these systems. To examine the effect of phosphorylation on protein stability, mPER2 and various mutants are expressed, and the cells are then treated with specific inhibitors of cellular kinases, phosphatases or the 26S proteasome. The cells are then harvested at different times points and the remaining protein levels are analyzed by immunoblotting.

The experiment presented below is designed to assess the role of CKI on PER2 stability. The data suggest that CKI ϵ phosphorylates PER2 targeting it for ubiquitin-mediated proteasomal degradation, whereas protein phosphatases dephosphorylate and therefore stabilize PER2. The first part of this experiment describes the assay of mPER2 stability after treatment of transfected

HEK 293 cells with the phosphatase inhibitor calyculin A. In the second part, we show how pretreatment with the CKIe inhibitor IC261 prevents calyculin A-induced mPER2 degradation.

IV. Induce mPER2 phosphorylation and degradation by phosphatase inhibition with calyculin A

1. Description

Myc-tagged PER2 (or specific mutants and fragments) is expressed in HEK 293 cells. The day after transfection, cells are treated with cycloheximide (CHX), a protein synthesis inhibitor, in order to block new protein synthesis. 20 minutes later, calyculin A is added to the growth medium. Cells are lysed at 0, 30 and 60 minutes and mPER2 protein abundance is analyzed by SDS-PAGE and western blotting.

2. Protocol

-Transfect HEK 293 cells (70% to 80% confluent) in a six-well dish with a plasmid encoding myc-mPER2(450–763).

-About 20 hours after the end of the transfection start pretreatment with CHX or vehicle. Aspirate the old media and replace it 1 ml with fresh DMEM containing CHX (25 µg/ml). Incubate for 20 minutes at 37° C. The concentration of cycloheximide may have to be adjusted depending on the sensitivity of specific cell types.

-To ensure that *de novo* protein synthesis remains inhibited throughout the experiment, the cycloheximide concentration should be altered as little as possible. To this end, a master mix of calyculin A should be prepared first. Dilute calyculin A in DMEM to a concentration sufficient so that only a small volume is required to give a final concentration of 80 nM after adding it to the 1 ml of medium already in the well.

-For each well, add the appropriate volume of the diluted calyculin A stock to the existing media (80 nM final concentration) and continue to incubate the cells at 37° C for 0, 30 or 60 minutes. The appropriate concentration of calyculin A may have to be empirically determined since prolonged exposure is toxic to tissue culture cells.

-Immediately after the addition of the calyculin A, take the 0 minute time point. To harvest the cells, aspirate the old media and wash with PBS. Then add 200 µl of lysis buffer (0.1 % NP40, 150 mM NaCl, 20 mM HEPES pH 7.5, 1 mM EDTA, and freshly added protease inhibitor cocktail and 2 mM DTT) and immediately place the dish on ice.

-Analyze protein abundance by SDS-PAGE and immunoblotting.

3. Testing degradation or mobility shift: alkaline phosphatase treatment

One confounding factor in the quantitation of highly phosphorylated proteins is the presence of a mobility shift. Thus, it is important to determine whether the apparent decrease in protein abundance is due to degradation or diffusion in the gel because of hyperphosphorylation. Treatment of cell lysates with calf alkaline phosphatase (CIP) should convert hyperphosphorylated species to a single unphosphorylated form. Therefore any previously phosphorylated mPER2 will resolve as a compact band after SDS-PAGE.

For the CIP assay, 100 µg of extracts from cells treated with calyculin A is mixed with CIP incubation buffer (final concentration 100 mM NaCl, 70 mM Tris-HCl pH 7.5 and 10 mM MgCl₂) in a reaction volume of 30 µl. Dephosphorylation is achieved by addition of 5 units of CIP (New England Biolabs) and incubation of the samples for 1 hour at 37° C. The reaction

is stopped by adding 5X SDS sample buffer and the samples are analyzed by SDS-PAGE and immunoblotting.

V. Analyzing mPER2 protein degradation in *Xenopus* egg extracts

Besides expression in mammalian cells, protein stability can also be analyzed in other systems. *Xenopus laevis* egg extract contains all the components necessary for degradation of protein substrates via the ubiquitin-proteasome pathway (Salic et al. 2000). One advantage of this system is that additional components such as inhibitors and recombinant proteins can be added and their effects on degradation can be easily assessed.

In this protocol, *in vitro* synthesized ^{35}S -methionine labeled mPER2 and luciferase (as a pipetting and gel loading control) is added to *Xenopus* egg extract. Okadaic acid is then added and at various times aliquots are taken and the amount of mPER2 remaining is analyzed by SDS-PAGE and autoradiography.

1. Protocol for preparing egg extract

-*Xenopus* eggs are dejellied in freshly prepared 2% L-cysteine, pH 8.2.

-Wash eggs 3–4 times in 0.2X MMR (dilute from a 10X stock, 100 mM NaCl, 2 mM KCl, 1 mM MgCl_2 , 2 mM CaCl_2).

-Wash eggs 3 times in 1X XB (100 mM KCl, 0.1 mM CaCl_2 , 1 mM MgCl_2 , 10 mM HEPES, pH 7.7, 50 mM sucrose). After the last wash, add a sufficient volume of XB to resuspend the eggs.

-Using a large bore transfer pipette, place the eggs in a 1.5 ml microfuge tube and spin at the lowest speed setting, 30 seconds at room temperature in a microcentrifuge. The eggs are very fragile and care must be taken to not break them at this point. After spinning, carefully remove the liquid supernatant.

-To depolymerize the microtubules, pipette 2 μl of cytochalasin B (10 mg/ml) directly into the packed eggs. Next, spin the eggs at full speed at 4°C for 10 min in a microcentrifuge. There should be three distinct layers: An upper yolk layer, an opaque cytoplasm layer and a dark debris layer at the bottom of the tube.

-Take a syringe with a 21 gauge needle and poke a hole in the side of the tube, just at the bottom of the cytoplasm layer. Carefully remove the cytoplasm, taking care to avoid the pelleted debris or yolk. Place the cytoplasm in a fresh microfuge tube and repeat the centrifugation steps two more times. After the third spin, the cytoplasm should be nearly clear but some particulate matter may still be present.

-Supplement the cytoplasm fraction with 1/20 volume energy mix (150 mM creatine phosphate, 20 mM ATP, pH 7.4, 2 mM EGTA, pH 7.7, 20 mM MgCl_2) and protease inhibitors, each at a final concentration of 10 $\mu\text{g}/\text{ml}$ (from a stock of leupeptin, pepstatin and chymostatin, 10 mg/ml each).

-Freeze extracts in liquid nitrogen and store at -80° .

2. Protocol for degradation assay

Since many of the components of the assay are present in small amounts, it is important to design the experiment such that master mixes can be used. This will diminish many sources of error, especially pipetting. All reactions should be assembled on ice to prevent premature

degradation of the protein of interest. The following protocol is based on previously described methods (Salic et al. 2000; Li et al. 2001)

- Determine the total volume of egg extract required for all experimental conditions. Although this will depend on the specific experimental design, a typical volume for a single degradation reaction is 15 μ l. To the total egg extract, add bovine ubiquitin (1 mg/ml stock dissolved in XB buffer) to a final concentration of 40 μ g/ml. Also add 1 μ l of 35 S-methionine-labeled luciferase to every 25 μ l of egg extract.

- Split the master mix into siliconized tubes, one for each degradation reaction.

- Add labeled protein of interest. Generally we add 3 μ l of 35 S-methionine-labeled protein synthesized from a programmed reticulocyte lysate.

- Further divide into smaller aliquots, usually a control and experimental tubes.

- To test the effect of phosphatase inhibition on mPER2 stability, add okadaic acid in DMSO to a final concentration of 1 μ M. An equal volume of DMSO is added to the control reactions. Mix the contents by gently flicking the side of the tube.

- Incubate at room temperature.

- At each desired time point, 3 μ l of the reaction is removed and added to a tube containing 25 μ l of 1X Laemmli sample preparation buffer and stored on ice or frozen at -20° until analysis.

- Resolve the proteins by SDS-PAGE and analyze by autoradiography or PhosphorImager.

VI. Concluding remarks

In vitro and transfection-based assays for the effects of protein kinases and phosphatases on circadian rhythm have the advantages of using the well established methods of biochemistry and signaling transduction fields. These methods allow straightforward structure function analysis of endpoints such as protein stability, protein-protein interaction and subcellular localization. The challenge in the circadian rhythm field is to move from these mechanistic findings to determine their role in circadian rhythm regulation in cycling systems.

References

- Dunlap JC. Molecular bases for circadian clocks. *Cell* 1999;96:271–90. [PubMed: 9988221]
- Ederly I, Zwiebel LJ, Dembinska ME, Rosbash M. Temporal phosphorylation of the *Drosophila* period protein. *Proc Natl Acad Sci U S A* 1994;91:2260–4. [PubMed: 8134384]
- Eide EJ, Vielhaber EL, Hinz WA, Virshup DM. The circadian regulatory proteins BMAL1 and cryptochromes are substrates of casein kinase Iepsilon. *J Biol Chem* 2002;277:17248–54. [PubMed: 11875063]
- Eide EJ, Virshup DM. Casein kinase I: another cog in the circadian clockworks. *Chronobiol Int* 2001;18:389–98. [PubMed: 11475410]
- Gietzen KF, Virshup DM. Identification of inhibitory autophosphorylation sites in casein kinase I epsilon. *J Biol Chem* 1999;274:32063–70. [PubMed: 10542239]
- Graves PR, Roach PJ. Role of COOH-terminal phosphorylation in the regulation of casein kinase I delta. *J Biol Chem* 1995;270:21689–94. [PubMed: 7665585]
- Kloss B, Price JL, Saez L, Blau J, Rothenfluh A, Wesley CS, Young MW. The *Drosophila* clock gene double-time encodes a protein closely related to human casein kinase Iepsilon. *Cell* 1998;94:97–107. [PubMed: 9674431]
- Lee C, Etchegaray JP, Cagampang FR, Loudon AS, Reppert SM. Posttranslational mechanisms regulate the mammalian circadian clock. *Cell* 2001;107:855–67. [PubMed: 11779462]

- Li X, Yost HJ, Virshup DM, Seeling JM. Protein phosphatase 2A and its B56 regulatory subunit inhibit Wnt signaling in *Xenopus*. *Embo J* 2001;20:4122–31. [PubMed: 11483515]
- Price JL, Blau J, Rothenfluh A, Abodeely M, Kloss B, Young MW. double-time is a novel *Drosophila* clock gene that regulates PERIOD protein accumulation. *Cell* 1998;94:83–95. [PubMed: 9674430]
- Reppert SM, Weaver DR. Coordination of circadian timing in mammals. *Nature* 2002;418:935–41. [PubMed: 12198538]
- Salic A, Lee E, Mayer L, Kirschner MW. Control of beta-catenin stability: reconstitution of the cytoplasmic steps of the wnt pathway in *Xenopus* egg extracts. *Mol Cell* 2000;5:523–32. [PubMed: 10882137]
- Vielhaber E, Eide E, Rivers A, Gao ZH, Virshup DM. Nuclear entry of the circadian regulator mPER1 is controlled by mammalian casein kinase I epsilon. *Mol Cell Biol* 2000;20:4888–99. [PubMed: 10848614]
- Vielhaber EL, Duricka D, Ullman KS, Virshup DM. Nuclear export of mammalian PERIOD proteins. *J Biol Chem* 2001;276:45921–7. [PubMed: 11591712]

Figure 1.
A. Purified thioredoxin-His-CKIε(Δ319) and MBP-mPER2(450–763). Proteins were purified as described and 5 μg analyzed by SDS-PAGE.

B. Recombinant CKI ϵ (Δ 319) phosphorylates mPER2 to high stoichiometry. Phosphorylation of mPER2(40 pmol) by CKI ϵ (Δ 319) (20 pmol) was carried out at 37°C in 30 mM HEPES (pH 7.5), 10 mM MgCl₂, 1 mM DTT, 1 μ g BSA, 250 μ M ATP, 200 μ Ci 32 P-[γ -³²P]ATP]. At each time point, the reaction was stopped by adding 1X SDS-PAGE sample buffer followed by boiling for 3 min at 95°C. The proteins in the reactions were separated on 12% SDS-PAGE. The phosphorylation of mPER2 was quantitated by Phosphor-Imager and ImageQuant software.

Figure 2.

Effect of kinase inhibitors on mPER2 stability. HEK 293 cells overexpressing myc-mPER2 (450–763) were pretreated with the CKIe inhibitor IC261 (50 μ M), MAPK inhibitor U0126 (30 μ M) or vehicle for 4 hours. Next, they were treated with cycloheximide (25 μ g/ml) for 20 minutes and with calyculin A 80 nM or DMSO for 0, 30 and 60 minutes. Cells lysates were analyzed by SDS-PAGE and immunoblotting with antibodies recognizing the myc epitope of PER2 and the actin proteins.