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Molecular Brain Research 136 (2005) 199–211
MOLECULAR BRAIN RESEARCH
www.elsevier.com/locate/molbrainres

Research report

The circadian clock-containing photoreceptor cells in *Xenopus laevis* express several isoforms of casein kinase I

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Accepted 3 February 2005
Available online 31 March 2005

Abstract

The frog (*Xenopus laevis*) retina has been an important model for the analysis of retinal circadian rhythms. In this paper, several isoforms of *X. laevis* casein kinase I (CKI) were analyzed to address whether they are involved in the phosphorylation and degradation of *period* protein (PER), as they are in the circadian oscillators of other species. cDNAs encoding two splice variants of CKIδ (a full-length form and deletion isoform, which is missing an exon that encodes a putative nuclear localization signal and two evolutionarily conserved protein kinase domains) were isolated and analyzed, together with a previously isolated CKIε isoform. Both CKIδ and CKIε were shown to be constitutively expressed in the photoreceptors of the retina, where a circadian clock has been localized. Both the full-length CKIδ and CKIε were shown to have kinase activity in vitro, and the full-length CKIδ phosphorylated and degraded *Drosophila* PER when expressed in *Drosophila* S2 cells. The expression and biochemical characteristics of these CKIs are consistent with an evolutionarily conserved role for CKI in the *Xenopus* retinal clock. The CKIδ deletion isoform did not exhibit kinase activity and did not trigger degradation of PER. Subcellular localization of both CKIδ isoforms was cytoplasmic in several cell culture lines, but the full-length CKIδ, and not the deletion CKIδ isoform, was localized to both the nucleus and the cytoplasm in *Drosophila* S2 cells. These results indicate that the sequences missing in the deletion CKIδ isoform are important for the nuclear localization and kinase activity of the full-length isoform and that one or both of these features are necessary for degradation of *Drosophila* PER.

Theme: Neural basis of behavior
Topic: Biological rhythms and sleep

Keywords: Doubletime; Protein phosphorylation; Protein stability; Nuclear localization; *Period* protein; Alternative splicing

1. Introduction

The circadian clock, a 24 h timekeeper that controls biochemical, physiological, and behavioral processes occurring at a particular time of day, can be found in organisms as diverse as fungi and humans. The endogenous rhythms generated by the clock persist in constant environmental conditions, but can adjust to environmental fluctuations through the process of entrainment. Entrainment of rhythms occurs by way of an input pathway that communicates environmental signals to a central oscillator, resulting in changes at both the cellular and organismal level (reviewed in [40]). It has been demonstrated in several organisms, including the marine mollusks *Bulla* and *Aplysia*, chickens, quails, *Xenopus laevis*, and in mammals, that a cultured retina can function as an independent circadian oscillator (reviewed in [19]).

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doi:10.1016/j.molbrainres.2005.02.009

Precise temporal processes are important to normal retinal function, as melatonin, serotonin, and dopamine synthesis, photoreceptor disc shedding and degradation, and retinomotor movements are controlled by the circadian clock (reviewed in [12]). Although rhythms are known to be present in retinas of all classes of vertebrates, including mammals [49], the *Xenopus* retina has been an important model for localizing the circadian oscillators that control retinal rhythms. *Xenopus* eyecups can easily be cultured, allowing for measurement of rhythmic outputs, such as retinal serotonin *N*-acetyl-transferase activity [7] and melatonin release [10]. Tissue reduction of the *Xenopus* retina has localized an independent circadian oscillator to the photoreceptor layer [11]. By creating a molecular lesion of the central oscillator specifically in *X. laevis* retinal photoreceptors, it was demonstrated that the circadian clock localized in the photoreceptors is necessary for rhythmic melatonin release [25].

By and large, the molecules that comprise the central oscillator and the pacemaker mechanisms have been conserved between invertebrate and vertebrate species (reviewed in [15,52]). In *Drosophila* and mammals, positive regulation of the *period* gene by the CLOCK and CYCLE/BMAL1 heterodimer and subsequent feedback of PER protein to inhibit its own transcription are essential for normal clock function (reviewed in [3]). The biochemical processes that comprise this autoregulatory feedback loop could occur in a short span of time, but a post-translational regulatory event is inherent to the 24 h rhythmicity of this cycle.

In both *Drosophila* and mammals, casein kinase I (CKI) ε/δs are involved in the circadian oscillator mechanism. The PER protein is a target for the kinase activity of these enzymes [1,13,16,26,27,29,34,37,41,43,48,50], which form complexes with PER [27,28,31,32,34,41], target it for degradation [26,29,37,42,43,48], regulate its subcellular localization [1,5,47,50], and enhance its repression of CLK/CYC mediated transcription [38]. CKI ε/δ shows a circadian oscillation in localization, with high nuclear levels during the night [28,31]. It is thought that the kinase activity delays the nuclear accumulation of PER in the early evening, when *per* mRNA levels are high. When PER does accumulate in the nucleus, it interacts with a transcription factor (CLK/CYC) to negatively regulate its own transcription, which is otherwise activated by CLK/CYC (reviewed in [3,51]). Hence, the delay in nuclear accumulation effected by CKI, as well as the temporal activity of several other kinases and phosphatases [2,33,35,46], allows the *per* mRNA to accumulate to high levels rather than to a low steady state level that would be produced by constitutive repression if PER nuclear accumulation were immediate. PER continues to be phosphorylated in the nucleus, and nuclear levels fall during the day, most likely in response to phosphorylation-mediated degradation [5,28]. The disappearance of PER relieves the negative feedback on *per* transcription and allows the reaccumulation of *per* mRNA. Hence, CKI activity is essential for the oscillations of *per* gene products at several different circadian times and in both the cytoplasmic and nuclear compartments.

The roles and extent of different isoforms of CKI in the circadian mechanism remain to be determined. In rodents, although genetic evidence for a circadian role only exists thus far for CKIε [34], both CKIδ and CKIε have been shown to form a complex with other circadian clock proteins, suggesting that both of these isoforms may be involved [13,31]. In this study, we have examined the tissue-specific and temporal expression of CKIδ isoforms as well as a CKIε isoform in order to determine if CKIδ/ε is expressed in a manner which would be consistent with a role in the *Xenopus* circadian system. Moreover, we have compared the enzymatic activity, capacity to degrade *period* protein, and subcellular localization of two CKIδ splice variants. We find that both *ckiδ* and *ckiε* are expressed constitutively in the photoreceptors of the retina, where a circadian clock has been localized. Full-length CKIδ phosphorylated and degraded *Drosophila* PER when the two proteins were coexpressed in *Drosophila* S2 cells and exhibits both cytoplasmic and nuclear localization in these cells. In contrast, a CKIδ isoform that lacks enzymatic activity does not trigger degradation of PER and is found predominantly in the cytoplasm in *Drosophila* S2 cells. These experiments identify sequences important for the kinase activity and localization of CKIδ and strongly suggest that one or both features of CKI function are required for degradation of *Drosophila* PER. In addition, the ability of a vertebrate CKIδ to degrade *Drosophila* PER demonstrates a high degree of evolutionarily conserved function for this kinase.

2. Materials and methods

2.1. Isolation of genes orthologous to dbt from a Xenopus cDNA library

A degenerate PCR approach (CODEHOP: *Co*nsensus *De*generate *H*ybrid *O*ligonucleotide *P*rimers; [44]) was used to isolate DNA fragments that encode fragments of *X. laevis* CKIδ and CKIε. The following primers were synthesized (the name indicates the *X. laevis* CKIδ amino acid number at which the 5′ end of the primer initiates; R = A or G, Y = T or C, N = A, G, C or T; D = G, A or T): 85F (CTGGGACCATCCCTGGARGAYYTNTT), 87F (GACCATCCCTGGAAGATCTGTTYAAYTTYTG), 175R (TTCCTGTCAGATTCTTATTTTCTCTGTANGGDATRTG), 205F (TGTACTTTAATCTGGGATCTCTGCCNTGGCARGG), 209F (GGGATCTCTGCCATGGCARGGNYT), 222R (TCTCTTAGTAGCAGCCTTCAGTCCYTGCCANGG), 223R (TTCTGTCTCTTAGTAGCAGCCTTCARNCCYTGCCA), 295R (ACTTCAGCATATTCCAATCAAACACRTARTCRTA).

DNA fragments that resulted from PCR where the oligonucleotide anchored to the 3′ end of the reading frame