^{57}Arg in the bHLH transcription factor DEC2 is essential for the suppression of CLOCK/BMAL2-mediated transactivation

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Abstract. The basic helix-loop-helix (bHLH) transcription factors, DEC2 and DEC1, play critical roles in the circadian rhythm of the suprachiasmatic nucleus (SCN). It is known that mammalian circadian rhythms are regulated by molecular clockwork systems based on a negative-feedback loop, and CLOCK/BMAL1 and CLOCK/BMAL2 enhance DEC2 transcription via CACGTG E-boxes. To understand the role of arginine 57 (^{57}Arg) within the basic region of DEC2, we examined the effect of substituting this residue into DEC2 on CLOCK/BMAL2-mediated transactivation. A luciferase assay showed that substituting ^{57}Arg for Ala or Lys in DEC2 diminished the suppressive activity of wild-type DEC2 on CLOCK/BMAL2-mediated transactivation, while substituting ^{48}Pro for Ala in DEC2 did not alter it, and the same was true for wild-type DEC2. We also showed that proteins which were wild-type and substitution mutants of DEC2 were expressed at nearly equivalent levels by Western blotting. These findings demonstrate that ^{57}Arg in the basic region of DEC2 is essential for its activity in suppressing CLOCK/BMAL2-mediated transactivation.

Introduction

Circadian rhythms are conserved by evolution from bacteria to humans (1). The clock mechanisms of the suprachiasmatic nucleus (SCN) and its periphery are similar and consist of a network of transcriptional/transnational feedback loops (2,3). In mammals, the clock genes *Clock*, *brain-muscle-arnt-like-protein* (*Bmal*) *1*, *period* (*Per*), *cryptochromes* (*Cry*) and their protein products comprise a molecular feedback loop in which a CLOCK/BMAL1 heterodimer binds to a CACGTG E-box and activates transcription of *Per* and *Cry* (4,5). We recently demonstrated that *Dec2* (*Sharp-1*) and *Dec1* (*Sharp-2/Stra13/Clast5*) were new regulators of the mammalian molecular clock which caused CLOCK/BMAL1-mediated transactivation (6). DEC2 and DEC1 are basic helix-loop-helix (bHLH) transcription factors (7,8) which bind to CACGTG E-boxes and BMAL1 to suppress transcription from target genes (6,9,10). The expression of DEC2 and DEC1 shows circadian rhythms in most organs, including the SCN (6,12), and the phases of the circadian rhythms for *Dec2* and *Dec1* were similar to those for *Per1, Per2, Per3* (13), *Rev-Erb α* (14) and were somewhat similar to *Cry1* (15).

It has been reported that BMAL2 regulated the circadian oscillation of the expression of the plasminogen activator inhibitor-1 gene (16). Recently, it was found that DEC2 regulated the molecular clock system by suppressing CLOCK/BMAL2-mediated transactivation (11), but the detailed mechanisms were unknown. We characterized some substitution mutants of DEC2 in order to analysis the transcriptional mechanisms by which DEC2 suppresses the transactivation of CLOCK/BMAL2.

In the present study, we demonstrated that the substitution of ^{57}Arg for Ala or Lys in the basic domain of DEC2 severely reduced the ability of wild-type DEC2 to suppress the transactivation of CLOCK/BMAL2, while the substitution of ^{48}Pro for Ala of DEC2 had no effect on the suppressive activity of wild-type DEC2 on CLOCK/BMAL2-mediated transactivation. We also showed that substitution mutants were expressed at nearly equivalent levels in NIH3T3 cells by Western blotting. These findings suggest that the region of ^{57}Arg, but not ^{48}Pro, in the basic domain of DEC2 was important for the suppression of CLOCK/BMAL2-mediated transactivation.

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Abbreviations: bHLH, basic helix-loop-helix; SCN, suprachiasmatic nucleus; mDEC2, mouse DEC2; TK, thymidine kinase

Key words: DEC2, CLOCK, BMAL2, bHLH, circadian rhythm

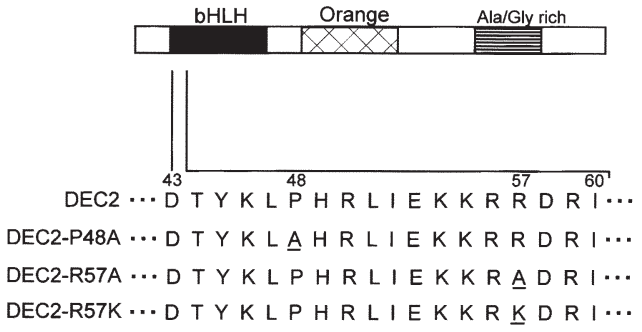

Figure 1. Substitution mutants of DEC2. The diagram shows the substitution mutants in the basic region of DEC2. DEC2-P48A: substituting ^{48}Pro for Ala in DEC2. DEC2-R57A: substituting ^{57}Arg for Ala in DEC2. DEC2-R57K: substituting ^{57}Arg for Lys in DEC2. All substitution mutants were subcloned into a pcDNA3.1/Zeo vector.

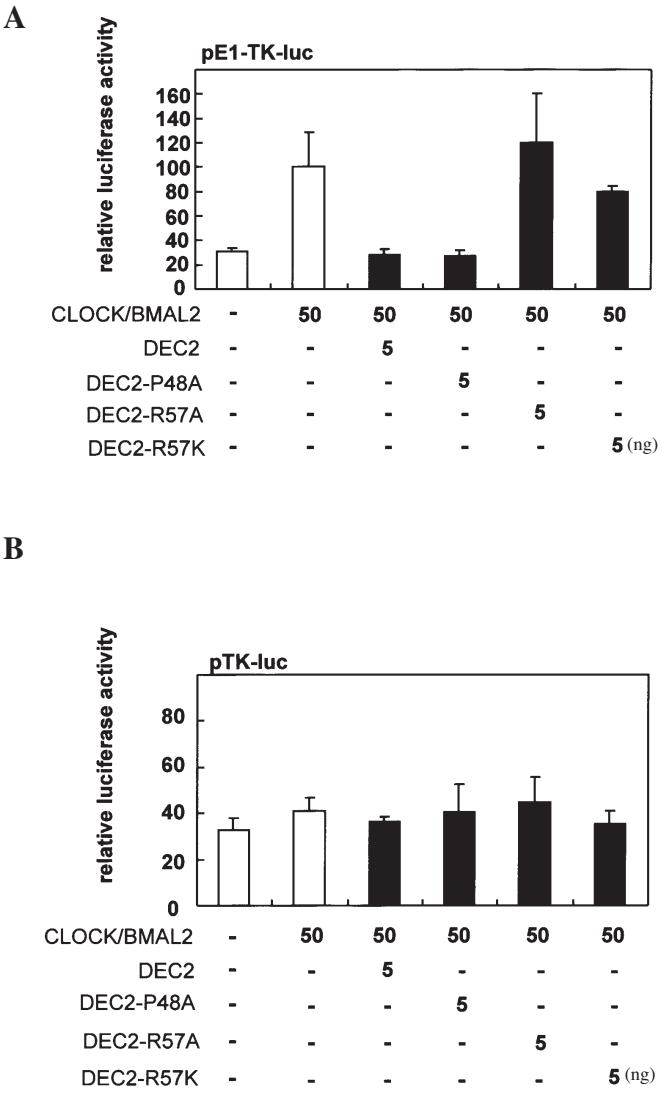

Figure 2. The suppressive activity of substitution mutants of DEC2 on CLOCK/BMAL2-mediated transactivation. (A) Substitution analysis of pE1-TK-Luc (containing three repeats of CACGTG E-box1) and (B) substitution analysis of pTK-Luc (containing no E-boxes) promoter activity. pE1-TK-Luc and pTK-Luc were co-transfected with expression vectors for CLOCK, BMAL2, wild-type and substitution mutants of DEC2 into NIH3T3 cells. The total amount of transfected DNA was adjusted to 375 ng per well with an empty vector (pcDNA3.1/Zeo). The relative luciferase activities of pE1-TK-Luc or pTK-Luc (mean ± SEM, n=6) are shown in the left-hand panel.

Materials and methods

Cell cultures. NIH3T3 cells were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA). Cells were cultured in Dulbecco's modified Eagle's medium-high glucose (Sigma Chemical Co., St. Louis, MO, USA) with L-glutamine and sodium bicarbonate supplemented with 10% fetal bovine serum.

Plasmid constructions. To obtain expression vectors for DEC2 mutants with a single amino acid-substitution, three sets of primers (5'-CCGCACAGATTAATAGAAAAGAAGAGA GCTGACCGA-3' and 5'-TAACTTGTAGGTATCCTTGGT ATCGTCTCG-3' for DEC2-R57A; 5'-CCGCACAGATTAA TAGAAAAGAAGAGAAAGGACCGA-3' and 5'-TAACTT GTAGGTATCCTTGGTATCGTCTCG-3' for DEC2-R57K; and 5'-TTAGCTCACAGATTAATAGAAAAGAAGAGAC GAGACCGA-3' and 5'-CTTGTAGGTATCCTTGGTATCG TCTCGCTTCAAGCTCCT-3' for DEC2-P48A) were used. The resulting PCR products were ligated to make circular forms of the plasmids and transformed into *E. coli DH5α*.

The cDNAs obtained from the transformants were confirmed by nucleotide sequencing and subcloned into a pcDNA3.1/Zeo vector. The expression vectors for CLOCK and BMAL2 were described previously (11).

Luciferase reporter assay. NIH3T3 cells were seeded at 1x10^4 cells per 16-mm well 24 h before transfection. The luciferase reporter plasmids, pE1-TK-Luc containing three repeats of E-box 1 (CACGTG), and pTK-Luc containing no E-boxes connected to the thymidine kinase promoter (11), were co-transfected with expression vectors for mouse CLOCK and BMAL2 (each 50 ng per well) together with an expression vector for mouse DEC2 (5 ng or the indicated amount per well) using PolyFect Transfection Reagent (Qiagen, Valencia, CA, USA) according to the manufacturer's directions. 0.2 ng of phRL-TK (Promega, Madison, WI, USA) was co-transfected for use as an internal standard. The total amount of transfected DNA was adjusted to 375 ng per well with an empty vector (pcDNA3.1/Zeo). The cells were incubated for 48 h and subjected to a luciferase reporter assay using the Dual Luciferase Reporter Assay System (Promega). Luciferase activities were normalized relative to the internal control activities. The experiments were repeated at least twice and the obtained data was combined to represent the mean ± SEM.

Western blot analysis. Rabbit antibodies to DEC2 were produced by immunizing the synthetic peptide fragment Cys-Lys-Pro-Lys-Arg-Ser-Leu-Lys-Arg-Asp-Asp-Thr-Lys-Asp.

The obtained antibodies (anti-mDEC2: 30-43) were purified by affinity column chromatography. NIH3T3 cells were seeded at 1x10^5 cells per 35-mm well 24 h before transfection. Expression plasmids (4.0 μg) were transfected into the cells using Lipofectamine 2000 Reagent (Invitrogen Corp., Carlsbad, CA, USA) according to the manufacturer's directions. Forty-eight hours after transfection, the cells were harvested and dissolved in 100 μl of SDS-sample buffer.

Figure 3. Western blot analysis with anti-DEC2 immunoglobulin. pcDNA and wild-type and substitution mutants of DEC2 were expressed in NIH3T3 cells. After 48 h, the cells were harvested and subjected to Western blot analysis using anti-DEC2 antibody.

Equal volumes of samples (10 μl) were subjected to SDS-PAGE and transferred onto a nylon membrane (Immobilion P, Millipore). DEC2 was detected using anti-DEC2: 30-43 antibodies.

Results

Substitution analysis of the suppressive activity of DEC2 on CLOCK/BMAL2-mediated transactivation. To determine which region in DEC2 is required for the suppression of CLOCK/BMAL2-mediated transactivation, we constructed the expression plasmids for DEC2 as shown in Fig. 1. DEC2 proteins were expressed in NIH3T3 cells together with CLOCK and BMAL2, and their activities were examined by luciferase assay. As shown in Fig. 2A, the promoter activity of pE1-TK-Luc was enhanced by CLOCK/BMAL2, and the increased activity was reduced by wild-type DEC2. We showed that substituting ^{57}Arg for Ala in DEC2 (DEC2-R57A) severely diminished the suppressive activity of wild-type DEC2 on CLOCK/BMAL2-mediated transactivation (Fig. 2A). We then showed that substituting ^{57}Arg for Lys in DEC2 (DEC2-R57K) diminished the suppressive activity of wild-type DEC2, as did substituting ^{57}Arg for Ala in DEC2, whereas substitution of ^{48}Pro for Ala of DEC2 (DEC2-P48A) did not alter the suppressive activity of wild-type DEC2 on CLOCK/BMAL2-mediated transactivation. We also showed that wild-type and substitution mutants of DEC2 and CLOCK/BMAL2 had little effect on the promoter activity of pTK-Luc, which contains no CACGTG E-box sequences (Fig. 2B). From these findings, we concluded that the conserved ^{57}Arg, but not ^{48}Pro, in DEC2 is essential for its suppressive activity on CLOCK/BMAL2-mediated transactivation.

The expression levels of DEC2 mutants were found to be comparable using Western blot analysis. To determine the significance of the promoter assay, we analyzed wild-type and substitution mutants of DEC2 protein expression in NIH3T3 cells. As shown in Fig. 3, the proteins were expressed at nearly equivalent levels to that of the promoter assay.

Discussion

In living cells, DEC2 transcription is up-regulated by CLOCK/BMAL1 and CLOCK/BMAL2 (11). BMAL2 is expressed at high levels in some tissues, including blood vessels (17), and binds to CLOCK to form a heterodimer for gene activation (16). Despite rapid advances in our understanding of the molecular basis for circadian rhythms, the relative contributions of DEC2 and BMAL2 to circadian rhythmicity remain unknown. Previously, luciferase assay showed that CLOCK/BMAL1 and CLOCK/BMAL2 increased the promoter activities of pE1-TK-Luc and p-1596-Luc reporters (11), but the detailed mechanism by which DEC2 mediated CLOCK/BMAL2-mediated transactivation was unknown.

In this study, we analyzed the mechanism involved in the molecular feedback loop through DEC2 transcription in the circadian clock system, and clarified that ^{57}Arg, but not ^{48}Pro, in DEC2 was essential for its suppressive activity on CLOCK/BMAL2-mediated transactivation. Mutation analysis demonstrated that the basic region in DEC2 essential for the suppressive activity coincided with the region in DEC1 as described previously (10). The amino acid residue, ^{57}Arg, in the basic region of DEC2 is conserved among the group B bHLH proteins, including USF, c-Myc, MAX and MAD (16), which can bind to the CACGTG E-box (9,17) and some other transcription factors (6,18). We have shown previously that ^{65}Arg in DEC1 was important for the interaction with CACGTG E-box and BMAL1 (10), while ^{91}Arg in the basic region of BMAL1 was also important for the interaction with E-box and CLOCK (21).

Using yeast two-hybrid and gel shift experiments, DEC2 has been shown to interact with BMAL1 and E-boxes (6,11,19). It is hence likely that this amino acid in the basic region of DEC2 is also important for the interaction with BMAL2 and the CACGTG E-box. We hypothesized that the two mechanisms by which CLOCK/BMAL2 regulated DEC2 expression are as follows: a) CLOCK/BMAL2 up-regulates DEC2 transcription through binding to CACGTG E-box elements in the DEC2 promoter and, by contrast, the increase in DEC2 protein down-regulates its own expression; and b) DEC2 protein interacts with BMAL2 and suppresses CLOCK/BMAL2-mediated transactivation. Probably, the two mechanisms work together in the clock system. Therefore, it is still necessary to confirm the direct interaction between DEC2 and BMAL2. The present study is the first to report that the region of ^{57}Arg in DEC2 is important for the suppression of CLOCK/BMAL2-mediated transactivation.

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