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Effects of NAD(P)H and its derivatives on the DNA-binding activity of NPAS2, a mammalian circadian transcription factor

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ABSTRACT

NPAS2 is a transcription factor that regulates mammalian circadian rhythms. It has been suggested that NPAS2 DNA-binding activity is regulated by the intracellular redox state of NAD(P)H, although the mechanism remains unclear. To investigate the NAD(P)H interaction site of murine NPAS2, we performed electrophoretic mobility shift assays using several truncation mutants of the NPAS2 bHLH domain. Among the mutants, NPAS2 containing the N-terminal 61 residues formed a heterodimer with BMAL1 to bind DNA, and NAD(P)H enhanced the binding activity, while NAD(P)H inhibited the DNA-binding activity of the BMAL1 homodimer in a dose-dependent manner. NAD(P)H derivatives such as 2′,5′-ADP, nicotinamide, nicotinic acid and nicotinic acid adenine dinucleotide (NAAD) did not affect the DNA-binding activity. Interestingly, $NAD(P)^+$, previously reported as an inhibitor, did not affect NPAS2 binding activity in the presence or absence of NAD(P)H in our system. These results suggest that NPAS2 DNA-binding activity is specifically enhanced by NAD(P)H independently of $NAD(P)^+$ and that the N-terminal 1–61 amino acids of NPAS2 are sufficient to sense NAD(P)H.

1. Introduction

Circadian rhythms are intrinsic mechanisms that are highly conserved in organisms ranging from cyanobacteria to humans and are entrained by the daily light–dark cycle in a 24 h period. These rhythms control behavioral and physiological events such as the sleep–wake cycle, locomotor activity, body temperature, hormone release and metabolism, and they autonomously continue even in constant darkness. In mammals, the master clock of circadian rhythms exists in the suprachiasmatic nucleus (SCN), and it coordinates peripheral clocks that exist in most tissues. At the molecular level, it is known that the intracellular positive and negative transcriptional/translational feedback loops of clock genes and proteins generate the oscillations of circadian clocks [1].

Neuronal PAS domain protein 2 (NPAS2) is a transcription factor regulating circadian rhythms, and it was originally identified as a homolog of CLOCK in the mammalian forebrain [2,3]. NPAS2, similar to CLOCK, forms a heterodimer with BMAL1 that binds to E-box sequences (CACGTG) located in the promoter regions of *Per* and *Cry* genes and enhances their transcription. When PER and CRY proteins accumulate, they negatively regulate their own expression by inhibiting NPAS2/BMAL1 or CLOCK/BMAL1 heterodimers. Although NPAS2 and CLOCK exhibit overlapping functions, NPAS2-deficient mice show particular difficulty in their adaptability to food restriction and sleep homeostasis [4,5]. NPAS2 contains a bHLH domain, two PAS domains (PASA and PASB) and a transactivation domain. A bHLH domain is a highly conserved basic DNA-binding domain that forms functional homodimer or heterodimer complexes with other bHLH proteins [6–8]. PAS domains have now been observed in over 2,000 proteins, including kinases, transcription factors, ion channels and other enzymes that have diverse functions in signal transduction, protein–protein interactions and transcription [9,10]. In NPAS2, each PAS domain binds heme as a prosthetic group, and the PASA domain acts as a gas sensor to regulate the DNA-binding activity of the NPAS2/BMAL1 heterodimer by CO and NO binding to the ferrous heme [11–15]. It has also been reported that NADH and NADPH enhance the DNA-binding activity of the NPAS2/BMAL1 heterodimer, whereas NAD^+ and $NADP^+$ inhibit its activity, suggesting regulation by the redox state of the NAD cofactor [16]. In that report, the N-terminal 116 amino acids (a.a.) of murine NPAS2 and a.a. 75–126 of human BMAL1 were demonstrated to be sufficient for responsiveness to NAD(P)H. Little is known, however, about their precise interaction sites and mechanisms. To explore these, we generated various truncated NPAS2 proteins and used electrophoretic mobility shift (EMS) assays to examine how their DNA-binding activities were affected by NAD(P)H cofactors and their derivatives.

Abbreviations: NPAS2, neuronal PAS domain protein 2; EMS, electrophoretic mobility shift; NAAD, nicotinic acid adenine dinucleotide; HAT, histone acetyltransferase.

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2. Materials and methods

2.1. Expression plasmids

The construction of expression plasmids for His-tagged murine NPAS2 bHLH-PASA (1–240 a.a.) and MBP-tagged murine BMAL1 bHLH-PASA-PASB (1–447 a.a.) was previously described [15]. To create the truncated mutants of NPAS2, NPAS2 bHLH (1–116 a.a.), bHLH (1–79 a.a.) and bHLH (1–61 a.a.), PCRs were performed using pET-28a(+) containing NPAS2-bHLH-PASA cDNA as the template. The primers used for PCR were 5′-CGGGATCCCATATGGACG AAGATGAGAAG-3′ as a sense primer for all mutants and 5′-CCCG TCGACCTAGAGAGGTGTGATAC-3′, 5′-CCCGTCGACCTAGAATGATG GCTTCC-3′ and 5′-CCCGTCGACCTATTCATTGTGTTTCTG-3′ as antisense primers for NPAS2-bHLH (1–116 a.a.), bHLH (1–79 a.a.) and bHLH (1–61 a.a.), respectively. The resulting products were subcloned into the *Nde*I and *Sal*I sites of pET-28a(+). To create BMAL1 bHLH-PASA (74–336 a.a.), PCR was performed using pMAL-c2X containing BMAL1 bHLH-PASA-PASB cDNA as the template. The primers used for PCR were 5′-CAGGATCCAGGGAGGCCCACAGTC-3′ as a sense primer and 5′-CGAGTCGACTATTTCACCCGTATTTC CCC-3′ as an antisense primer. The resulting product was subcloned into the *Bam*HI and *Sal*I sites of pMAL-c2X. The desired constructs were confirmed by sequencing. The domain structures of these proteins are shown in Figs. 1 A and 2 A.

2.2. Expression and purification of the isolated bHLH-PASA and bHLH proteins of NPAS2

Expression and purification of the isolated bHLH-PASA or bHLH proteins of NPAS2 were performed essentially as described [14,15]. Briefly, cell extract was applied to a Ni–NTA agarose column pre-equilibrated with buffer A (50 mM sodium phosphate pH 7.8, 50 mM NaCl, 10% [v/v] glycerol, 2 μg/ml aprotinin, 2 μg/ml leupeptin, 2 μg/ml pepstatin A, 1 mM PMSF and 0.2 mM DTT). The column was washed sequentially with buffer A containing 20 mM and 70 mM imidazole for His-NPAS2 bHLH-PASA or 50 mM and 100 mM imidazole for the truncated mutants. The His-NPAS2 bHLH-PASA and the truncated mutants were then eluted with buffer A containing 150 mM and 250 mM imidazole, respectively. The protein fractions were pooled, concentrated and applied to a Sephadex G-25 column pre-equilibrated with buffer B (50 mM HEPES pH 7.5, 10% [v/v] glycerol and 1 mM DTT) to remove the imidazole and to exchange the buffer with buffer B. His-NPAS2 bHLH-PASA was prepared as holo-protein of bHLH-PASA by recon-

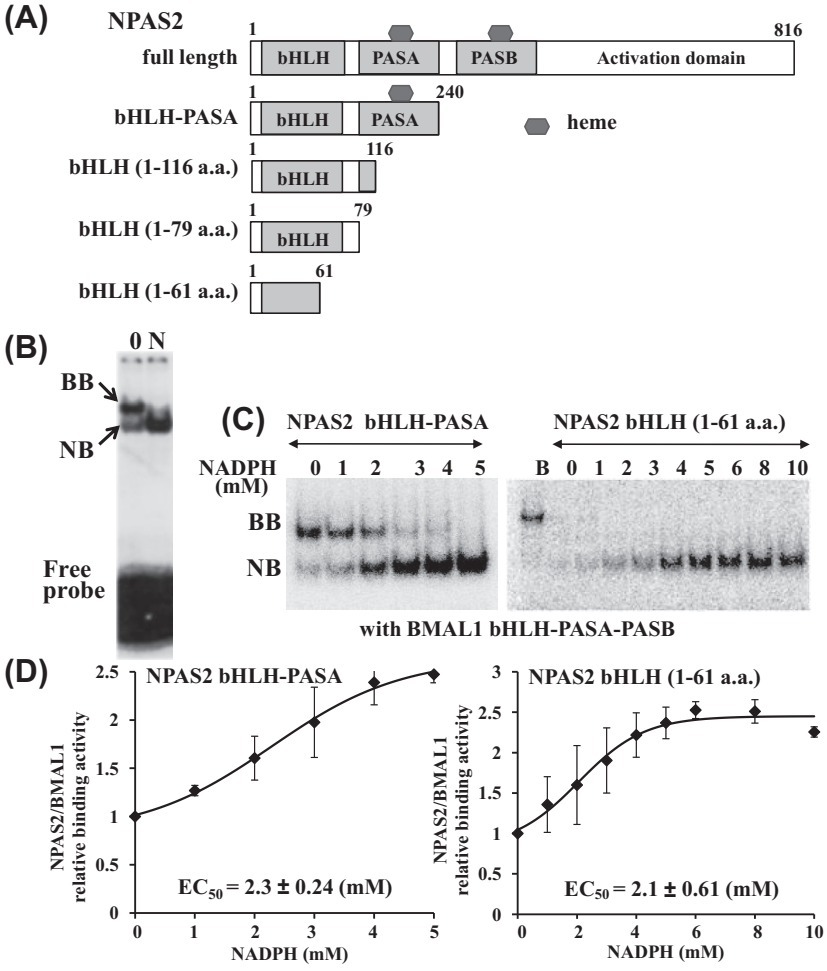

Fig. 1. Effects of NADPH on the DNA-binding activity of NPAS2 bHLH-PASA and bHLH mutants. (A) Domain structures of NPAS2. (B) the autoradiograph of EMS assay-gel using NPAS2 bHLH-PASA and BMAL1 bHLH-PASA-PASB in the absence (0) and presence (N) of 5 mM NADPH. BB, BMAL1/BMAL1 homodimer with ^{32}P-labelled DNA probe; NB, NPAS2/BMAL1 heterodimer with the DNA probe. (C) The DNA-binding activities of these proteins were analyzed in the presence of various amounts of NADPH. The label "B" at the top of the gel indicates an EMS assay in which BMAL1 protein was incubated with DNA in the absence of NPAS2. (D) Relationship between DNA-binding activity and NADPH. The DNA-binding activity of the NPAS2/BMAL1 heterodimer shown in (C) was quantified by Multi Gauge v. 2.1 and represents a relative value normalized to the value obtained in the absence of NADPH. Each dot is the mean of at least three independent experiments ± SD. EC_{50} was estimated from a sigmoidal plot fitted to the data.

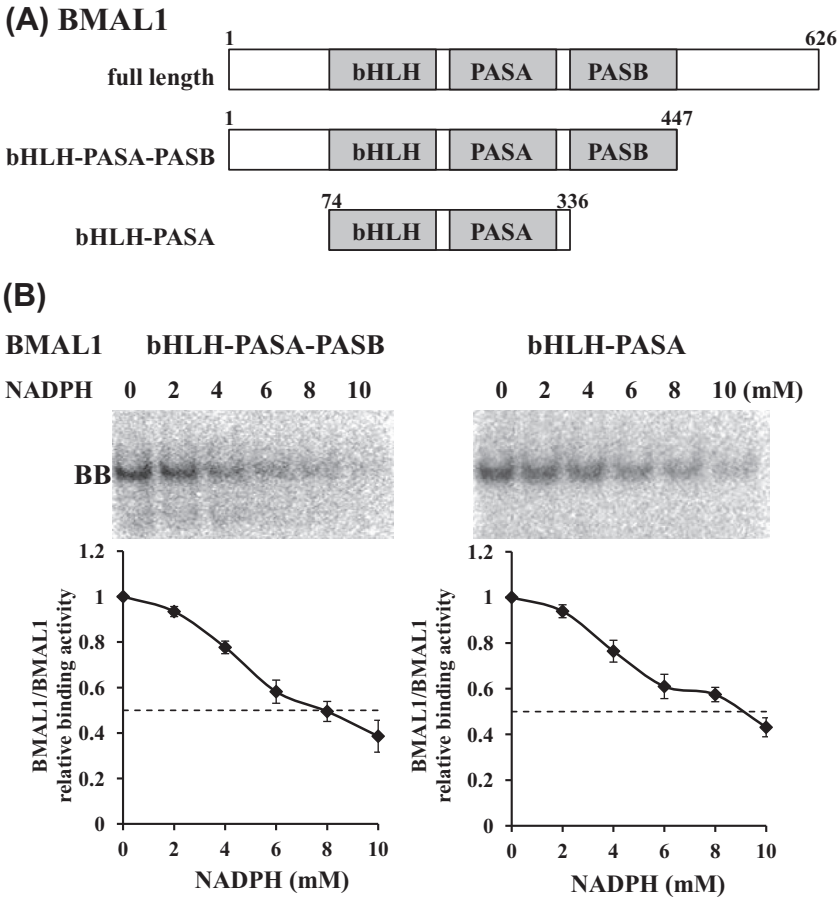

Fig. 2. Effects of NADPH on the DNA-binding activity of the MBP-BMAL1 bHLH-PASA-PASB and MBP-BMAL1 bHLH-PASA proteins. (A) Domain structures of BMAL1. (B) The DNA-binding activity of the BMAL1/BMAL1 homodimer. The activity was quantified by Multi Gauge v. 2.1, and represents a relative value normalized to the value obtained in the absence of NADPH. Each dot is the mean of at least three independent experiments ± SD.

stituting with hemin from apo-protein as described previously [14,15].

2.3. Expression and purification of the isolated bHLH-PASA-PASB and bHLH-PASA domains of BMAL1

Expression and purification of the isolated bHLH-PASA-PASB and bHLH-PASA domains of BMAL1 were performed as described previously [15]. The purified proteins were applied to a Sephadex G-25 column pre-equilibrated with buffer B to exchange the buffer.

2.4. EMS assay

The double-stranded oligonucleotide containing a canonical E-box sequence (5′-GGGGCGCCACGTGAGAGG-3′ and 5′-GGCCTCTCACGTGGCGCC-3′) was used as an EMS probe after end-labeling with [^{32}P] dCTP by Klenow enzyme. The DNA binding reactions were performed for 30 min on ice in 6 μl of the reaction mixture (0.3 or 0.6 μM His-NPAS2, 0.6 μM MBP-BMAL1, 0.1 μM ^{32}P-labeled E-box, 50 mM HEPES pH 7.5, 50 mM NaCl, 1.2 mM $MgCl_2$, 10% [v/v] glycerol, 0.5% [w/v] *n*-octyl-glycoside, 0.12 mg/ml BSA, 0.05 mg/ml poly-dI-dC and 2 mM DTT). To analyze the effects of NAD(P)H on the DNA-binding activity of NPAS2/BMAL1 heterodimer or BMAL1 homodimer, various concentrations of NAD(P)H were added to the reaction mixture. In competition assays between NADPH and its derivatives, the concentration of NADPH was fixed at 2 mM and the derivatives were added to the reaction mixture at various concentrations. Because $NADP^+$, nicotinic acid and NAAD have acidic pH values in aqueous solution, the stock solutions of these compounds were pH-adjusted to 7.5 just before the experiments. The reaction mixtures were electrophoretically separated on 5% (w/v) native acrylamide gels in buffer C (20 mM Tris–acetate and 0.5 mM EDTA) for 2.5 h at 100 V and 4 °C. After electrophoresis, the gels were dried and analyzed using a BAS-1800 II Image Analyzer with Multi Gauge v. 2.1. To estimate EC_{50} values, the binding data were fitted to variable-slope sigmoidal dose–response curves with Igor Pro (chi-squared values 0.007–0.069).

3. Results and discussion

3.1. Protein expression and purification

All of the purified proteins were analyzed by SDS–PAGE (Fig. S1). All the His-NPAS2 proteins were more than 95% homogeneous, except for His-NPAS2 bHLH (1–79 a.a.), which was approximately 60% pure with lower yield. These results suggest that NPAS2 bHLH (1–61 a.a.) has a more stable domain structure than bHLH (1–79 a.a.). Each of bHLH proteins showed a typical α-helical structure in circular dichroism spectra, indicating that it was properly folded (Data not shown). The UV–vis absorption spectra of the Fe(III), Fe(II) and Fe(II)–CO complexes of His-NPAS2 bHLH-PASA were obtained as previously reported, confirming the structure of the PASA domain [14]. The MBP-BMAL1 proteins used in this study were more than 85% homogeneous.

3.2. Effects of NAD(P)H on the DNA-binding activity of truncated NPAS2 mutants

To identify the sites of NPAS2 that interact with NADPH, the effects of NADPH on DNA-binding activity were examined in an EMS assay using various truncated NPAS2-bHLH-PASA proteins (Fig. 1A). As shown in Fig. 1B, two protein bands were observed as retarded bands by DNA probe binding in the presence of both NPAS2 bHLH-PASA and BMAL1 bHLH-PASA-PASB proteins in the absence of NADPH. The addition of 5 mM NADPH resulted in decrease of the upper band and increase of the lower band. The estimated molecular sizes indicate that the upper and lower bands correspond to the BMAL1/BMAL1 homodimer and the NPAS2/BMAL1 heterodimer, respectively. The super-shift analysis using an anti-His-tag antibody confirmed that the lower band contained His-NPAS2 bHLH-PASA and corresponded to the NPAS2/BMAL1 heterodimer [15]. It also indicates that the complex of NPAS2/NPAS2 homodimer with DNA was not formed or not observed under the experimental conditions of our EMS assay as previously reported [15]. As shown in Fig. 1C, the DNA-binding activity of the heterodimer increased with NADPH addition in a dose-dependent manner. The EC_{50} of NADPH for NPAS2 bHLH-PASA (1–240 a.a.) was estimated to be 2.3 mM (Fig. 1D), consistent with the results for NPAS2 bHLH-PASA-PASB (1–416 a.a.) in Rutter et al. [16]. NADH also enhanced the binding of the heterodimer to DNA with an EC_{50} value of 3.5 mM.

The truncated mutants of His-NPAS2 bHLH were examined under the same conditions for cofactor effects. The mutants NPAS2 bHLH (1–116 a.a.), bHLH (1–79 a.a.) and bHLH (1–61 a.a.) each formed a heterodimer with BMAL1 that bound to E-box DNA, and all their DNA-binding activities were fully enhanced by NADPH (Figs. 1C and D, S2). The EC_{50} of NADPH for NPAS2 bHLH (1–116 a.a.), bHLH (1–79 a.a.) and bHLH (1–61 a.a.) were 3.9 ± 0.29, 3.6 ± 0.23 and 2.1 ± 0.61 mM, respectively. These results indicated that the N-terminal 61 a.a. of NPAS2 were sufficient for heterodimer formation with BMAL1 bHLH-PASA-PASB, E-box binding and the effect of NADPH. Similar results were obtained with MBP-BMAL1 bHLH-PASA (Fig. S3) and with untagged proteins. It was surprising that the bHLH (1–61 a.a.) mutant showed a response to NADPH, as this mutant is the minimum size for a bHLH DNA-binding domain as estimated from a domain search (Fig. S4). The EC_{50} value of the bHLH (1–61 a.a.) mutant was lower than those of the other truncated proteins, suggesting that the bHLH (1–61 a.a.) fragment could contain a site for the interaction between NPAS2 and NADPH. It is also noticed that the addition of NPAS2 bHLH (1–61 a.a.) resulted in the drastic decrease of the complex of BMAL1/BMAL1 homodimer with DNA in the absence of NADPH. Similar results were obtained for NPAS2 bHLH (1–116 a.a.) and NPAS2 bHLH (1–79 a.a.) (Fig. S2), but not for NPAS2 bHLH-PASA (Fig. 1 C). These results suggest that the heterodimer of NPAS2 bHLH and BMAL1 bHLH-PASA-PASB was predominantly formed than the homodimer of BMAL1, but in the absence of NADPH the affinity of the heterodimer to DNA-probe was not enough to see as a retarded-band. Further, the PASA domain of NPAS2 appears to reduce the formation of NPAS2/BMAL1 heterodimer. We need further experiments to elucidate the precise kinetics of the BMAL1/BMAL1 homodimer and the NPAS2/BMAL1 heterodimer formations and their DNA-binding affinities.

3.3. NADPH effects on the DNA-binding activity of the BMAL1 homodimer

The effects of NADPH on the BMAL1 homodimer were examined by EMS assays using the purified BMAL1 bHLH-PASA-PASB and bHLH-PASA proteins in the absence of NPAS2. As shown in Fig. 2 , NADPH inhibited the DNA-binding activity of the BMAL1/BMAL1 homodimer in a dose-dependent manner. The DNA-binding activities of BMAL1 bHLH-PASA-PASB and bHLH-PASA were 50% inhibited (IC_{50}) by addition of 7.6 and 8.4 mM NADPH, respectively. NADPH oppositely affected the DNA-binding activities of the NPAS2/BMAL1 heterodimer and the BMAL1/BMAL1 homodimer. The inhibition of BMAL1/BMAL1 homodimer activity by NADPH may facilitate NPAS2/BMAL1 heterodimer formation at higher NADPH concentrations. These results indicate that the enhancement of DNA-binding activity by NADPH was specific for NPAS2/BMAL1 heterodimer formation and/or the interaction between the heterodimer and DNA. These results also suggest that the

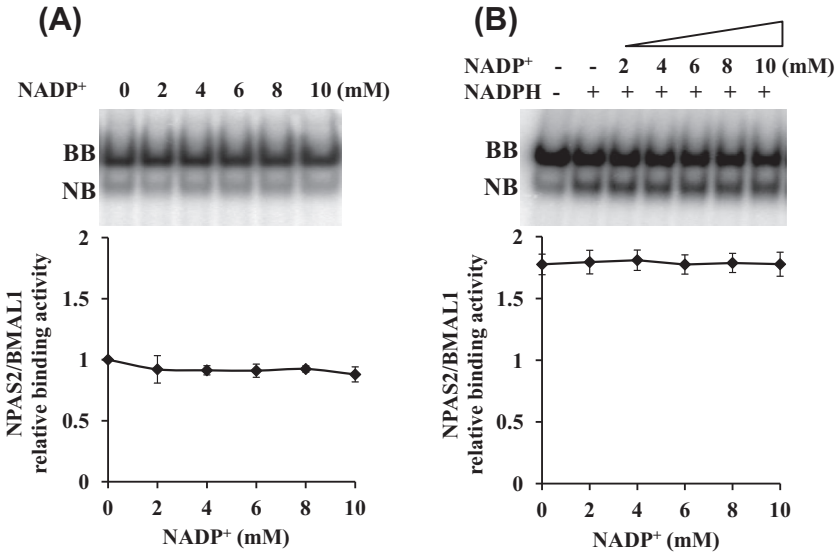

Fig. 3. Effects of $NADP^+$ on the DNA-binding activity of NPAS2 bHLH-PASA and BMAL1 bHLH-PASA-PASB in the presence or absence of NADPH. (A) The DNA-binding activity of NPAS2/BMAL1 heterodimer was not affected by addition of $NADP^+$. (B) $NADP^+$ did not compete with NADPH in the DNA-binding activity of the NPAS2/BMAL1 heterodimer. The independent experiments in the presence of 2 mM NADPH were conducted at least three times, and DNA-binding activity relative to that obtained without NADPH is shown as the mean ± SD ($n = 3$).

MBP-tag and BMAL1 protein were not sites for activation by NADPH.

3.4. Effects of NAD(P)H derivatives

We examined the effects of various NAD(P)H derivatives on the DNA-binding activity of NPAS2, and we also used them to perform competition assays with NAD(P)H (Fig. S5). Rutter et. al. reported that $NADP^+$ inhibited the DNA-binding activity of the NPAS2/BMAL1 heterodimer and competed with NADPH at an IC_{50} of 0.56 mM [16]. In their system, even equimolar addition of $NADP^+$ to a reaction mixture containing 2 mM NADPH completely inhibited the formation of the NPAS2/BMAL1/DNA complex. Unexpectedly, in our system, $NADP^+$ itself had no effect on the DNA-binding activity of the heterodimer (Fig. 3 A), and it did not compete with NADPH for the effect, even with the addition of a fivefold (Fig. 3 B) or 10-fold (data not shown) excess. Similarly, NAD^+ neither affected the activity of NPAS2/BMAL1 heterodimer nor competed with NADH (Fig. S6). The reasons for these inconsistencies are not clear, because our experimental conditions for EMS assay are very similar to those described by Rutter et al. [16], except that they used the proteins purified from inclusion bodies, while our proteins were purified from soluble fraction.

Other NADPH derivatives, namely 2′,5′-ADP, nicotinamide, nicotinic acid and NAAD, were also examined for effects on DNA-binding activity. As shown in Fig. 4 A, none of these compounds affected the DNA-binding activity of NPAS2/BMAL1 heterodimer when added individually. As with $NADP^+$, they also did not compete with NADPH by addition of a 5-fold excess (Fig. 4 B). Competition assays using NPAS2 bHLH (1–61 a.a.) showed similar results (data not shown). In all experiments, the pH values of the NADPH derivative solutions were adjusted to 7.5 immediately before use, and we carefully confirmed that the pH of the assay solution was not changed by the addition of excess derivatives during the assays, as some of them were highly acidic in aqueous solution. These results suggest that the reduced form of the nicotinamide moiety was critical for the effect of NAD(P)H. All the reaction mixtures for EMS assays contained 2 mM DTT, suggesting that NAD(P)H may not work as a reducing agent but rather that its structure is important for its effects.

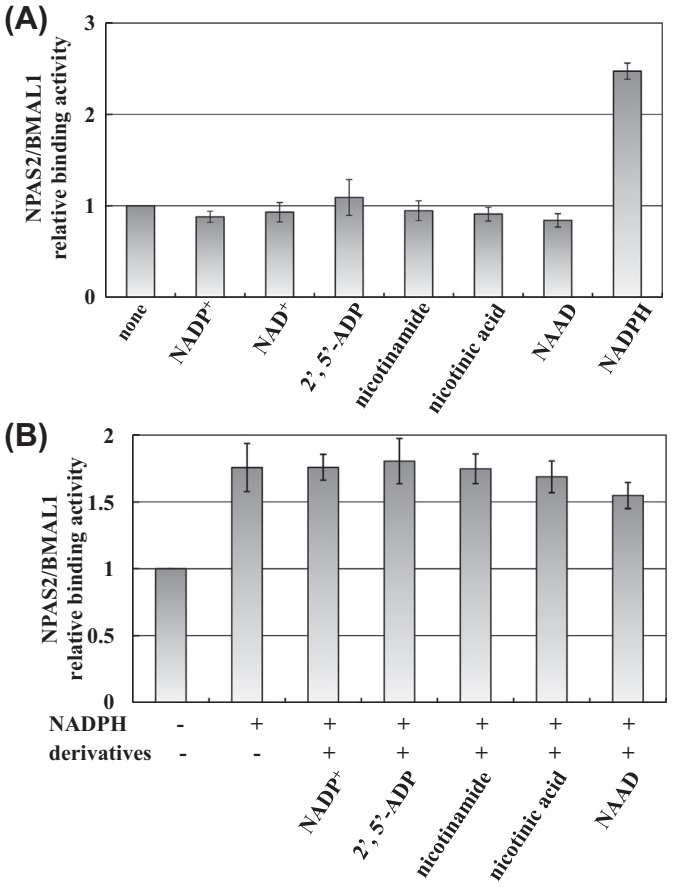

Fig. 4. Effects of NAD(P)H derivatives on the DNA-binding activity of the NPAS2 bHLH-PASA and BMAL1 bHLH-PASA-PASB heterodimer. (A) Only NADPH had an effect; $NADP^+$, NAD^+, 2′,5′-ADP, nicotinamide, nicotinic acid, and nicotinic acid adenine dinucleotide (NAAD) had no effect on the DNA-binding activity of the NPAS2/BMAL1 heterodimer. All derivatives were examined at 10 mM. (B) Competition assays with NAD(P)H derivatives in the presence of 2 mM NADPH. 10 mM of NAD(P)H derivatives were added to the reaction; other conditions are described in Materials and methods.

NAD(P)H also enhanced DNA-binding activity of the CLOCK/BMAL1 heterodimer [16]. Recently, the crystal structures of the bHLH-PASA-PASB domains of the murine CLOCK/BMAL1 heterodimer [17] and the bHLH domains of the human CLOCK/BMAL1 heterodimer bound to E-box DNA have been reported [18]. As observed in other bHLH proteins, the N-terminal halves of the α1 helices of both CLOCK and BMAL1 contain many basic residues to bind DNA, and the C-terminal halves of the α1 helices form a four-helix bundle with the α2 helices to stabilize the CLOCK/BMAL1 heterodimer. Because the residues in the bHLH domain of CLOCK are highly conserved in the bHLH domain of NPAS2, the NPAS2/BMAL1 heterodimer is expected to bind to E-box DNA with a similar conformation. It is interesting to note that residues 1–61 of NPAS2 are sufficient to form the four-helix bundle of the heterodimer (Fig. S4). Considering the structural requirements for DNA binding and heterodimer formation, the binding sites of NAD(P)H on NPAS2 may exist in the loop region. Further experiments are required to elucidate the molecular mechanism by which NADPH affects the DNA-binding activity of the NPAS2/BMAL1 heterodimer or the BMAL1/BMAL1 homodimer.

CLOCK is reported to be a histone acetyltransferase (HAT) whose activity is counterbalanced by SIRT1, an NAD^+-dependent histone deacetylase [19,20]. The CLOCK/BMAL1 heterodimer activates transcription of *Nampt*, which encodes a rate-limiting enzyme in the NAD^+ salvage pathway [21]. SIRT1 is recruited with the CLOCK/BMAL1 heterodimer and inhibits the transcription of *Nampt*. Thus, intracellular NAD^+ levels are regulated in a 24-h cycle in a circadian manner; inversely, cell metabolism could regulate CLOCK function. Although NPAS2, unlike CLOCK, does not contain any regions with homology to HATs, another HAT, p300, exhibits a circadian time-dependent association with NPAS2 [22]. These results suggest that both CLOCK and NPAS2 could be regulated by cellular NAD^+/NADH levels via HAT-dependent chromatin remodeling. Therefore, the direct regulation of NPAS2 and CLOCK DNA-binding activity indicated in this study may act as an acute system that responds to fluctuations in intracellular NAD(P)H levels.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2013.06.086.

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