FEBS Letters 587 (2013) 2131–2136

FEBS Letters
journal homepage: www.FEBSLetters.org

Imaging of heme/hemeproteins in nucleus of the living cells expressing heme-binding nuclear receptors

Ryuhei Itoh [a], Ken-ichi Fujita [a], Anfeng Mu [a], Dao Hoang Thien Kim [a], Tran Tien Tai [a], Ikuko Sagami [b], Shigeru Taketani [a,c,*]

[a] Department of Biotechnology, Kyoto Institute of Technology, Kyoto 606-8585, Japan
[b] Graduate School of Life and Environmental Sciences, Kyoto Prefectural University, Sakyo-ku, Kyoto 606-8522, Japan
[c] Insect Biomedical Center, Kyoto Institute of Technology, Kyoto 606-8585, Japan

ARTICLE INFO

Article history:
Received 28 March 2013
Revised 30 April 2013
Accepted 9 May 2013
Available online 2 June 2013

Edited by Stuart Ferguson

Keywords:
Hemeprotein
Cytoglobin
Neuronal PAS2
REV-erbα
2′,7′-Dichlorodihydrofluorescin diacetate

ABSTRACT

Several factors involved in the core circadian rhythm are PAS domain proteins, one of which, neuronal PAS2 (NPAS2), contains a heme-binding motif. It is thought that heme controls the transcriptional activity of core circadian factors BMAL1-NPAS2, and that the heme-binding nuclear receptor REV-erbα negatively regulates the expression of BMAL1. To examine the role of heme in the nucleus, we expressed nuclear hemeproteins including the nuclear localization signal-added cytoglobin, NPAS2 and REV-erbα. Then, the living cells expressing these proteins were treated with 2′,7′-dichlorodihydrofluorescin diacetate (DCFH-DA). The fluorescent signal derived from DCFH-DA was observed in the nucleus. When the cells were cultured with hemin, the signal of heme in the nucleus increased. Considering that DCFH-DA reacted with heme, we propose that the use of DCFH-DA could be useful in detection of the heme moiety of hemeprotein in vivo.

Clock and **Bmal1** colocalize by fluorescence microscopy (View interaction)
Npas2 and **Bmal1** colocalize by fluorescence microscopy (View interaction)

1. Introduction

2′,7′-Dichlorodihydrofluorescin (DCFH) is a fluorogenic probe that has been widely used in the detection of oxidative stress in cells. The diacetate ester form of DCFH, 2′,7′-dichlorodihydrofluorescin diacetate (DCFH-DA), is relatively resistant to oxidation, but when taken up by cells, is de-acetylated to form DCFH. The fluorescent product of DCFH was shown to be formed as a 2-electron oxidation product, dichlorodihydrofluorescein (DCF), in reactive oxygen species (ROS)-liberated cells [1,2]. The generation of ROS as well as radical reaction occurs upon injury to various tissues and red blood cells, and the production of hydroxyl radical from hydrogen peroxide by the release of heme from hemoglobin and myoglobin follows cell membrane damage [3]. Both heme and iron can be involved in the generation of potentially harmful ROS [3]. On the other hand, we found that the fluorescence from DCFH-DA markedly increased in hemin-treated cells, and occurred independently of the generation of ROS [4]. Namely, hemeproteins such as hemoglobin and cytochrome c are potent catalysts of DCFH oxidation, and can be detected by the formation of DCF as a fluorescent compound with the reduction of the oxidized form of hemeprotein/heme [4,5].

Circadian rhythm is a fundamental regulatory factor for various aspects of physiological functions including sleep/wake cycles, blood pressure, temperature and metabolism [6,7]. Cellular rhythms are controlled and maintained through interconnected transcriptional feedback of clock genes. The cycle involves two transcriptional factors, BMAL1 and CLOCK, which heterodimerize to activate a number of circadian genes [8]. Recently, it was found that transcriptional factors including neuronal PAS2 (NPAS2_, REV-erbα and Bach1 are capable of binding heme, and control the heme-dependent transcriptional activity of the corresponding gene [9–11]. NPAS2, a transcriptional factor homologous to CLOCK, has been shown to bind heme through the PAS domains and to function as a gas sensor [12]. In addition, an orphan nuclear receptor, REV-erbα, can bind heme and regulates the

Abbreviations: NPAS2, neuronal PAS2; DCFH-DA, 2′,7′-dichlorodihydrofluorescin diacetate; DCFH, dichlorodihydrofluorescin; DCF, dichlorodihydrofluorescein; ROS, reactive oxygen species; NLS, nuclear localization signal; FCS, fetal calf serum; DMEM, Dulbecco's modified Eagle's medium; SDS, sodium dodecylsulfate; PBS, phosphate-buffered saline; BSA, bovine serum albumin; ALAS1, δ-aminolevulinic acid synthase 1

* Corresponding author. Fax: +81 75 724 7760.
E-mail address: taketani@kit.ac.jp (S. Taketani).

0014-5793/$36.00
http://dx.doi.org/10.1016/j.febslet.2013.05.036

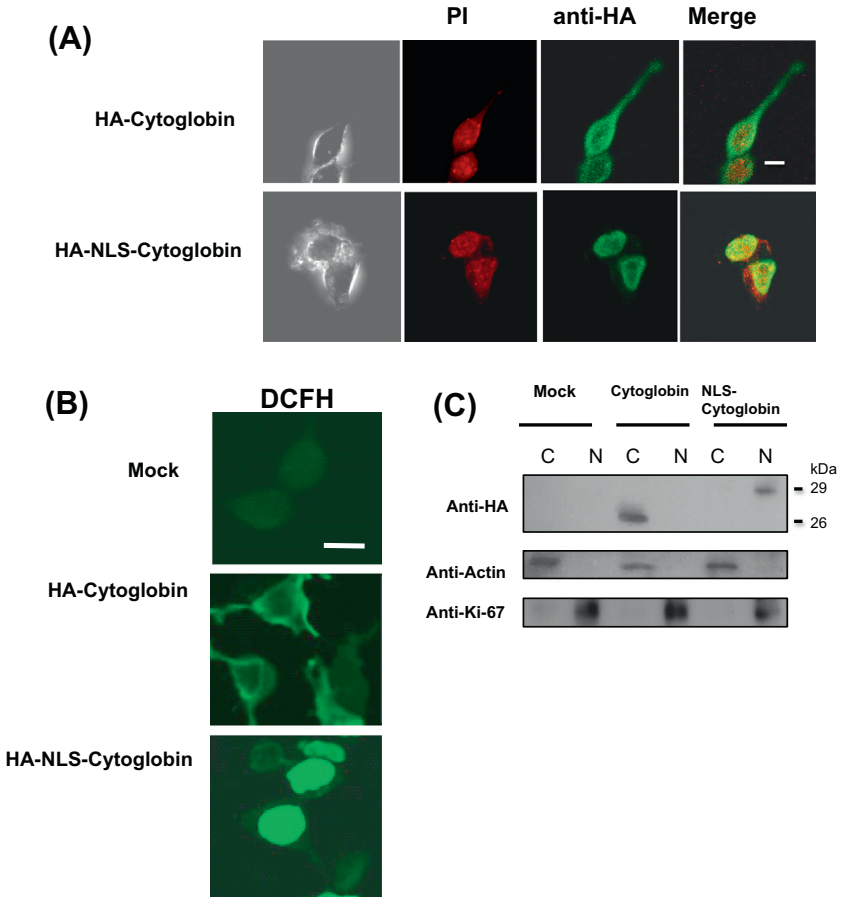

Fig. 1. Expression of cytoglobin and NLS-cytoglobin in HEK293T cells. (A) Localization of cytoglobin and NLS-cytoglobin in HEK293T cells. HEK293T cells were transfected with pCG-HA-cytoglobin (Upper panel) and pCG-HA-NLS-cytoglobin (Lower panel) and cultured for 24 h. After the cells were fixed, they were incubated with anti-HA antibody, followed by incubation with cy2-conjugated anti-mouse *Ig*. The nuclei of the cells were also stained with propidium iodide (PI). Phase contrast images of the cells are shown at left side. (B) Microscopic observation of DCF fluorescence in cytoglobin- and NLS-cytoglobin-expressing HEK293T cells. Cells transfected with mock DNA, pCG-HA-cytoglobin and pCG-HA-NLS-cytoglobin, as above were treated with 10 μM DCFH-DA for 10 min. After the cells were washed with DMEM, they were visualized by confocal microscopy. White bars: 10 μm. (C) Immunoblot analysis. The nuclear fraction (N) of the transfected and control HEK cells was separated from the cytosolic fraction (C). Proteins of both fractions were analyzed and immunoblotted using anti-REV-erbα, actin (cytosolic marker) and Ki-67 (nuclear marker).

transcription of BMAL1 [10]. However, heme-binding properties of these transcriptional factors were demonstrated with purified recombinant proteins after they were synthesized in *Escherichia coli* [10–12]. None of these factors was shown to utilize a heme moiety in vivo. Owing to the low expression of these nuclear factors in mammalian cells, it is actually unclear whether the nuclear receptors function by binding heme in the nucleus of mammalian cells. As a control with well-characterized hemeprotein, when cytoglobin fused to nuclear localization signal (NLS) was expressed in the nucleus, heme moiety of cytoglobin was detected with DCFH-DA in vivo. Then, to clarify the binding of heme to the nuclear factors in the nucleus, we overexpressed BMAL1/NPAS2 complex in the nucleus and found that the nuclear localization of heme depended on the expression in living cells. Stable transfectant expressing REV-erbα also showed augmentation of heme signal in the nucleus.

2. Materials and methods

2.1. Materials

DCFH-DA was from Molecular Probes Co (Eugene, OR). Fetal calf serum (FCS) and Dulbecco's modified Eagle's medium (DMEM) medium were from GE Healthcare (Buckinghamshire, UK). Anti-BMAL1 and anti-Rev-erba antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-HA was a product of Nacalai-Tesque (Kyoto, Japan). Cy2-conjugated anti-goat and anti-mouse Ig were products of Millipore Co. (Tokyo, Japan). Hemin was dissolved in dimethyl sulfoxide at a concentration of 20 mM, then diluted 20-fold with phosphate-buffered saline (PBS) containing 10 mg/ml bovine serum albumin (BSA). All other chemicals used were of analytical grade.

2.2. Plasmids

Plasmids pCG-HA-NPAS2 (mouse NPAS2), pcDNA3-BMAL1 (mouse BMAL1), and pCG-HA-CLOCK (mouse CLOCK) were as described previously [12]. To construct pCG-HA-NLS-cytoglobin, the NLS fragment (590–732 bp) of PLAGL2 [13] was amplified by PCR. Primers 5′-AATCTAGAAGGCGGTTCTATACTCG-3′ and 5′-AATCTAGACTGTGAGTGGCTCTTCT-3′ were used. Amplified cDNAs were digested with *Xba*I and ligated into *Xba*I-digested pCG-HA-cytoglobin [14]. To obtain the full-length cDNA fragment of human REV-erbα, PCR reaction was performed with the following primers: 5′-AAAAGCTTACGACCCCTGGACTCCAA-3 and 5′-AATCTAGATCACTGGGCGTCCACCC-3′ for REV-erbα and human kidney cDNA library as a template. Then, to make mammalian expression vector carrying REV-erbα, the amplified cDNA was digested with

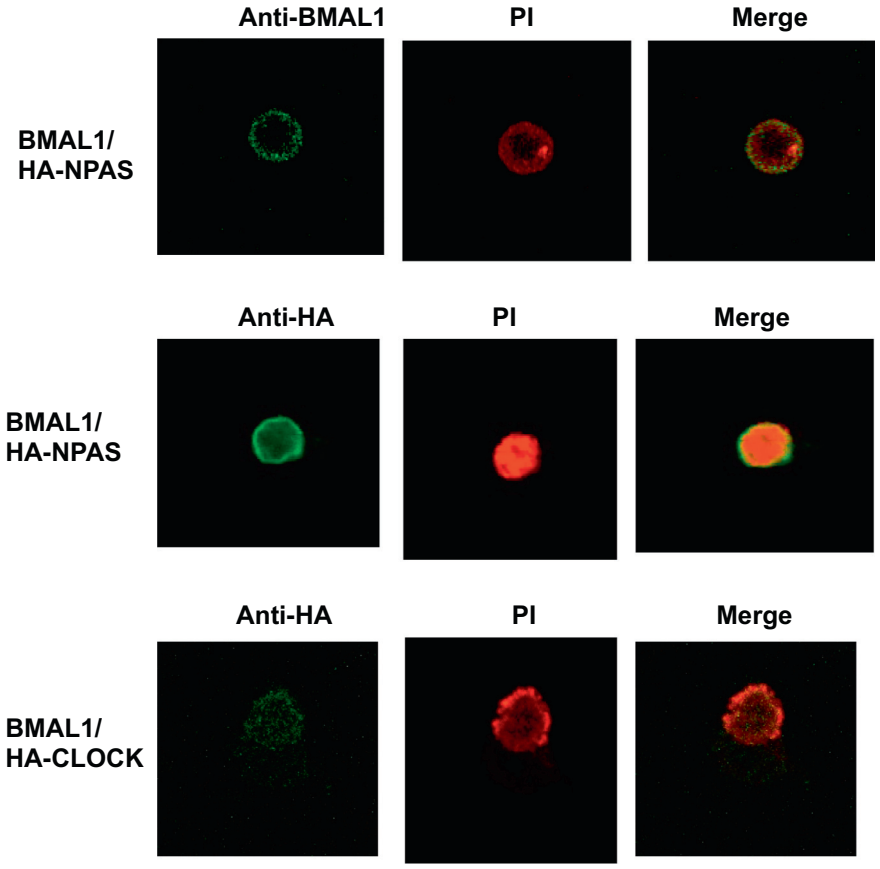

Fig. 2. Nuclear localization of BMAL1/CLOCK and BMAL1/NPAS2 in HEK 293T cells. HEK293T cells were co-transfected with pcDNA3-BMAL1 and pCG-HA-CLOCK or pcDNA3-BMAL1 and pCG-HA-NPAS2, and cultured for 24 h. After the cells were fixed, they were incubated with anti-HA, followed by incubation with cy2-conjugated anti-mouse *Ig*. PI: propidium iodide staining. White bars: 10 μm.

*Hind*III/*Xba*I and ligated into the *Hind*III/*Xba*I site of the pEF-vector (Life Technologies, Carlsbad, CA).

2.3. Cell culture

HeLa cells and HEK293T cells were maintained in DMEM supplemented with 10% FCS, 50 U/ml penicillin, and 50 μg/ml streptomycin. The cells were transfected with pCG-HA-cytoglobin [14], pCG-HA-NLS-cytoglobin, pCG-HA-NPAS2, pCG-HA-CLOCK or pCDNA3-BMAL1 by the lipofection method [13] and cultured for 24 h. To obtain HeLa cells constitutively expressing REV-erbα, pEF-REV-erbα (5.0 μg) was transfected with calcium phosphate, as described previously [15]. For selection, G418 (Wako Chemicals, Tokyo, Japan) at a final concentration of 300 μg/ml was added to the culture medium. After 5 days, colonies of the G418-resistant cells were trypsinized, seeded in a 24-well tissue culture plate and cultured in medium containing G418 (300 μg/ml). Individual clones were isolated and examined for the expression of human REV-erbα by immunoblotting, using anti-REV-erbα antibodies. Three REV-erbα-expressing clones were obtained, mixed to avoid clonal variation and maintained in DMEM containing 7% FCS and antibiotics. The cells were treated for 16 h in the medium supplemented with 7% FCS containing 20 μM hemin.

2.4. Microscopy

The cells in 3.5 cm dishes were washed with PBS (+) (PBS containing 1 mM $CaCl_2$ and 0.5 mM $MgCl_2$), fixed with 4% paraformaldehyde for 20 min, and permeabilized in 0.1% TritonX-100 with PBS (+) for 1 h. After blocking with 2% FCS in PBS (+), incubation with the primary antibodies was carried out, followed by incubation with a fluorolink Cy2-conjugated rabbit anti-mouse *Ig* [13,15]. The localization of the antigens in the cells was visualized using a Zeiss confocal microscope or a Nikon fluorescence microscope Model ECLIPSE E600 (Tokyo, Japan) [13]. To obtain the image of heme in cells, living cells were treated with 10 μM DCFH-DA for 10 min, washed three times with PBS, and the localization of fluorescence in the cells was examined by confocal microscopy.

2.5. Immunoblotting

The cellular proteins were separated by sodium dodecylsulfate (SDS)–polyacrylamide gel electrophoresis and transferred onto a polyvinylidene difluoride membrane (Bio-Rad Laboratories, Hercules, CA). Immunoblotting was carried out with antibodies for HA, REV-erbα and actin, as the primary antibodies [14,15]. The nuclear fraction of HEK cells was separated from the cytosolic fraction, as described previously [16].

2.6. Fluorescence spectrophotometry

Cells suspended in PBS were lysed by sonication. The lysates were then treated with 10 μM DCFH-DA at 37 °C. The aliquots of the sample were withdrawn and the DCF fluorescence was measured using spectrofluorometer with excitation at 500 nm and emission by scanning from 510 to 550 nm [4,5]. The content of heme in cells was determined by fluorescence spectrophotometry after the conversion of heme to protoporphyrin [4,16].

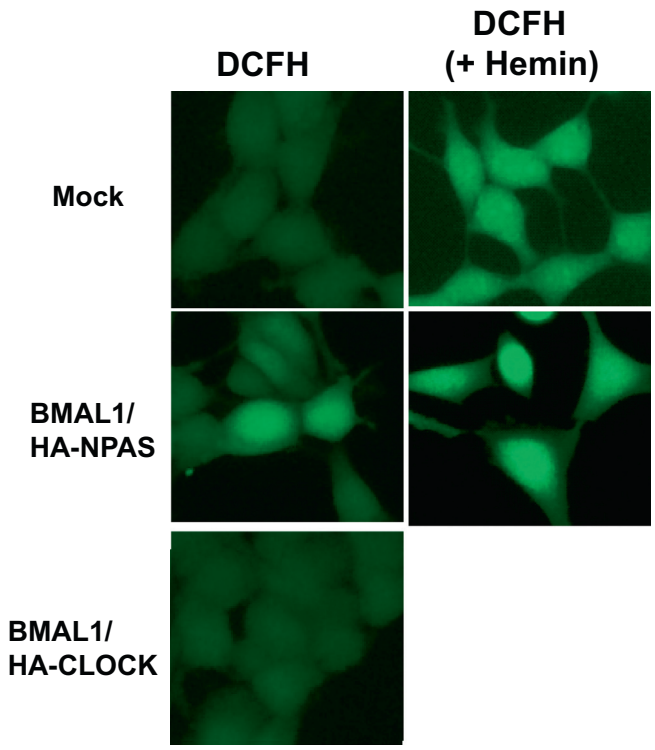

Fig. 3. DCF fluorescence in BMAL1/CLOCK- and BMAL1/NPAS2-expressing HEK293T cells. The cells expressing BMAL1/CLOCK and BMAL1/NPAS2 were cultured without or with 20 μM hemin for 16 h. The cells were then treated with 10 μM DCFH-DA for 10 min. After washing the cells, they were then visualized by confocal microscopy. White bars: 10 μm.

3. Results

3.1. Expression of cytoglobin in cytosol and nucleus

Cytoglobin is known to be a cytosolic hemeprotein [14]. To examine the localization of cytoglobin, pCG-HA-cytoglobin was transfected into HEK293T cells and cultured for 16 h. Indirect immunofluorescence microscopy with the transfected HEK cells revealed that the staining of HA-cytoglobin occurred in the cytosol (Fig. 1A). To evaluate cytoglobin/heme in cells, the living cells transiently expressing cytoglobin were treated with 10 μM DCFH-DA for 10 min. The DCF-fluorescence was mainly found in the cytosol (Fig. 1B). Then, the NLS of a nuclear protein, PLAGL2 [13], was fused to the N-terminal of cytoglobin and the resulting NLS-cytoglobin was expressed in HEK cells. Immunofluorescence with anti-HA antibody revealed that NLS-cytoglobin had become localized in the nucleus. Immunoblots analysis confirmed the localization of cytoglobin (26 kDa) in the cytosol and NLS-cytoglobin (29 kDa) in the nucleus (Fig. 1C). The treatment of living cells expressing NLS-cytoglobin with DCFH-DA resulted in a strong fluorescent signal in the nucleus. When the cells were fixed, the intensity of DCF fluorescence in the cells diminished. These results indicated that DCFH-DA could detect NLS-cytoglobin/heme expressed in the nuclei of living cells.

3.2. The detection of heme-binding nuclear factors by DCFH-DA

NPAS2 is known to be a heme-binding rhythmic factor that forms a complex with BMAL1 in the nucleus [9,12]. Plasmids carrying HA-tagged NPAS2 and BMAL1 were co-transfected into HEK cells, and the cellular localization of BMAL1/NPAS2 was examined with anti-BMAL1 and -HA. As shown in Fig. 2, BMAL1 and HA-tag NPAS2 were found in the nucleus. Then, we examined the localization of heme in the BMAL1/NPAS2-transiently expressing cells. The fluorescence derived from DCFH was predominantly found in the nuclei of living cells (Fig. 3), suggesting that NPAS2 expressed in the nucleus can bind heme. As a control experiment, we expressed CLOCK, which is another partner of BMAL1 and was recently found to bind heme in vitro, although there has been no information on the function of its heme binding [17]. When the BMAL1/CLOCK complex was expressed in the nucleus and heme staining with DCFH-DA was examined in living cells, no clear localization of heme was found in these cells (Fig. 3). When the cells expressing BMAL1/NPAS2 were treated with 20 μM hemin, intense DCFH-derived fluorescence in the nucleus was found, while the fluorescence of cells expressing CLOCK with hemin was weakly observed in whole cells (Fig. 3B). REV-erbα is also reported to be a heme-binding nuclear receptor [10]. We generated HeLa cells stably expressing human REV-erbα. Immunoblot analysis showed the expression of REV-erbα with a mass of 80 kDa in REV-erbα transfectant (Fig. 4A). The expression of REV-erbα in the nucleus was observed by indirect immunofluorescence study with anti-REV-erbα (Fig. 4B). Microscopic observations revealed that REV-erbα was localized in the nucleus, where most cells were stained by DCFH-DA (Fig. 4C). The incubation of these cells with 20 μM hemin led to an increase in the intensity of the fluorescence in the nucleus. These results indicate that the DCF fluorescence reflected heme binding to REV-erbα in the nucleus.

4. Discussion

The present study first demonstrated the imaging of heme in the nucleus of hemeprotein-expressing cells. DCFH has proved to be a good marker for measuring hemeproteins as well as ROS. In a model experiment, we generated cytoglobin fused to NLS, and the resulting NLS-cytoglobin was expressed in the nucleus (Fig. 1). The heme binding of NLS-cytoglobin in the nuclei of living cells was observed by a strong fluorescent signal from DCFH-DA. We [5] previously found that hemeproteins including hemoglobin, myoglobin and lactoperoxidase were detected by the formation of DCF as a fluorescent compound with the reduction of the oxidized form of hemeprotein/heme. Consistent with the observations that an increase in the DCF fluorescence with cell lysates of the cells expressing cytoglobin and NLS-cytoglobin was more than that of control cells (Fig. S1) and that the level of intracellular heme in cytoglobin-expressing cells was more than that in control cells (Fig. S2), DCFH-DA can be oxidized by heme/hemeprotein. However, the mechanism involved in the heme-dependent fluorescence from DCFH-DA remains unexplored. Hemeproteins, NPAS2 and REV-erbα, present in the nucleus bound heme, a finding similar to that for cells containing a large amount of myeloperoxidase and hemoglobin [18,19]. When the cells expressing NPAS2 or REV-erbα were cultured in the presence of hemin, the intensity of the DCF fluorescence in the nucleus increased (Figs. 3 and 4). Thus, the microscopic observation by imaging the DCFH-derived fluorescence is a sensitive method to estimate the intracellular level and localization of hemeproteins of cells. From these observations, it is also implied that native NPAS2 and REV-erbα bind heme in the nucleus, which regulates their transcriptional activities.

Because nuclear receptors including NPAS2 and REV-erbα have been shown to bind heme in vitro [10,11,20], a question arose of whether heme binding to these factors is biologically relevant. Furthermore, recent studies showed the heme binding of various proteins with no known role for such binding [17,21–23]. Heme is hydrophobic and is known to aggregate in aqueous solution and to require detergents to maintain monodispersity. It should be

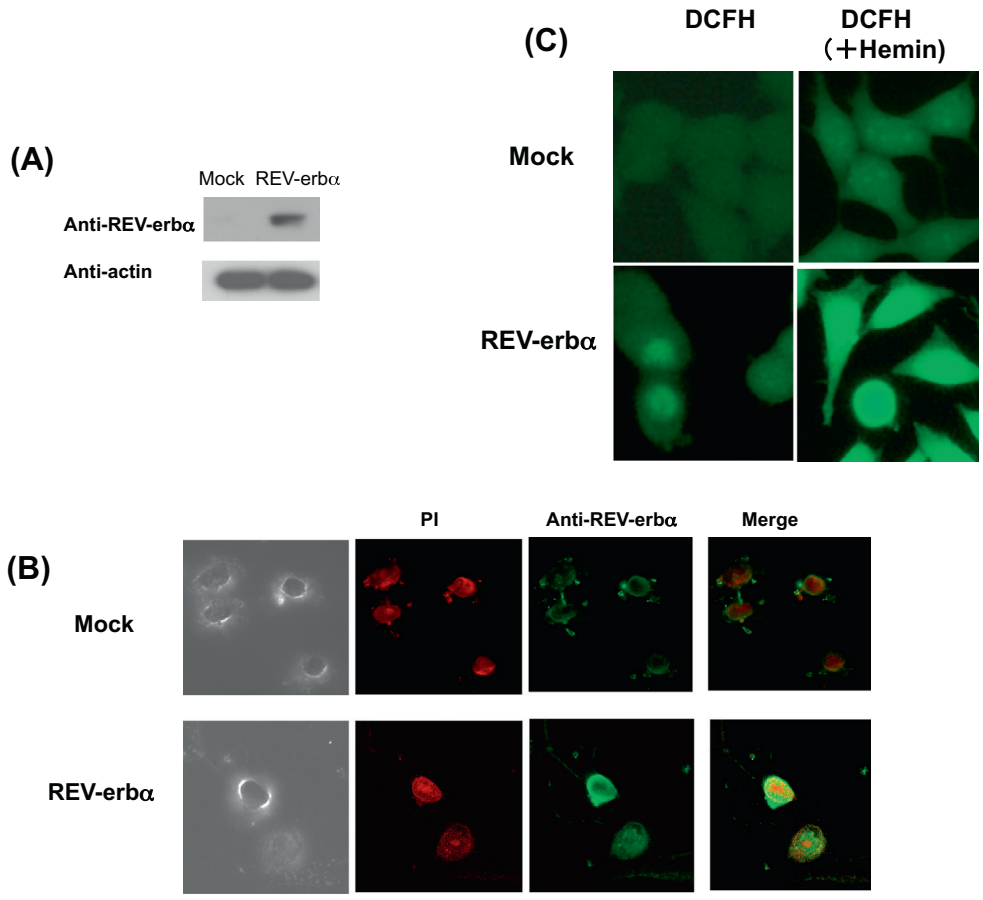

Fig. 4. Nuclear localization of REV-erbα and heme in REV-erbα-stably-expressing HeLa cells. (A) Immunoblot analysis. Cellular proteins in REV-erbα-stably-expressing and control HeLa cells were analyzed. Immunoblotting was performed with REV-erbα and anti-actin. (B) Indirect immunofluorescence of REV-erbα. REV-erbα-expressing transfectant and control cells were fixed, and were immunostained with anti-REV-erbα. Phase contrast images of the cells are shown at left side. PI: propidium iodide staining. (C) Heme imaging of REV-erbα. The cells were cultured without or with 20 μM hemin for 16 h. The DCF fluorescence of living transfectant and control cells was observed with DCFH-DA. White bars: 10 μm.

noted that investigations with in vitro heme binding face a marked risk of artificial binding when heme binding is examined with purified proteins [24]. To overcome these obstacles, direct evidence of heme binding to these proteins in vivo is needed. The expressed NPAS2 and REV-erbα were shown to bind heme, and exogenously added hemin can be utilized as a heme moiety of nuclear heme-binding factors in mammlian cells (Figs. 3 and 4C). The heme moiety of CLOCK was not found in BMAL1/CLOCK-expressing cells. The reason for the discrepancy in the results of heme binding of CLOCK between the present findings and a previous study [17] is unknown, but it may be explained by the difference between in vitro and in vivo observations. We have now shown that the treatment of cells with DCFH-DA leads to the generation of the fluorescence with heme/hemin, and microscopic examination with living cells revealed the localization of heme/hemeprotein in vivo.

δ-Aminolevulinic acid synthase 1 (ALAS1), a rate-limiting enzyme of heme biosynthesis, is under the circadian control and is regulated by clock transcription factors, NPAS2 and the co-activator PGC-1α [9]. NPAS2 suppresses heme biosynthesis by lowering the transcription of ALAS1 gene since the heme-dependent repression of ALAS1 was attenuated in the liver of NPAS2-mutated mice [11]. On the other hand, REV-erbα suppresses the biosynthesis of its ligand, heme, by the down-regulation of PGC-1α [25]. Conversely, lowering of the intracellular level of heme reduced the suppressive function of REV-erbα, and enhanced the PGC-1α-dependent stimulation of the transcription of ALAS1 [25]. However, the precise mechanisms involved in the suppression of ALAS1 expression by heme have yet to be established. On the basis of the present observations that heme reacted with DCFH in the nucleus, it is possible that change in the activity of nuclear heme-binding factors by heme leads to suppression of the expression of the ALAS1 gene via REV-erbα. Considering that sensing of the redox state of heme and gases including O_2, CO and NO by nuclear receptors basically controls the functions of their activities [9,26], more detailed studies on the movement of nuclear heme are required for clarification of regulation of gene expression in terms associated with the circadian rhythm.

Acknowledgements

This study was supported in part by Grants from the Ministry of Health, Labor and Welfare of Japan, and from the Ministry of Education, Science, Sports, and Culture of Japan. The authors are also grateful to Asako Kawazoe, Koji Fukuda, and Ayaka Furukawa for their technical support.

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.febslet.2013.05.036.

References

[1] Puntarulo, S. and Cederbaum, A.I. (1997) Inhibition of ferritin-stimulated microsomal production of reactive oxygen intermediates by nitric oxide. Arch. Biochem. Biophys. 340, 19–26.

[2] Rhee, S.G., Chang, T.S., Jeong, W. and Kang, D. (2010) Methods for detection and measurement of hydrogen peroxide inside and outside of cells. Mol. Cells 29, 539–549.
[3] Belcher, J.D., Beckman, J.D., Balla, G., Balla, J. and Vercellotti, G. (2010) Heme degradation and vascular injury. Antioxid. Redox. Signal. 12, 233–248.
[4] Ohashi, T., Mizutani, A., Murakami, A., Kojo, S., Ishii, T. and Taketani, S. (2002) Rapid oxidation of dichlorodihydrofluorescin with heme and hemoproteins: formation of the fluorescein is independent of the generation of reactive oxygen species. FEBS Lett. 511, 21–27.
[5] Ohashi, T., Kakimoto, K., Sokawa, Y. and Taketani, S. (2002) Semi-quantitative estimation of heme/hemoprotein with dichlorodihydrofluorescin diacetate. Anal. Biochem. 308, 392–395.
[6] Zheng, B., Albrecht, U., Kaasik, K., Sage, M., Lu, W., Vaishnav, S., Li, Q., Sun, Z.S., Eichele, G., Bradley, A. and Lee, C.C. (2001) Non-redundant roles of the mPer1 and mPer2 genes in the mammalian circadian clock. Cell 105, 683–694.
[7] Feng, D. and Lazar, M.A. (2012) Clocks, metabolism, and the epigenome. Mol. Cell 47, 58–67.
[8] Bass, J. and Takahashi, J.S. (2010) Circadian integration of metabolism and energetics. Science 330, 1349–1354.
[9] Taketani, S. (2005) Aquisition, mobilization and utilization of cellular iron and heme: endless findings and growing evidence of tight regulation. Tohoku J. Exp. Med. 205, 297–318.
[10] Raghuram, S., Stayrook, K.R., Huang, P., Rogers, P.M., Nosie, A.K., McClure, D.B., Burris, L.L., Khorasanizadeh, S., Burris, T.P. and Rastinejad, F. (2007) Identification of heme as the ligand for the orphan nuclear receptors REV-ERBalpha and REV-ERBbeta. Nat. Struct. Mol. Biol. 14, 1207–1213.
[11] Kaasik, K. and Lee, C.C. (2004) Reciprocal regulation of haem biosynthesis and the circadian clock in mammals. Nature 430, 467–471.
[12] Ishida, M., Ueha, T. and Sagami, I. (2008) Effects of mutations in the heme domain on the transcriptional activity and DNA-binding activity of NPAS2. Biochem. Biophys. Res. Commun. 368, 292–297.
[13] Mizutani, A., Furukawa, T., Adachi, Y., Ikehara, S. and Taketani, S. (2002) A zinc-finger protein, PLAGL2, induces the expression of a proapoptotic protein Nip3, leading to cellular apoptosis. J. Biol. Chem. 277, 15851–15858.
[14] Shigematsu, A., Adachi, Y., Matsubara, J., Mukaide, H., Koike-Kiriyama, N., Minamino, K., Shi, M., Yanai, S., Imamura, M., Taketani, S. and Ikehara, S. (2008) Analyses of expression of cytoglobin by immunohistochemical studies in human tissues. Hemoglobin 32, 287–296.
[15] Sawamoto, M., Imai, T., Umeda, M., Fukuda, K., Kataoka, T. and Taketani, S. (2012) The p53-dependent expression of frataxin controls 5-aminolevulinic acid-induced accumulation of protoporphyrin IX and photo-damage in cancerous cells. Photochem. Photobiol. 89, 163–172.
[16] Gotoh, S., Ohgari, Y., Nakamura, T., Osumi, T. and Taketani, S. (2008) Heme-binding to the nuclear receptor retinoid X receptor alpha (RXRalpha) leads to the inhibition of the transcriptional activity. Gene 423, 207–214.
[17] Lukat-Rodgers, G.S., Correia, C., Botuyan, M.V., Mer, G. and Rodgers, K.R. (2010) Heme-based sensing by the mammalian circadian protein CLOCK. Inorg. Chem. 49, 6348–6365.
[18] Taketani, S., Kohno, H. and Tokunaga, R. (1987) Cell surface receptor for hemopexin in human leukemia HL60 cells. Specific binding, affinity labeling, and fate of heme. J. Biol. Chem. 262, 4639–4643.
[19] Jeannesson, P., Lahlil, R., Chenais, B., Devy, L., Gillet, R., Aries, A., Morceau, F. and Trentesaux, C. (1997) Anthracyclines as tumor cell differentiating agents: effects on the regulation of erythroid gene expression. Leuk. Lymphoma. 26, 575–587.
[20] Dioum, E.M., Rutter, J., Tuckerman, J.R., Gonzalez, G., Gilles-Gonzalez, M.A. and McKnight, S.L. (2002) NPAS2: a gas-responsive transcription factor. Science 298, 2385–2387.
[21] Airola, M.V., Du, J., Dawson, J.H. and Crane, B.R. (2010) Heme binding to the mammalian circadian clock protein period 2 is nonspecific. Biochemistry 49, 4327–4338.
[22] Dycke, C., Bougault, C., Gaillard, J., Andrieu, J.P., Pantopoulos, K. and Moulis, J.M. (2007) Human iron regulatory protein 2 is easily cleaved in its specific domain: consequences for the haem binding properties of the protein. Biochem. J. 408, 429–439.
[23] Guenthner, C.J., Bickar, D. and Harrington, M.E. (2009) Heme reversibly damps PERIOD2 rhythms in mouse suprachiasmatic nucleus explants. Neuroscience 164, 832–841.
[24] Muller-Eberhard, U. and Fraig, M. (1993) Bioactivity of heme and its containment. Am. J. Hematol. 42, 59–62.
[25] Wu, N., Yin, L., Hanniman, E.A., Joshi, S. and Lazar, M.A. (2009) Negative feedback maintenance of heme homeostasis by its receptor Rev-erbalpha. Genes Dev. 23, 2201–2209.
[26] Ascenzi, P., Bocedi, A., Leoni, L., Visca, P., Zennaro, E., Milani, M. and Bolognesi, M. (2004) CO sniffing through heme-based sensor proteins. IUBMB Life 56, 309–315.