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

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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

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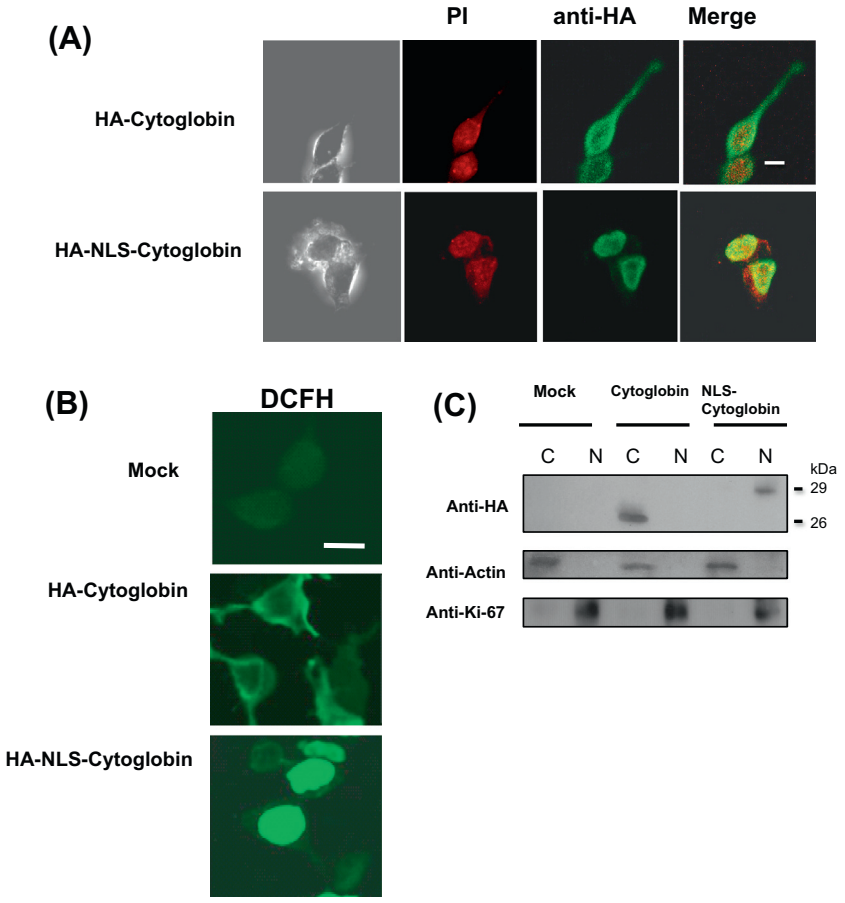

Fig. 1. Expression of cytoglobin and NLS-cytoglobin in HEK293T cells. (A) Localization of cytoglobin and NLS-cytoglobin in HEK293T cells. HEK293T cells were transfected with pCG-HA-cytoglobin (Upper panel) and pCG-HA-NLS-cytoglobin (Lower panel) and cultured for 24 h. After the cells were fixed, they 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