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Eel green fluorescent protein is associated with resistance to oxidative stress

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ABSTRACT

Green fluorescent protein (GFP) from eel (*Anguilla japonica*) muscle (eelGFP) is unique in the vertebrates and requires bilirubin as a ligand to emit fluorescence. This study was performed to clarify the physiological function of the unique GFP. Investigation of susceptibility to oxidative stress was carried out using three types of cell lines including jellyfish (*Aequorea coerulescens*) GFP (jfGFP)-, or eel GFP (eelGFP)-expressing HEK293 cells, and control vector-transfected HEK293 cells. Binding of eelGFP to bilirubin was confirmed by the observation of green fluorescence in HEK293-eelGFP cells. The growth rate was compared with the three types of cells in the presence or absence of phenol red which possessed antioxidant activity. The growth rates of HEK293-CV and HEK293-jfGFP under phenol red-free conditions were reduced to 52 and 31% of those under phenol red. Under the phenol red-free condition, HEK293-eelGFP had a growth rate of approximately 70% of the phenol red-containing condition. The eelGFP-expressing cells were approximately 2-fold resistant to oxidative stress such as H_2O_2 exposure. The fluorescence intensity partially decreased or disappeared after exposure to H_2O_2, and heterogeneous intensity of fluorescence was also observed in isolated eel skeletal muscle cells. These results suggested eelGFP, but not jfGFP, coupled with bilirubin provided the antioxidant activity to the cells as compared to non-bound free bilirubin.

1. Introduction

The 27 kDa green fluorescent protein (GFP) from jellyfish *Aequorea Victoria* (jfGFP), which excitation and emission maxima were 475 and 508 nm, respectively, was discovered and isolated by Shimomura (1979). The jfGFP contains a chromophore induced from the amino acid sequence of -Ser-Tyr-Gly- within the protein structure by cyclization, dehydration, and aerial oxidation (Tsien, 1998; Miyawaki et al., 2003).

We (Hayashi and Toda, 2009) have discovered the green fluorescent protein from eel (*Anguilla japonica*) muscle (eelGFP), with excitation and emission maxima of 493 and 527 nm, respectively. The deduced amino-acid sequences of eelGFP contained no chromophore such as -Ser-Tyr-Gly-, but exhibited a high similarity to the fatty acid binding protein (FABP) family. Additionally, both eelGFP and FABPs were a lower molecular weight of 14–16 kDa (Hertzel and Bernlohr, 2000; Zimmerman and Veerkamp, 2002; Kumagai et al., 2013) than jfGFP. The FABP family consists of FABP1 - FABP9 which act on different organs and functions (Zimmerman and Veerkamp, 2002). FABPs possess multi-functions including intercellular uptake and transport of fatty acids, regulation of gene transcription and enzyme activity, oxidation of peroxisome and mitochondria (Furuhashi and Hotamisligil, 2008). Thereafter, Kumagai et al. (2013) indicated that *A. japonica* UnaG, which was identical to eelGFP, required hydrophobic antioxidant bilirubin as a ligand with strongly high affinity, Kd = 98 pM, and specificity, to emit green fluorescence. Bilirubin is an endogenous product of heme metabolism in vertebrates. Heme is degraded by heme oxygenase to biliverdin, which is then reduced by biliverdin reductase to form uncojugated bilirubin (Maines, 1988). In mammals, bilirubin forms a complex with albumin, Kd = 87 nM (Kumagai et al., 2013), in blood circulation, and is transported to the liver (Brodersen, 1979). Since bilirubin is a hydrophobic compound, it is conjugated by glucuronyl

Abbreviation: APF, 2-[6-(4′-amino)phenoxy-3 H-xanthen-3-on-9-yl]benzoic acid; eelGFP, eel green fluorescent protein; E-MEM, Eagle's Minimum Essential Medium; FABP, fatty acid binding protein; FBS, fetal bovine serum; hROS, highly oxidative reactive oxygen species; Gly, glysine; jfGFP, jellyfish green fluorescent protein; H2O2, hydrogen peroxide; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide; OH, hydroxyl radical; ROS, reactive oxygen species; Ser, serine; Tyr, tyrosine; UnaG, unagi (eel) green fluorescent protein.

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transferase to a hydrophilic compound, which possess solubility in the bile for excreation (Ostrow and Schmid, 1963; Zucker et al., 1994; Patra and Pai, 1997). The accumulation of excess bilirubin in the circulation after opposite vectorial transport from hepatocytes to the blood causes a jaundice in human, especially in neonates, resulting in basal ganglia with neurologic dysfunction (Dennery et al., 2001). On the other hand, it is suggested that bilirubin possess a physiologically important role to protect lipids (Frei et al., 1988; Hulea et al., 1995), proteins (Neuzil et al., 1993; Minetti et al., 1998), and nucleotides both intracellularly and extracellularly (Sedlak et al., 2009) against oxidative stress as a potent antioxidant. Interestingly, the antioxidant activity of bilirubin coupled with serum albumin is higher than that of non-bound free style bilirubin (Wu et al., 1991; Kapitulnik, 2004). The eelGFP-bound bilirubin is also suggested to possess an antioxidant activity, however, there is no information concerned with the functional properties of eelGFP. Hence, we investigated the effect of the eelGFP coupled with bilirubin on exposure to oxidative stress in the eelGFP-expressing cells.

2. Materials and methods

2.1. Preparation of vector and transfection

Total RNA was extracted from the muscle of eel *Anguilla japonica* and used for cDNA synthesis. The 417-bp target of eelGFP (AB731138) was amplified from cDNA by using the forward primer (5′-AAAACTCGAGATGGTCGAGAAATTTGTT-3′) and reverse primer (5′-AAAACTCGAGTCATTCCGTCGCCCTCCG-3′) (underlined letters show *Xho*I recognition site).

The PCR fragment was ligated into the *Xho*I - digested pcDNA3.1(+) mammalian expression vector (Invitorgen Corp., Carlsbad, CA USA). Human embryonic kidney cells (HEK293) were transfected with eelGFP-pcDNA3.1(+) vector (HEK293-eelGFP) using Lipofectamine™ 2000 (Thermo Fisher Scientific Inc., MA, USA) according to the manufacturer's directions. As a control, HEK293 cells were transfected with empty pcDNA3.1(+) vector (HEK293-CV). In addition, HEK293 cells were transfected with pEGFP-C2 vector (Clontech, Mountain View, CA, USA) (HEK293-jfGFP), and then employed as a further comparison with the functional property of HEK293-eelGFP cells. Stable transfectants were selected and subcloned in the presence of 400 μg/ml G418.

2.2. Cell culture

HEK293-eelGFP, HEK293-jfGFP, and HEK293-CV cells were cultured in Eagle's Minimum Essential Medium (E-MEM) supplemented with 10% fetal calf serum (FCS), 100 units/ml penicillin, 100 μg/ml streptomycin, and 400 μg/ml G418 for 3–5 days under 100% humidity and 5% CO_2 at 37 °C.

2.3. Cell growth rate measurement

Cells were seeded at 2.0×10^5 cells/well (3 mL/well) using 12-well plates, and cultured in Dulbecco's Modified Medium (D-MEM) containing 10% FCS, 100 units/ml penicillin, 100 μg/ml streptomycin, and 6.0 mM L-glutamine with or without phenol red. The number of cells were counted at 0, 24, 48, 72, and 96 h to measure the growth rate of the cells.

2.4. Investigation of sensitivity to oxidative stress

HEK293-eelGFP (1.3×10^4 cells/well), HEK293-jfGFP (1.0×10^4 cells/well), and HEK293-CV (1.0×10^4 cells/well) were seeded into 96-well plates and cultured in D-MEM-10% FCS without phenol red at 37 °C under 5% CO_2 for 48 h. After exposure to H_2O_2 serially diluted from 0 to 200 μM, the cells were cultured at 37 °C under 5% CO_2 for 72 h.

The cell viability was measured to assess the sensitivity of the cells to H_2O_2, and determined by colorimetric assay using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) as described previously (Carmichael et al., 1987). The survival fractions (%) of three independent experiments were calculated from the ratio of cell numbers in each concentration of H_2O_2 to H_2O_2-free controls. The IC_{50} values were measured as the concentration of H_2O_2 that reduced the number of cells to 50% of that in control medium.

2.5. Isolation of muscle cells and observation of green fluorescence

Muscle cells were isolated by the collagenase digestion method (Rosenblatt et al., 1995; Alam et al., 2004) described in our previous report (Hayashi and Toda, 2009). The green fluorescence of cultured eel muscle cells was then observed using a fluorescence stereomicroscope (MZ10F, Leica, Wetzler, Germany).

2.6. Conversion of H_2O_2 to the highly oxidative reactive oxygen species (hROS) such as hydroxyl radical

Non fluorescent aminophenyl fluorescein (APF) is converted to the green fluorescent compound fluorescein after reaction with hROS such as hydroxyl radical, but is not detectable with H_2O_2 (Setsukinai et al., 2003). HEK293-CV cells (1.2×10^4 cells/well) were seeded into 96-well plates and cultured for 24 h. Then, cells were exposed to 200 μM H_2O_2 for 4 h in the CO_2 incubator. After exposure to H_2O_2, cells were additionally treated with the hROS specific detection probe APF for 24 h in the CO_2 incubator. We tried to detect the hROS such as hydroxyl radical after conversion mediated by the Fenton reaction (Imlay et al., 1988) from H_2O_2 in the HEK293-CV cells. The green fluorescence of the fluorescein product was observed using EVOS Floid imaging station (Thermo Fisher Scientific Inc., MA, USA).

3. Results

3.1. Cloning of eelGFP

We have successfully cloned eelGFP mRNA (GenBank accession no. AB731138) from Japanese eel muscle. The open reading frame sequence of eelGFP mRNA was completely identical to that of UnaG mRNA (GenBank accession no. AB763906) (Kumagai et al., 2013).

3.2. FCS-dependent expression of green fluorescence of eelGFP

Detection of green fluorescence expression using HEK293-eelGFP cells was carried out after cell culture for 120 h in medium containing

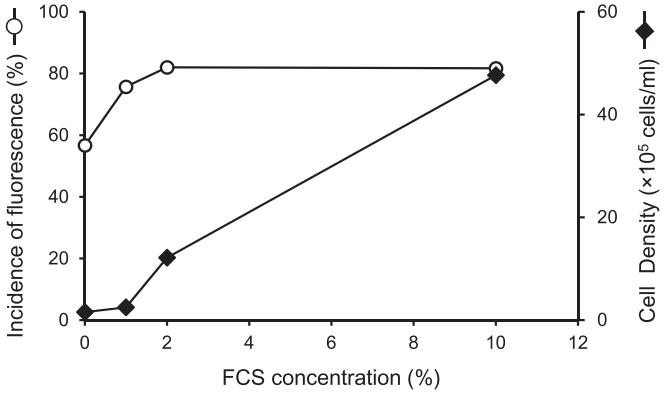

Fig. 1. The relationship between cell density and fluorescent expression in HEK293-eelGFP cells depends on FCS concentration. The FCS concentration (0, 1, 2, 10%)-dependent green fluorescence expression was measured using a Tali Image Cytometer (Thermo Fisher Scientific Inc., MA, USA) after cell culture for 120 h.

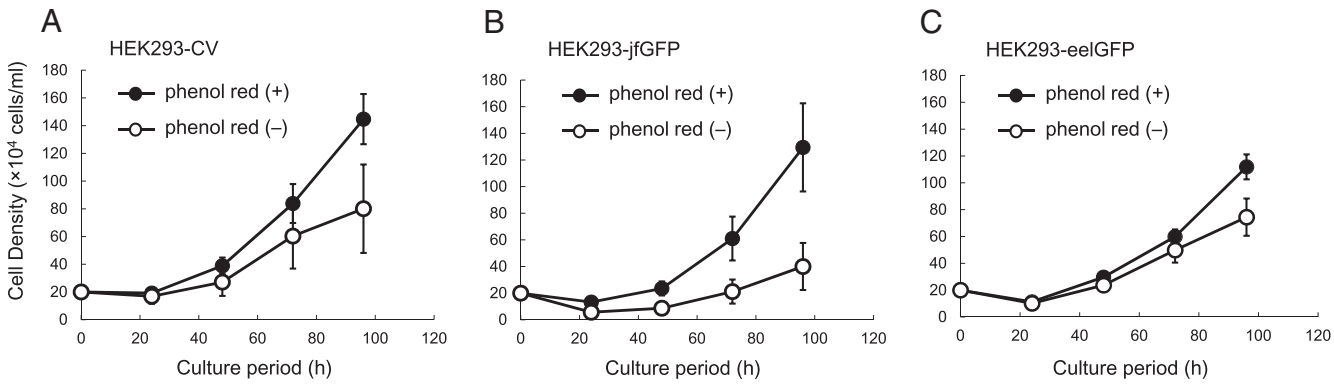

Fig. 2. Comparisons of cell growth rates of HEK293-CV (A), HEK293-jfGFP (B), and HEK293-eelGFP (C) cells. Cells were cultured in D-MEM-10% FCS with or without phenol-red, and the cell densities were counted at 0, 24, 48, 72, and 96 h. The data represent the mean values ± SD of three independent experiments each performed in triplicate.

10% FCS. The formation of eelGFP-bilirubin complex was confirmed by green fluorescence detection (Fig. 1). The FCS concentration-dependent green fluorescence expression was measured using a Tali Image Cytometer (Thermo Fisher Scientific Inc., MA, USA). The ratios of green fluorescence expression in HEK293-eelGFP cells were increased in a FCS concentration dependent manner up to 2% with increasing cell densities, and saturated around 80% cells under the condition of over 2% up to 10% FCS concentrations. The bright green fluorescence expression was obtained above 2% FCS concentrations. Therefore, 10% FCS concentration was employed to optimize cell proliferation and fluorescent expression.

3.3. Effect of eelGFP expression on the cell growth rate

The effect of phenol red on growth rate was evaluated using HEK293-CV, HEK293-jfGFP, and HEK293-eelGFP cells (Fig. 2). When cells were cultured for 96 h, the growth rates of HEK293-CV and HEK293-jfGFP in phenol red-free medium were reduced to 52 and 31% on average, compared to those in phenol red medium (Fig. 2A and 2B). However, the growth rate of HEK293-eelGFP was approximately 70% between phenol red-free and phenol red medium (Fig. 2C). The fluorescent expression as an index of eelGFP-bilirubin complex was observed in cells of HEK293-eelGFP with or without phenol red. The growth rate was kept in HEK293-eelGFP more than HEK293-CV and HEK293-jfGFP cells under the condition of phenol red-free.

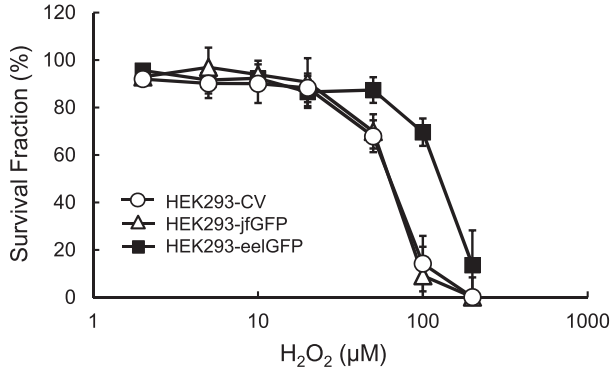

Fig. 3. Effect of H_2O_2 on the sensitivity of HEK293-CV, HEK293-jfGFP and HEK293-eelGFP cells. Cells were cultured in D-MEM-10% FCS without phenol red for 48 h, and then final concentrations of 0, 2, 5, 10, 20, 50, 100, and 200 μM of H_2O_2 were added. After incubation for 72 h, cell viability was measured by the MTT assay. The data represent the mean values ± SD of three independent experiments each performed in triplicate.

3.4. Resistance of the cells to oxidative stress

The sensitivity of cells to H_2O_2 exposure was measured to assess whether the eelGFP-bilirubin complex protects the cells from oxidative stress. After exposure to H_2O_2 serially diluted from 0 to 200 μM, cells were cultured in phenol red-free medium for 72 h. The survival fraction of HEK293-CV and HEK293-jfGFP was almost equally sensitive to H_2O_2 exposure, in contrast, HEK293-eelGFP cells were approximately 2-fold resistant to H_2O_2 than HEK293-CV and HEK293-jfGFP cells (Fig. 3). As shown in Table 1, IC_{50} values of HEK293-CV, HEK293-jfGFP, and HEK293-eelGFP cells were 66.4, 66.7 and 134.4 μM H_2O_2, respectively. At a concentration of 200 μM H_2O_2, the survival fraction of HEK293-CV and HEK293-jfGFP was negligible, while that of HEK293-eelGFP cells was 13%.

3.5. Heterogeneous fluorescence intensity of eelGFP

Isolated eel muscle cells expressed heterogeneous expression of green fluorescence intensity (Fig 4A), and good agreement with our previous report (Hayashi and Toda, 2009).

The green fluorescent intensity also seemed to be heterogeneous in the HEK293-eelGFP (Fig. 4B). In contrast, HEK293-CV cells expressed non-fluorescence (Fig. 4B). When HEK293-eelGFP cells were exposed to 200 μM H_2O_2 for 24 h, green fluorescence intensity partially decreased or disappeared (Fig. 4B).

3.6. Detection of hROS

We tried to detect the highly oxidative reactive oxygen species (hROS) such as hydroxyl radical (•OH) after exposure to 200 μM H_2O_2 for a total of 28 h using the HEK293-CV cells, since HEK293-eelGFP and HEK293-jfGFP cells were already green. The hROS was detected using the green fluorescent probe APF, suggesting that H_2O_2 was converted to •OH mediated probably by the Fenton reaction (Imlay et al., 1988) in the HEK293-CV cells (Fig. 5). These results indicated

Table 1
Sensitivity of HEK293-CV, HEK293-jfGFP, and HEK293-eelGFP cells to H_2O_2 exposure.

	IC_{50} (μM)
HEK293-CV	66.4 ± 3.7
HEK293-jfGFP	66.7 ± 5.1
HEK293-eelGFP	134.4 ± 9.1*

The data represent the mean values ± SD of each cell performed in triplicate; *$p < 0.05$, as compared with control by Mann–Whitney U test.

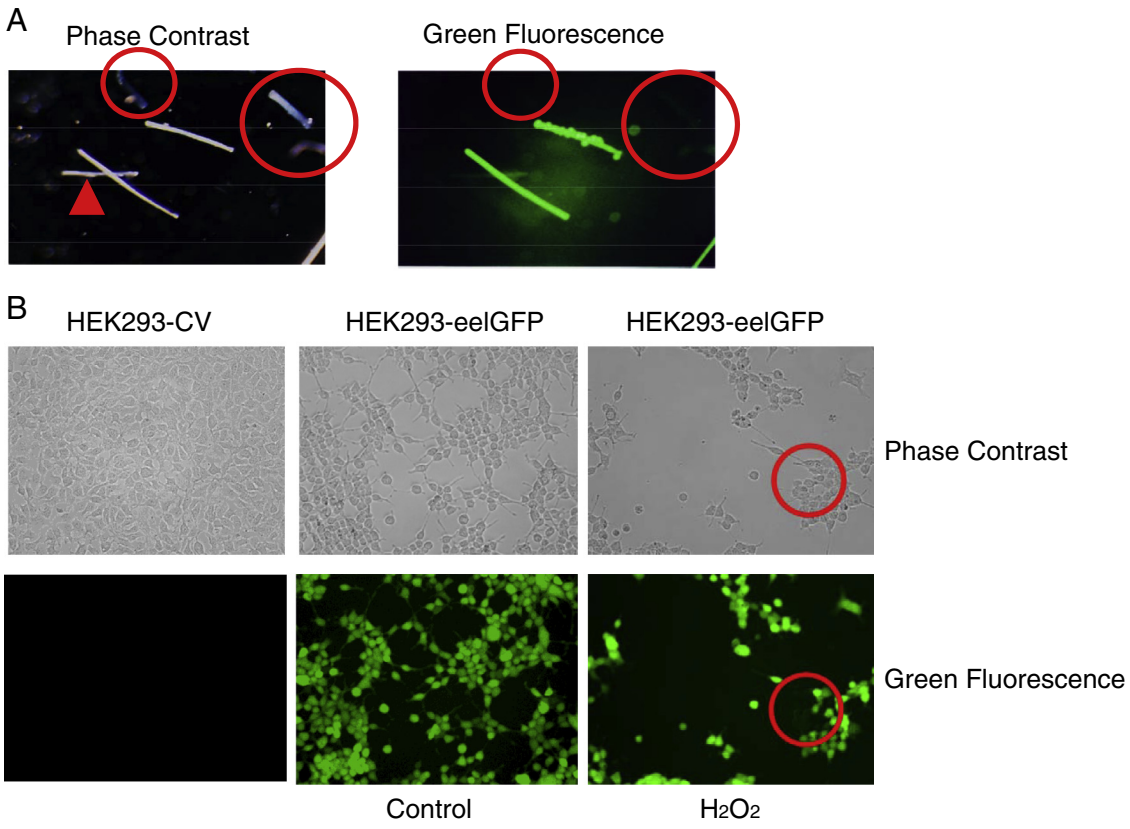

Fig. 4. Detection of heterogeneous green fluorescence in the eel skeletal muscle and HEK293-eelGFP cells. Red colored circles and the arrow head indicate cells with weak or non-intensity of green fluorescence.

that the eelGFP-bilirubin complex possessed the scavenging activity to at least hydrogen peroxide and/or hydroxyl radical.

4. Discussion

This study was performed to clarify the physiological functions of the unique eelGFP from a viewpoint of the functional properties. The eelGFP requires bilirubin as a ligand to emit fluorescence, and the bilirubin possesses a physiological role to protect cells (Sedlak et al., 2009) against oxidative stress. Bilirubin binds to albumin in the blood of mammals (Carmichael et al., 1987), and is also included in FCS. In general, and this study is also the case, FCS was a supplement added to the culture medium. The stable but not homogeneous green fluorescent expression required FCS above 2% concentration. The culture condition we employed was 10% FCS concentration to optimize cell proliferation and high fluorescent emission. However, unstable green fluorescent emission was detectable under the FCS-free condition, suggesting that the bilirubin, which was included in pre-culture medium-derived FCS, was intracellularly distributed to a limited extent.

Phenol red is supplementarily added to cell culture medium as a pH indicator in general, and also possesses a strong antioxidant activity with the phenol group (Lewinska et al., 2007). After 96 h-culture, cell densities of HEK293-CV and HEK293-jfGFP cells under phenol red-free condition were reduced to 52 and 31%, respectively, when compared to those with phenol red. The reduction of the cell densities in HEK293-eelGFP cells under the phenol red-free condition was to approximately 70%, which was higher than that of HEK293-CV and HEK293-jfGFP cells. Therefore, the eelGFP-bilirubin complex was suggested to provide the antioxidant activity in the HEK293-eelGFP cells. The sensitivity to H_2O_2 also indicated IC_{50} values of 66.4, 66.6, and 134.4 μM H_2O_2 for HEK293-CV, HEK293-jfGFP, and HEK293-eelGFP cells, respectively. These results suggested that cells containing

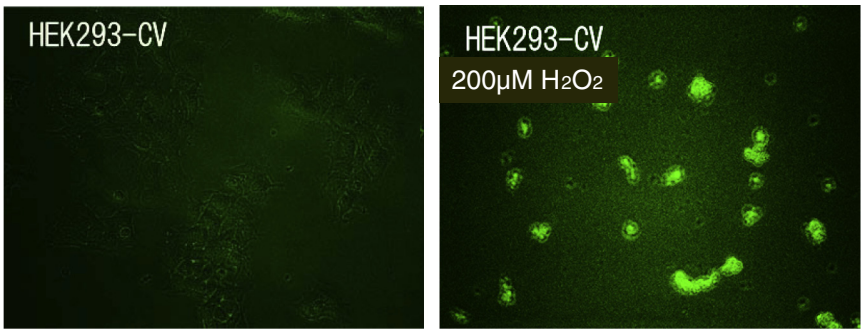

Fig. 5. Detection of highly oxidative reactive oxygen species (hROS) after exposure to H_2O_2. Non-fluorescent HEK293-CV cells were treated with 200 μM H_2O_2 for a total of 28 h, and the hROS was detected by APF.

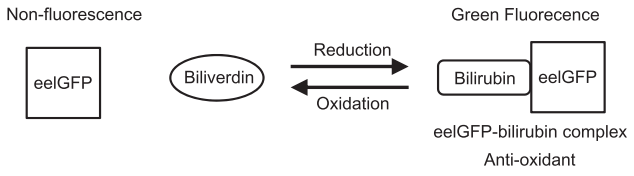

Fig. 6. Proposed mechanism of heterogeneous green fluorescent expression of eelGFP. Biliverdin, which is a main and abundant form of heme metabolite in the eel blood, is taken-up into the skeletal muscle cells. The biliverdin is enzymatically reduced to bilirubin intracellularly. The bilirubin binds to eelGFP with high affinity and expresses bright green fluorescence. When skeletal muscle cells are exposed to oxidative stress, the eelGFP-bilirubin complex functions as a scavenger of ROS, and bilirubin is oxidized to biliverdin. The formed biliverdin is removed from eelGFP, and is transported to the blood circulation. The apo eelGFP then loses green fluorescence.

eelGFP-bilirubin complex were less susceptible to damage caused by oxidative stress.

It is reported that when bilirubin reacts with ROS including superoxide, hydrogen peroxide, and hydroxyl radical, the bilirubin is easily oxidized to be converted into biliverdin (Inoue, 2001). Three distinct forms, non-bound bilirubin, lipoproteins-bound bilirubin, and albumin-bound bilirubin that is a main form in the blood, are known to possess antioxidant activity (Stocker et al., 1987; Hulea et al., 1995; Inoue, 2001; Kaur et al., 2003). In the human blood plasma, the albumin-bound bilirubin can donate two hydrogens to scavenge the ROS, and yields the reaction product, albumin-bound biliverdin which keeps binding to human albumin (Ahlfors, 1981; Stocker et al., 1987). However, eelGFP possess specific binding ability to only bilirubin with strong high affinity and specificity, but not to biliverdin, suggesting that biliverdin is freely dissociated from eelGFP after the cells are exposed to oxidative stress. Additionally, the antioxidant ability of albumin-bilirubin complex protects cells and lipoproteins effectively more against the ROS than that of non-coupled free bilirubin (Wu et al., 1991; Hulea et al., 1995). The UnaG, identical to eelGFP, has an approximately 1000-fold higher affinity for bilirubin than albumin (Kumagai et al., 2013). The eel blood plasma is held in low concentration of bilirubin and in high concentration of biliverdin, unlike human plasma. (Ellis and Poluhowich, 1981; Endo et al., 1992).

The eel of genus *Anguilla* have a habit of catadromous migration. The juvenile inhabits rivers, lakes, and estuaries, and the grown eel goes back to open ocean in order to spawn. The eel makes migration from hundreds to thousands of kilometers away from the growth habitat (Kuroki et al., 2006; Aoyama, 2009). The muscle cells in the eel are subjected to oxidative stress for a long time. It is most likely that the muscle cells are protected against oxidative stress by the eelGFP-bilirubin complex. We assume that the eel GFP needs to store a lot of bilirubin for long distance migration, and the eelGFP-bilirubin complex protects the muscle cell from oxidative stress.

In conclusion, we propose a mechanism of heterogeneous green fluorescence expression of eelGFP (Fig. 6). Biliverdin, which is a main and abundant form of heme metabolite in the eel blood, is taken-up into the skeletal muscle cells. The biliverdin is enzymatically reduced to bilirubin intracellularly. The bilirubin binds to eelGFP with strong high affinity and specificity, and expresses bright green fluorescence. When skeletal muscle cells are exposed to oxidative stress, eelGFP-bilirubin complex functions as a scavenger of ROS, and bilirubin is oxidized to biliverdin. The formed biliverdin is removed from eelGFP, and is transported to the blood circulation. Thus, the eelGFP reversibly loss fluorescence which is replenished by further binding of bilirubins.

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