Bioluminescent Indicators for Ca^{2+} Based on Split *Renilla* Luciferase Complementation in Living Cells

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Genetically encoded bioluminescent indicators for intracellular Ca^{2+} are described here with CaM-M13 interaction-induced complementation of split *Renilla* luciferase. The Ca^{2+} -induced interaction between CaM and M13 leads to complementation of the N- and C-terminal halves of split *Renilla* luciferase in living cells. This intramolecular interaction results in the spontaneous and simultaneous emission of bioluminescence split *Renilla* luciferase. This is how intracellular Ca^{2+} is illuminated with the intramolecular complementation of split *Renilla* luciferase. The Ca^{2+} -dependent spontaneous and simultaneous emission of bioluminescence promises to reveal Ca^{2+} dynamics in living cells, and also *in vivo* using the present indicators.

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Introduction

In living cells, calcium ion (Ca^{2+}) acts as an important messenger for secretion and synthesis, cell growth and differentiation. Calmodulin is a ubiquitous protein that regulates many cellular and physiological processes by monitoring Ca^{2+} levels in living cells. The binding of Ca^{2+} to CaM simultaneously leads to a conformational change of CaM, which results in an interaction between CaM and its various target proteins, such as myosin light chain kinase (MLCK), CaM-dependent kinases, protein phosphatase calcineurin, phosphodiesterase, nitric oxide synthase, Ca^{2+} -ATPase pumps and cytoskeletal proteins. Several synthetic fluorescent indicators for Ca^{2+} have been developed to reveal the Ca^{2+} dynamics involved in biological functions.^{1,2} These synthetic fluorescent indicators are easily observed under a fluorescence microscope, but hard to target to subcellular locations of interest. To combine the advantages of fluorescence readout and subcellular targeting, a genetically encoded fluorescent indicator for intracellular Ca^{2+} , “cameleon” was developed.³ This indicator is based on fluorescence resonance energy transfer, and detects the Ca^{2+} -induced protein interaction between calmodulin (CaM) and M13.^{3,4} M13 is a synthetic peptide with a sequence of a CaM-binding domain of skeletal muscle MLCK (residues 577 – 602).^{5–8} This ratiometric measurement with the changes in the ratio of the intensities of emission at two wavelengths has a limitation concerning sensitivity. Although “cameleon” visualized Ca^{2+} dynamics in transparent living cells, the fluorescent indicator suffers from much autofluorescence when the indicator is applied *in vivo*.

Aequorin is known to emit bioluminescence signals upon binding to Ca^{2+} in the presence of its substrate, coelenterazine.⁸ Aequorin is genetically targetable to subcellular locations. Also, aequorin does not suffer from autofluorescence when applied *in vivo*. However, aequorin is irreversibly destroyed by Ca^{2+} and cannot be used for monitoring intracellular Ca^{2+} .

In the present paper, bioluminescent indicators for intracellular Ca^{2+} are presented. The principle of the present bioluminescent indicator is shown in Fig. 1. The Ca^{2+} -induced interaction between CaM and M13 leads to complementation of the N- and C-terminal halves of split *Renilla* luciferase in living cells.^{9,10} This intramolecular interaction results in the spontaneous and simultaneous emission of bioluminescence from split *Renilla* luciferase domains with coelenterazine as a substrate.

Fig. 1 Bioluminescent indicator for intracellular Ca^{2+} concentrations. The CaM-M13 fusion was sandwiched between the N- and C-terminal halves of split *Renilla* luciferase. The Ca^{2+} -induced protein interaction between CaM and M13 leads to the complementation of split *Renilla* luciferase, which results in the spontaneous emission of luminescence in living mammalian cells.

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Fig. 2 Schematic representations of the plasmid constructs. All of these plasmids have a CMV-promoter sequence upstream of the start codon. hRLn and hRLc are the N- and C-terminal halves of full-length *Renilla* luciferase C124A.

Table 1 Luminescence intensity of *Renilla* luciferase mutants

	RLU/ μ g protein	RLU/RLU(hRL)
hRL	$1.19 \times 10^{10} \pm 0.46 \times 10^{10}$	1
hRL C124A	$4.81 \times 10^{11} \pm 0.84 \times 10^{11}$	$4.04 \times 10 \pm 0.7 \times 10$

Experimental

Reagents and chemicals

Restriction enzymes, modification enzymes and ligase were purchased from Takara Biomedicals (Tokyo, Japan). A synthetic *Renilla* luciferase gene vector (hRL-CMV) coding *Renilla* luciferase with most frequently used codons in mammals and *Renilla* luciferase assay system were purchased from Promega Co. (Madison, WI). The mammalian expression vector, pcDNA3.1(+) was obtained from Invitrogen (Groningen, Netherlands, Alto, CA). Minimum essential Eagle's medium (MEM), fetal bovine serum (FBS), Hank's balanced buffer solution (HBSS) and LipofectAMINE2000 were obtained from Gibco BRL (Rockville, MD).

Plasmid construction

The *E. coli* strain DH5 α was used as a bacterial host for all plasmid construction subcloning. All plasmids were verified by sequencing with a genetic analyzer ABI prism310 (PE Biosystems, Tokyo, Japan). Figure 2 shows the plasmid constructs of synthetic *Renilla* luciferase mutants. Figures 4a and 5a show plasmid constructions of the bioluminescent indicators for Ca²⁺. CaM, C124A, hRLn and hRLc were prepared as described previously.⁹⁻¹²

Cell culture and transfection

MCF-7 cells were cultured in MEM supplemented with 10% heat-inactivated fetal calf serum, 1 mM sodium pyruvate, 100 μ M MEM non-essential amino-acid solution, 100 unit/mL penicillin and 100 μ g/mL streptomycin. Cells were maintained in 5% CO₂ at 37°C. Transfection was performed using a LipofectAMINE2000.

Luminescence assay

Luminescence intensities were measured with a *Renilla* luciferase assay system. Whole cell lysates were subjected to centrifugation at 15000g for 30 s at 4°C. A portion of the supernatant was measured for the luminescence intensity with a luminometer Minilumat LB9506 (Berthold GmbH & Co. KG, Wildbad, Germany) for 10 s. The protein concentration in the

Fig. 3 Emission spectra of the luminescence with *Renilla* luciferase mutants. The luminescent spectra of hRL, hRL C124A and N64C were measured upon addition with a substrate coelenterazine. Cysteine 124 in *Renilla* luciferase was replaced by an alanine in the mutant "hRL C124A". The "N64C" has a flexible-64 amino acid-peptide linker between the N- and C-terminal halves of split *Renilla* luciferase C124A.

Table 2 Luminescence intensity of split *Renilla* luciferases

	RLU/ μ g protein	RLU/(hRL C124A)
hRL C124A	$4.81 \times 10^{11} \pm 0.84 \times 10^{11}$	1
N	$6.74 \times 10^7 \pm 0.92 \times 10^7$	$1.4 \times 10^{-4} \pm 0.4 \times 10^{-4}$
C	$3.86 \times 10^6 \pm 0.04 \times 10^6$	$7.9 \times 10^{-6} \pm 1.5 \times 10^{-6}$
N64C	$1.63 \times 10^{10} \pm 0.08 \times 10^{10}$	$3.2 \times 10^{-2} \pm 0.8 \times 10^{-2}$

supernatant was assessed by the Bradford method. The time course of the luminescence intensities of the living cells was detected with a luminometer Mithrus 940 (Berthold GmbH & Co. KG, Germany). Solutions containing a substrate and a stimulus (ATP and histamine) were injected into wells of a 96-wellmicroplate. Coelenterazine, the substrate for *Renilla* luciferase, was injected at time 0 s, and the cells were stimulated with ATP or histamine at 180 s. The luminescence intensities were obtained with a 50-ms exposure time.

Results and Discussion

Improvement of *Renilla* luciferase luminescence intensity

We replaced cysteine 124 in *Renilla* luciferase with an alanine (C124A) to increase the luminescence intensity of *Renilla* luciferase in living cells.⁹⁻¹¹ The mutation of cysteine 124 in *Renilla* luciferase increased its enzymatic activity, and its luminescence was 4.1×10 fold higher than that with wild hRL (Table 1). The luminescence spectrum of this mutant C124A was similar to that of wild-type *Renilla* luciferase (Fig. 3).

Fig. 4 (a) Bioluminescent indicator for intracellular Ca^{2+} , N(C20M)C. The CaM-M13 fusion was sandwiched between N- and C-terminal halves of split *Renilla* luciferase C124A. A flexible-20 amino acid-peptide-linker (GNGGG GNGGN GGNNN NGGGS) was inserted between CaM and M13. The interaction between CaM and M13 leads to intramolecular complementation of the split *Renilla* luciferase. (b) Time course of the luminescence intensities with the cells expressing N(C20M)C. The substrate of *Renilla* luciferase, coelenterazine, was injected at -300 s. The cells were stimulated, respectively, with $50 \mu\text{M}$ ATP and $10 \mu\text{M}$ histamine at 0 s. The luminescence intensities from -30 s to 60 s are shown.

Characterization of luminescence with split *Renilla* luciferase

To confirm that neither N- nor C-terminal of split *Renilla* luciferase has any enzymatic activity, N and C were constructed. N has the N-terminal half (1–91 a.a.) of split *Renilla* luciferase C124A and C has the C-terminal half (92–311 a.a.) of split *Renilla* luciferase C124A. N and C were separately expressed in MCF-7 cells. The luminescence intensities of the cells are given in Table 2. The “hRL” was expressed with a synthetic gene encoding *Renilla* luciferase with the most frequently used codons in mammals. The 124-cysteine residue in *Renilla* luciferase was replaced with alanine in the mutant “C124A”. The luminescence intensity of the cells expressing hRL, hRL C124A were assessed. They were normalized with their protein concentration. Also, their relative luminescence intensities normalized with the luminescence intensities of hRL were shown. The result indicates that the N- and C-terminal of split *Renilla* luciferase completely lost their luminescence activity.

To test a complementation-induced emission of split *Renilla* luciferase, linker-inserted split *Renilla* luciferase, N64C, was constructed. The N- and C-terminal of split *Renilla* luciferase were connected with a 64 amino acids flexible linker, as a substitution for an interaction protein pair. The luminescence intensities of the cells expressed with hRL C124A, N, C and N64C are given in Table 2. The “N” is 1–91 amino acid residues of hRL C124A. The “C” is 92–311 amino acid residues of hRL C124A. The “N64C” respectively inserted flexible- or 64 amino acid-peptide linkers containing N-G repeats between N- and C-terminal halves of split encoding *Renilla* luciferase. They were normalized with their protein concentration. Also, their relative luminescence intensities normalized with the luminescence intensities of hRL C124A

Fig. 5 (a) Bioluminescent indicator for intracellular Ca^{2+} , N20(C20M)25C. The CaM-M13 fusion was sandwiched between the N- and C-terminal halves of split *Renilla* luciferase C124A. A flexible-20 amino acid-peptide-linker (GNGGG GNGGN GGNNN NGGGS) was inserted between CaM and M13. A flexible-25 amino acid-peptide-linker (LHNGG DNGGG NGGNG NGNLG) was inserted between the N-terminal half of split *Renilla* luciferase and CaM. A flexible-25 amino acid-peptide-linker (GGNGG GNGGG GNNGG NNDVM AIAAN) was inserted between M13 and the C-terminal half of split *Renilla* luciferase. The interaction between CaM and M13 leads to intramolecular complementation of the split *Renilla* luciferase. (b) Time course of the luminescence intensities with the cells expressing N25(C20M)30C. The substrate of *Renilla* luciferase, coelenterazine, was injected at -300 s. The cells were stimulated, respectively, with $50 \mu\text{M}$ ATP and $10 \mu\text{M}$ histamine at 0 s. The luminescence intensities from -30 to 60 s are shown.

were shown. The luminescent intensity of N64C corresponds to a 2–3% luminescence intensity of hRL C124A, and also corresponds to about a 10000-fold luminescence intensity of the N- and C-terminal of split *Renilla* luciferase. In addition to an analysis of the luminescence intensities, the spectrum of the linker-inserted split *Renilla* luciferase was assessed. The luminescence spectrum of N64C is similar to that of the full-length *Renilla* luciferase with a broad band (400–630 nm) covering a tissue-transparent near-infrared region (Fig. 3). The result confirms that split *Renilla* luciferase emits luminescence signals upon its complementation in a single polypeptide.

Construction of bioluminescent indicators for intracellular Ca^{2+}

The present split *Renilla* luciferase-based bioluminescent indicator contains a tandem connected CaM-M13 fusion between the N- and C-terminal of split *Renilla* luciferase (Fig. 4a). The Ca^{2+} -induced interaction between CaM and M13 induces an intramolecular complementation of split *Renilla* luciferase, which results in the emission of bioluminescence.

To examine the efficiency of association and dissociation between the N- and C-terminal halves of split *Renilla* luciferase, two bioluminescent indicators were constructed with various flexible-amino acid-peptide linkers (Figs. 4a and 5a). The C-terminus of CaM was fused to M13 with a flexible-20 amino acid-peptide linker, and this tandemly connected CaM-M13 fusion was inserted between the N- and C-terminal of split

Renilla luciferase, N(C20M)C (Fig. 4a). A CaM-M13 fusion with a flexible-20 amino acid-peptide linker was inserted between the N- and C-terminal halves of split *Renilla* luciferase with a flexible-20 amino acid-peptide linker and a flexible-25 amino acid-peptide linker, N20(C20M)25C (Fig. 5a).

Evaluation of bioluminescent indicators for intracellular Ca²⁺

The present bioluminescent indicators for Ca²⁺ were, respectively, expressed in MCF-7 cells to examine their performances. The cells were respectively stimulated with 10 μ M histamine and 50 μ M ATP. Histamine and ATP are well known to rapidly and transiently increase the Ca²⁺ concentrations in a wide range of living cells, including MCF-7 cells.

The observed luminescence intensities of the cells respectively expressing N(C20M)C are shown in Fig. 4b. Stimulation with ATP or histamine rapidly increased the luminescence intensities of the cells. However, the luminescences of the cells did not decrease afterward. This indicates that the N- and C-terminal of split *Renilla* luciferase in N(C20M)C do not easily dissociate once they complement in an intramolecular manner. Figure 5b shows the time courses of the luminescence intensities with the cells expressing N20(C20M)25C. These luminescence intensities were rapidly increased just after stimulation of ATP or histamine, and then decreased afterward. The time course obtained with N20(C20M)25C is very consistent well with that obtained with fluorescent Ca²⁺ indicators. The results indicate that the bioluminescent indicator based on split *Renilla* luciferase complementation with comfortable flexible linkers allows one to monitor fluctuating, that is, increasing and decreasing concentrations of Ca²⁺ in living cells.

In summary, a bioluminescent indicator for intracellular Ca²⁺ was developed with a CaM-M13 interaction-induced complementation of split *Renilla* luciferase. The spontaneous and simultaneous conversion of intracellular Ca²⁺ into

bioluminescence promises to reveal the dynamics of Ca²⁺ in living cells, and also *in vivo*.

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