Spectral measurements

To measure bioluminescence spectra in living cells, NIH3T3 cells were transfected with 2 μg of pCMV-ELuc (pox) or pCMV-SLR (nuc), and cultured for 1 day. The medium was replaced with DMEM without phenol red (Gibco-BRL, Grand Island, NY, USA) supplemented with 10 % FBS, 25 mM *N*-(2-hydroxyethyl)piperazine-*N*′-ethanesulfonic acid (Hepes)/NaOH (pH 7.0; Sigma-Aldrich), and 200 μM D-luciferin potassium salt (Toyobo). The dish was placed on the sample stage of a spectrophotometer (AB-1850, ATTO, Tokyo, Japan), and spectral measurements were performed noninvasively for 1 min with a slit width of 1 mm.

Bioluminescence imaging

For bioluminescence imaging, NIH3T3 cells were transfected with reporter plasmid, and were seeded in 35-mm glass-bottom dishes (Iwaki) at 5×10^5 cells per dish and incubated for 1 day. The culture medium was replaced with DMEM without phenol red (Gibco) supplemented with 10 % FBS, 25 mM Hepes/NaOH (pH 7.0; Sigma-Aldrich), and 500 μM D-luciferin potassium salt (Toyobo), and was overlaid with 2 ml of mineral oil (Sigma-Aldrich) to prevent evaporation. For tumor necrosis factor α (TNFα) treatment, TNFα (Wako, Tokyo, Japan) at 10 ng/ml was added to the DMEM described earlier. Bioluminescence imaging was performed using a CellGraph (ATTO) luminescence microscope at 37 °C. CCD images were acquired using a ×20 or ×40 objective lens (numerical aperture, 0.9; Nikon, Tokyo, Japan) at 1×1 binning of the 512×512 pixel array in the absence or presence of BG39 short-pass (SCHOTT, Duryea, PA, USA) and R62 long-pass (HOYA, Tokyo, Japan) optical filters. Luminescence intensity was quantified using CellGraph Viewer (ATTO) and Metamorph (Universal Imaging, Brandywine, PA, USA).

Real-time measurement of ELuc and SLR luminescence using a luminometer

NIH3T3 cells were seeded in 35-mm dishes (Iwaki) at 5×10^5 cells 1 day before transfection. NIH3T3 cells were cotransfected with 1 μg of reporter plasmid pCMV-ELuc (pox) and 2 μg of pNFκB-TK-SLR (nuc). One day after the transfection, the culture medium was replaced with DMEM without phenol red supplemented with 10 % FBS, 25 mM Hepes/NaOH (pH 7.0; Sigma-Aldrich), 200 μM D-luciferin potassium salt (Toyobo), and TNFα at 10 ng/ml. Bioluminescence was measured and calculated as reported previously [26]. Briefly, bioluminescence was recorded for 1 min at intervals of 10 min in the absence or presence of the R62 long-pass filter at 37 °C using an AB2500 Kronos (ATTO) dish-type luminometer. Bioluminescence intensity was expressed as counts per minute.

Results and discussion

Subcellular bioluminescence imaging using green-emitting ELuc and red-emitting SLR

The bioluminescent reporters used in this study were the green-emitting luciferase ELuc (λ_{max}=538 nm) from a Brazilian click beetle (*Pyrearinus termitilluminans*) [12, 35] and the red-emitting luciferase SLR (λ_{max}=630 nm) from a railroad worm (*Phrixothrix hirtus*) [18, 36] (Fig. 1). We chose these luciferases as reporters for the following reasons: (1) ELuc and SLR display the most blueshifted and redshifted spectra, respectively, among the beetle luciferases, which are most easily separable emissions; (2) these luciferases emit light with a single luciferin (D-luciferin), allowing simultaneous emission; and (3) the emission colors are not affected by intracellular pH changes [35, 36] and remain unchanged even with prolonged incubation in the cells [26]. To perform dual-color bioluminescence imaging at the subcellular level, ELuc was targeted to a peroxisome by utilizing its own peroxisomal targeting signal at the extreme C-terminus. SLR was targeted to a nucleus by fusing the nuclear localization signal from SV40 large T antigen at the C-terminus. To capture their emissions with a CCD camera, we chose the BG39 short-pass filter (maximum transmittance, 82 %; Fig. 1, dotted blue line) and the R62 long-pass filter (maximum transmittance, 92 %; Fig. 1, dotted purple line) for ELuc and SLR, respectively.

To determine whether subcellular bioluminescence imaging is possible using these luciferases as reporters, peroxisome-targeted ELuc and nuclear-targeted SLR were

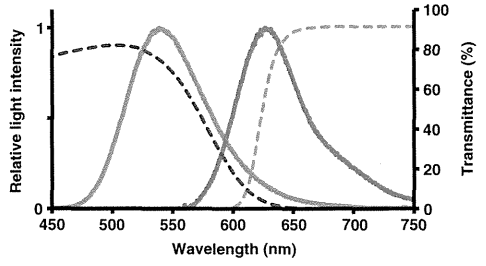

Fig. 1 Emission spectra of luciferases in viable cells and transmission spectra of the optical filters used in dual-color bioluminescence imaging. NIH3T3 cells were transfected with 2 μg of reporter plasmid pCMV-ELuc (pox) or pCMV-SLR (nuc), and the spectra were measured without destroying the cells. The bioluminescence spectra of peroxisome-targeted Emerald Luc (ELuc) and nuclear-targeted Stable Luciferase Red (SLR) are indicated by the *green line* and the *red line*, respectively. The transmission spectra of BG39 short-pass and R62 long-pass filters are shown by the *dotted blue line* and the *dotted purple line*, respectively

independently expressed in NIH3T3 cells under the control of the CMV promoter, and their images were captured using the ×40 objective lens without binning. As shown on the left in Fig. 2, peroxisome-localized ELuc exhibited a typical peroxisomal dot-like pattern, as reported previously [12]. The bioluminescence signal from nuclei was also clearly imaged using nuclear-targeted SLR (Fig. 2, right). We have previously demonstrated that bioluminescence imaging at the subcellular level with a high-magnification objective lens requires high signal intensity from living cells, and if the light intensity is insufficient, it would be difficult to acquire images at high temporal and/or spatial resolution, and this would result in low-resolution images [12]. We preliminarily estimated the light intensities of peroxisome-targeted ELuc and SLR from living cells to be 5×10^7 and 4×10^5 photons per second, respectively (data not shown), when measurements were conducted under the same conditions (same promoter and vector backbone) with a luminometer whose absolute responsivity was calibrated as reported previously [37]. Thus, although the light intensity of peroxisome-expressed SLR was approximately 1 % of that of peroxisome-expressed ELuc in living cells, we could acquire clear images of SLR in the nucleus (Fig. 2, right) by localizing SLR to the nucleus. Together, the results indicate that ELuc and SLR display sufficient light output intensity to make possible bioluminescence imaging of the peroxisome and the nucleus, respectively, in cells and that their emissions allow simultaneous imaging at subcellular resolution.

Next, we determined whether optical filters are appropriate to separate ELuc and SLR emissions. Peroxisome-targeted ELuc and nuclear-targeted SLR were expressed independently in NIH3T3 cells, and images were acquired in the absence or presence of optical filters. We obtained clear images of the peroxisome-localized ELuc luminescence with the BG39 short-pass filter (Fig. 3a, left), even though the light intensity was slightly lower (56.8±0.4 %) than that of the image taken in the absence of the filter, owing to emission loss caused by

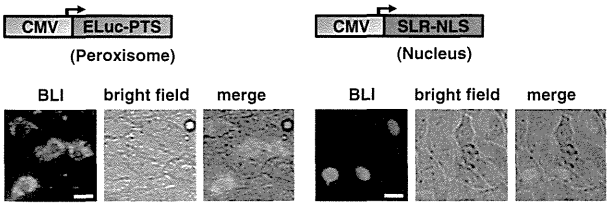

Fig. 2 Representative bioluminescence imaging of peroxisome-localized ELuc and nuclear-localized SLR in NIH3T3 cells. NIH3T3 cells were transfected independently with 2 μg of reporter plasmid pCMV-ELuc (pox) or pCMV-SLR (nuc). Luminescence images were acquired using 3-min exposure time and a ×40 objective lens without binning (*scale bar* 20 μm). A luminescence image, a bright-field photograph, and a merged photograph are shown in the panels on the *left*, in the *middle*, and on the *right*, respectively. Schematic drawings of the plasmids are shown at the *top*. *BLI* bioluminescence imaging. *CMV* cytomegalovirus promoter, *NLS* nuclear localization signal, *PTS* peroxisomal targeting signal

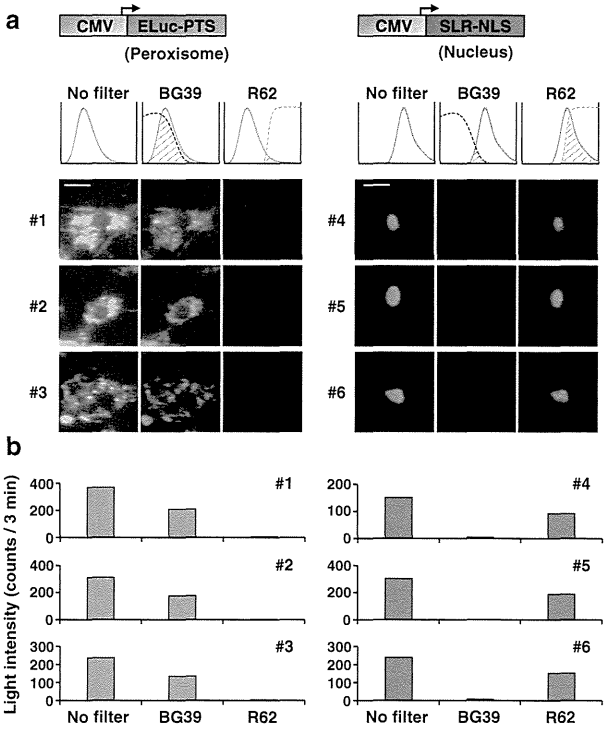

Fig. 3 Representative bioluminescence imaging of peroxisome-localized ELuc and nuclear-localized SLR in NIH3T3 cells using optical filters. **a** NIH3T3 cells were transfected independently with 2 μg of reporter plasmid pCMV-ELuc (pox) or pCMV-SLR (nuc). Luminescence images were acquired using 3-min exposure time and a ×40 objective lens without binning in the absence (indicated by *no filter*) or presence of BG39 and R62 filters (*scale bar* 20 μm). Schematic drawings of the plasmids are shown at the *top*. Schematic diagrams of the bioluminescence spectra of luciferases and the transmission spectra of the BG39 and R62 filters (*dotted lines*) are shown in the *middle*. Luminescence images of three representative cells with or without filters are shown at the *bottom*. **b** Quantified luminescence intensity of peroxisome-localized ELuc and nuclear-localized SLR shown in **a**

the filter. No remarkable luminescence signals were captured with the R62 long-pass filter (Fig. 3a and b, left). In contrast, clear images of the nuclear localization of SLR were obtained with the R62 long-pass filter, the light intensity of which was 62.5±1.5 % of that in the absence of the filter, but not with the BG39 short-pass filter (Fig. 3a and b, right). The results suggest that the BG39 and R62 filters are appropriate to capture the luminescence of ELuc and SLR, respectively, and that dual-color bioluminescence imaging at the subcellular level can be accomplished with these optical filters.

Dual-color bioluminescence imaging at the subcellular level

Next, we attempted to perform dual-color bioluminescence imaging. NIH3T3 cells were cotransfected with peroxisome-targeted ELuc and nuclear-targeted SLR, and images were taken using the ×40 objective lens in the presence of BG39 short-pass and R62 long-pass filters. One day after the

transfection, ELuc emission through the BG39 filter and subsequent SLR emission through the R62 filter were captured in real time. Figure 4 shows representative images of three cells. As expected, we captured clearly and simultaneously ELuc luminescence from peroxisomes using the BG39 short-pass filter (Fig. 4, left) and SLR luminescence from nuclei using the R62 long-pass filter (Fig. 4, middle) even when these luciferases were co-expressed in a single cell. The merged photographs demonstrate that the respective luciferases are accurately localized to peroxisomes and nuclei in a cell (see also Movies S1, S2, S3). The results indicate that dual-color bioluminescence imaging at subcellular resolution can be performed using green-emitting ELuc and red-emitting SLR and the combined use of optical filters. Furthermore, time-lapse imaging demonstrated that the luminescence of peroxisome-localized ELuc and nuclear-localized SLR could be tracked continuously and simultaneously for a long time. These results appear to indicate that the dual-color bioluminescence imaging developed in this study makes possible quantitative imaging of gene expression changes in a single living cell at subcellular resolution.

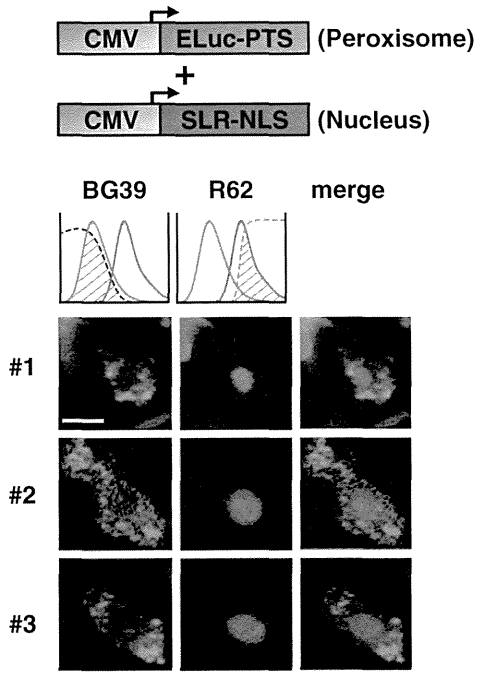

Fig. 4 Representative dual-color bioluminescence imaging of peroxisome-localized ELuc and nuclear-localized SLR in NIH3T3 cells. NIH3T3 cells were cotransfected with 1 μg of each reporter plasmid—pCMV-ELuc (pox) and pCMV-SLR (nuc)—and incubated for 1 day. Luminescence images were acquired using 3-min exposure time and a ×40 objective lens with BG39 and R62 filters (*scale bar* 20 μm). Images were taken without binning. Schematic drawings of the plasmids are shown at the *top*. Schematic diagrams of the bioluminescence spectra of the luciferases and the transmission spectra of the BG39 and R62 filters (*dotted lines*) are shown in the *middle*. Luminescence images acquired using the BG39 (green) and R62 (red) filters and merged images of three representative cells are shown at the *bottom*

Time-lapse dual-color imaging assay of NF-κB-dependent transactivation

To explore the possibility of quantitative imaging of the expressions of two genes by time-lapse dual-color imaging, we attempted to track the TNFα-induced activation of NF-κB, which is activated by cytokines, including TNFα, growth factors, and Toll-like receptor signaling [38, 39]. NF-κB exists in an inactive form in the cytoplasm owing to its interaction with IκB, an inhibitor of NF-κB. After activation of IκB kinase by TNFα, IκB is phosphorylated, ubiquitinated, and degraded by proteasomes. The dissociated NF-κB becomes free to translocate to the nucleus, where it activates specific target genes through selective binding to the NF-κB RE. Through such ways, NF-κB regulates the expression of a wide variety of genes, including cytokines, chemokines, and antiapoptotic genes [40].

For time-lapse dual-color bioluminescence imaging, we used nuclear-targeted SLR as the test reporter, whose expression is regulated by the NF-κB RE upstream of the thymidine kinase promoter. On the other hand, we used a reporter plasmid in which the peroxisome-targeted ELuc is expressed under the control of the CMV promoter, as the internal control reporter. NIH3T3 cells were cotransfected with these reporter plasmids. One day after the transfection, the medium was replaced with DMEM containing TNFα and D-luciferin. Time-lapse imaging was performed using the ×40 objective lens in the presence of BG39 and R62 filters, and images were captured for 3 min with 15-min intervals for 12 h. Figure 5a (see also Fig. S1a and Movies S4, S5, S6) shows representative images of SLR luminescence from the nucleus and ELuc luminescence from the peroxisomes of the TNFα-treated cells. After TNFα treatment, the intensity of the SLR signal that passed through the R62 long-pass filter gradually increased, whereas the intensity of the ELuc signal that passed through the BG39 filter slightly decreased or rapidly decayed after an abrupt increase, which might be due to cell death caused by TNFα stimulation. On the other hand, we could not detect distinct SLR signals from the nuclei of the TNFα-untreated cells even after 10 h, although ELuc luminescence from peroxisomes could be captured clearly (Fig. S1b). The results indicate that the increase in intensity of the SLR signal from the nucleus of TNFα-treated cells shown in Fig. 5a specifically monitors NF-κB RE-dependent transactivation via NF-κB activation pathways triggered by TNFα.

To estimate NF-κB RE-dependent transactivation, we quantified the SLR signals that passed through the R62 filter and the ELuc signals that passed through the BG39 filter (Fig. 5b, top), and the SLR signal intensity was normalized to the ELuc signal intensity, as analyzed by a typical dual-reporter assay (Fig. 5b, bottom). In two of three cells (cells 1 and 2), NF-κB RE-dependent transcription was initially rapidly activated but became constant after TNFα treatment for

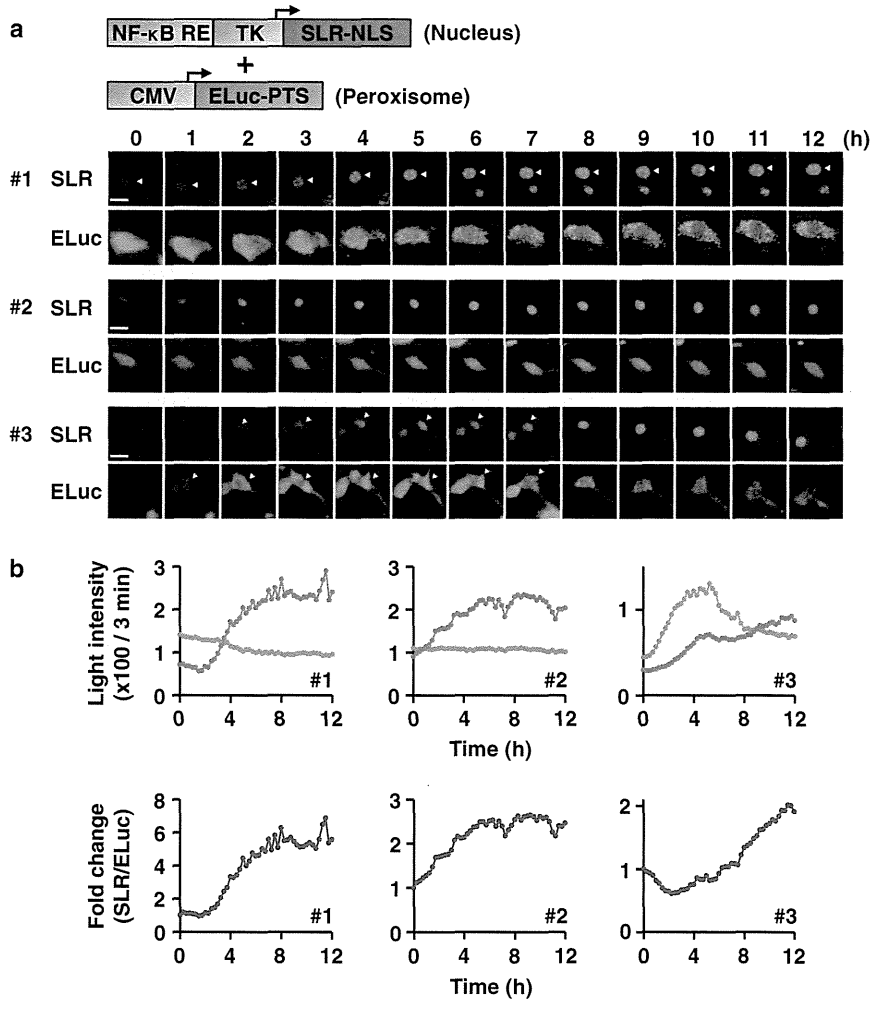

Fig. 5 Time-lapse dual-color bioluminescence imaging of tumor necrosis factor α (TNFα)-induced nuclear factor κB response element (NF-κB RE)-dependent transactivation in NIH3T3 cells. **a** NIH3T3 cells were cotransfected with 1 μg of reporter plasmid pCMV-ELuc (pox) and 2 μg of pNFκB-TK-SLR (nuc). One day after the transfection, the culture medium was replaced with Dulbecco's modified Eagle's medium supplemented with TNFα at 10 ng/ml. CCD images were acquired using 3-min exposure time at 15-min intervals for 12 h with a ×40 objective lens without binning (*scale bar* 20 μm). Representative serial luminescence images acquired using R62 (red, indicated as *SLR*) and BG39 (green, indicated as *ELuc*) filters at 1-h intervals are shown at the *bottom*. *White triangles* in the images indicate quantified organelle. Schematic drawings of the plasmids are shown at the *top*. **b** Recordings of ELuc (*green lines*) and SLR (*red lines*) luminescence shown in **a** (*top*), and fold change (*blue lines*), where the SLR intensity was normalized to the ELuc intensity (*bottom*). The fold change at time zero is set to 1. *TK* thymidine kinase promoter

8 h, whereas the NF-κB RE-dependent transcription of one cell (cell 3) was continuously activated. We assume that the differences in activation kinetics among the cells reflect differences in sensitivity to TNFα among the cells. We also confirmed the activation kinetics by means of real-time photomultiplier recording, in which the overall average of gene expressions was measured, under the same conditions as those for Fig. 5. After treatment with TNFα, the cells were acutely activated, and the bioluminescence intensity reached a maximum approximately 9 h after treatment and remained at this level until 12 h after treatment (Fig. S2), which was similar to the kinetics of cells 1 and 2. Thus, the kinetics of gene expression and activation measured by the two monitoring systems were very similar, suggesting the suitability of the bioluminescence imaging system developed in this work.

Thus, we have successfully used subcellular localized green-emitting ELuc and red-emitting SLR to visualize and quantify gene expression. This work is the first to demonstrate the time-lapse simultaneous imaging of the expressions of two genes by bioluminescence imaging at the subcellular level.

Conclusions

We have developed a dual-color bioluminescence imaging system in which peroxisome-targeted green-emitting ELuc and nuclear-targeted red-emitting SLR are used as reporters. This system allows us not only to track the subcellular localization of luciferases in a single cell with high spatial resolution, but also to analyze two gene expressions with high temporal resolution. The system offers several advantages for high spatiotemporal imaging for the continuous visualization of the intracellular localization of protein and gene expression at the subcellular level. This system can be easily used to analyze the intracellular trafficking of proteins, such as the nuclear entry of transcription factors, and the concomitant change of gene expression. Furthermore, the system can be also applied to subcellular imaging of intracellular dynamics of Ca^{2+} and ATP by combined use of aequorin, a blue-emitting Ca^{2+}-binding photoprotein, and SLR [41, 42], thereby making possible the simultaneous analysis of multiple cellular events.

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Highly sensitive luciferase reporter assay using a potent destabilization sequence of calpain 3

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ABSTRACT

Reporter assays that use luciferases are widely employed for monitoring cellular events associated with gene expression in vitro and in vivo. To improve the response of the luciferase reporter to acute changes of gene expression, a destabilization sequence is frequently used to reduce the stability of luciferase protein in the cells, which results in an increase of sensitivity of the luciferase reporter assay. In this study, we identified a potent destabilization sequence (referred to as the C9 fragment) consisting of 42 amino acid residues from human calpain 3 (CAPN3). Whereas the half-life of Emerald Luc (ELuc) from the Brazilian click beetle *Pyrearinus termitilluminans* was reduced by fusing PEST ($t_{1/2}$ = 9.8 to 2.8 h), the half-life of C9-fused ELuc was significantly shorter ($t_{1/2}$ = 1.0 h) than that of PEST-fused ELuc when measurements were conducted at 37 °C. In addition, firefly luciferase (*luc2*) was also markedly destabilized by the C9 fragment compared with the humanized PEST sequence. These results indicate that the C9 fragment from CAPN3 is a much more potent destabilization sequence than the PEST sequence. Furthermore, real-time bioluminescence recording of the activation kinetics of nuclear factor-κB after transient treatment with tumor necrosis factor α revealed that the response of C9-fused ELuc is significantly greater than that of PEST-fused ELuc, demonstrating that the use of the C9 fragment realizes a luciferase reporter assay that has faster response speed compared with that provided by the PEST sequence.

1. Introduction

Reporter assay systems are widely used to study various biological functions, such as gene expression, post-translational modification, and protein–protein interaction, in vitro and in vivo (Greer and Szalay, 2002; Gross and Piwnica-Worms, 2005; Wilson and Hastings, 1998). Of the reporter genes known to date, luciferases that emit light by oxidizing their substrates are frequently employed because their sensitivity and range of linear response are superior to those of other typical reporters, including β-galactosidase, chloramphenicol acetyltransferase, and fluorescent proteins (Naylor, 1999). Thus, luciferases are the most suitable reporter genes for the quantitative measurement of cellular events, including gene expression.

In a typical luciferase reporter assay that uses firefly and *Renilla* luciferases, a luciferase-expressing object, such as a cell, is destroyed at a particular time point, called the endpoint assay, enabling conventional and high-throughput assay. On the other hand, *Gaussia*, *Metridia*, and *Cypridina* luciferases are secreted from cells by utilizing their own secretion signals, thereby allowing us to continuously monitor luciferase activity in the culture medium.

Abbreviations: CAPN3, calpain 3; ELuc, Emerald Luc from Brazilian click beetle *Pyrearinus termitilluminans*; CCD, charged-coupled device; *luc2*, firefly luciferase from *Photinus pyralis*; EGFP, enhanced green fluorescent protein; TK, thymidine kinase; NF-κB, nuclear factor-κB; TNFα, tumor necrosis factor α; MI-HAC, multi-integrase human artificial chromosome; DMEM, Dulbecco's Modified Eagle's Medium; FBS, fetal bovine serum; FISH, fluorescence in situ hybridization.

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Recent improvements of both the luciferases and the detection systems have enabled us to detect noninvasively bioluminescence from cells, tissues, and whole organisms in real time by using a photomultiplier tube or a highly sensitive charged-coupled device (CCD) camera (Luker and Luker, 2008; Nakajima and Ohmiya, 2010; Welsh and Kay, 2005). Such monitoring systems permit the kinetic analysis of gene expression and change of second messenger levels, such as ATP and Ca^{2+} (Grygorczyk et al., 2013; Kwon et al., 2012).

In general, bioluminescence detection sensitivity increases proportionally with the stability of luciferase protein in the cells due to the accumulation of residual luciferase protein. Conversely, it is known that a stable luciferase is inappropriate for the high-sensitivity detection of acute changes of cellular response, owing to the residual photonic contribution of the pre-existing luciferase protein (Leclerc et al., 2000). In this regard, for monitoring acute or transient changes of cellular response, a destabilization sequence is frequently used to reduce the stability of the luciferase protein in the cells. The most typical destabilization sequence is the PEST motif from the C-terminal region of ornithine decarboxylase. The PEST sequence, which consists of 41 amino acid residues, contains regions rich in proline, glutamate, serine, and threonine residues, and participates in proteolytic degradation (Rechsteiner and Rogers, 1996). By fusing the PEST sequence to the C-termini of luciferases, including firefly luciferase from *Photinus pyralis*, *Renilla* luciferase from *Renilla reinformis*, and Emerald Luc (ELuc) from *Pyrearinus termitilluminans*, the half-lives of those luciferases became significantly shorter than those of unmodified luciferases (Leclerc et al., 2000; Nakajima et al., 2010; Voon et al., 2005). Although the PEST sequence enables highly sensitive detection of rapid up- and downregulation of gene expression, a much more potent destabilization sequence is required to improve assay sensitivity. For this purpose, an mRNA degradation signal, such as an AU-rich element from an immediate early gene, such as c-fos or c-myc, is introduced into the 3′ untranslated region of the firefly and *Renilla* luciferase genes, in combination with the PEST sequence, to drastically destabilize mRNA and protein expression (Voon et al., 2005). However, the interpretation of the results of the luciferase assay would be more straightforward when the stability of luciferase protein is simply reduced by fusing the destabilization sequence only.

Calpain 3 (CAPN3, also called p94), a member of the calpain superfamily, is a calcium- or sodium-dependent cysteine protease mainly expressed in skeletal muscle, and is involved in many cellular functions, including cytoskeletal remodeling processes, cell differentiation, and apoptosis (Duguez et al., 2006; Sorimachi et al., 2011). CAPN3 is also known to be a short-lived protein. In vitro experiments demonstrated that CAPN3 was almost completely degraded within 10 min by autolysis (Fanin et al., 2007; Sorimachi et al., 1993). We therefore thought that a more rapid method for the destabilization of luciferase than the destabilization by PEST sequence could be achieved by fusing partial fragments of CAPN3 protein.

In this study, we identified a potent destabilization sequence consisting of 42 amino acid residues from CAPN3, which achieved more rapid degradation of luciferase in the cells than the PEST sequence. In addition, we successfully monitored the acute activation of nuclear factor-κB (NF-κB) by treatment with tumor necrosis factor α (TNFα), and confirmed that the sensitivity of the luciferase assay was markedly improved by using CAPN3-fused luciferase rather than PEST-fused luciferase.

2. Materials and methods

2.1. Plasmid construction

In the present study, the destabilization sequences were fused in-frame to the C-terminus of ELuc from *P. termitilluminans* (Nakajima et al., 2010). To construct an expression plasmid carrying cytosol-targeting ELuc, cDNA sequence in which the peroxisome-targeting signal (Ser-Lys-Leu) at the extreme C-terminus of ELuc was deleted by the polymerase chain reaction (PCR) with the pELuc-test (TOYOBO, Osaka, Japan) was used as the template, with the primer set SLGOR-T-F1 and ELuc(-PTS)-R-XbaI (Table S1). The amplified product was ligated into the *Nco*I/*Xba*I site of expression vector pGVC2 (TOYO Inc., Tokyo, Japan), from which the firefly luciferase was removed, resulting in pSV40-ELuc (cyto). PEST-fused ELuc, in which the PEST sequence is fused in-frame to the C-terminus of ELuc whose peroxisome-targeting signal has been removed, was prepared as reported previously (Nakajima et al., 2010). Briefly, the PEST sequence of mouse ornithine decarboxylase (in which the *Nco*I site at the C-terminal end was deleted without changing the deduced amino acid sequence) was PCR-amplified with pd1EGFP-N1 (Clontech, Palo Alto, CA) as the template using the primer set PEST-F-EcoRVm and PEST-R-XbaI, and the amplified product was ligated downstream of ELuc (cyto). The PEST-fused ELuc (cyto) was then replaced with the *Nco*I and *Xba*I fragment of pGVC2, from which the firefly luciferase was removed, resulting in pSV40-ELuc::PEST.

To generate an expression plasmid carrying CAPN3-fused ELuc, a partial sequence of human CAPN3 (hCAPN3) was PCR-amplified with FLJ40082 plasmid (TOYOBO) as the template using the primer set Capn3-F-SmaI and Capn3-R-XbaI, and the amplified product was ligated into the *Eco*RV/*Xba*I site of pSV40-ELuc::PEST, from which the PEST sequence was removed, resulting in pSV40-ELuc::CAPN3. Expression plasmids carrying CAPN3 fragments N1-, C1-, C2-, and C3-fused ELuc were prepared by inverse PCR using a KOD -Plus- Mutagenesis Kit (TOYOBO) with pSV40-ELuc::CAPN3 as the template, and primer sets Capn3-R1 and SV40-pA-F (for the N1 fragment), ELuc(-PTS,-Stop)-R-EcoRV and Capn3-F1 (for the C1 fragment), ELuc(-PTS,-Stop)-R-EcoRV and Capn3-F2 (for the C2 fragment), and Capn3-R2 and SV40-pA-F (for the C3 fragment), respectively, resulting in pSV40-ELuc::CAPN3-N1, pSV40-ELuc::CAPN3-C1, pSV40-ELuc::CAPN3-C2, and pSV40-ELuc::CAPN3-C3. The C4 to C9 fragments of CAPN3 were amplified by PCR with the FLJ40082 plasmid as the template using primer sets Capn3-F1 and Capn3-R3-XbaI (for the C4 fragment), Capn3-F4 and V-R4-XbaI (for the C5 fragment), Capn3-F5 and Capn3-R5-XbaI (for the C6 fragment), Capn3-F2 and Capn3-R6-XbaI (for the C7 fragment), Capn3-F6 and Capn3-R7-XbaI (for the C8 fragment), and Capn3-F7 and Capn3-R-XbaI (for the C9 fragment), respectively. The amplified fragments were digested with *Xba*I and ligated into the *Eco*RV/*Xba*I site of pSV40-ELuc::PEST, from which the PEST sequence was removed, resulting in pSV40-ELuc::CAPN3-C3, pSV40-ELuc::CAPN3-C4, pSV40-ELuc::CAPN3-C5, pSV40-ELuc::CAPN3-C6, pSV40-ELuc::CAPN3-C7, pSV40-ELuc::CAPN3-C8, and pSV40-ELuc::CAPN3-C9. The C10, C11, and C12 fragments of CAPN3 were prepared by annealing the following sets of 5′-phosphorylated oligonucleotides: Capn3-C10F and Capn3-C10R (for the C10 fragment), Capn3-C11F and Capn3-C11R (for the C11 fragment), and Capn3-C12F and Capn3-C12R (for the C12 fragment), respectively. Phosphorylated double-strand DNAs were ligated into pSV40-ELuc::CAPN3, from which the CAPN3 sequence was removed by inverse PCR using the primer set ELuc(-PTS,-Stop)-R-EcoRV and SV40-pA-F, resulting in pSV40-ELuc::CAPN3-C10, pSV40-ELuc::CAPN3-C11, and pSV40-ELuc::CAPN3-C12.

To generate an expression plasmid carrying humanized PEST (hPEST)-fused firefly luciferase (*luc2*), hPEST-fused *luc2* was excised with *Nco*I and *Xba*I from pGL4.37 (Promega, Madison, WI), and the fragment was ligated into the *Nco*I/*Xba*I site of pSV40-ELuc::CAPN3-C9 from which ELuc::CAPN3-C9 fragment was removed, resulting in pSV40-luc2::hPEST. An expression plasmid carrying CAPN3-C9-fused *luc2* was generated by using the In

Fusion System (Clontech). First, the CAPN3-C9 fragment was PCR-amplified with pSV40-ELuc::CAPN3-C9 as the template using the primer set Capn3-13F-luc2-EcoRV and Capn3-13R-pGVC2. Next, *luc2* cDNA without the hPEST sequence was PCR-amplified with pGL4.37 as the template using the primer set luc2-F-pGVC2-HindIII and luc2-R-Capn3c9-EcoRV. The amplified products were simultaneously ligated into the *Hind*III/*Xba*I site of pSV40-ELuc::CAPN3-C9 from which the ELuc::CAPN3-C9 fragment was removed, using the In Fusion System according to the manufacturer's instructions, resulting in pSV40-luc2::CAPN3-C9.

To generate an expression plasmid carrying C9-fused enhanced green fluorescent protein (EGFP), the CAPN3-C9 fragment was PCR-amplified with pSV40-ELuc::CAPN3-C9 as the template using the primer set Capn3-F7 and Capn3-R-XbaI, and the fragment was ligated into the *Sma*I/*Xba*I site of pEGFP-C2 (Clontech) downstream of EGFP, resulting in pCMV-EGFP::CAPN3-C9.

To generate NF-κB reporter plasmids, oligonucleotides containing six tandem repeats of the NF-κB response element (5′-CGGAAAGTCCCA-3′) were ligated into the *Xho*I/*Bgl*II site of pSLR-HSVtk Control (TOYOBO), immediately upstream of the herpes simplex virus thymidine kinase (TK) promoter. The SLR gene was replaced with *Nco*I and *Xba*I fragment of pSV40-ELuc (cyto), pSV40-ELuc::PEST, and pSV40-ELuc::CAPN3-C9. Then, expression cassettes consisting of the NF-κB response element, the HSV-TK promoter, ELuc or destabilized ELuc, and polyA signal were amplified by PCR using the primer set SLGOR-T-F2 and SLGOR-T-R1, and the amplified products were cloned into pENTR-D-TOPO (Invitrogen). The expression cassettes were recombined into pNeo-φC31 attB (Yamaguchi et al., 2011) by the LR reaction using LR Clonase II Plus Enzyme Mix (Invitrogen), yielding pφC31-Neo-NFκB-ELuc, pφC31-Neo-NFκB-ELuc::PEST, and pφC31-Neo-NFκB-ELuc::CAPN3-C9.

2.2. Cell culture

Mouse NIH3T3 cells (Riken Cell Bank 1862) and A9 cells harboring the multi-integrase human artificial chromosome (MI-HAC) vector (Yamaguchi et al., 2011) were grown in Dulbecco's Modified Eagle's Medium (DMEM, Sigma–Aldrich, St. Louis, MO) supplemented with 10% fetal bovine serum (FBS, ICN Biochemicals, Aurora, OH) in a humidified atmosphere containing 5% CO_2 at 37 °C.

2.3. Measurement of stability of luciferases in NIH3T3 cells

NIH3T3 cells were seeded in 96-well black clear-bottom plates (Nunc, Wiesbaden, Germany) at 2×10^4 cells per well one day before transfection. One hundred nanograms of reporter plasmid was transfected using Lipofectamine PLUS (Invitrogen) according to the manufacturer's instructions. One day after transfection, the culture medium was replaced with DMEM without phenol red (Gibco-BRL, Grand Island, NY) but supplemented with 10% FBS, 200 μM D-luciferin potassium salt (TOYOBO) that had been dissolved in distilled water, and 100 μM cycloheximide (Nacalai Tesque), and overlaid with 50 μl of mineral oil (Sigma–Aldrich) to prevent evaporation. Bioluminescence was recorded in real time for 10 s at 10-min intervals for 9.6 h at 37 °C using a microplate-type luminometer (AB-2350 Phelios, ATTO, Tokyo, Japan) unless otherwise noted.

2.4. Immunoblot analysis

NIH3T3 cells were seeded in six-well plates at the density of 5×10^5 cells per well one day before transfection. Two micrograms of expression plasmid was transfected into the cells, and the cells were incubated for two days. Thereafter, the culture medium was replaced with DMEM supplemented with 10% FBS and 100 μM cycloheximide. The cells were incubated at 37 °C under 5% CO_2 atmosphere, and lysed with M-PER extraction reagent (Pierce Biotechnology, Rockford, IL) supplemented with protease inhibitor cocktail (Roche, Basel, Switzerland). For the detection of ELuc and EGFP, we used rabbit anti-ELuc and anti-EGFP polyclonal antibodies that were raised against purified recombinant ELuc (Nakajima et al., 2010) and EGFP. For the detection of tubulin, mouse anti-α-tubulin (Sigma–Aldrich) was used as the primary antibody. We used horseradish peroxidase-conjugated anti-rabbit IgG (Jackson ImmunoResearch, West Grove, PA) and anti-mouse IgG (BioRad, Hercules, CA) as the secondary antibodies. The antibodies were diluted with Can Get Signal solution (TOYOBO) (1/20,000, 1/20,000, and 1/3,000 dilution for anti-ELuc, anti-EGFP, and anti-tubulin antibodies, respectively). Immunoreactive bands were detected with the ECL Plus kit (GE Healthcare, Freiburg, Germany) according to the manufacturer's instructions using an image analyzer (LAS-3000 mini, Fuji Photo Film, Tokyo, Japan).

2.5. Generation of stable cell lines

Mouse fibroblast A9 cells harboring the MI-HAC vector were seeded in six-well plates at 6×10^5 cells per well one day before transfection. Three micrograms of reporter plasmid, pφC31-Neo-NFκB-ELuc, pφC31-Neo-NFκB-ELuc::PEST or pφC31-Neo-NFκB-ELuc::CAPN3-C9, was co-transfected with 1 μg of φC31 recombinase expression plasmid pCMV-φC31 (Yamaguchi et al., 2011) using Lipofectamine PLUS (Invitrogen) according to the manufacturer's instructions. The transfected cells were seeded in 10-cm dishes one day after transfection, and subcultured for selection with 800 μg/ml G418 (Nacalai Tesque). Integration of transgene into the corresponding site on the HAC vector was confirmed by genomic PCR using the primer set PGK5 and G418 3AS.

2.6. Fluorescence in situ hybridization (FISH) analysis

Preparation of metaphase chromosome from exponentially growing cell culture and FISH were performed as reported previously (Tomizuka et al., 1997). Briefly, FISH was carried out using digoxigenin-labeled (Roche, Basel, Switzerland) human COT1 DNA (Invitrogen) and biotin-labeled reporter plasmid pφC31-Neo-NFκB-ELuc. Chromosome DNA was counterstained with DAPI (Sigma–Aldrich). Images were captured with a fluorescence microscope BX51 (Olympus, Tokyo, Japan).

2.7. Real-time bioluminescence measurement of TNFα-induced NF-κB activation

The A9 stable cell lines in which the NF-κB reporter plasmid was integrated into the MI-HAC vector were seeded in 35-mm dishes at 6×10^5 cells per dish. After one day, the medium was replaced with DMEM without phenol red (Gibco-BRL) and supplemented with 10% FBS and 10 ng/ml TNFα (Wako, Tokyo, Japan), and incubated for 15 min. The medium was again replaced with DMEM without phenol red (Gibco-BRL) but containing 10% FBS and 200 μM D-luciferin potassium salt (TOYOBO) that had been dissolved in distilled water, and overlaid with mineral oil (Sigma–Aldrich) to prevent evaporation. Bioluminescence was recorded for 1 min at 15-min intervals under a 5% CO_2 atmosphere at 37 °C using a dish-type luminometer (AB2500 Kronos, ATTO).

2.8. Time-lapse bioluminescence imaging

The A9 stable cell lines in which the NF-κB-ELuc::CAPN3-C9 reporter plasmid was integrated into the MI-HAC vector were seeded in 35-mm glass-bottom dishes (Iwaki, Tokyo, Japan) at 6×10^5 cells per dish. After one day, the medium was replaced with

DMEM without phenol red (Gibco-BRL) but supplemented with 10% FBS, 25 mM Hepes/NaOH (pH 7.0; Sigma–Aldrich), 500 μM D-luciferin potassium salt (TOYOBO) that had been dissolved in distilled water, and 10 ng/ml TNFα (Wako), and was sealed with Parafilm (American National Can, Menasha, IL) to prevent evaporation. Bioluminescence imaging was performed using the luminescence microscope CellGraph (ATTO) at 37 °C. CCD images were acquired with a 10-min exposure time at 15-min intervals with a 4× objective lens (NA, 0.5; ATTO) at 1 × 1 binning of the 512 pixel × 512 pixel array. Luminescence intensity was quantified with CellGraph Viewer (ATTO) and Metamorph (Universal Imaging, Brandywine, PA).

3. Results

3.1. CAPN3 significantly destabilizes luciferase protein in cells more than PEST sequence

In this study, we used the FLJ40082 clone (GenBank accession number AK097401) as hCAPN3 cDNA, which is a close homologue, and the partial sequence of the entire hCAPN3 (GenBank accession number AF209502), which corresponded to amino acid residues between 104 and 513 of hCAPN3 (Fig. S1, hereinafter referred to as CAPN3), and ELuc (Nakajima et al., 2010) as reporter. As ELuc exhibits brighter luminescence in cells compared with other beetle luciferases, including the firefly luciferase, because of the high stability of ELuc in the cells, it allowed us to reliably estimate the destabilization effect of CAPN3. The functional half-life of destabilized ELuc was estimated as the half-decay time of the luminescence intensity recorded over time in the presence of the protein synthesis inhibitor cycloheximide.

To determine whether the stability of ELuc can be reduced by CAPN3, CAPN3 cDNA was fused in-frame to the C-terminus of ELuc (Fig. S2). As shown in Fig. 1, whereas the functional half-life of unmodified ELuc in NIH3T3 cells was 9.83 h under the experimental conditions used in this study, the half-life of CAPN3-fused ELuc was significantly shortened (1.72 h). Furthermore, the half-life of CAPN3-fused ELuc was shorter than that of ELuc fused to the typical destabilizing motif PEST sequence from mouse ornithine decarboxylase (2.80 h), suggesting that CAPN3 contains a very effective destabilizing motif.

Western blot analysis using anti-ELuc antibody revealed that the amount of residual CAPN3-fused ELuc protein in the cells at time 0 was much smaller than that of the unmodified ELuc and the PEST-fused ELuc, and that the CAPN3-fused ELuc protein was rapidly degraded during incubation in the presence of cycloheximide (Fig. 1b). The results indicate that the decay of bioluminescence kinetics and light intensity caused by fusing CAPN3 is due not to the inhibition of the luciferin-luciferase reaction, but to the degradation of ELuc protein in the cells.

3.2. Identification of minimum destabilization sequence in CAPN3

We identified the minimum CAPN3 sequence that can effectively destabilize luciferase protein in the cells, as the minimum fragment size would reduce the risk of loss of luciferase activity due to interference with the active conformation and/or the luciferin-luciferase reaction when the fragment is fused to luciferase. First, CAPN3 was divided into two fragments (N1 and C1 fragments), and the half-lives and the light intensities of ELuc fused at C-terminus to those fragments in the NIH3T3 cells were determined by real-time bioluminescence measurements (Figs. 2, S3, and S4). We examined the stability and the light intensity of ELuc fused to CAPN3 fragments using a 96-well plate format, which enables a direct comparison to a control experiment with PEST sequence performed in parallel. Both the half-life and the light

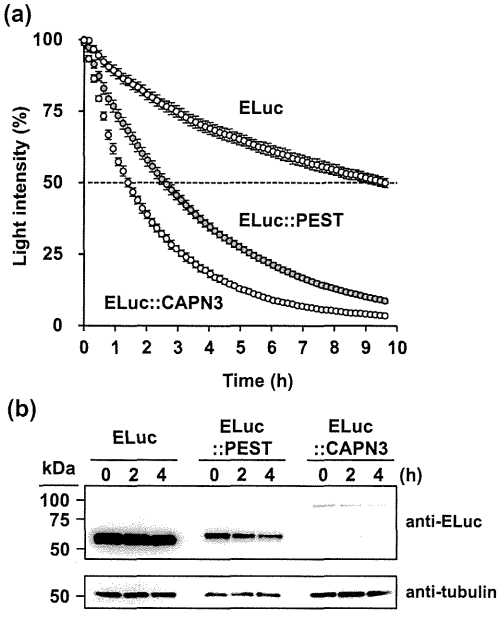

Fig. 1. Stability of ELuc, PEST-fused ELuc, and CAPN3-fused ELuc in NIH3T3 cells. (a) Functional half-lives of ELuc, PEST-fused ELuc, and CAPN3-fused ELuc. Expression plasmids pSV40-ELuc (cyto), pSV40-ELuc (cyto)::PEST, and pSV40-ELuc (cyto)::CAPN3 were independently transfected into NIH3T3 cells seeded in 96-well plates. One day after transfection, the culture medium was replaced with DMEM supplemented with 10% FBS, 200 μM D-luciferin, and 100 μM cycloheximide. Bioluminescence was recorded in real time for 10 s at 10-min intervals for 9.6 h at 37 °C. The maximum peak values were set to 100%. Error bars indicate the standard deviation ($n = 4$). (b) Western blot analysis of unmodified ELuc, PEST-fused ELuc, and CAPN3-fused ELuc in NIH3T3 cells. The expression plasmids were transfected into NIH3T3 cells, and incubation was carried out for two days. The culture medium was replaced with DMEM supplemented with 10% FBS and 100 μM cycloheximide, and incubation was carried out for 0, 2, and 4 h. Luciferase was detected using the anti-ELuc antibody. Tubulin was used as the internal control. The positions of molecular weight markers are indicated on the left of each panel.

intensity of N1-fused ELuc were severely reduced (0.57 h and 1.6% of PEST-fused ELuc (light intensity of PEST-fused ELuc was set to 100%), respectively), making it inappropriate for use in the reporter gene assay due to insufficient light output. We therefore chose the C1 fragment (half-life and light intensity were 1.83 h and 37.0%, respectively) to optimize the minimum sequence. The C1 fragment was further divided into two fragments (C2 and C3). The half-life of C3-fused ELuc was shorter (1.18 h) than that of C2-fused ELuc (2.55 h). Although the C2 fragment was further divided into three fragments (C4, C5, and C6), the half-lives of ELuc fused to those fragments were longer than that of C2-fused ELuc, indicating that an effective destabilization sequence does not exist in the C2 fragment and the sequence is included in the C3 fragment. Next, the C3 fragment was divided into three fragments (C7, C8, and C9 fragments). The half-lives of ELuc fused to the C7 and C8 fragments were longer (6.10 and 7.83 h for C7 and C8 fragments, respectively), whereas that of C9-fused ELuc was slightly shorter (0.99 h) than the half-life of the original C3-fused ELuc. To minimize the amino acid sequence of the C9 fragment (42 amino acid residues, Figs. 2b and S3), the C9 fragment was further divided into two fragments (C10 and C11), but the half-lives of ELuc fused to those fragments were longer than that of C9-fused ELuc. Finally, we made the C12 fragment by deleting the N-terminal ten residues of C9, but the half-life of C12-fused ELuc was also longer. Correlations between the half-lives and the light intensities indicate that the total amount of ELuc protein existing in cells is affected by the half-lives (Fig. S5).

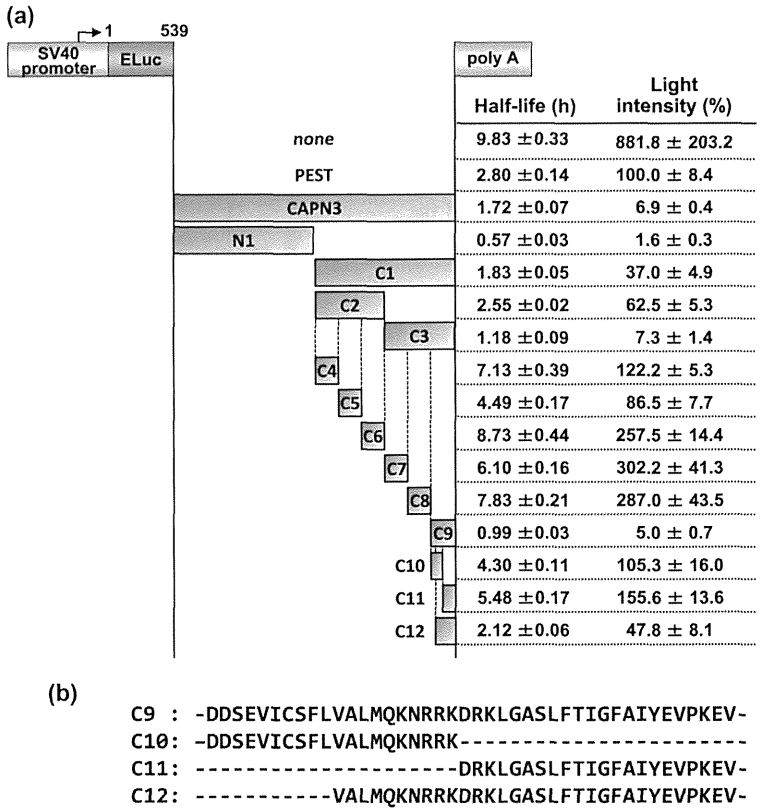

Fig. 2. Half-lives and light intensities of ELuc destabilized by fusing CAPN3 fragments. (a) Half-lives and light intensities of CAPN3-fused ELuc. Transfection and bioluminescence measurements were performed as described in the legend of Fig. 1 (see also Section 2). Light intensity was calculated from the integrated values of each count, and the light intensity of PEST-fused ELuc of that was set to 100%. Values are expressed as means ± standard deviation ($n = 4$). Schematic representation of expression plasmids is shown on the left. CAPN3 and its fragments are shown between ELuc and polyA, where N and C indicate N- and C-termini, respectively, and digits indicate fragment number. (b) Deduced amino acid sequences of C9, C10, C11, and C12 fragments of CAPN3.

To confirm whether the rapid reduction of the light intensity of ELuc by fusion to the C9 fragment was accompanied by the degradation of the ELuc protein in the cells, we performed western blot analysis using anti-ELuc. As shown in Fig. 3a, the immunoreactive bands corresponding to C9-fused ELuc became thinner over time after the cells were treated with cycloheximide (left panel), and its degradation kinetics (right panel) was similar to the luminescence decay kinetics (Fig. 3b), indicating that the ELuc protein was destabilized by fusion to the C9 fragment. We decided, therefore, that the C9 fragment, which consists of 42 amino acid residues corresponding to the amino acid residues from 472 to 512 of hCAPN3 (GenBank accession number AF209502), except for valine at the extreme C-terminus of C9 (Fig. S1), is the minimum destabilization sequence, and used it in the following experiments.

3.3. *Destabilization of firefly luciferase and green fluorescent protein by C9 fragment*

To confirm whether the C9 fragment identified as the minimum destabilization sequence of CAPN3 could destabilize other reporter proteins in the cells, we fused the C9 fragment to the C-terminus of firefly luciferase (*luc2*), the most popular luciferase reporter gene, and EGFP. First, we compared the functional half-lives of hPEST-fused and C9-fused *luc2* in NIH3T3 cells. An expression plasmid was transfected into NIH3T3 cells, and bioluminescence intensity was measured in real time in the presence of cycloheximide. As shown in Fig. 4a, the half-life of C9-fused *luc2* (0.18 h) was markedly short compared with that of hPEST-fused *luc2* (1.62 h).

Next, we examined the stability of unmodified and C9-fused EGFP in NIH3T3 cells. An expression plasmid was transfected into the cells, and the cells were incubated in the presence of cycloheximide for 4 h. EGFP protein levels were analyzed by western blotting using anti-EGFP antibody. As shown in Fig. 4b, whereas the immunoreactive bands corresponding to unmodified EGFP showed no changes, the bands corresponding to C9-fused EGFP became thinner over time after the cells were treated with cycloheximide. In addition, the amount of residual C9-fused EGFP protein in the cells at time 0 was much smaller than that of the unmodified EGFP, indicating that EGFP can be significantly destabilized by fusing the C9 fragment in the cells. The results together suggest that C9 fragment identified in this study can destabilize not only luciferase proteins but also other reporter proteins, including fluorescent proteins.

3.4. *C9 fragment of CAPN3 improves sensitivity of luciferase assay for acute gene expression*

To examine whether the C9 fragment of CAPN3 can monitor acute gene expression more sensitively than the PEST sequence when fused to ELuc, we examined the TNFα-induced transactivation of NF-κB, a transcription factor involved in the regulation of inflammation, immune response, and apoptosis (Tornatore et al., 2012), by means of real-time bioluminescence measurements

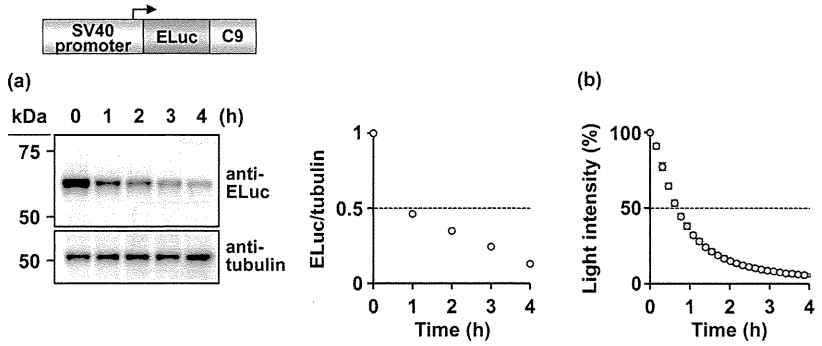

Fig. 3. Rapid decay of protein amount and activity of CAPN3-C9-fused ELuc in NIH3T3 cells. Expression plasmid SV40-ELuc::CAPN3-C9 was transfected into NIH3T3 cells. One day after transfection, the medium was replaced with DMEM supplemented with 10% FBS, 200 μM D-luciferin, and 100 μM cycloheximide. (a) Western blot analysis (left panel) and quantified data (right panel) of CAPN3-C9-fused ELuc. The transfected cells were harvested 0, 1, 2, 3, and 4 h after the medium change. Luciferase was detected using anti-ELuc antibody. Tubulin was used as the internal control. The positions of molecular weight markers are indicated on the left of the panel. To quantify the change in protein amount, the intensity of the bands for ELuc was normalized to that for tubulin, and time 0 was set to 1. (b) Functional half-life of CAPN3-C9-fused ELuc (also shown in Fig. S4). Schematic drawing of the plasmid is shown at the top.

(Fig. 5). NF-κB exists in an inactive form in the cytoplasm due to its interaction with IκB. After activation of the IκB kinase by TNFα, IκB is phosphorylated, ubiquitinated, and degraded. The dissociated NF-κB becomes free to translocate to the nucleus where it acutely activates specific target genes through selective binding to the NF-κB response element (Ling and Kumar, 2012).

To verify the effects of destabilization sequences on the sensitivity of the luciferase reporter assay using unmodified or destabilized ELuc, we inserted reporter plasmids into a multi-integrase human artificial chromosome (MI-HAC) vector (Yamaguchi et al., 2011) (Fig. 5a), which was generated by deleting all of the endogenous genes of human chromosome 21, and was stably maintained as a single copy in the host cells (Katoh et al., 2004; Kazuki et al., 2011).

Reporter plasmids carrying unmodified or destabilized ELuc under control of six tandem repeats of the NF-κB response element and the TK promoter were inserted into the φC31 attB site of the MI-HAC vector in mouse fibroblast A9 cells, in which the expression cassette was flanked by a tandem repeat of the HS4 insulator to prevent promoter interference (Fig. 5a). The site-specific insertion of the reporter plasmids into the MI-HAC vector was verified by genomic PCR (Fig. S6a) and FISH analysis (Fig. 5b). In the FISH analysis, signals from biotin-labeled reporter plasmids (green) were observed only on the MI-HAC vector that was labeled with digoxigenin (red), and not on the chromosome stained with DAPI (blue). Then, we examined again the half-lives of unmodified, PEST-fused, and C9-fused ELuc. Those luciferases were inserted into the MI-HAC vector in A9 cells (Fig. S6b). Consistent with their half-lives in NIH3T3 cells (Fig. 2a), we confirmed that C9-fused ELuc displayed the shortest half-life (0.43 h) compared with unmodified ELuc (5.90 h) and PEST-fused ELuc (1.48 h). The half-lives of all the luciferases in A9 cells were approximately half of those in NIH3T3 cells in which luciferase genes were introduced by transient transfection. We assume that those differences are due to differences in the plasmid copy number and the amount of luciferase expressed in the cells, because one copy of the luciferase gene is kept in the MI-HAC vector in A9 cells, whereas it was maintained at a high copy number in NIH3T3 cells.

The A9 stable cell lines carrying the NF-κB reporter plasmid on the MI-HAC vector were transiently treated with TNFα for 15 min, and bioluminescence was recorded in real time. The

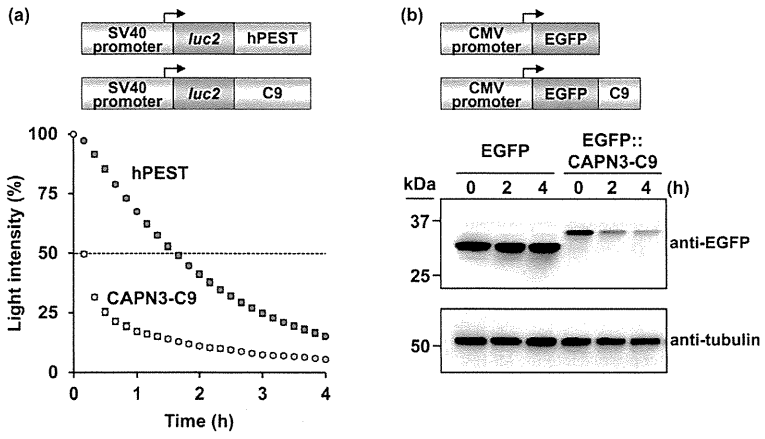

Fig. 4. Destabilization of firefly luciferase (*luc2*) and EGFP by C9 fragment in NIH3T3 cells. (a) Stability of hPEST-fused *luc2*, and CAPN3-C9-fused *luc2* in NIH3T3 cells. Expression plasmid pSV40-luc2::hPEST or pSV40-luc2::CAPN3-C9 was transfected into NIH3T3 cells seeded in 96-well plates. One day after transfection, the culture medium was replaced with DMEM supplemented with 10% FBS, 200 μM D-luciferin, and 100 μM cycloheximide. Bioluminescence was recorded in real time for 10 s at 10-min intervals for 4 h at 37 °C. The maximum peak values were set to 100%. Error bars indicate the standard deviation ($n = 6$). (b) Western blot analysis of unmodified EGFP and CAPN3-C9-fused EGFP in NIH3T3 cells. The expression plasmid pEFGP-C2 or pCMV-EGFP::CAPN3-C9 was transfected into NIH3T3 cells, and incubation was carried out for two days. The culture medium was replaced with DMEM supplemented with 10% FBS and 100 μM cycloheximide, and incubation was carried out for 0, 2, and 4 h. EGFP was detected using the anti-EGFP antibody. Tubulin was used as the internal control. The positions of the molecular weight markers are indicated on the left of each panel. Schematic drawings of the plasmids are shown at the top.

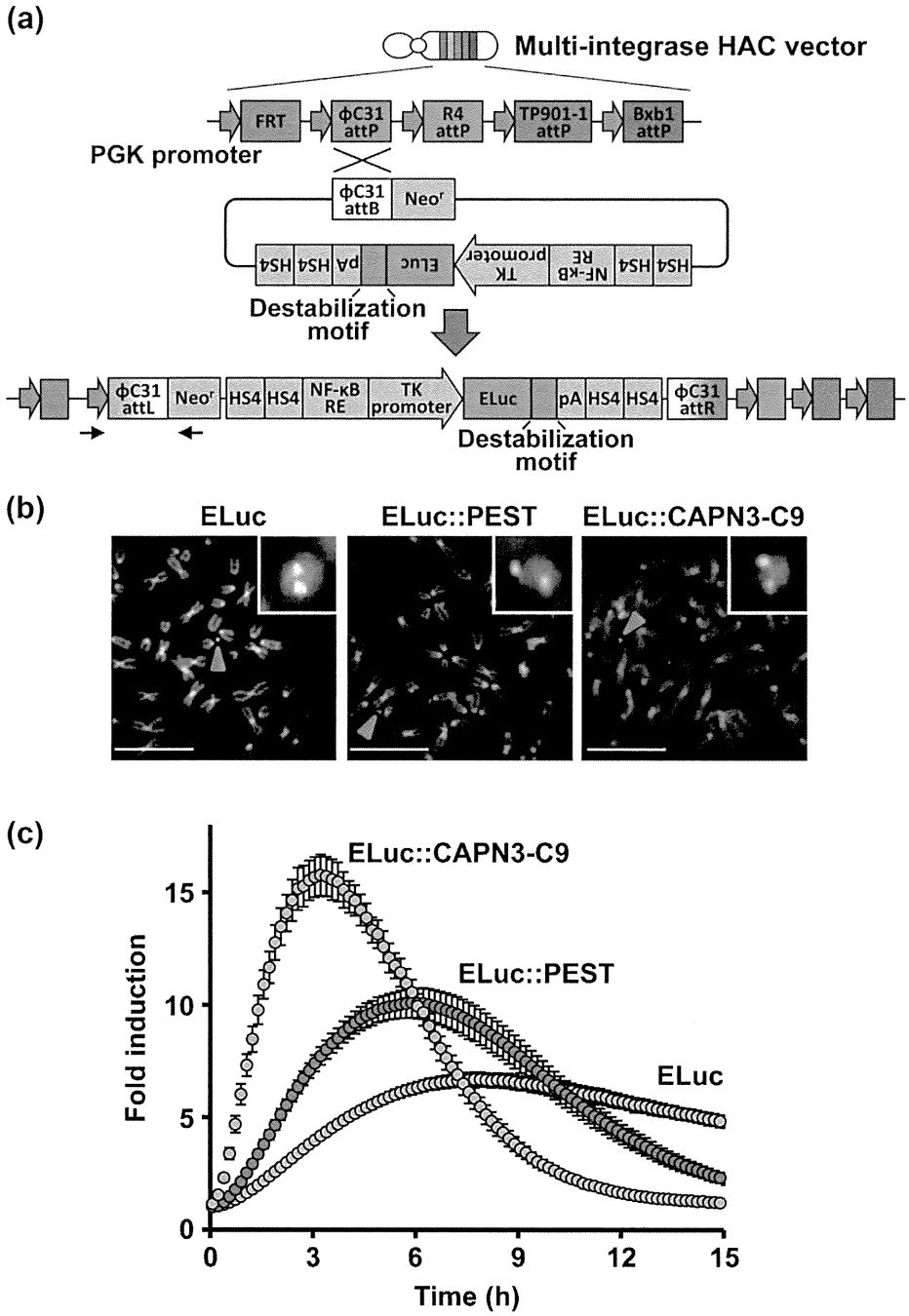

Fig. 5. Real-time bioluminescence recording of TNFα-induced transactivation of NF-κB with destabilized ELuc in A9 cells harboring the MI-HAC vector. (a) Schematic diagram of the site-specific insertion of the NF-κB reporter plasmid into the MI-HAC vector. Schematic diagrams of the MI-HAC vector and the multi-integrase platform are shown on top. NF-κB reporter plasmid was inserted into the φC31 attP site of the MI-HAC vector by φC31 recombinase-mediated homologous recombination in A9 cells. Neo^r, HS4, and NF-κB represent neomycin resistance gene, HS4 insulator, and six tandem repeats of the NF-κB response element, respectively. Arrowheads indicate primers used for genomic PCR to verify insertion of the reporter plasmid into the MI-HAC vector. (b) FISH analysis of A9 cells harboring the MI-HAC vector into which NFκB-ELuc (left panel), NFκB-ELuc::PEST (middle panel), and NFκB-ELuc::CAPN3-C9 (right panel) reporter plasmids were inserted. The A9 cells during metaphase were probed with digoxigenin-labeled human COT-1 (red) that hybridized with the HAC vector, and the biotin-labeled NF-κB reporter plasmid (green). Arrowheads indicate the HAC vector, and insets show enlarged images. Chromosome DNA was counter-stained with DAPI (scale bar, 10 μm). (c) Real-time bioluminescence recording of TNFα-induced transactivation of NF-κB, monitored by unmodified ELuc (green circles), PEST-fused ELuc (blue circles), and CAPN3-C9-fused ELuc (orange circles). The A9 cells harboring the MI-HAC vector into which the NF-κB reporter plasmid was integrated were seeded in 35-mm dishes, and treated with 10 ng/ml TNFα for 15 min. Then, the medium was replaced with DMEM without phenol red supplemented with 10% FBS and 200 μM D-luciferin. Bioluminescence was recorded in real time for 1 min at 10-min intervals for 15 h. Counts of TNFα-treated cells were divided by that of untreated cells, and the initial values (time = 0) were set to 1. Error bars indicate standard deviation ($n = 4$).

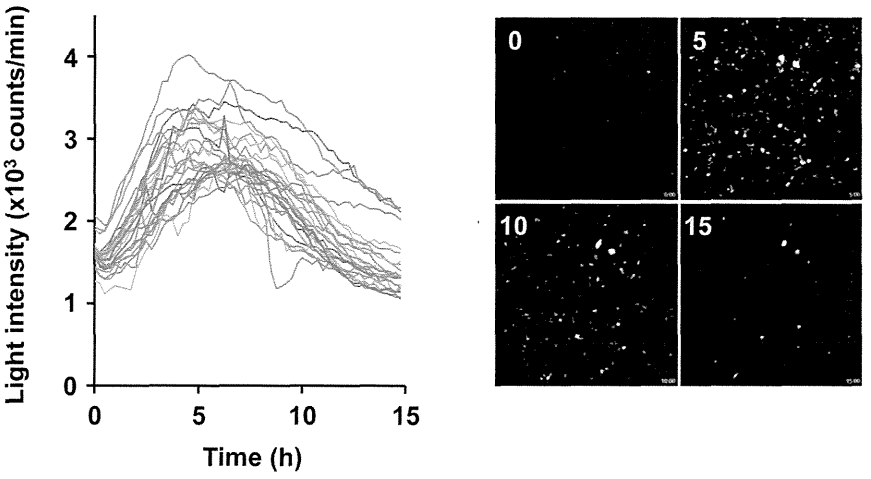

Fig. 6. Time-lapse bioluminescence imaging of TNFα-induced transactivation of NF-κB in A9 cells using CAPN3-C9-fused ELuc. A9 cells harboring the MI-HAC vector into which the NF-κB-ELuc::CAPN3-C9 reporter plasmid was integrated were seeded in 35-mm glass-bottom dishes, and treated with 10 ng/ml TNFα for 15 min. Then, the medium was replaced with DMEM supplemented with 10% FBS and 500 μM D-luciferin. CCD images were acquired with a 10-min exposure time at 15-min intervals for 15 h with a 4× objective lens. Recordings of luminescence from 25 cells and serial CCD images at 5-h intervals are shown on left and right panels, respectively.

time-dependent change of NF-κB activation was expressed as fold induction where bioluminescence intensity of the TNFα-treated cells was normalized to that of the TNFα-untreated cells at each time point. As shown in Fig. 5c, the NF-κB activation by TNFα monitored by unmodified ELuc was slowly increased and reached a maximum (6.69 ± 0.32-fold, mean ± SD) at 7.68 ± 0.10 h. This was followed by a very slow decay owing to residual luciferase protein in the cells (green circles). The fold induction and the peak time monitored by PEST-fused ELuc were 10.01 ± 0.66-fold and 5.11 ± 0.22 h, respectively (blue circles), indicating more sensitive monitoring of the activation than the unmodified ELuc. As expected, the most rapid activation and the subsequent rapid decay of bioluminescence could be monitored by using ELuc fused to the CAPN3 C9 fragment, and the fold induction and the peak time were 15.78 ± 0.92-fold and 3.47 ± 0.18 h, respectively (orange circles). Although the light intensity of C9-fused ELuc was 1.0% of that of PEST-fused ELuc (Fig. S6c), we were able to reliably quantify the activation kinetics of NF-κB, demonstrating that the C9 fragment is a suitable destabilization sequence for monitoring gene expression. Our results indicate that the CAPN3 C9 fragment identified in this study acts as rapid degradation signal compared with the PEST sequence of ornithine decarboxylase, enabling highly sensitive monitoring of cellular response using the luciferase reporter gene.

3.5. Time-lapse single-cell bioluminescence imaging using CAPN3-C9-fused ELuc

Recent advances in luciferase technology have enabled the quantitative visualization of gene expression at single-cell resolution by monitoring luminescence in real time using a highly sensitive CCD camera (Nakajima et al., 2010; Roda et al., 2009; Welsh et al., 2010). Although fluorescence imaging techniques that use fluorescent proteins as probes have contributed immensely to the advancement of cell biology and are used as powerful probes to monitor an extensive array of entities, bioluminescence imaging is rapidly emerging as a new and quantitative approach to understanding cell physiology. We therefore verify whether bioluminescence imaging can be performed using C9-fused ELuc. As shown in Fig. 5c, A9 cells carrying a cassette consisting of NF-κB response element, TK promoter, and C9-fused ELuc on the MI-HAC vector were transiently treated with TNFα for 15 min. CCD images were captured for 10 min at 15-min intervals for 15 h using a luminescence microscope, and light intensities from 25 cells were quantified. As shown in Fig. 6, after treatment with TNFα, the luminescence intensities rapidly increased, reached a maximum, and subsequently decreased, and this tendency was similar to what was observed in the photomultiplier recordings shown in Fig. 5c. The peak times of the 25 quantified cells varied from 3 to 8 h, which might be due to differences in sensitivity to TNFα of the cells. Thus, we were able to clearly capture and quantify acute gene expression in a cell by means of time-lapse bioluminescence imaging with C9-fused ELuc.

4. Discussion

We have identified a destabilization sequence consisting of 42 amino acid residues from the C9 fragment of hCAPN3 (Fig. 2b), and succeeded in destabilizing two luciferases, ELuc and *luc2*, in the cells by fusing the C9 fragment (Figs. 2a and 4a). Moreover, we have demonstrated that a highly sensitive luciferase reporter assay could be achieved by using C9-fused ELuc rather than PEST-fused ELuc, in which acute gene expression was tracked by means of real-time bioluminescence recording and single-cell bioluminescence imaging (Figs. 5c and 6). Although the light intensity of C9-fused ELuc was reduced to less than 1% of the light intensity of unmodified ELuc, the light intensity is enough to produce reliable results, and the speed and magnitude of the activation kinetics of NF-κB could be monitored with high sensitivity using the C9-fused ELuc.

The PEST sequence from ornithine decarboxylase is widely used to reduce the stability of reporter gene in cells. Previous studies have demonstrated that the degradation rate of firefly luciferase, the most typical beetle luciferase reporter gene, is increased by 3–4-fold by fusing PEST in T-47D cells (Leclerc et al., 2000) and HeLa cells (Voon et al., 2005), being consistent with our study in which measurements were carried out in NIH3T3 (Fig. 2) and A9 (Fig. S6b) cells using ELuc. An even more rapid destabilization of firefly luciferase was accomplished by the combined use of PEST and RNA degradation signal, which resulted in an approximately 6-fold higher degradation rate than that of wild-type luciferase in HeLa cells (Voon et al., 2005). As shown in Figs. 2a and S6b, the degradation rates of ELuc fused to the C9 fragment were 10- and

14-fold of that of unmodified ELuc in NIH3T3 and A9 cells, respectively. Although we cannot make a direct comparison because the luciferases, cells, and measurement systems used were not identical between this study and previous studies, it may be reasonable to assume that the C9 fragment is a much more potent destabilization sequence that reduces the stability of luciferase in the cells than the combination of PEST and RNA degradation signal.

Western blot analysis demonstrated that C9-fused ELuc is rapidly reduced in cells in the presence of the protein synthesis inhibitor cycloheximide (Fig. 3a), indicating that the loss of luminescence intensity by fusing the C9 fragment is due not to interference with the luciferin-luciferase reaction, but to the acceleration of the proteolytic degradation of luciferase in the cells. CAPN3, which has four domains, is a calcium- or sodium-dependent cysteine protease, and its half-life is very short due to autoproteolytic degradation (Sorimachi et al., 2011). In this study, the identified C9 fragment is a partial sequence of domain III. Domain II, or the so-called autolytic activation domain, plays a role in the autoproteolysis of CAPN3, whereas domain III, or the so-called C2-like domain, participates in the binding of calcium and phospholipid. Both are required for promoting calpain activation, but are not directly involved in autolysis (Duguez et al., 2006). Thus, even though the C9 fragment dramatically reduces the stability of luciferase, further study is needed to elucidate the mechanism of the induction of the rapid degradation of luciferase in the cells by fusing the C9 fragment.

To verify the effects of PEST and C9 fragment on the sensitivity of the luciferase reporter assay, we used stable A9 cells harboring the MI-HAC vector, in which a reporter plasmid was inserted into the target site of the vector. Although the transient transfection of reporter gene is conventionally used to introduce a reporter gene into the host cells and to monitor cellular response, heterogeneities in the transfection efficiency and the plasmid copy number among the transfected cells sometimes result in differences in the amount of expressed reporter protein, and consequently differences in cellular response measured by the reporter. Even though stable cell lines can be generated by the conventional method in which a reporter gene is randomly integrated into the chromosome of host cells, it is impossible to avoid the positional effect and to regulate the copy number of the reporter gene. To resolve these issues, we inserted a reporter plasmid into a specific site of the MI-HAC vector, where both copy number and integration site of the reporter plasmid could be accurately regulated. By doing so, the responsiveness of cells monitored by luciferases having different stabilities in the cells could be precisely compared. Thus, the MI-HAC vector is useful not only for carrying transgene as a reporter vector but also for evaluating the characteristic properties of luciferase in the cells.

We also found that the C9 fragment destabilized EGFP after its fusion to the C-terminus of EGFP in NIH3T3 cells, as demonstrated by immunoblot analysis using anti-EGFP antibody (Fig. 4b). Together, the results demonstrate that the C9 fragment is broadly applicable to not only the luciferase reporter assay, including real-time recording and bioluminescence imaging, but also fluorescence imaging. Thus, the destabilization sequence identified in this study should be useful for a broad range of sensitive reporter assays that require measurement of acute response kinetics in the cells, including cell-based and cell-chip assays.

Disclosure statement

Patent registration for this work is under way.

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.jbiotec.2014.12.004.

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