Journal of Biotechnology 194 (2015) 115–123

Contents lists available at ScienceDirect

Journal of Biotechnology

journal homepage: www.elsevier.com/locate/jbiotec

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

Mayu Yasunaga[a], Kazutoshi Murotomi[a], Hiroko Abe[a], Tomomi Yamazaki[b], Shigeaki Nishii[b], Tetsuya Ohbayashi[c], Mitsuo Oshimura[d,e], Takako Noguchi[f], Kazuki Niwa[g], Yoshihiro Ohmiya[h], Yoshihiro Nakajima[a,*]

[a] Health Research Institute, National Institute of Advanced Industrial Science and Technology (AIST), Takamatsu, Kagawa 761-0395, Japan
[b] Tsuruga Institute of Biotechnology, TOYOBO Corporation Ltd., Tsuruga, Fukui 914-0047, Japan
[c] Division of Functional Genomics, Research Center for Bioscience and Technology, Tottori University, Yonago, Tottori 683-8503, Japan
[d] Chromosome Engineering Research Center, Tottori University, Yonago, Tottori 683-8503, Japan
[e] Department of Biomedical Science, Institute of Regenerative Medicine and Biofunction, Graduate School of Medical Sciences, Tottori University, Yonago, Tottori 683-8503, Japan
[f] Department of Psychiatry and Center for Circadian Biology, University of California, San Diego, La Jolla, CA 92093-0603, USA
[g] National Metrology Institute of Japan (NMIJ), National Institute of Advanced Industrial Science and Technology (AIST), Tsukuba, Ibaraki 305-8063, Japan
[h] DAILAB, Biomedical Research Institute, National Institute of Advanced Industrial Science and Technology (AIST), Tsukuba, Ibaraki 305-8063, Japan

ARTICLE INFO

Article history:
Received 13 July 2014
Received in revised form
18 November 2014
Accepted 10 December 2014
Available online 18 December 2014

Keywords:
Luciferase
Destabilization sequence
Calpain 3
Real-time monitoring

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.

* Corresponding author. Tel.: +81 87 869 3525; fax: +81 87 869 4178.
E-mail address: y-nakajima@aist.go.jp (Y. Nakajima).

http://dx.doi.org/10.1016/j.jbiotec.2014.12.004
0168-1656/

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 *NcoI* and *XbaI* 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