FEBS Letters 581 (2007) 4551–4556

Real-time bioluminescence imaging of a protein secretory pathway in living mammalian cells using *Gaussia* luciferase

Takahiro Suzuki[a,1], Shinsuke Usuda[a], Hiroshi Ichinose[a], Satoshi Inouye[b,*]

[a] *Faculty of Bioscience and Biotechnology, Tokyo Institute of Technology, Yokohama 226-8501, Japan*
[b] *Yokohama Research Center, Chisso Corporation, Yokohama 236-8605, Japan*

Received 5 July 2007; revised 20 August 2007; accepted 20 August 2007

Available online 27 August 2007

Edited by Peter Brzezinski

Abstract **Using photon counting and charge-coupled device (CCD) cameras, we have applied the method of real-time bioluminescence imaging to investigate protein trafficking in mammalian cells. In the living cells of Chinese hamster ovary and PC12D cells, exocytotic secretion of protein and protein targeting on the cell surface were visualized using the secreted *Gaussia* luciferase (GLase) as a reporter protein in a minute. After incubation of the cells with luciferin (coelenterazine) for 10 min, luciferin was imported into the cells and the vesicle transport network in the cells could be shown by luminescence images of GLase activity. Further, we demonstrate that GLase with a heterologous signal peptide sequence is targeted to the cell surface in neuronally differentiated PC12D cells and luminescence signals could be detected in a few seconds.**

Keywords: Coelenterazine; Secretion; Protein targeting; Protein refolding

1. Introduction

Light emission produced by an enzymatic reaction of a luciferase (enzyme) with a luciferin (substrate) is known as bioluminescence and has been used to monitor cellular events such as the regulation of gene expression. Recent advances in photon detection devices have accelerated the progress of biological imaging and the resulting real-time images will give the valuable information on biological functions [1]. The methods of imaging based on a luciferase-luciferin reaction are simple, sensitive and convenient, and have been applied to tumor imaging in mice with firefly luciferase and *Renilla* luciferase as reporters [2,3]. Previously, we visualized the site-specific protein secretion from Chinese hamster ovary (CHO) cells using a secreted luminescent reporter enzyme (527 aa; 59.5 kDa), *Vargula* luciferase [4]. The secretion of *Vargula* luciferase from a live mouse preimplantation embryo was also visualized with an image intensifying system [5].

The marine copepod *Gaussia princeps* secretes a luciferase that catalyzes the oxidation of luciferin (coelenterazine; CTZ) to emit blue light (λ_{max} = ~480 nm, Fig. 1A). The cDNA of secreted *Gaussia* luciferase (GLase) was cloned and the luciferase consists of 185 amino acid residues (19.9 kDa) including a putative signal peptide sequence for secretion [6]. Thus, the luminescence reaction of GLase is simple and is a good candidate for a reporter protein in real-time imaging of the protein secretion.

To characterize protein trafficking from inside to outside of living mammalian cells, a smaller secreted GLase was introduced and the secretory pathway including protein targeting on the cell surface was visualized.

2. Materials and methods

2.1. Plasmids

The codon-optimized gene of GLase (GLuc), pcDNA3-GLuc (Prolume Ltd., Pinetop, AZ) with the signal peptide sequence (sp), was used for expression in mammalian cells. To construct the expression vector of GLase lacking the signal peptide sequence (ΔSP-GLuc), a *Bam*HI-*Not*I fragment was obtained from pcDNA3-GLuc by PCR using primer GL1 (5′-GGATCC AGCCACC ATG AAG CCC ACC GAG AAC AAC GAA-3′) and primer GL2 (5′-GCGGCCGC TTA GTC ACC AC-3′), cloned into a blunt end-cloning vector, and then the fragment was replaced with the *Bam*HI-*Not*I fragment in pcDNA3-GLuc, to give pcDNA3-ΔSP-GLuc. For expression of GLase with the signal peptide sequence of human DBH, a vector pcDNA3-DBHsp-GLuc was constructed by replacement with the signal peptide sequence in pcDNA3-GLuc as follows; a new *Eco*RI site was introduced into the *Bam*HI-*Not*I region of pcDNA3-GLuc by PCR using primer GL2 and primer GL3 (5′-GGATCC A GAATTC AAG CCC ACC GAG AAC AAC GAA-3′, *Eco*RI site underlined) to give pcDNA3-GLuc-BE having the *Bam*HI-*Eco*RI site in pcDNA3-ΔSP-GLuc. The *Bam*HI-*Eco*RI fragment containing the signal peptide sequence of hDBH from pBluescript KS (+)-hDBH [7] was obtained by PCR using a primer set of primer DBH1 (5′-GGATCC AGCCACC ATG CCC GCC CTC AGT CGC TGG GCC AGC C-3′) and primer DBH2 (5′-GGATCC A GAATTC AAG CCC ACC GAG AAC AAC GAA-3′) and inserted into *Bam*HI-*Eco*RI sites in pcDNA3-GLuc-BE to give pcDNA3-DBHsp-GLuc (DBH-GLuc).

2.2. Cell culture and transfection

CHO-K1 cells transfected with the GLuc expression vectors using Polyfect (Qiagen, Hilden, Germany) were cultured in α-MEM (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum (Equitech-Bio. Inc., Kerrville, TX). To establish the stable transformant in CHO-K1 cell line expressing GLuc, cells were selected in G418 (800 μg/ml, Invitrogen). A clonal transformant showing the highest luminescence (CHO-K1-GLuc cells; clone #4) was used. PC12D

*Corresponding author. Fax: +81 45 786 5512.
E-mail address: sinouye@chisso.co.jp (S. Inouye).

[1]Present address: Department of Biochemistry, School of Dentistry, Aichi-Gakuin University, Nagoya 464-8650, Japan.

Abbreviations: GLase, *Gaussia* luciferase; CTZ, coelenterazine; GLuc, the codon-optimized gene of GLase; CCD, charge-coupled device; CHO cell, Chinese hamster ovary cell; DBH, dopamine β-hydroxylase

0014-5793/$32.00
doi:10.1016/j.febslet.2007.08.036

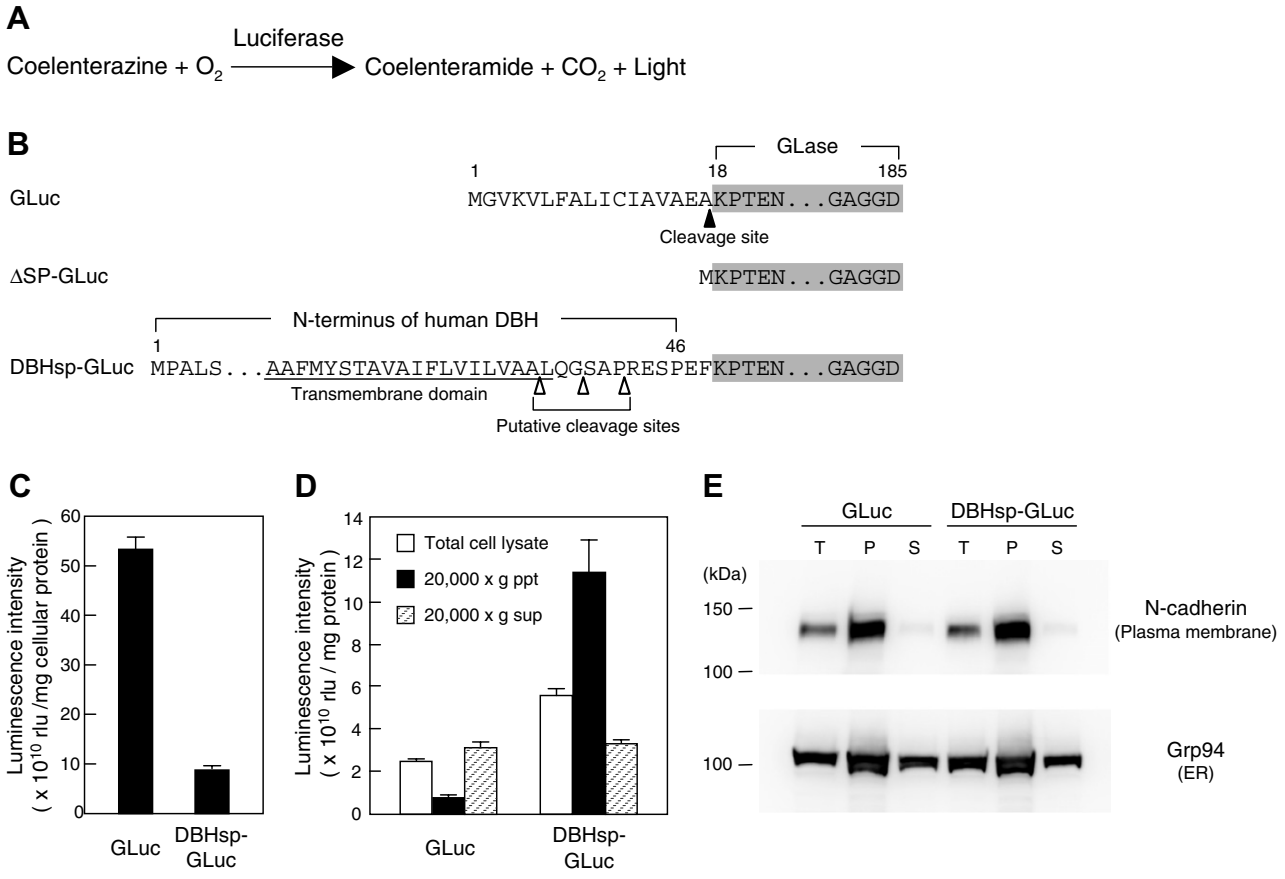

Fig. 1. Characterization of GLase expressed in mammalian cells. (A) Luminescence reaction of GLase with coelenterazine (CTZ). (B) A schematic representation of various GLuc expression vectors. GLuc (pcDNA3-GLuc); the signal peptide sequence with GLase, ΔSP-GLuc (pcDNA3-ΔSP-GLuc); lacking of the signal peptide sequence of GLase, DBHsp-GLuc (pcDNA3-DBHsp-GLuc); GLase fused to N-terminal sequence of human DBH. The transmembrane domain of DBH is underlined and putative cleavage sites are assigned from previous report of bovine DBH [9]. (C) GLase activity in the conditioned medium of NGF-differentiated PC12D cells. After transfection with GLuc or DBHsp-GLuc for 24 h, the luminescence activity in the conditioned medium was determined. (D) Subcellular fractionation of GLase expressed in cells. Cells used are the same as in C. (E) Western blot analysis of subcellular fractions using anti-*N*-cadherin and anti-Grp94 antibodies. *T*; total cell lysate, *P*; 20000 × *g* ppt, S; 20000 × *g* sup in D.

cells were cultured in DMEM (Invitrogen) supplemented with 5% fetal bovine serum and 10% horse serum (Sigma, St. Louis, MO) and were transfected with the vectors using LipofectAMINE2000 (Invitrogen) on Falcon poly-D-lysine coated dishes (BD Bioscience, Bedford, MA). After transfection, PC12D cells were immediately treated with rat recombinant β-NGF (50 ng/ml, Sigma) for differentiation.

2.3. Measurement of GLase activity using a luminometer

For measurement of luminescence intensity, an aliquot of the conditioned medium or cell lysate was added into 0.2 ml of 20 mM Tris–HCl (pH7.4)/150 mM NaCl containing coelenterazine (CTZ: 5 μg/ml, Chisso Co., Tokyo, Japan) and luminescence intensity was immediately measured with a Lumat LB9507 luminometer (Berthold, Bad Wildbad, Germany).

2.4. Preparation of conditioned medium and cell lysate from cells expressing GLase

The conditioned medium was collected by centrifugation at 300 × *g* for 5 min at 4 °C to remove detached cells. For preparing cell lysate, cells were washed twice and scraped in ice-cooled PBS containing a protease inhibitor cocktail (Sigma) and was disrupted by sonication for 5 s at 4 °C. After centrifugation at 300 × *g* for 10 min, the supernatant was used as the total cell lysate. For further fractionation, the cell lysate was centrifuged at 20000 × *g* for 10 min at 4 °C. Protein concentration was determined by the method of Bradford using a protein assay kit (Bio-Rad, Hercules, CA) with bovine γ-globulin as a standard. To determine subcellular contents in fractions, Western blot analysis was performed using anti-*N*-cadherin (Sigma) and anti-Grp94 (Stressgen, Ann Arbor, MI) antibodies as markers for plasma membrane and ER lumen, respectively. Briefly, proteins (7 μg) were separated by SDS–PAGE (4–20% gel, Daiichi Pure Chemicals, Tokyo, Japan) under reducing conditions and then transferred to a PVDF membrane (Bio-Rad). After incubation with HRP-conjugated antibodies (GE Healthcare UK Ltd), proteins on the membrane were visualized with an Immobilon Western blotting kit (Millipore, Billerica, MA) and a LAS-3000 mini image analyzer (Fuji film, Tokyo, Japan).

2.5. Bioluminescence imaging

In imaging experiments, the cells were cultured on a 35 mm glass-bottom dish (Mat-Tek Co., Ashland, MA). To obtain the luminescence image, the cells were washed twice with 3 ml of PBS and then were soaked with 1 ml of serum-free culture medium containing 20 mM HEPES (pH 7.4) and CTZ (1 μg/ml). Luminescence signals were taken at room temperature (25–30 °C) using a model IX71 microscope (Olympus Co., Tokyo, Japan), equipped with a VIM photon-counting camera (model C2741-35A; Hamamatsu photonics, K.K., Japan) or an ORCAII-ER-1394 CCD camera (model C4742-98-24ERG; Hamamatsu photonics) in a dark box. Luminescence signals were processed using AQUACOSMOS software version 2.6 (Hamamatsu photonics) and converted to pseudo-colored images.