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Bioluminescent technologies to detect calicivirus protease activity in cell-free system and in infected cells

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\textbf{A B S T R A C T}

Feline calicivirus (FCV) is an important veterinary pathogen and causes respiratory disease in cats. Because it grows well in cell culture, FCV is often used as a model virus of non-culturable caliciviruses. In this study, a cell-free and two cell culture-based biosensor assay systems were established to detect FCV protease activity. The assays utilize luciferase sensor technology or second-generation bioluminescence resonance energy transfer (BRET\textsuperscript{2}). A luciferase sensor was designed to contain an FCV protease cleavage motif within the permuted luciferase (GloSensor). The BRET\textsuperscript{2}-based probe contained the same cleavage motif flanked by a renilla luciferase and a variant of green fluorescent protein. To confirm the specificity of these assay systems, GloSensor or a BRET\textsuperscript{2}-based probe containing a mutation in the cleavage motif was also constructed. In a cell-free assay, GloSensor showed increased luminescence in proportion to the amount of FCV protease, while no signal change was observed when the construct harboring the mutant cleavage motif was used. A feline cell line stably expressing GloSensor or the BRET\textsuperscript{2}-based probe was established. Increased levels of GloSensor luminescence, and decreased levels of BRET\textsuperscript{2} signals were observed according to input FCV titers. In contrast, no significant signal change was observed in the cells stably expressing the mutant cleavage motif. GloSensor and the BRET\textsuperscript{2}-based probe were capable of detecting the inhibitory activity of ribavirin in FCV-infected cells. Our results demonstrate that these biosensors are useful to detect FCV protease activity induced in infected cells, and well worth consideration for screening of anti-FCV protease compounds in cell-free system as well as anti-FCV compounds in cultured cells.

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1. Introduction

Feline caliciviruses (FCV), a member of the genus Vesivirus in the family Caliciviridae, is an important pathogen and cause upper respiratory tract diseases in cats. Prevalence of FCV was 29.4\% in clinically diseased cats (Abd-Eldaim et al., 2009), and 43.1\% in respiratory tract diseases in cats. Prevalence of FCV was 29.4\% in cat population of a rescue shelter (Zicola et al., 2009). At present, vaccination is administered worldwide to prevent FCV infection in pet cats. However, new highly virulent FCV strains that cause systemic hemorrhagic disease and significant mortality up to 67\% have recently been reported (Foley et al., 2006). Current vaccines may not protect against these new strains, highlighting the importance of antivirals (Radford et al., 2007). Sensitive methods for detecting FCV infection would be extremely useful to identify such inhibitors.

The FCV genome encodes three open reading frames (ORFs) of approximately 7.7 kb. The viral protease, encoded in ORF1, is initially synthesized as a part of the ORF1 polyprotein, and responsible for the cleavage among ORF1 proteins to generate functional non-structural proteins. This protease also functions to cleave an ORF2-encoded protein to generate the capsid protein VP1 (Clarke and Lambden, 2000; Sosnovtsev et al., 1998, 2002). The FCV protease cleaves peptide bonds downstream of a specific glutamic acid (E) in the precursor protein (Green, 2007; Oka et al., 2009; Sosnovtsev et al., 1998, 2002). This processing is essential for the formation of mature infectious virus particles (Sosnovtsev et al., 1998, 2002). Therefore, FCV protease is an attractive target to develop anti-FCV inhibitors, and its activity can also be a useful marker of FCV infection and replication in cultured cells. At present, no FCV-specific growth inhibitor has been developed. However,
ribavirin, a broad spectrum anti-virus compound, has been shown to inhibit FCV growth in cultured cells (Belliot et al., 2005; Povey, 1978b).

In the current study, two bioluminescence technologies based on a genetically engineered luciferase (GloSensor) (Fan et al., 2008; Wigdal et al., 2008) and a second-generation bioluminescence resonance energy transfer (BRET$^2$) (Pfleger and Eidne, 2006) were developed to assay FCV protease activity in cell-free system and in infected cells. Furthermore, we evaluated the inhibitory effect of ribavirin in cell-free system and in FCV-infected cells using these systems.

2. Materials and methods

2.1. Virus and cells

FCV F4 strain (GenBank accession no. D31836) isolated from a cat with signs of respiratory tract infection was used throughout the experiments (Makino et al., 2006; Takahashi et al., 1971). Cran-dell Rees feline kidney (CRFK) cells were obtained from the Japanese Collection of Research Bioresources and cultured in Eagle’s minimal essential medium (MEM) (Sigma–Aldrich, St. Louis, MO) supplemented with 5% heat-inactivated fetal bovine serum (Invitrogen, Carlsbad, CA) and antibiotics (100 U/mL penicillin and 100 μg/mL streptomycin) (Invitrogen). The virus was propagated and titrated in CRFK cells with a conventional assay. Briefly, viral suspensions were fivefold serially diluted (5$^{-1}$–5$^{-10}$) in 100 μL of MEM medium, and then added to CRFK culture wells in 96-well plates (total volume of 200 μL/well). Eight wells were used per dilution. Cultures were maintained at 37°C in 5% CO$_2$, and after 3 days, the cytopathic effect was visualized by methylene-blue staining. The 50% cell-culture infectious dose (CCID$_{50}$) was calculated by the Kärber formula (WHO, 2004).

2.2. Construction of the GloSensor plasmid to detect protease activity in vitro

A forward oligonucleotide, 5'-ctagccCGCTTCTCCATGAGGGAGGCGATGGGATGATCATGcG-3' and a reverse oligonucleotide, 5'-gatccGATGGATCCATCATCGGcGcgcCAATCGGAAGAGGGGc-3' were synthesized. These oligonucleotides corresponded to 12 amino acids, PLFRLE/ADDGSI (P6–P6$^\prime$), and a reverse oligonucleotide, 5'-gtatcGATGGATCCATCATCGGgGCGGca-3'. The resulting mixture was incubated with 30 μL of nuclease-free water. The resulting mixture was then added to CRFK culture wells in 96-well plates (total volume of 200 μL/well). Eight wells were used per dilution. Cultures were maintained at 37°C in 5% CO$_2$, and after 3 days, the cytopathic effect was visualized by methylene-blue staining. The 50% cell-culture infectious dose (CCID$_{50}$) was calculated by the Kärber formula (WHO, 2004).

2.3. Construction of the GloSensor plasmid to detect protease activity in FCV-infected cells

To express the GloSensor protein, a substrate to detect protease activity in FCV-infected CRFK cells, the sensor-encoding region was amplified with a forward primer, 5'-CGAGCTGATCATGATACCAATGGACACCGCTATCTC-3', and a reverse primer, 5'-TTATCTAGGCCTCCCGTTAAACCGGGATGATC-3'. The amplified PCR fragment was purified with a QIAquick Gel Extraction Kit (Qiagen, Hilden, Germany) and cloned into an EcoRV–NotI (New England Biolabs, Ipswich, MA) site in the pIRENneo3 vector (TakaraBio Inc., Shiga, Japan) using an In-fusion Advantage PCR Cloning system (TakaraBio Inc.) according to the manufacturer’s instructions. E. coli Fusion-Blue Competent Cells (TakaraBio Inc.) were used for transformation, and the plasmids were purified with a MARIGEN Maxi Plasmid Purification Kit (OriGene USA). The resulting plasmids were designated as CellGlo–FCV-Cut and -Uncut, respectively.

2.4. Generation of the BRET$^2$-based probe

A short DNA molecule encoding the FCV protease cleavage motif was prepared with a forward synthetic oligo, 5'-agcttgCCGCTTCTCCATGAGGGAGGCGATGGGATGATCATGcG-3' and a reverse synthetic oligo, 5'-gtatcGATGGATCCATCATCGGgGCGGca-3', as described above. Another short DNA molecule encoding a mutant FCV protease cleavage motif was prepared similarly with a forward oligo, 5'-agcttgCCGCTTCTCCATGAGGGAGGCGATGGGATGATCATGcG-3' and a reverse oligo, 5'-gtatcGATGGATCCATCATCGGgGCGGca-3'. Each DNA was cloned into the HindIII–BglIII site in pGFP$^2$–MCS–RLuc vector (PerkinElmer, Waltham, MA), an in-frame site of the green fluorescent protein 2 (GFP$^2$) and renilla luciferase (RLuc) ORF, by a Quick Ligation Kit (New England Biolabs). E. coli DH5α-T1 (Invitrogen) was used for transformation and the plasmids were purified with a MARIGEN Maxi Plasmid Purification Kit (OriGene, USA). The fusion protein was placed under the control of the cytomegalovirus (CMV) promoter. The resulting plasmids were referred to as BRET2–FCV-Cut and BRET2–FCV-Uncut. Sequencing analyses were performed to confirm the cleavage motif and its frame between the GFP$^2$ and RLuc encoding region.

2.5. In vitro translation

GloSensor proteins were expressed in an SP6 promotor-coupled transcription–translation system in wheat germ using a TNT SP6 High-Yield Wheat Germ Protein Expression System (Promega) with InvitroGlo–FCV-Cut and -Uncut as a template. A 50 μL aliquot of the reaction mixture including 2 μg of the plasmid was mixed with 30 μL of TNT SP6 High Yield Master Mix (Promega) and nuclease-free water, and incubated at 25°C for 3 h. FCV protease was prepared with in vitro T7 polymerase-coupled transcription–translation in rabbit reticulocytes using the TNT T7 Quick for PCR DNA Kit (Promega). In brief, the DNA fragment corresponding to the entire ORF of the FCV F4 genome was amplified with a forward primer (5'-GATCTAAATCCTCAGTATGGAAAGCCACCATGTTCACACATCCAGTTCG-3'), an antisense primer (5'-TGTTATGATCATTGAGGACGCTACATCAAGGTCGTCATAC-3'), and a template PCR fragment of the pUC19/FCV F4 full-length that contains the full-length FCV F4 cDNA or the plasmid “FCV F4 full-C1193A/ORF1” that encodes inactive protease (Oka et al., 2007) as the template. 5 μL of thePCR mixture, 40 μL of TNT T7 PCR Quick Master Mix (Promega), 1 μL of 1 mM methionine (Promega), and 4 μL of nuclease-free water. The resulting mixture was incubated with GloSensor protein at 30°C for 3 h to achieve in vitro trans cleavage as described previously (Oka et al., 2009).
2.6. Detection of the cleavage signal in cell-free system

To detect the GloSensor signal in cell-free system, 20 μL of the in vitro transcription–translation reaction mixture containing the substrate GloSensor protein, with either the native or mutant FCV protease cleavage motif, was mixed with 20 μL of the in vitro transcription–translation reaction mixture (twofold serial dilutions from 1:4 to 1:1024 with PBS) containing either native or mutant FCV protease. The resultant mixture, which was 40 μL in total was incubated at 30 °C for 1 h. Then 10 μL of the reaction mixture was mixed with 90 μL of PBS in a 96-well plate (ViewPlate black-96; PerkinElmer), and 100 μL of Bright-Glo Luciferase Assay Substrate (Promega) was added to each well. After a 5-min incubation, the luminescent signal was detected for 1 s/well with a 2030 Multilabel Reader ARVO-X3 (PerkinElmer).

2.7. Establishment of biosensor-expressing cells

To establish a stable cell line expressing the GloSensor protein or BRET2-based probe, subconfluent CRFK cells in a six-well plate were transfected with 1 μg of CellGlo-FCV-Cut, CellGlo-FCV-Uncut, BRET2-FCV-Cut, or BRET2-FCV-Uncut plasmid and Effectene transfection reagent (Qiagen) according to the manufacturer’s instructions. After 48 h of transfection, the transfected cells were subjected to G418 (Merk KGaA, Darmstadt, Germany) at 500 μg/mL or Zeocin (Invitrogen) at 62.5 μg/mL, respectively. The G418- or Zeocin-resistant CRFK cell lines were further isolated by limiting dilutions with 96-well plates. Each clone was maintained under the same conditions.

2.8. Detection of the GloSensor signal in infected cells

CRFK cells constitutively expressing CellGlo-FCV-Cut or CellGlo-FCV-Uncut were seeded into 96-well plates (ViewPlate white-96; PerkinElmer) at a density of approximately 5000 cells/well and cultured in 200 μL of medium without G418 for 48 h. The culture medium was then aspirated, and 100 μL of medium containing 10-fold serial diluted FCV ranging from 10^3 to 10^8 CCID50/100 μL, corresponding to approximate multiplicities of infection (MOIs) of 0.1–0.0001 or the culture medium without virus was added. After 20 h of infection, 100 μL of Bright-Glo Luciferase Assay Substrate was added to each well and incubated for 5 min, and then the luminescent signal was measured for 1 s/well with a 2030 Multilabel Reader ARVO-X3 (PerkinElmer).

2.9. Detection of the bioluminescence resonance energy transfer signal in infected cells

CRFK cells constitutively expressing BRET2-FCV-Cut or BRET2-FCV-Uncut protein were seeded into 96-well plates (ViewPlate white-96; PerkinElmer) at a density of approximately 5000 cells/well and cultured in 200 μL of the medium without Zeocin for 48 h. The culture medium was aspirated, and 100 μL of medium containing 10-fold serially diluted FCV ranging from 10^4 to 10^1 CCID50/100 μL, corresponding to approximate MOIs of 1–0.001 or the culture medium without virus was added. After 20 h of infection, the medium was aspirated, and washed once with 250 μL of MEM-alpha (Invitrogen). The BRET2 signal was detected by sequential dual luminescence measurements mode for each well immediately after adding 50 μL of MEM-alpha containing 2 ng/μL Coelenterazine 400A (also known as DeepBlue C) (Biotium Inc., Hayward, CA) using 2030 Multilabel Reader ARVO-X3 and as equipment linked liquid-dispenser (PerkinElmer). For signal detection, a BRET2 filter set composed of an RLuc emission filter (410-nm bandpass 80 nm) and GFP emission filter (515-nm bandpass 30 nm) (PerkinElmer) were used. The BRET2 signal ratio was calculated from GFP emission (515 nm) divided by RLuc emission (410 nm).

2.10. Inhibitory effect of ribavirin in cell-free system and in infected cells

Ribavirin (1-β-D-ribofuranosyl-1,2,4-triazole-3-carboxamide) (MP Biomedicals LLC, Illkirch, France) was solubilized in water, and aliquots of 100 mM stock were stored at 4 °C. Immediately before use, the stock was diluted to the desired final concentration with PBS (–).

A 20 μL aliquot of in vitro transcription–translation reaction mixture containing the native GloSensor protein was mixed with 4 μL of ribavirin and 20 μL of in vitro transcription–translation reaction mixture (1:10, 1:50, or 1:100 dilution with PBS (–)) containing native FCV protease. The resulting 44 μL of the mixture (final ribavirin concentration of 400, 200, 100, or 50 μM or mock) was incubated at 30 °C for 1 h, and the luminescent signal was detected as described above.

CRFK cells constitutively expressing CellGlo-FCV-Cut or BRET2-FCV-Cut were seeded into 96-well plates and cultured for 48 h as described above. The culture medium was then aspirated, and 100 μL of medium containing FCV was added. An approximate MOI of 0.01 and 1 was used for CellGlo-FCV-Cut and BRET2-FCV-Cut, respectively. The same medium was used for the negative control. After 1 h of infection, the culture medium was aspirated, and 100 μL of medium containing ribavirin (final concentration of 400, 200, 100, or 50 μM) or without ribavirin (mock) was added. After 20 h of infection, the signals of GloSensor or the BRET2-based probe were measured as described above.

The cytotoxic effect of ribavirin on CRFK cells was measured by a CellTiter Glo Luminescent Cell Viability Assay Kit (Promega) according to the manufacturer’s instructions at 20 h and 48 h after treatment.

2.11. Western blotting

The expression and cleavage of the BRET2-based probe were visualized by Western blotting. The BRET2-FCV-Cut- or -Uncut-expressing CRFK cells were infected with FCV with various MOIs (1, 0.1, 0.01, and 0.001) or mock (each fourth wells of 96-well plate), and BRET2 signals were measured. The cells were collected in 200 μL of SDS–PAGE sample buffer (62.5 mM Tris–HCl, pH 6.8, 5% (w/v) sucrose, 2% (w/v) SDS, 0.002% (w/v) bromophenol blue with 5% (v/v) 2-mercaptoethanol), heated at 95 °C for 5 min, and subjected to electrophoresis in 5–20% SDS–PAGE gels. Proteins in the gel were transferred to a polyvinylidene difluoride filter (Immobilon-P; Millipore, Billerica, MA) and the blocking was performed in PBS containing 5% skim milk (Invitrogen). The filters were incubated with rabbit anti-GFP antibody (Living Colors Full-length A.v. Polyclonal Antibody; Clontech, Palo Alto, CA) (1:1500 dilution in PBS containing 0.05% Tween 20 (Bio-Rad Laboratories, Inc., Hercules, CA) and 0.5% skim milk, respectively) at room temperature for 2 h. The filters were washed with PBS containing 0.1% Tween 20 five times for 10 min each, and then incubated with stabilized goat anti-rabbit IgG antibodies conjugated with horseradish peroxidase (Thermo Fisher Scientific, Waltham, MA) (1:1500 dilution in PBS containing 0.05% Tween 20 and 0.5% skim milk) at room temperature for 1 h. The filters were washed with PBS containing 0.1% Tween 20 five times for 10 min each, and then treated with Amer sham ECL Western Blotting Detection Reagents (GE Healthcare UK, Ltd., Little Chalfont, England) for the detection of the signal. The signal was detected with an image analyzer LAS-3000 (FujiFilm, Tokyo, Japan).
3. Results

3.1. GloSensor detects FCV protease activity in cell-free system

To test the GloSensor approach for detecting FCV protease activity in cell-free system, we developed two constructs: InvitroGlo-FCV-Cut and InvitroGlo-FCV-Uncut (Fig. 1). The FCV protease-recognition sequence was cloned into a circularly permuted form of firefly (Photinus pyralis) luciferase (GloSensor; Promega) (Fan et al., 2008; Wigdal et al., 2008). Cleavage of the protease recognition sequence by the cognate protease activates the luciferase, and results in light emission when luciferase substrate is added (Fig. 1).

The boundary linker region contains 12 amino acids (aa), PLFRLE/ADDGS, corresponding to the P6–P6′ positions of the cleavage site in the precursor capsid protein, VP1, of the FCV F4 strain (Fig. 1b; InvitroGlo-FCV-Cut). As a negative control, a construct containing a mutant cleavage motif (PLFRLA/ADDGS) that cannot be cleaved by the FCV protease was generated (Fig. 1b; InvitroGlo-FCV-Uncut). The InvitroGlo-FCV-Cut protein exhibited increased luminescence in a dose-dependent manner when incubated with different concentrations of in vitro translated FCV protease (Fig. 2). The ratio of the luminescence signal reached the maximum of 193-fold when 1:4 diluted FCV protease generated in vitro was incubated with twofold serially diluted (1/4–1/1024 dilution) in vitro translated FCV protease or phosphate-buffered saline. The ratios of the intensity of the signal between the incubation with protease and that without protease are depicted. At least two separate experiments were performed.

3.2. GloSensor detects FCV protease activity in infected cells

GloSensor was tested for its activity in cultured cells. For this purpose, the GloSensor containing the cleavage motif was isolated and transferred into a vector harboring the CMV promoter because the original vector, the pGloSensor-10F linear vector, was designed for in vitro transcription–translation by the SP6 promoter. We developed two constructs, CellGlo-FCV-Cut and CellGlo-FCV-Uncut (Fig. 1b), and used them to establish CRFK cell lines stably expressing the substrate. In CRFK cells expressing the CellGlo-FCV-Cut GloSensor protein, the ratio of the luminescence signal decreased 37.9-, 38.3-, and 10.3-fold after FCV infection at MOIs of 0.1, 0.01, and 0.001, respectively, whereas cells expressing the CellGlo-FCV-Uncut protein showed no decrease in luminescence at any MOI (Fig. 3). These results demonstrate that CRFK cell lines stably expressing the CellGlo-FCV-Cut protein are valuable and sensitive enough to monitor FCV infection in an MOI-dependent manner.

3.3. BRET$^2$-based probe detects FCV protease activity in infected cells

The biosensors BRET$^2$–FCV-Cut and -Uncut are based on the BRET$^2$ principle. BRET$^2$ uses a luciferase (RLuc) isolated from the sea pansy Renilla reniformis as the donor, and a GFP variant (GFP$^2$) as the acceptor (Fig. 4a). The energy transfer efficiencies between RLuc and GFP$^2$, upon DeepBlueC addition, were determined ratiometrically by dividing the intensity of the acceptor emission by that of the donor emission. This ratiometric measurement is referred to as the BRET$^2$ signal and reflects the proximity of RLuc to GFP$^2$.
The FCV protease recognition sequence, PLFRLE/ADDGSi, was inserted between the donor and acceptor of a BRET2 pair (Fig. 4b). As a negative control, a construct containing a mutant cleavage motif (PLFRLA/ADDGSi) that cannot be cleaved by the FCV protease was also generated (Fig. 4b). When the FCV protease cleaves the recognition sequence, the GFP2 and RLuc proteins drift away from each other, and the energy transfer to GFP2 is lost (Fig. 4a). CRFK cells expressing BRET2-FCV-Cut and BRET2-FCV-Uncut showed a BRET2 ratio of approximately 1.0 without FCV infection, whereas CRFK cells expressing BRET2-Cut exhibited declining BRET2 ratios (GFP2/RLuc) of 0.22, 0.48, and 0.82 when FCV infection was performed at MOIs of 1, 0.1, and 0.01, respectively (Fig. 5a). In contrast, CRFK cells expressing the BRET2-FCV-Uncut showed no detectable BRET2 signal change regardless of MOI (Fig. 5a). The expression of BRET2-FCV-Cut and -Uncut proteins in the cells, and a cleaved GFP2 product generated in BRET2-FCV-Cut-expressing cells were confirmed by immunoblotting (Fig. 5b). These results demonstrate that the BRET2-FCV-Cut-expressing CRFK cell line is useful for detecting the protease activity after FCV infection.

3.4. Effects of ribavirin in cell-free system and in infected cells

Ribavirin, a purine analog, is taken up and phosphorylated within cells, where it interferes with viral replication (Leyssen et al., 2008; Parker, 2005). The inhibitory effect of ribavirin on FCV growth in cultured cells has been reported (Belliot et al., 2005; Povey, 1978b), however, it is unknown whether ribavirin has the effect on FCV protease activity in cell-free system.

Various concentrations, 50–400 μM, of ribavirin had negligible effects on FCV protease activity when the in vitro GloSensor system was examined with 1:50 diluted protease (Fig. 6a). Similar results were obtained with 1:100 and 1:10 diluted protease (data not shown). However, we observed a clear dose-dependent inhibitory effect when we performed the assay in CellGlo-FCV-Cut-expressing cells infected with FCV, as shown in Fig. 6b, where 37.2%, 82.3%, 97.9%, and 99.9% inhibition were observed at concentrations of 50, 100, 200, and 400 μM ribavirin, respectively. BRET2-FCV-Cut-expressing cells infected with FCV also demonstrated a similar inhibition effect: 27.3%, 61.4%, 90.9%, and 100% inhibition at 50, 100, 200, and 400 μM ribavirin, respectively (Fig. 6c).
inhibition of the cleavage of BRET²-FCV-Cut was also confirmed by immunoblotting (Fig. 6d). The mean 50% inhibitory concentration (IC₅₀) of the ribavirin was 61 μM in the GloSensor system and 79 μM in the BRET²-based probe system. Ribavirin appeared to have negligible cytotoxic effects at a concentration of 400 μM, the maximum concentration of this study (data not shown). These results clearly demonstrated that ribavirin inhibits FCV infection and/or growth in cultured cells, although an inhibitory effect was not observed in a cell-free FCV protease activity detection system.

4. Discussion

In this study, we employed sensor technology and the BRET² effect to construct assay systems for monitoring FCV protease activity in a cell-free system and in an FCV-infected cell culture. The GloSensor has been used to detect protease activity in vitro for recombinant severe acute respiratory syndrome virus ( Coronaviridae ), rhinovirus ( Picornaviridae ), or tobacco etch virus ( Potyviridae ) ( Fan et al., 2008 ). In this study, we demonstrate that this system can also detect the protease activity of FCV ( genus Vesivirus and the family Caliciviridae ). We selected the P6–P6’ amino acid sequence of the cleavage site in the precursor VP1 to construct the GloSensor for the FCV protease. We showed the critical role of the P1 amino acid, E, for the cleavage by FCV protease ( Figs. 1b and 2 ), as reported previously ( Green, 2007 ; Oka et al., 2009 ; Sosnovtsev et al., 1998, 2002 ). Therefore, this system may also be useful to determine the substrate specificity of FCV protease, by which the length of essential sequences and/or specific amino acids is identified.

The in vitro GloSensor system works in a complete cell-free system which provides advantages for the identification of protease inhibitors in chemical compound libraries. Furthermore, the assay can easily be used in laboratories that do not have specialized equipment for virus research.

In addition to the cell-free system, we used the GloSensor for the first time to detect viral protease activity in cultured cells.

We could not provide the data for the cleavage of InvitroGlo-FCV-Cut and CellGlo-FCV-Cut because antibodies to detect the GloSensor are not available (personal communication from the Technical Services Center of Promega Corporation). However, no signal change was noted in the InvitroGlo-FCV-Uncut and CellGlo-FCV-Uncut even when the FCV protease was present ( Figs. 2 and 3 ), and an addition of the inactive FCV protease to InvitroGlo-FCV-Cut did not result in a detectable increase in the luminescence (data not shown). These results suggest that the increased luminescent signal in cell-free system and in FCV-infected cells depends on the cleavage of GloSensor.
We also constructed another detection system for the FCV protease activity based on the BRET²-based probe (Bacart et al., 2008; Pfleger and Eidne, 2006). The original BRET method with Coelenterazine as a substrate is called BRET¹. It is characterized by strong signals and a long lifetime. In BRET², the donor and acceptor emission peaks are more clearly separated. Thus, BRET² is a better choice for screening when high signal-to-noise ratios are required. The BRET² signal is much shorter than that of BRET¹ (Bacart et al., 2008). Previously, the BRET² sensor was used to detect thrombin activity in vitro, and HIV protease was expressed with a plasmid vector in the cells (Dacres et al., 2009; Hu et al., 2005). To our knowledge, this is the first report of the use of a BRET²-based probe for the detection of a viral protease during natural viral infection and growth.

The sensitivity of the CellGlo system was at least 10-fold greater than that of the BRET² system, because viral protease activity was detectable in infected cells at an MOI of 0.001 in the CellGlo system, and an MOI of 0.01 was needed in the BRET² system (Figs. 3 and 5a). In addition, the CellGlo system may be more useful than the BRET² system for high-throughput screening of FCV protease activity, because a medium change is not needed before the cells are harvested: the reagent is simply added to the well.

The BRET² system requires a washing step before the signal is measured to eliminate serum and phenol-red, and the BRET² signal is measured with sequential dual luminescence measurements: one for the donor and one for the acceptor for each microplate well, because of the short lifetime of the light emission. Therefore, the donor and acceptor light output must be measured consecutively or simultaneously before moving to the next microplate well. Nevertheless, the BRET² system has merits. The BRET² signal is a ratiometric measurement value, which eliminates the data variability caused by fluctuations due to assay volume and the number of cells per well. We can also confirm the expression of this sensor protein by fluoromicroscopy by detecting GFP² (data not shown) and immunoblotting (Fig. 5b).

Our CellGlo-FCV-Cut and BRET²-FCV-Cut assays detect the viral protease activity as a marker of FCV infection and growth, so that they can be used to test not only protease inhibitors but also inhibitors of viral or cellular proteins associated with any stage of viral replication.

Ribavirin was used more than 30 years ago for FCV infection in cats, but it was found to be too toxic (Povey, 1978a). However, combinations of ribavirin and interferon-α are now clinically available for hepatitis C virus treatment. Re-evaluation of this drug and its mechanism of FCV growth inhibition would be interesting topics for future study.

FCV could grow in cultured cells efficiently, and showed clear CPE. However, CPE observation with microscopy is time-consuming and difficult to quantify the infection/growth inhibitory effect especially when a large number of compounds are screened. Instead, our screening system that measure bioluminescence signals described in this study is worth to obtain quantitative data speedily using multi-well plate reader.

Cell-based systems may also facilitate evaluation of the cytotoxicity of candidate compounds. Furthermore, screening systems based on infected cells offer advantages for the identification of novel host factors associated with the virus replication cycle that would not have been discovered in target protein-oriented assays.

The FCV has provided a good model of the calicivirus, especially of that infecting humans, because the human calicivirus still cannot be cultivated. Since the calicivirus proteases of different genera have structural and functional similarities (Oka et al., 2007, 2009), the biosensor systems established in this study might also be useful for screening protease inhibitors of other uncultivable caliciviruses that infect animals or humans by combination with a plasmid expressing viral protease and a biosensor harboring the protease recognition sequence.

In conclusion, we developed cell-based biosensor systems, GloSensor and the BRET²-based probe, to monitor FCV protease activity during a natural viral infection. We demonstrated that our biosensor system is capable of detecting the inhibitory effect of ribavirin in the FCV-infected cells. The systems developed in this study will be useful for future studies involving the screening of chemical compound libraries, including those for protease inhibitors and antiviral drugs.

Acknowledgements

This work was supported in part by a Grant-in-Aid for Young Scientists (B) from the Ministry of Education, Culture, Sports, Science and Technology of Japan, a grant from The Japan Health Science Foundation for Research on Health Sciences Focusing on Drug Innovation, and grants for Research on Emerging and Re-emerging Infectious Diseases, as well as Research on Food Safety, from the Ministry of Health, Labour, and Welfare of Japan.

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