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

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ABSTRACT

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^2). A luciferase sensor was designed to contain an FCV protease cleavage motif within the permutated luciferase (GloSensor). The BRET^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^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^2-based probe was established. Increased levels of GloSensor luminescence, and decreased levels of BRET^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^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.

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 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,

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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). Crandell 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′-ctagcCCGCTCTTCCGATTGGAGGCCGATGATGGATCCATCg-3′, and a reverse oligonucleotide, 5′-gatccGATGGATCCATCATCGGCCTCCAATCGGAAGAGCGGg-3′ were synthesized. These oligonucleotides corresponded to 12 amino acids, PLFRLE/ADDGSI (P6–P6′ in the precursor capsid protein, VP1, of the FCV F4 strain), including the FCV protease cleavage motif. They were annealed and cloned into pGloSensor-10F linear vector (Promega, Madison, WI) with a Fast DNA Ligation Kit (Promega) according to the manufacturer's instructions. Another set of oligomers, a forward oligonucleotide, 5′-ctagcCCGCTCTTCCGATTGgcgGCCGATGATGGATCCATCg-3′, and a reverse oligonucleotide, 5′-gatccGATGGATCCATCATCGGCcgcCAATCGGAAGAGCGGg-3′, corresponding to a mutant FCV protease cleavage motif, PLFRLA/ADDGSI, were synthesized and cloned similarly into the same vector.

Transformation was performed with *Escherichia coli* DH5α-T1 (Invitrogen), and the plasmid was purified with a MARIGEN Maxi plasmid purification Kit (OriGene USA, Rockville, MD). The resultant plasmids were designated as InvitroGlo-FCV-Cut and -Uncut, respectively. These clones were verified by sequencing analysis.

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′-CGAGCTCGGATCGATATCACC**ATGGACACCGCTATCCTC**-3′, and a reverse primer, 5′-GTTATCTATGCGGCCGC**TTAAACGGGGATGATCTGG**-3′ (the underlined portions indicate restriction enzyme sites, and the boldface portions correspond to the region encoding GloSensor) with the InvitroGlo-FCV-Cut or -Uncut plasmid as a template. 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 pIRESneo3 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′-agcttgCCGCTCTTCCGATTGGAGGCCGATGATGGATCCATCa-3′, and a reverse synthetic oligo, 5′-gatctGATGGATCCATCATCGGCCTCCAATCGGAAGAGCGGca-3′, as described above. Another short DNA molecule encoding a mutant FCV protease cleavage motif was prepared similarly with a forward oligo, 5′-agcttgCCGCTCTTCCGATTGgcgGCCGATGATGGATCCATCa-3′, and a reverse oligo, 5′-gatctGATGGATCCATCATCGGCcgcCAATCGGAAGAGCGGca-3′.

Each DNA was cloned into the HindIII–BglII 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 BRET^2-FCV-Cut and BRET^2-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 promoter-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 ORF1 of the FCV F4 genome was amplified with a forward primer (5′-GGATCCTAATACGACTCACTATAGGGAACAGCCACCATGTCTCAAACTCTGAGCTTCGTG-3′), an antisense primer (5′-T_{30}TTATCAAACTTCGAACACATCACAGTG-3′) (Oka et al., 2007, 2009), 100 ng of either the plasmid "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 the PCR 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 BRET^2-based probe, subconfluent CRFK cells in a six-well plate were transfected with 1 μg of CellGlo-FCV-Cut, CellGlo-FCV-Uncut, BRET^2-FCV-Cut, or BRET^2-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 10^0 $CCID_{50}$/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 BRET^2-FCV-Cut or BRET^2-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 $CCID_{50}$/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 BRET^2 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 BRET^2 filter set composed of an RLuc emission filter (410-nm bandpass 80 nm) and GFP^2 emission filter (515-nm bandpass 30 nm) (PerkinElmer) were used. The BRET^2 signal ratio was calculated from GFP^2 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, lllkirch, 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 BRET^2-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 BRET^2-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 BRET^2-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 BRET^2-based probe were visualized by Western blotting. The BRET^2-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 BRET^2 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 Amersham 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).

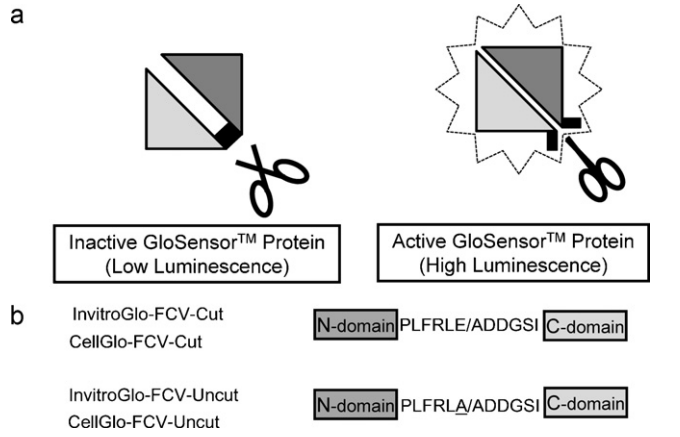

Fig. 1. Native and mutant GloSensor proteins for detection of FCV protease activity. (a) Schematic representation of the genetically modified luciferase biosensor assay for FCV protease. The N-domain of the GloSensor is colored dark-gray and the C-domain of the GloSensor is colored light-gray. The polypeptide linker, a small black square, inhibits the formation of the closed conformation and yields low levels of luminescence (left). Cleavage by the FCV protease relieves this constraint, activating the GloSensor and resulting in high levels of luminescence (right). (b) Schematic diagram of the GloSensor. The 12 amino acid sequences of the FCV protease cleavage motif were located within the genetically modified firefly luciferase. The cleavage site is indicated by a slash, and the mutated amino acid is underlined.

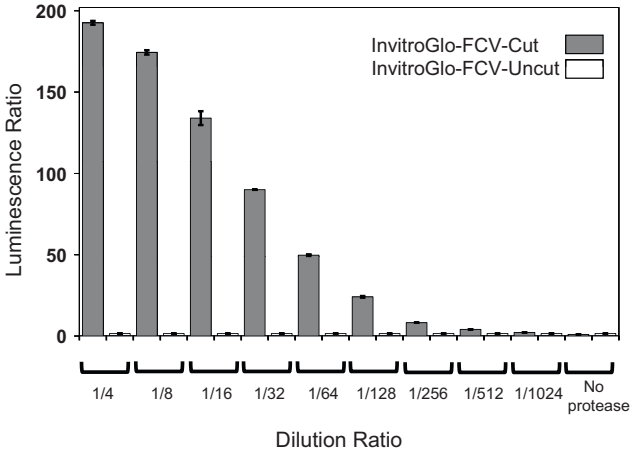

Fig. 2. Luminescence signals in the *in vitro* GloSensor assay. The InvitroGlo-FCV-Cut (light-gray bar) or InvitroGlo-FCV-Uncut (white bar) protein expressed *in vitro* was incubated with twofold serially diluted (1/4–1/1024 dilution) *in vitro* translated FCV protease or phosphate-buffered saline. The 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. 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 permutated 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/ADDGSI, 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/ADDGSI) 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 cell-free system was added. InvitroGlo-FCV-Uncut did not show any increase in luminescence at any concentration of the FCV protease (Fig. 2). In addition, no increased luminescence was detected when InvitroGlo-FCV-Cut or -Uncut was incubated with FCV protease containing a Cys to Ala mutation in the catalytic site (data not shown). These results demonstrate that the increase of the luminescence signal is dependent on FCV protease activity, and the Glu at the P1 position in the cleavage motif is critical for this cleavage.

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 cells 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

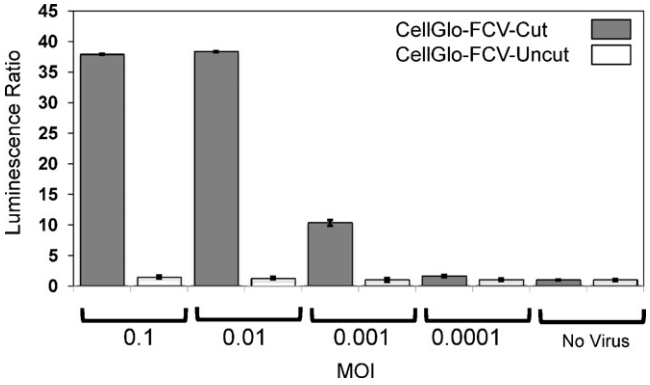

Fig. 3. Luminescence signals in FCV-infected cells. CellGlo-FCV-Cut- (light-gray bar) and CellGlo-FCV-Uncut-expressing CRFK cells (white bar) in a 96-well plate were infected with FCV at the indicated MOI, and incubated for 20 h. The ratios of the intensity of the signal between the incubation with FCV and the incubation without FCV are shown. Error bars represent the standard error of the mean ($n = 4$). At least two separate experiments were performed.

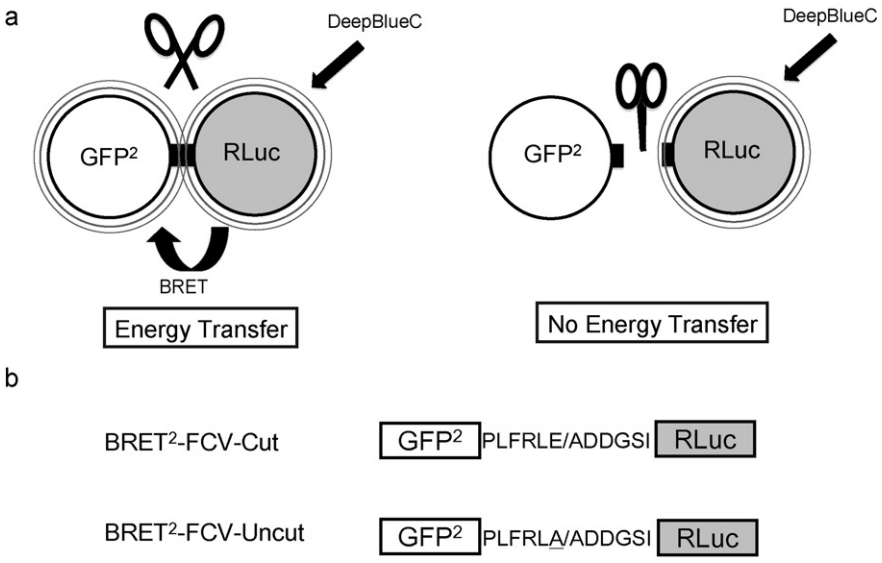

Fig. 4. Native and mutant BRET^2-based probe for detection of FCV protease activity. (a) Schematic representation of the BRET^2-based probe for FCV protease. A BRET^2 signal is generated due to the proximity of the GFP^2 and RLuc molecules (left), but it declines significantly when they are separated by FCV protease (right). (b) Schematic diagram of the FCV protease substrate. The 12 amino acid sequences of the FCV protease cleavage motifs between GFP^2 and RLuc are shown. The cleavage site is indicated by a slash, and the mutated amino acid is underlined.

(Pfleger and Eidne, 2006). The FCV protease recognition sequence, PLFRLE/ADDGSI, was inserted between the donor and acceptor of a BRET^2 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 GFP^2 and RLuc proteins drift away from each other, and the energy transfer to GFP^2 is lost (Fig. 4a). CRFK cells expressing BRET^2-FCV-Cut and BRET^2-FCV-Uncut showed a BRET^2 ratio of approximately 1.0 without FCV infection, whereas CRFK cells expressing BRET^2-FCV-Cut exhibited declining BRET^2 ratios (GFP^2/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 BRET^2-FCV-Uncut showed no detectable BRET^2 signal change regardless of MOI (Fig. 5a). The expression of BRET^2-FCV-Cut and -Uncut proteins in the cells, and a cleaved GFP^2 product generated in BRET^2-FCV-Cut-expressing cells were confirmed by immunoblotting (Fig. 5b). These results demonstrate that the BRET^2-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. BRET^2-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). Dose-dependent

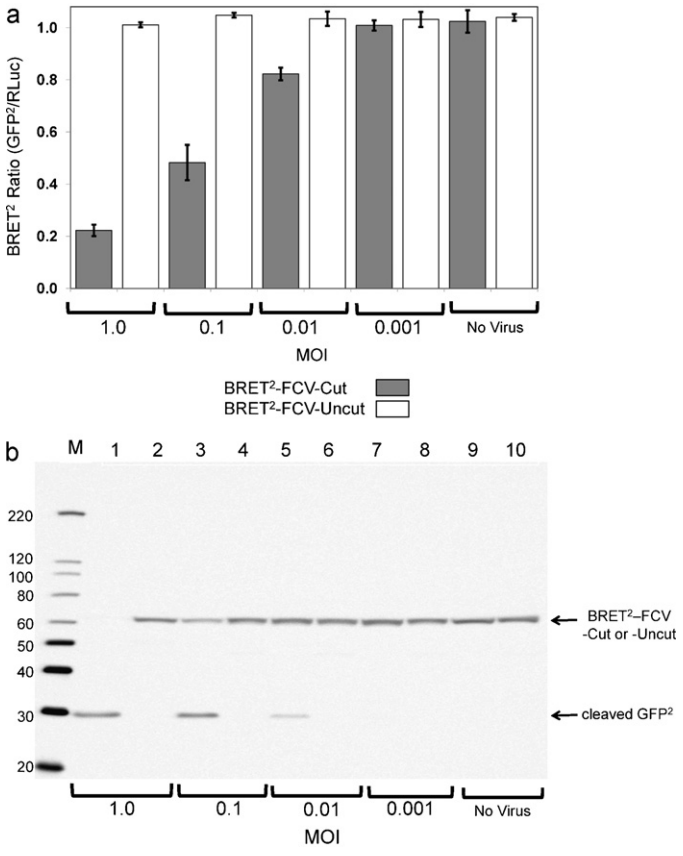

Fig. 5. BRET^2 assays in infected cells. (a) BRET^2 signals in FCV-infected cells. BRET^2-FCV-Cut- and BRET^2-FCV-Uncut-expressing CRFK cells in a 96-well plate were infected with FCV at the indicated MOI, and incubated for 20 h. The BRET^2 ratio was defined as the intensity of GFP^2 emission at 515 nm divided by that of RLuc at 410 nm. Error bars represent the standard error of the mean ($n = 4$). At least two separate experiments were performed. (b) Cleavage products of BRET^2-FCV-Cut- (lanes 1, 3, 5, 7, and 9) and BRET^2-FCV-Uncut-expressing (lanes 2, 4, 6, 8, and 10) CRFK cells detected by Western blotting. M: molecular size marker (MagicMark XP: Invitrogen).

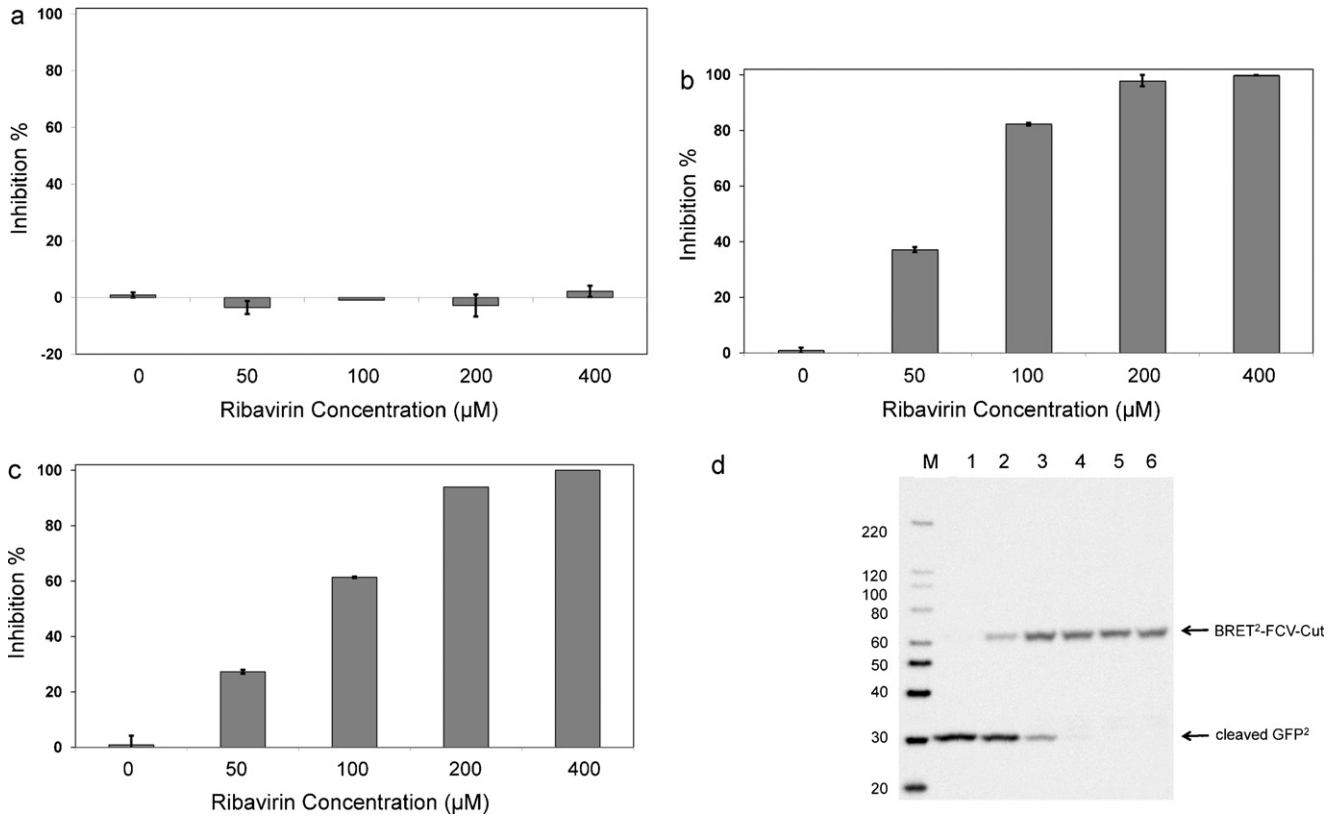

Fig. 6. Effect of ribavirin in cell-free system and in FCV-infected cells. (a) InvitroGlo-FCV-Cut was expressed by *in vitro* translation and incubated with FCV protease diluted 50-fold in the presence of ribavirin at final concentrations of 50, 100, 200, and 400 μM. After 1 h incubation, bioluminescent signals were measured and the inhibitory effect relative to the ribavirin-untreated control was calculated. (b) CellGlo-FCV-Cut protein-expressing CRFK cells in a 96-well plate were infected with FCV at an MOI of 0.01. The medium was aspirated and replaced with medium containing ribavirin at a concentration of 50, 100, 200, or 400 μM. After 20 h, the bioluminescent signals were measured. Inhibition was calculated as the percentage decrease of luminescence relative to the ribavirin-untreated control. Error bars represent the standard error of the mean ($n = 4$). At least two separate experiments were performed. (c) BRET^2-FCV-Cut protein-expressing CRFK cells in a 96-well plate were infected with FCV at an MOI of 1. The medium was aspirated and replaced with medium containing ribavirin at a concentration of 50, 100, 200, or 400 μM. After 20 h, the bioluminescent signals were measured. Inhibition was calculated as the percentage decrease of the multiplicative inverse of the recovered BRET^2 signal ratio relative to the ribavirin-untreated control. Error bars represent the standard error of the mean ($n = 4$). At least two separate experiments were performed. (d) Cleavage of BRET^2-FCV-Cut protein in FCV-infected cells with ribavirin (lanes 1–5: 0, 50, 100, 200, and 400 μM) and uninfected cells (lane 6). M: molecular size marker (MagicMark XP; Invitrogen).

inhibition of the cleavage of BRET^2-FCV-Cut was also confirmed by immunoblotting (Fig. 6d). The mean 50% inhibitory concentration (IC_{50}) of the ribavirin was 61 μM in the GloSensor system and 79 μM in the BRET^2-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 BRET^2 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 BRET^2-based probe (Bacart et al., 2008; Pfleger and Eidne, 2006). The original BRET method with Coelenterazine as a substrate is called BRET^1. It is characterized by strong signals and a long lifetime. In BRET^2, the donor and acceptor emission peaks are more clearly separated. Thus, BRET^2 is a better choice for screening when high signal-to-noise ratios are required. The BRET^2 signal is much shorter than that of BRET^1 (Bacart et al., 2008). Previously, the BRET^2 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 BRET^2-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 BRET^2 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 BRET^2 system (Figs. 3 and 5a). In addition, the CellGlo system may be more useful than the BRET^2 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 BRET^2 system requires a washing step before the signal is measured to eliminate serum and phenol-red, and the BRET^2 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 BRET^2 system has merits. The BRET^2 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 GFP^2 (data not shown) and immunoblotting (Fig. 5b).

Our CellGlo-FCV-Cut and BRET^2-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 speedy 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 BRET^2-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.

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