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Fluorescence Resonance Energy Transfer-Based Assay for Characterization of Hepatitis C Virus NS3-4A Protease Activity in Live Cells[▽]

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The NS3/4A protease from hepatitis C virus (HCV) plays a key role in viral replication. We report a system for monitoring the activity of this enzyme in single living mammalian cells. We constructed a fluorescence resonance energy transfer (FRET) probe that consists of an enhanced cyan fluorescent protein-citrine fusion, with a cleavage site for HCV NS3/4A protease embedded within the linker between them. Expression of the biosensor in mammalian cells resulted in a FRET signal, and cotransfection with the NS3/4A expression vector produced a significant reduction in FRET, indicating that the cleavage site was processed. Western blot and spectrofluorimetry analysis confirmed the physical cleavage of the fusion probe by the NS3/4A protease. As the level of FRET decay was a function of the protease activity, the system allowed testing of NS3/4A protease variants with different catalytic efficiencies. This FRET probe could be adapted for high-throughput screening of new HCV NS3/4 protease inhibitors.

It is estimated that nearly 200 million people are infected with the hepatitis C virus (HCV) (27). In >70% of infected individuals, HCV establishes a persistent infection over decades that may lead to cirrhosis and hepatocellular carcinoma. There is no effective vaccine to prevent HCV infection, and the only therapy available consists of pegylated interferon plus ribavirin. However, this treatment only achieves a sustained virological response in ~55% of treated patients, with significant differences between HCV genotypes (2). Therefore, both new therapies and strategies to search for effective HCV inhibitors are needed.

HCV is an enveloped virus classified in the *Flaviviridae* family. The RNA genome, ~9.6 kb in length, encodes a single polypeptide that is cleaved to produce four structural and six nonstructural (NS) proteins, designated C, E1, E2, and p7 and NS2, NS3, NS4A, NS4B, NS5A, and NS5B, respectively. The 181 amino-terminal amino acid residues of the NS3 protein form a serine protease that cleaves at the NS3/4A junction in *cis*, followed by cleavage at the NS4A/B, NS4B/5A, and NS5A/B sites in *trans.* The NS3 serine protease requires an accessory viral protein, NS4A, for optimal cleavage activity (8). Due to its essential role in viral replication and its effects on the physiology of the infected cell, the NS3/4A protease is a good target to develop new HCV antivirals. Many NS3/4A protease inhibitors are in various phases of research, including clinical trials (23–26). However, drug-resistant mutants have also been described and partially characterized. Therefore, in addition to identifying new inhibitor molecules, it may be important to develop assays to phenotypically characterize NS3/4A protease variants isolated from HCV-infected patients.

Fluorescence methods, such as Förster resonance energy transfer (6), allow real-time monitoring of cell processes in their physiological cell environment and are amenable for high-throughput screening. Fluorescence resonance energy transfer (FRET) between a donor and an acceptor fluorophore can provide a measure of molecular proximity at nanometer resolution because of its dependence on the inverse sixth power of distance (28). The use of FRET between spectral variants of green fluorescent protein to assay protease action was one of the first biological applications of these proteins (10, 21, 30). In these studies, a FRET donor fluorescent protein was linked to an acceptor fluorescent protein by a short peptide containing a protease recognition site. Cleavage resulted in separation of the donor and acceptor fluorochromes by diffusion and decrease in FRET rate. Recently, sensitive energy transfer-based reporter assays for viral proteases have also been described, in particular for human immunodeficiency virus type 1 (12) and enterovirus (11).

Recent publications report FRET-based in vitro assays for HCV protease (14, 19). Here, we describe for the first time an HCV NS3/4A protease biosensor assay based on FRET in live cells. The assay was validated by using variants of the NS3/4A protease, which are of known enzymatic activity. We show that this system is quantitative, sensitive, and appropriate for characterizing the activity of NS3/4A variants within mammalian cells. In addition, this assay has the potential to be used for pharmacological screening.

MATERIALS AND METHODS

Construction of plasmids. Oligonucleotide primers (Bonsai Technologies) used in this study are listed in Table 1. We verified all constructs by nucleotide sequencing.

Plasmid constructs pC-CS$_{wt}$-Y and pC-CS$_{mut}$-Y for mammalian expression of the FRET sensor. The FRET sensor pC-CS$_{wt}$-Y, in which C stands for enhanced cyan fluorescent protein (ECFP), CS$_{wt}$ for wild-type cleavage site, and Y for the

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TABLE 1. Sequences of DNA oligonucleotides used for cloning NS3-4A protease substrates and NS3-4A protease expressors

Name	Sequence (5′ to 3′)
CS_wt_s	ACCCAAGCTTATGGTGAGCAAGGG
CS_wt_as	ATGCGGCCGCCGGCGCCTGTCCACGTATA GGACATTGAGCAGCAGACGACGTCCTC ACTAGCGCGTCCCTTGTACAGCTCGTCC ATGCCGAGAGTGATCC
CS_mut	AGTGAGGACGTCGTCGGCGGCTCAATGTC CTAT
Nt_scNS3/4A	ACTTAAGCTTGCCACCATGGCTCCTATTGG ATCTGTTG
Ct_scNS3/4A	GGGGAGGGGCTCGAGTCAAG
N-t-protease-24	ACTTAAGCTTGCCACCATGGCTCCTATTGG CAGCGTGG
N-t-protease-i	ACTTAAGCTTGCCACCATGGCTCCTATTGG CTGCGTTG
C-t-protease-24	GGGGAGGGGCTCGAGTCATG

enhanced yellow fluorescent protein (EYFP) citrine, was constructed by replacing the linker of pcDNA3-ECFP-23-Citrine (5) with an NS3/4A protease substrate sequence. First, to avoid dimerization of cleaved donor and acceptor fluorescent proteins, the mutation A206K was introduced into both ECFP and citrine (32) by site-directed mutagenesis (QuikChange multisite-directed mutagenesis kit; Stratagene). In a second step, DNA encoding ECFP was obtained by digestion with HindIII and BamHI. This fragment was used as template for PCR amplification using the primers CS_wt_s and CS_wt_as; the latter codifies the NS5A/B cleavage recognition sequence EDVVCCSMSYTWTG and a NotI sequence. This PCR product and the parental pcDNA3-ECFP-23-Citrine were digested with HindIII and NotI. Finally, the fragment containing the ECFP cleavage site was inserted in frame into the pcDNA3-citrine fragment.

To prepare a molecular FRET sensor insensitive to NS3/4A protease (pC-CS_{mut}-Y), a mutant cleavage site (EDVVGGSMSYTWTG) was also constructed by site-directed mutagenesis, using primer CS_mut with plasmid pC-CS_{wt}-Y as a template (QuikChange multisite-directed mutagenesis kit; Stratagene).

Construction of NS3/4A expressors in mammalian cell vectors. We used an $NS3_{2\text{-}181}/4_{21\text{-}34}$ protease construct containing NS4 residues 21 to 34 fused in frame via a GG linker to the amino-terminal protease domain (residues 2 to 181) of NS3 (Fig. 1C). This single-chain NS3 protease (scNS3/4A protease) is fully active, with kinetic parameters virtually identical to those of the NS3/NS4A noncovalent complex (4).

Bacterial expression vectors (20) were used as templates for PCR amplification of NS3/4A protease variants. The forward primer contained a HindIII restriction site, a Kozak sequence, and the first seven codons of scNS3/4A. Templates carrying sequences of proteases of different genotypes, in which the NS4A sequence varies, were also PCR amplified. The reverse primer contained the final codons of the NS3 protease domain and an XhoI sequence. The resulting amplicons were digested and ligated into the HindIII- and XhoI-treated pcDNA3 plasmid to obtain the different expression vectors. In pcDNA3 vectors (Invitrogen), expression in mammalian cells is driven by human cytomegalovirus immediate-early promoter. scNS3/4A from patient 1 (scNS3/$4A_{Pt1}$) (20), scNS3/4A-A156V, scNS3/4A-A156T, and scNS3/4A-I389 were amplified with oligonucleotides Nt_scNS3/4A and Ct_scNS3/4A. Variant scNS3/4A-24 (genotype 4d) (7) was amplified with oligonucleotides N-t-protease-24 and C-t-protease-24. Variant scNS3/4A-i (genotype 3a) (7) was amplified with oligonucleotides N-t-protease-i and Ct_scNS3/4A.

The variants scNS3/$4A_{Pt1}$ (genotype 1b), scNS3/4A-24 (genotype 4d), and scNS3/4A-i (genotype 3a) are master sequences obtained from serum samples of HCV-infected patients. Protease scNS3/4A-S139A contained a single-point mutation (based on scNS3/$4A_{Pt1}$) within the catalytic triad that abolishes the proteolytic activity of the enzyme. In turn, scNS3/4A-I389 was obtained from the subgenomic HCV-1b replicon system, whereas scNS3/4A-A156V and -T were resistance variants with single-point mutations of Ala 156 that were generated by site-directed mutagenesis.

Cell culture and transfection. HeLa cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum, 2 mM L-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin sulfate at 37°C in 5% CO_2. Huh-7 cells (a gift from Jose J. Ramirez) were cultured with the same medium, supplemented with nonessential amino acids (1×) and 1 mM pyruvate. Transfection was performed with Lipofectamine 2000 (Invitrogen) according to the manufacturer's protocol. Posttransfection (24 h) cells were used for imaging experiments or lysed to obtain cell extracts. Huh-7 cells were more difficult to transfect than HeLa cells and were imaged 72 h after transfection to obtain a higher percentage of stained cells.

Western blotting. Cells were collected by centrifugation at 500 × *g* for 10 min and treated with lysis buffer (10 mM Tris-HCl, pH 7.5, 100 mM NaCl, 1 mM EDTA, 0.5% Igepal, 1 mM phenylmethylsulfonyl fluoride, 20 μg/ml leupeptin, and 20 μg/ml aprotinin) on ice for 45 min. Cell lysates were centrifuged at 14,000 × *g* for 30 min. Protein contents in the cytosolic supernatant extracts were quantified by using a micro-Bradford assay (Bio-Rad). Equal amounts of protein samples were applied in 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Proteins in the gel were then transferred to Hybond-P membrane (Amersham Biosciences) using a semidry method (Bio-Rad). The membrane was immunoblotted with mouse anti-green fluorescent protein antibody (1:3,000) (Covance). The immunoblot was then probed with sheep anti-mouse immunoglobulin G-horseradish peroxidase conjugate (1:2,000) (Santa Cruz). As a loading control, primary antitubulin antibody (Santa Cruz) was used.

Fluorescence spectroscopic analysis. The cytosolic extracts of cells transfected with biosensor and the corresponding protease variant (as indicated) were diluted in lysis buffer. The excitation wavelength was set at 430 nm (excitation peak of ECFP) to obtain fluorescence emission spectra (460 to 600 nm) in an LS-50B spectrofluorometer (Perkin-Elmer).

Imaging. HeLa cells were cultured in 24-mm-diameter glass coverslips and transfected with the chimeric proteins (biosensor-protease variant). After transfection (24 h), the coverslips were placed in a microscopy chamber (Attofluor; Molecular Probes) and observed in an epifluorescence inverted microscope (DMIRE-2; Leica Microsystems) with oil immersion objectives (40× PlanApo with a numerical aperture of 1.25 and 16× PlanFluotar with a numerical aperture of 0.5). Interference filters and dichroic mirrors (described under "FRET analysis") were from Omega Optical, Chroma Technology, and Semrock. A polychromator source with a Xe lamp (Hamamatsu Photonics) was used for excitation. The emission filter wheel was controlled by a Lambda-10 device (Sutter Instruments). The detector was a cooled charge-coupled device (C9100-13 EM-CCD; Hamamatsu Photonics). The polychromator, camera, and filter wheel were controlled and all images were acquired and analyzed by using

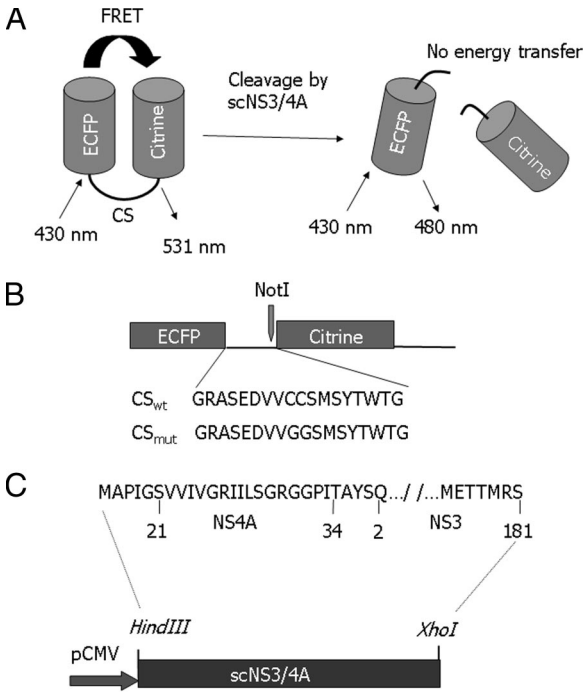

FIG. 1. Experimental design. (A) Schematic representation of the FRET assay for NS3 protease. (B) Schematic diagram of the NS3 substrate. The amino acid sequences of the NS3 cleavage motif (CS_{wt}) and the mutant motif (CS_{mut}) in the linker between ECFP and citrine are shown. (C) Diagram of the scNS3/$4A_{Pt1}$ construct.

AquaCosmos 2.6 software (Hamamatsu Photonics). A binning of two was used to improve the signal-to-noise ratio and to minimize photobleaching. Background correction was made by subtracting from the raw images the averaged value of three regions of interest taken in areas devoid of cells from the same field.

FRET analysis. A simple ratio of a FRET image (donor excitation and acceptor emission) and donor image (donor excitation and donor emission) (both images had the background subtracted) was used as a FRET index as previously described (5). Furthermore, the apparent FRET efficiency (E_{app}) and the ratio of the donor-to-acceptor fluorescent protein concentration ([D]/[A] ratio) in living cells were calculated with the "3-cube FRET" method as described previously (3). E_{app} depends on the product of the intrinsic FRET efficiency and the fraction of the donor involved in FRET. In our FRET assay, as the sensor is cleaved by NS3/4A protease, donor and acceptor moieties diffuse and the fraction of donor involved in FRET decreases, causing E_{app} to drop. The hardware settings and filters used in the "3-cube FRET" method were as follows. For the acceptor channel, polychromator excitation at 500 nm; excitation filter, 500/24; dichroic, 520LP; and emission filter, 542/27 (Brightline HC-YFP cube; Semrock). For the donor channel, polychromator excitation at 430 nm; excitation filter, 440/20; dichroic, 455DRLP; and emission filter, 480/30. For the FRET channel, polychromator excitation at 430 nm; excitation filter, 440/20; dichroic 455DRLP; and emission filter, 535/26. The bandpass interference filters above are named as center wavelength/bandwidth in nm.

Statistical analysis. The statistical significance of differences between control and treated samples was determined by one-way analysis of variance (ANOVA) followed by Dunnet's posttest or by Student's *t* test, using GraphPad Prism software.

Nuclease sequence accession numbers. Accession numbers for the above genotype isolates are AF510039 for scNS3/4A$_{Pt1}$ (genotype 1b); AJ242654 for scNS3/4A-I389 (replicon 1b); EF363559 for scNS3/4A-i (genotype 3a); and DQ516083 for scNS3/4A-24 (genotype 4d).

RESULTS

Experimental strategy and assay validation. A substrate for HCV NS3/4A protease was designed to obtain a FRET-based assay of protease activity in living mammalian cells (Fig. 1A). This FRET-based assay contains the fluorescent proteins ECFP (FRET donor) and YFP variant citrine (FRET acceptor) linked by a protease recognition sequence such that cleavage results in diffusion of donor and acceptor fluorochromes and a decrease in the E_{app} (5). We generated a recombinant construct, pC-CS$_{wt}$-Y, encoding an ECFP-citrine fusion protein joined by a 22-amino-acid linker containing the NS5A/5B cleavage motif for NS3/4A protease (Fig. 1B). In addition, a plasmid containing a mutated cleavage site lacking two Cys residues critical for proteolysis (pC-CS$_{mut}$-Y) was also constructed (Fig. 1B).

First, we analyzed the validity of the assay and target specificity by cotransfecting the biosensor expression plasmids with plasmid pscNS3/4A$_{Pt1}$ (Fig. 2). Fluorescence was cytoplasmic and nuclear in transfected HeLa cells (Fig. 2A). A ratio of FRET image/donor image was used as a FRET index. The pseudocolored ratio images (Fig. 2A) indicated that scNS3/4A$_{Pt1}$ protease cleaved the ECFP-CS$_{wt}$-citrine fusion protein very efficiently. The specificity of this reaction was demonstrated by using a mutated form of the scNS3/4A protease (scNS3/4A-S139A) or a sensor containing a mutated cleavage site (Fig. 2A). No change in FRET index was observed with either scNS3/4A-S139A protease coexpressed with wt sensor or wt protease coexpressed with mutated sensor.

To confirm these results, cytosolic extracts from transfected HeLa cells were analyzed by Western blotting. The Western blot results (Fig. 2B) showed that the cleavage products (~28.3 kDa) only appeared when pC-CS$_{wt}$-Y and pscNS3/4A were cotransfected. Thus, the full-length fusion (~56.7 kDa) remained intact when a mutated cleavage site sensor or a mutated inactive protease (pscNS3/4A-S139A) was used.

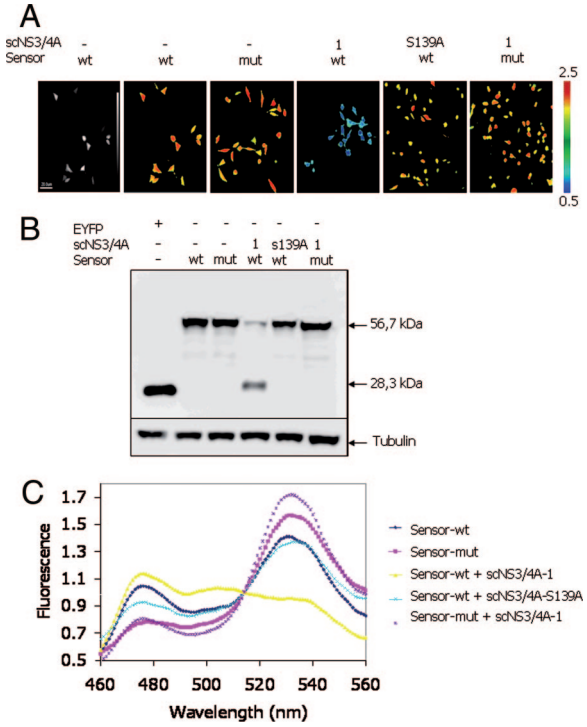

FIG. 2. Cleavage of fluorescent sensor mediated by HCV scNS3/4A$_{Pt1}$ (scNS3/4A-1) as determined by FRET imaging in live cells, Western blot, and fluorimetry of cell extracts. (A) FRET imaging of live cells. Fluorescence (leftmost panel) and pseudocolored ratio images (FRET channel/ECFP donor channel, with pseudocolor scale shown on the right) of HeLa cells 24 h after transfection are shown. (B) Western blot of cytosolic extracts of HeLa cells transfected with the indicated expression plasmids. EYFP was used to monitor the size of a free fluorescent protein. The bands corresponding to the full-size fusion protein (56.7 kDa) and its cleavage products (28.3 kDa) are indicated. (C) Emission spectra of cytosolic extracts show that the sensor with CS$_{wt}$ coexpressed with scNS3/4A$_{Pt1}$ had a negligible FRET rate (decrease in emission peak at 531 nm). Neither the CS$_{mut}$ sensor nor mutated protease (NS3/4A-S139A) displayed a decrease in FRET. +, present; −, absent.

Fluorescence energy transfer in the cytosolic extracts was also analyzed by spectrofluorimetry (Fig. 2C). When extracts of cells transfected with pC-CS$_{wt}$-Y or pC-CS$_{mut}$-Y were excited at 430 nm (donor excitation), an ECFP emission peak was observed at 475 nm, and an enhanced acceptor (citrine) emission peak was recorded at 531 nm, demonstrating energy transfer from ECFP to citrine. A large decrease of fluorescence emission at 531 nm was observed when pC-CS$_{wt}$-Y was cotransfected with an scNS3/4A$_{Pt1}$ expression vector, but not when pC-CS$_{mut}$-Y was used. These results indicated that the protease cleaves the fusion protein C-CS$_{wt}$-Y but cannot cleave the C-CS$_{mut}$-Y protein. On the other hand, there was efficient energy transfer when the expression vector pscNS3/4A-S139A was cotransfected with pC-CS$_{wt}$-Y, showing that a mutated protease was unable to cleave the biosensor. The fluorimetry results confirm the conclusions obtained with FRET imaging of single cells (Fig. 2A) and Western blot analysis of cell extracts (Fig. 2B).

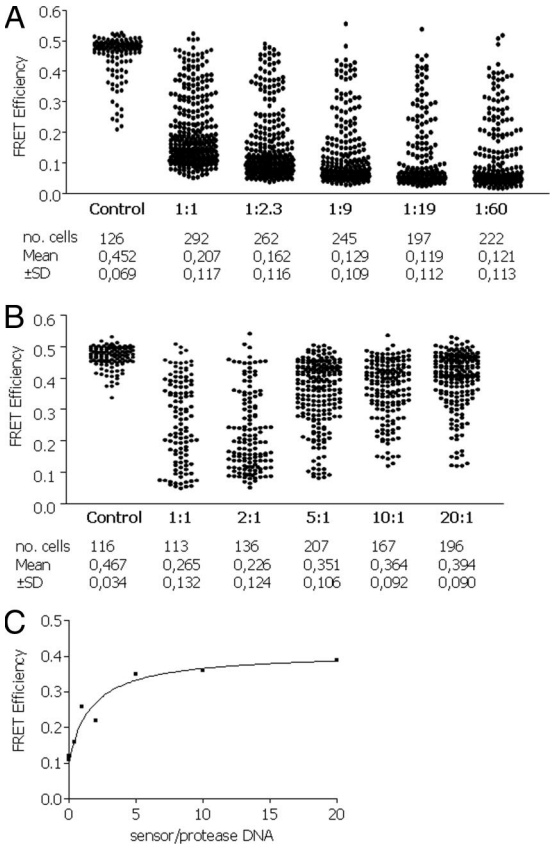

FIG. 3. HCV NS3/4A protease catalytic efficiency in HeLa cells as a function of the ratio of sensor (ECFP-CS_{wt}-citrine) to protease (pscNS3/$4A_{Pt1}$) DNA in the transfection mix. The scatter plots show the distribution of E_{app} found in single cells. The results for the experimental samples were compared to those for the control (sensor alone without scNS3/4A) by using one-way ANOVA with Dunnett's posttest. The P value was <0.01 for all comparisons. (A) The ratio of pECFP-CS_{wt}-Citrine to pscNS3/$4A_{Pt1}$ DNA in the transfection mix was decreased (from 1/1 to 1/60); therefore, the protease expression level was expected to increase. (B) The ratio of pECFP-CS_{wt}-Citrine to pscNS3/$4A_{Pt1}$ DNA in the transfection mix was increased (from 1/1 to 20/1); therefore, the protease expression level was expected to decrease. (C) E_{app} decreased with the expected expression level of protease NS3/4A at low sensor/protease DNA ratios.

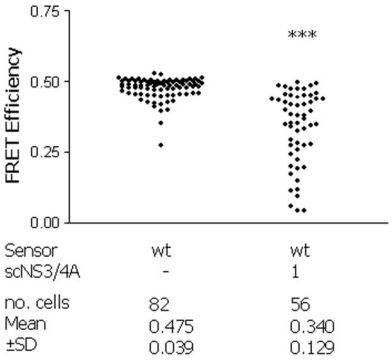

FIG. 4. Catalytic efficiency of HCV scNS3/$4A_{Pt1}$ in hepatocyte-derived Huh-7 cells expressing the sensor ECFP-CS_{wt}-citrine 72 h after transfection. The ratio of sensor/protease expression plasmid DNA used was 1:1. Two amounts of plasmid DNA per coverslip, 1 μg and 2 μg, were assayed (results were pooled). Each point of the scatter plot represents the E_{app} of a single cell. The results for experimental samples were compared to those for the control (wt −; sensor alone without scNS3/4A protease) by using Student's t test. ***, $P < 0.0001$.

Functional assay of the FRET probe in living cells—FRET analysis. We explored the possibility of measuring E_{app}, rather than the FRET ratio as shown in Fig. 2A, by using the "3-cube FRET" method as described previously (3). Furthermore, a dose-response study of substrate sensor and protease was attempted by varying the DNA ratios of the expression vectors in the transfection mixture (from a 20-fold excess of sensor to a 60-fold excess of protease DNA). Although the expression levels of each protein are probably not linearly related to the amount of plasmid DNA used, an inverse correlation between FRET and the amount of scNS3/4A plasmid in the transfection mix was expected.

We represented the E_{app} of each individual cell measured on a scatter plot in order to visually show the means and the variations in the data (Fig. 3). Control HeLa cells expressing the sensor alone showed a FRET efficiency of 0.46 (Fig. 3A and B). Cells cotransfected with sensor and scNS3/$4A_{Pt1}$ expression vectors showed a significant FRET decrement (Fig. 3A). The FRET rate presented a direct dose-response relationship with the sensor-to-protease DNA ratio in the transfection mix (Fig. 3A to C). At high sensor/protease DNA ratios (>5/1) (Fig. 3B), less protease was expected to be present in cells expressing the fluorescent sensor and the FRET rate approached that found in the absence of protease. The opposite was seen with low sensor/protease DNA ratios in the transfection mix (Fig. 3A and C), reaching an efficiency value of 0.12 at the lowest. In summary, the transfected HeLa cells exhibited reductions of FRET efficiency with lower sensor-to-protease DNA ratios used in the transfection mix, which is attributable to a higher expression of protease relative to sensor.

Since HCV is a hepatotropic virus, we also tested the ability of the FRET assay to detect NS3/4A protease activity in the cell line Huh-7, derived from a human hepatocellular carcinoma. As is shown in Fig. 4, the coexpression of sensor and scNS3/$4A_{Pt1}$ protease significantly reduced the FRET efficiency, demonstrating that NS3/4A protease activity can also be quantified in Huh-7 cells.

Catalytic efficiencies of scNS3/4A variants. To explore whether this mammalian-cell-based assay is useful to characterize HCV NS3/4 proteases with different proteolytic activities, we assayed proteases whose catalytic efficiencies have previously been characterized by us (7, 20). The activities of variant proteases were compared to that of the replicon system 1b (scNS3/4A-I389) and expressed as [(FRET of sensor alone − FRET of variant)/(FRET of sensor alone − FRET of scNS3/4A-I389)] × 100. The catalytic efficiencies of scNS3/4A-i and scNS3/4A-24 were found to be 61% and 43%, respectively, of the catalytic efficiency of wt scNS3/4A-I389 (Fig. 5A). In contrast, the scNS3/$4A_{Pt1}$ protease displayed 207% (~twofold) of the activity of the protease from replicon 1b. As expected, the most-active protease (7, 20) rendered the lowest E_{app} (most cleavage).

Two specific mutations, A156V and A156T, have been shown to confer high-level resistance to different HCV NS3/4A protease inhibitors (BILN-2061 and VX-950) (9, 18). The calculated catalytic efficiencies of mutants scNS3/4A-A156V and

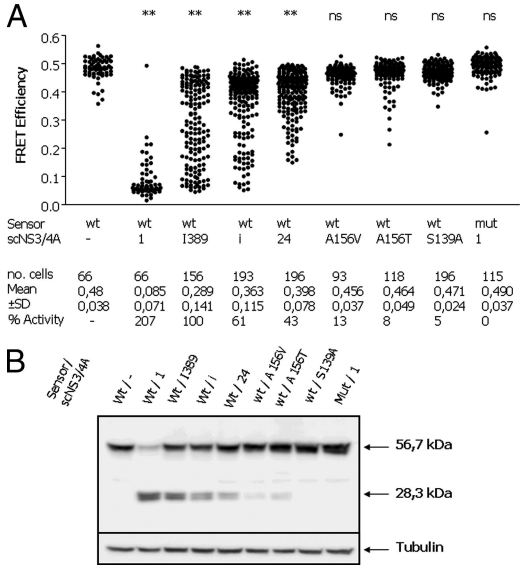

FIG. 5. Comparison of the catalytic efficiencies of HCV NS3/4A variants in HeLa cells coexpressing the sensor (ECFP-CS_{wt}-citrine). The ratio of sensor/protease expression plasmid DNA used was 1:9 to ensure that most cells expressing the sensor had protease expression as well. (A) Each point of the scatter plot represents the E_{app} of a single cell. The results for the experimental samples were compared to those for the control (wt −; sensor without scNS3/4A) by using one-way ANOVA with Dunnett's posttest. **, $P < 0.01$; ns, not significant; SD, standard deviation. (B) Western blot of the cytosolic extracts of transfected cells. The bands corresponding to full-size fusion protein (56.7 kDa) and its cleavage products (28.3 kDa) are indicated. wt/−, control.

-A156T in our assay were 13% and 8%, respectively, of that of the replicon 1b protease (scNS3/4A-I389) (Fig. 5A). A reduced catalytic efficiency was previously observed using an in vitro assay, in which the A156T mutant NS3/4A protease showed a decrease in *trans* cleavage activity and, therein, the polyprotein-processing step compared with the results for wt NS3/4A-I389 protease (31). In addition, a reduction in replication capacity, using a genotype 1b HCV replicon system, has also been described with these mutants (9).

A single-point mutation in Ser 139 within the catalytic triad (scNS3/4A-S139A) resulted in no protease activity (Fig. 5A), showing the specificity of the cleavage reaction. This was further demonstrated by the coexpression of a mutated sensor, C-CS_{mut}-Y, with scNS3/$4A_{Pt1}$ protease. As shown in Fig. 5A, the mutant recognition site abrogated cleavage by the NS3/4A protease.

The relative enzymatic activities of all the scNS3/4A protease variants described above were confirmed by Western blotting of the cytosolic extracts (Fig. 5B). Faint reaction products (28.3-kDa bands) were observed with the resistance variants A156T and A156V. Overall, these results demonstrate the robustness of the FRET-based assay to detect and quantify the enzymatic activity of the HCV NS3/4A protease.

Decrease in the [D]/[A] ratio by NS3/4A protease. The implementation of the "3-cube FRET" method allowed the measurement of E_{app} (Fig. 3 to 5) and, also, an accurate estimation of the [D]/[A] ratio in each cell (3). The E_{app} and [D]/[A] ratio of HeLa cells transfected with the sensor alone (ECFP-CS_{wt}-citrine) or cotransfected with scNS3/$4A_{Pt1}$ were determined

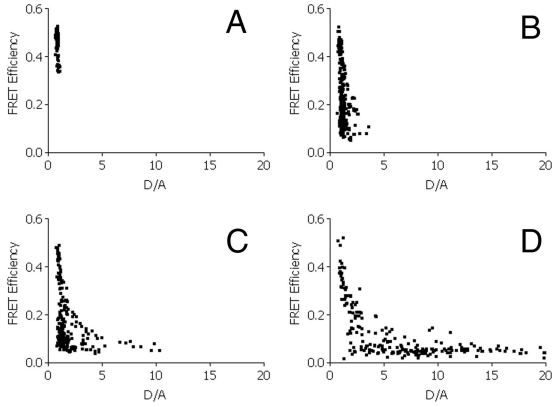

FIG. 6. E_{app} and [D]/[A] ratio (D/A) in HeLa cells cotransfected with sensor (pECFP-CS_{wt}-Citrine) and protease (pscNS3/$4A_{Pt1}$), as determined with the "3-cube FRET" method. (A) Sensor was expressed alone. (B) Cotransfection of sensor and protease at 0.5 μg of each plasmid (ratio, 1:1). (C) Cotransfection of 0.3 μg of sensor and 0.7 μg of protease (ratio, 1:2.3). (D) Cotransfection of 0.05 μg of sensor and 3 μg of protease (ratio, 1:60). The [D]/[A] ratio increased when the ratio of sensor/protease expression plasmid DNA was lowered (higher expression of protease relative to sensor was expected).

(Fig. 6). The mean [D]/[A] ratio measured for cells expressing ECFP-CS_{wt}-citrine alone was 0.891 ± 0.066 (mean ± standard deviation) (Fig. 6A), which was very close to the expected value of 1 for a chimera containing equimolar ECFP and citrine. The following different ratios of pECFP-CS_{wt}-Citrine and pscNS3/$4A_{Pt1}$ expression plasmid DNA were tested in the cotransfection experiments: 0.5/0.5 μg (Fig. 6B), 0.3/0.7 μg (Fig. 6C), and 0.05/3 μg (Fig. 6D) sensor/protease. The [D]/[A] ratio increased when protease expression plasmid DNA was in excess (higher expression of protease relative to sensor is likely to occur) (Fig. 6B to D). This result indicates that after cleavage by the protease, the acceptor moiety (citrine with the sequence SMSYTWTG in its amino-terminal end) is being degraded at a higher rate than the donor (ECFP with the sequence GRA SEDVVCC in its carboxyl-terminal end). Why the acceptor should be more labile than the donor after cleavage is presently unknown, given the overall stability of *Aequorea victoria*-derived fluorescent proteins (such as ECFP and citrine) (13). Interestingly, this result suggests that, in our assay, determination of the [D]/[A] ratio may be a readout mode to detect NS3/4A protease activity in addition to the FRET rate.

DISCUSSION

The clinical success of inhibitors designed against the human immunodeficiency virus type 1 protease and the fact that the HCV NS3/4A protease has an essential role in both viral replication and pathogenesis have made this protease a promising target for therapeutic intervention. The development of therapeutics against HCV has been hampered by the lack of an efficient cell culture system and a small-animal model for this virus. Lohmann et al. partially solved this problem (17) by developing a reliable cell culture HCV replication system based on a subgenomic HCV or replicon. Nonetheless, this system was restricted to replicons derived from genotype 1 isolates, thus limiting the ability to evaluate potential inhibitors

across a spectrum of clinically relevant genotypes, and the system only mimicked the authentic replication cycle of HCV, without production of infectious particles. In 2005, the first cell culture replication system based on an HCV genome of genotype 2a, that of the JFH1 strain, was published (16, 29, 33). However, there is not yet a cell culture system for the most-prevalent genotypes worldwide, 1a and 1b. Although the replicon system has some limitations, it is currently the preferred standard to evaluate HCV antivirals.

Several alternative mammalian-cell-based systems for monitoring the activity of NS3/4A serine protease, based on reporter substrates fused to a cleavage sequence, have been reported (1, 15, 22). We have constructed a genetically coded FRET probe that detects NS3/4A protease activity in living cultured human cells. This system is simple and allows the characterization of large cohorts of samples of different genotypes. Because fluorescence techniques are very sensitive, the assay can potentially be used to evaluate inhibitors against proteases of several genotypes and can be adapted to assay formats suitable for high-throughput pharmacological screening. In contrast with the replicon system, this approach allows the evaluation of the protease activity alone, without interference from other viral components. This is important in order to demonstrate that the effect of inhibitors is exerted on the target enzyme, the NS3/4A protease. The FRET-based assay described here can be seen as a complement to the HCV replicon or tissue-grown HCV systems. We did not attempt as yet to introduce the FRET substrate into these systems, and thus we have not addressed whether the sensitivity of the assay would be sufficient to detect potentially lower protease levels resulting from viral RNA replication.

A limitation for assessing protease activity in transfected mammalian cells is that the relative concentration of substrate sensor and NS3/4A enzyme will likely vary from cell to cell. Interestingly, energy transfer did not reach zero at sensor/protease DNA ratios as low as 1/60 (Fig. 3C). The expression levels of the fluorescent sensor within cells have bell-shaped histograms (number of cells versus fluorescence intensity) (data not shown). If the expression of the protease is similarly distributed, cells having limiting protease amounts will tend to show a higher apparent FRET rate than the average population. However, the ability of the present assay to quantify protease activity on a cell-by-cell basis provides unique advantages. For instance, a DNA library of NS3/4A protease variants could be transfected and cells sorted according to FRET rate.

In summary, we have developed a mammalian-cell-based assay which allows measurement of the catalytic efficiencies of HCV NS3/4A protease variants from different HCV genotypes. It will likely complement in vitro assays and methods based on the subgenomic replicon to fully understand sequence/activity relationships of NS3/4A proteases and their interaction with host cell factors and could be used for screening of inhibitory molecules.

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R.S. had the idea for the project, designed, cloned and sequenced mammalian expression constructs, did Western blots and statistical analysis, and drafted and edited the manuscript. F.P. performed all imaging experiments and fluorimetry and helped with cloning and statistics. B.D. set up FRET imaging methods and controls and, together with F.P., analyzed FRET data. S.F. cloned protease variants in bacterial expression vectors. M.-A.M. advised on NS3 protease biochemistry and variants and helped with writing. J.L. supervised project design, experiments, and data analysis and wrote the paper.

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