Enzymatic Characterization of Membrane-Associated Hepatitis C Virus NS3-4A Heterocomplex Serine Protease Activity Expressed in Human Cells†

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Received December 9, 2004; Revised Manuscript Received February 3, 2005

ABSTRACT: The hepatitis C virus (HCV) nonstructural (NS)3-NS4A serine protease heterocomplex is a prime target for development of novel HCV therapies, due to its essential role in maturation of the viral polyprotein. While the mode of substrate/inhibitor recognition of the HCV NS3/NS4A serine protease has been extensively studied in vitro, important molecular aspects of the mechanism of action for this membrane-bound multifunctional enzyme remain unresolved in vivo. In particular, what influence does membrane association exert on the specificity and catalysis of NS3-4A protease? To carry out this study, we developed a specific and sensitive protease assay using a unique internally quenched fluorogenic substrate (IQFS). Our IQFS enables for the first time the direct, specific detection of NS3-4A protease activity within membrane fractions isolated from human cells expressing NS3-4A and the determination of its steady-state kinetic parameters, which were found to be $K_m = 51 \pm 3$ μM and $k_{cat} = 0.39$ min^{-1}. We also show that our fluorescence-based bioassay can be used to evaluate specifically the potency and mode of action of NS3-4A directed inhibitors, such as in the case of a known NS3-4A substrate-analogue inhibitor ($K_i = 22$ nM). Our results indicate that the membrane anchoring of NS3 by NS4A does not affect the substrate/inhibitor recognition by the NS3-4A protease domain. Further investigation may reveal whether membrane association could be important for regulating other enzymatic activities associated with NS3 (e.g., helicase and/or ATPase) and/or regulating the recently proposed cross-talk between the protease and helicase activities.

Hepatitis C virus (HCV)[1] is a serious worldwide health concern owing to the large numbers of infected individuals globally (~170 million) and the lack of a treatment regime which is effective against all HCV genotypes. Roughly 50% of infected individuals develop chronic HCV infection, which is a leading cause of serious liver disease, including cirrhosis and/or hepatocellular carcinoma (*1*).

HCV is a member of the Flaviviridae family, possessing a positive-sense, single-stranded RNA genome (approximately 9.6 kb) that encodes a single, large polyprotein, which is translated by host cell ribosomes in the cytoplasm. The polyprotein must then be proteolytically cleaved to yield individual functional viral proteins (*2*), a process that occurs both co- and posttranslationally and is mediated by a combination of cellular and viral proteases to generate at least 10 viral proteins (*2*). The HCV structural proteins are located in the amino-terminal end of the polyprotein and are cleaved by cellular signal peptidases within the endoplasmic reticulum (ER) (*3*−*5*). The nonstructural (NS) NS2, NS3, NS4A, NS4B, NS5A and NS5B proteins comprise the remainder of the polyprotein and are involved in viral replication. The junction between the NS2 and NS3 proteins is cleaved by a poorly understood rapid autoproteolytic event (*6*−*8*), followed by cleavage of the NS3/4A junction, which occurs posttranslationally and is mediated by the serine protease domain of the NS3 protein. This releases the NS3 and NS4A proteins, which form a protease heterocomplex that mediates the cleavage of all the other downstream junctions (*9*−*11*).

NS3 is a multifunctional enzyme, containing a serine protease domain in its N-terminal region and an ATPase/helicase domain located in its C-terminal half (*9*−*12*). It forms a stable complex with NS4A, a 54 amino acid protein which acts as a protease cofactor, greatly enhancing the proteolytic activity of NS3 and being an absolute requirement for cleavage of the NS3/4A and NS4B/5A junctions (*13*). The interaction between NS3 and NS4A is also believed to tether NS3 to the ER membrane by virtue of the hydrophobic α-helix domain at the N-terminus of NS4A (*14*). Functional NS3-4A serine protease is essential for infectivity in a chimpanzee model of HCV infection (*15*), and importantly, validation of NS3 as a effective target for anti-HCV therapy was recently shown by Lamarre et al., who demonstrated a decrease in viral RNA levels in the plasma of HCV-infected patients treated with a small molecule NS3 inhibitor (*16*).

HCV NS3-4A protease has been extensively characterized in vitro; however, it is possible that its enzymatic characteristics may differ in vivo. It has been shown that the nonstructural proteins NS3 to NS5B associate with the ER

† This work was supported by a Canadian Institutes of Health Research Initiative on Hepatitis C scholarship and Grants EOP38153/EOP67273 (to F.J.).

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[1] Abbreviations: Abz, *O*-aminobenzoyl; ER, endoplasmic reticulum; *E. coli*, *Escherichia coli*; FRET, fluorescence resonance energy transfer; HCV, hepatitis C virus; IQFS, internally quenched fluorogenic substrate; MF, membrane fraction; MALDI-TOF, matrix-assisted laser desorption/ionization time of flight; NS, nonstructural; TFA, trifluoroacetic acid.

10.1021/bi047408j CCC: $30.25 © 2005 American Chemical Society
Published on Web 04/08/2005

membrane, forming the viral "replicase" complex (*14*, *17*−*23*), and in addition, numerous physical interactions between the HCV nonstructural replicase proteins, including NS3-4A, have been reported as occurring in vitro (*3*, *6*, *24*−*27*). Hence, it may be important to investigate the effect that the association of NS3-4A with cellular membranes and other viral replicase or cellular components may have upon its enzymatic properties in order to further our understanding of HCV RNA replication and to evaluate novel antiviral compound candidates.

Recent advances in the development of HCV replicon systems (reviewed in ref *28*), which allow the expression of subgenomic or full-length HCV RNA in mammalian cells, have led to the development of assays to detect HCV RNA replication in whole cell lysates or membrane fractions isolated from HCV replicon-containing hepatoma cells (*29*−*31*). Also recently, cell-based assays that use reporter protein systems to indirectly detect HCV NS3-4A protease activity have been described (*32*, *33*). However, as yet no continuous in vitro assay to allow the direct and specific detection of protease activity from NS3-4A expressed in mammalian cells has been reported.

In this paper, we report the development of a continuous fluoresence assay for the detection of membrane-associated HCV NS3-4A serine protease activity using a novel internally quenched fluorogenic substrate (IQFS). We demonstrate that our IQFS allows the specific detection and enzymatic characterization of NS3-4A protease activity and have used this system to determine, for the first time, the kinetic parameters of membrane-associated NS3-4A protease within membrane fractions isolated from a human cell line that expresses the full-length NS3−full-length NS4A heterocomplex (*14*).

MATERIALS AND METHODS

Cell Culture. UNS3-4A cells (*14*) were grown at 37 °C in Dulbecco's modified Eagle medium supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 1 μg/mL puromycin, 500 μg/mL G418, 50 units/mL penicillin, 50 μg/mL streptomycin, and 1 μg/mL tetracycline. To induce expression of NS3-4A protease, monolayers of cells at greater than 50% confluency were washed twice with phosphate-buffered saline (PBS), and fresh medium lacking tetracycline was added.

Synthesis of Peptides. Internally quenched fluorescent substrate-1 (IQFS-1) [*O*-aminobenzoyl (Abz)-Asp-Asp-Ile-Val-Pro-Cys-Ser-Met-Ser-Tyr(3-NO_2)-Thr-NH_2] and Abz standard peptide (Abz-Asp-Asp-Ile-Val-Pro-Cys-OH) were synthesized by the Protein Service Laboratory (University of British Columbia) using Fmoc [*N*-(9-fluorenyl)methoxycarbonyl] methodology. A noncleavable decapeptidyl NS3 inhibitor peptide, PepInh-1 (fluorescein-Asp-D-Glu-Leu-Ile-Cha-Cys-Pro-Cha-Asp-Leu-NH_2, where Cha is β-cyclohexylalanine), was synthesized by Anaspec Inc. (San Jose, CA). In all cases, peptide purity and composition were demonstrated by reverse-phase high-pressure liquid chromatography (RP-HPLC) and matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry, respectively. Peptide stocks were also quantified by amino acid analysis (Advanced Protein Technology Centre, HSC, Toronto).

Preparation of Purified Recombinant NS3-4A. The bacterial expression plasmid for NS3-4A (pET NS3/4A) was kindly provided by Dr. Paola Gallinari (*34*). The expression in *Escherichia coli* and subsequent purification of full-length NS3-4A will be described elsewhere (M. J. Richer et al., manuscript in preparation).

Immunofluorescence Microscopy. UNS3-4A cells were grown on glass coverslips in 16 mm wells seeded with 1×10^5 cells/well. At different time points following removal of tetracycline from the growth medium, cells were fixed using 2% formaldehyde in PBS for 30 min at room temperature and then permeabilized using PBS containing 0.05% saponin and 3% bovine serum albumin (BSA) for 30 min at room temperature. Cells were incubated with primary antibody [monoclonal anti-NS3 (1:100, Novocastra)], monoclonal anti-NS4A (1:100, Anogen), or goat anti-NS4A (1:100, Biodesign International) diluted in PBS containing 0.05% saponin and 3% BSA for 1 h at room temperature and then washed using 2% saponin in PBS. Cells were incubated with Alexa Fluor 488-conjugated anti-mouse or Alexa Fluor-568 conjugated anti-goat secondary antibody (1:100, Molecular Probes) diluted in PBS containing 0.05% saponin and 3% BSA for 1 h at room temperature in the dark and washed as previously described. Air-dried coverslips were mounted in 2.5% DABCO anti-fading reagent and viewed under a 63× oil immersion lens using a Bio-Rad Radiance confocal microscope.

Western Blotting. UNS3-4A cells grown for 48 h in either the presence or absence of tetracycline were lysed by addition of lysis buffer [150 mM NaCl, 1% Igepal CA630 (Sigma), 0.5% deoxycholate, 0.1% SDS, 50 mM Tris (pH 8), and 1 complete EDTA-free protease inhibitor tablet (Roche) in a total volume of 50 mL] followed by incubation on ice for 15 min. Protein (20 μg) from 0, 12, 24, and 48 h cell extracts was then resolved by SDS−polyacrylamide gel electrophoresis on a 15% gel and transferred to a nitrocellulose membrane. The membrane was probed with an anti-NS3 monoclonal primary antibody (1:1000, Novocastra) and HRP-conjugated anti-mouse secondary antibody (1:2000, Amersham Biosciences). For Western analysis of membrane fractions, equal amounts of membrane from (+)- and (−)-tet UNS3-4A cells were separated by SDS−PAGE. Membranes were then probed using either anti-NS3 monoclonal (1:1000), anti-NS4A monoclonal (Dr. Alla Kusch, 1:300), or anti-HSP47 monoclonal (Stressgen, 1:1000) followed by the appropriate HRP-conjugated secondary antibody (1:1000).

Cellular Membrane Fractionation. Cellular membrane fractions were isolated as described in Hardy et al. (*29*). Briefly, cells were lysed by resuspension in ice-cold hypotonic lysis buffer [10 mM Tris (pH 7.8), 10 mM NaCl] for 20 min followed by disruption using a Dounce homogenizer. Lysates were centrifuged at 900*g* for 5 min at 4 °C to remove the nuclei, and the resulting supernatants were subjected to further centrifugation at 15000*g* for 20 min at 4 °C to pellet the NS3-4A-containing cellular membranes. Membrane pellets were resuspended in lysis buffer containing 15% glycerol. Pellets isolated from 2×10^7 cells were resuspended in a final volume of 200 μL, aliquotted, snap frozen, and stored at −80 °C.

NS3-4A Protease Enzymatic Assay. UNS3-4A cells grown for 48 h in either the presence or absence of tetracycline

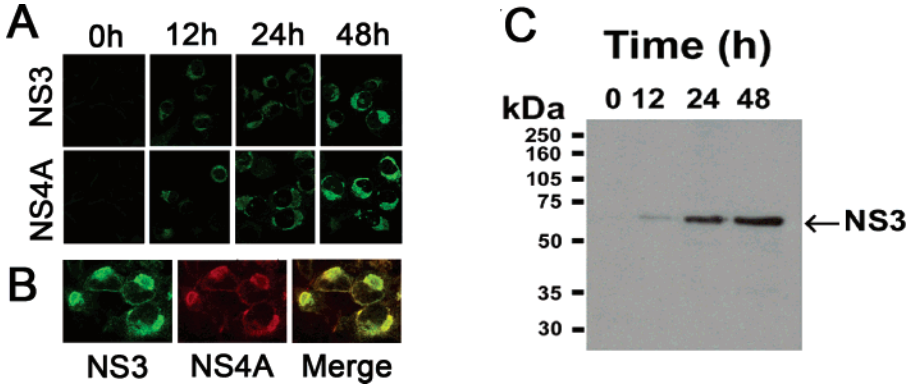

FIGURE 1: Expression of NS3 and NS4A in UNS3-4A cells. (A) UNS3-4A cells were grown in medium lacking tetracycline to induce NS3-4A expression and then fixed at the time points indicated following tetracycline withdrawal. Monolayers were then stained using either NS3- or NS4A-specific monoclonal antibodies followed by Alexa Fluor 488-conjugated anti-mouse antibody and then viewed by confocal microscopy. (B) Colocalization of NS3 and NS4A at 48 h post-tetracycline removal in UNS3-4A cells was detected by confocal fluorescence microscopy using an NS3-specific monoclonal and an NS4A-specific polyclonal. The merged image is shown in the right-hand panel. (C) Detection of NS3 in lysate from UNS3-4A cells grown in the absence of tetracycline for the durations of time indicated. Whole cell lysates were resolved by SDS−PAGE on a 12% gel and analyzed by Western blotting using an NS3-specific antibody.

were harvested as described for Western blotting. For assays using whole cell lysate, cells were lysed as described for Western blotting, and aliquots representing 0.5×10^5 cells were added to protease assay reactions of total volume 100 μL in 96-well plates. For assays containing membrane fractions, aliquots representing 1×10^6 cells were used. For assays containing recombinant, purified NS3-4A, 0.11 μM protein was added to each reaction. Protease assay buffer consisted of 50 mM Hepes (pH 7.3), 100 mM NaCl, 0.1% Triton X-100, and 10 mM dithiothreitol. Following addition of 0.1 mM IQFS-1 substrate, protease activity within cell extracts was monitored by detection of fluorescence emitted from each well in real time at 30 °C using a SpectraMax Gemini XS spectrofluorometer equipped with a temperature-controlled 96-well plate reader, set at excitation and emission wavelengths of 320 and 420 nm, respectively (Molecular Devices). To confirm the scissile bond cleaved by recombinant NS3-4A, reactions were carried out as described except that Triton X-100 was omitted from the buffer. Reactions were stopped by the addition of 0.3% formic acid and then analyzed by MALDI-TOF MS using negative mode (Genome BC Proteomics Centre, Victoria, BC, Canada).

Calculation of the Inhibition Constant K_i and Kinetic Parameters K_m and V_{max}. Whole cell lysates, cellular membrane fractions, or recombinant NS3-4A was preincubated with varying concentrations of PepInh-1 peptide inhibitor and assay buffer constituents at 30 °C for 15 min. The residual protease activity was then determined by the addition of 0.1 mM IQFS-1 per reaction and performing protease assays as described above. Estimation of the inhibition constant (K_i) of PepInh-1 against NS3-4A protease was calculated by fitting the data (v_i and I) to the equation $v_i = SA\{E_0 - 0.5[(E_0 + I + K_i) - [(E_0 + I + K_i)^2 - 4E_0I]^{1/2}]\}$, where v_i = initial velocity, SA = specific activity, E_0 = active enzyme concentration, and I = inhibitor concentration by nonlinear regression using the ENZFITTER program (Elsevier-Biosoft, Cambridge, U.K.) (*35*). Initial rate values for both recombinant NS3-4A or (−)-tet UNS3-4A cell membrane fractions were obtained by performing protease assays in the presence of a range of IQFS-1 substrate concentrations. The kinetic parameters K_m and V_{max} were then calculated by fitting these values to the hyperbolic Michaelis−Menten rate equation using the Sigma Plot 2000 Enzyme Kinetic Module (Systat Software Inc.). The amounts of cleaved IQFS-1 product generated were determined by extrapolation of values from a standard curve of fluorescence as a function of increasing concentration using Abz-DDI-VPC-OH standard peptide.

RP-HPLC. Whole protease assay reactions were resolved by RP-HPLC on a Varian Microsorb MV C8 column (whole cell lysates) or Vydac monomeric C18 column (membrane fractions) using a Polaris 212 system equipped with a model 363 fluorescence detector (Varian Inc.). Samples were resolved using a linear gradient of 5−70% of acetonitrile/0.1% TFA over 65 min (1 mL/min). Eluted proteins were detected by UV absorbance at 225 nm, and fluorescent IQFS cleavage products were detected using excitation and emission wavelengths of 320 and 420 nm, respectively.

RESULTS

Expression of HCV NS3-4A in Tetracycline-Regulated Cells. UNS3-4A cells, first described by Wölk et al. (*14*), are human osteosarcoma cells that inducibly express the HCV NS3-4A protease heterocomplex derived from genotype 1b. Expression of viral protein is tightly regulated under the control of a tetracycline-sensitive promoter. Expression of viral protein is repressed when cells are grown in tetracycline-containing medium but is triggered following removal of tetracycline and increases over time until a steady state is reached at 48 h.

To establish that the cells behaved as previously reported, in our hands, we performed immunofluorescence and Western blotting analysis of UNS3-4A cells to detect NS3 and NS4A at different time points following removal of tetracycline from the growth medium (0, 12, 24, and 48 h). UNS3-4A cell monolayers stained with NS3- and/or NS4A-specific antibodies were examined by confocal fluorescence microscopy (Figure 1A,B). As expected, no signal corresponding to either NS3 or NS4A was detected in cells at 0 h post-tetracycline removal, but the amounts of NS3 and NS4A detected increased up to 48 h following tetracycline removal. Similarly, Western blotting analysis of UNS3-4A

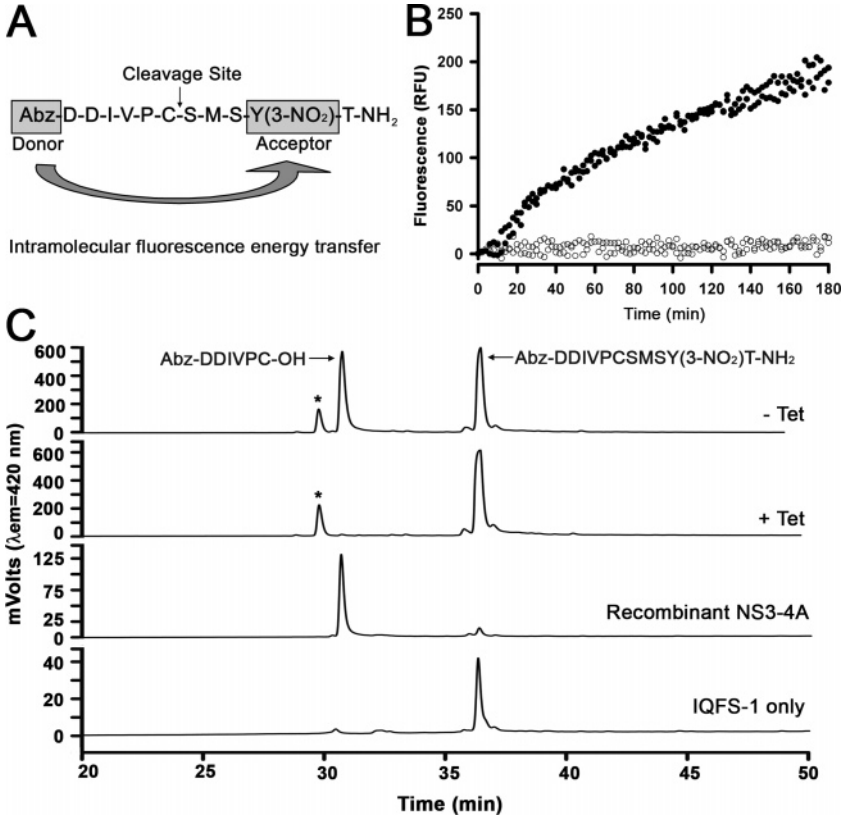

FIGURE 2: Detection of NS3-4A-specific protease activity in whole cell extracts from UNS3-4A cells. (A) The amino acid sequence and features of the IQFS-1 substrate used in protease assays to detect NS3-4A protease activity are shown. (B) On-line assay showing protease activity in UNS3-4A cells. Whole cell lysates from UNS3-4A cells grown in the presence [(+)-tet] or absence [(−)-tet] of tetracycline for 48 h were assayed for their ability to cleave IQFS-1. Lysates were mixed with protease assay constituents, and assays were initiated by addition of 0.1 mM IQFS-1. The fluorescence (RFU, $\lambda_{ex} = 320$ nm; $\lambda_{em} = 420$ nm) detected from duplicate reactions containing either (+)-tet (open circles) or (−)-tet (closed circles) UNS3-4A cell lysate is plotted against time. (C) RP-HPLC chromatograms showing the separation of fluorescent IQFS digestion products produced in protease assays containing (from top to bottom) (−)-tet NS3-4A cell lysate, (+)-tet NS3-4A cell lysate, recombinant NS3-4A, or IQFS-1 only. Fluorescent peptides were detected following elution using a fluorescence detector set at excitation and emission wavelengths of 320 and 420 nm, respectively. Peaks corresponding to cleaved IQFS-1 (Abz-DDIVPC-OH) and uncleaved IQFS-1 [Abz-DDIVPCSMSY(3-NO_2)T-NH_2] are indicated. A smaller nonspecific peak common to both (+)- and (−)-tet cell lysate-containing reactions is labeled with an asterisk.

whole cell lysates resolved by SDS−PAGE using an anti-NS3 monoclonal antibody (Figure 1C) revealed that no NS3 is detected at 0 h following tetracycline removal but an increasing signal corresponding to NS3 is detected in cell lysates from 12 to 48 h after induction of NS3-4A expression.

Having confirmed that the tetracycline regulation of NS3-4A expression was robust and that, as previously reported, levels of expression of both proteins increase over time following tetracycline removal, reaching a steady state at 48 h (*14*), we chose to harvest cells grown for 48 h in the absence of tetracycline to use in our assays aimed at detecting NS3-4A protease activity.

Detection of NS3-4A Protease Activity in the Lysate of UNS3-4A Cells Grown in the Absence of Tetracycline. Since the tetracycline-regulated UNS3-4A cell line provides a reliable system for inducible and high-level expression of NS3-4A, we sought to determine whether we could detect NS3-4A-specific protease activity in cells grown in the absence of tetracycline. We recently developed an in vitro assay to monitor purified, recombinant NS3-4A protease activity that depends on intramolecular resonance energy transfer within NS3-4A protease peptide substrates (internally quenched fluorogenic substrates; IQFS). The peptides correspond to an 11-residue sequence encompassing the cleavage junctions between the nonstructural proteins in the HCV polyprotein and are characterized by a fluorescent donor group (*O*-aminobenzoyl) at one end and an acceptor, or quenching, group at the other end (3-nitrotyrosine) (*36*, *37*).

We used an IQFS corresponding to the NS5A/NS5B cleavage junction sequence in the HCV polyprotein (IQFS-1), with the following exception: the cysteine residue at the P2 position in the original sequence was substituted with a proline to avoid disulfide linkage between vicinal cysteines in solution, as originally described by Kakiuchi et al. (*38*). The IQFS was also amidated to stabilize the peptide and prevent its degradation by carboxypeptidases present in UNS3-4A cell lysates (*39*). The sequence and features of IQFS-1 are shown in Figure 2A.

Whole cell extracts of UNS3-4A cells grown for 48 h in either the presence [(+)-tet] or absence of tetracycline [(−)-tet] were tested for their ability to cleave the IQFS-1 substrate in our continuous protease assay. Cell lysates were mixed with protease assay constituents, and the assay was started by addition of 0.1 mM IQFS-1 substrate to each reaction. A linear increase in fluorescence was detected in reactions containing lysates from cells induced to express viral proteins [(−)-tet]; however, no significant increase in fluorescence was detected in (+)-tet cell lysate-containing reactions over the 3 h assay period (Figure 2B), indicating that IQFS-1 was cleaved by lysate from cells expressing NS3-4A but that no

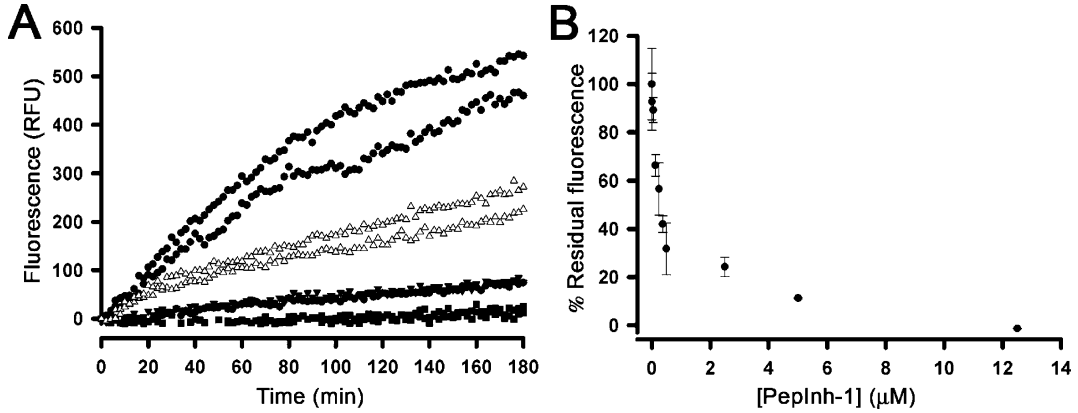

FIGURE 3: Protease activity detected in (−)-tet UNS3-4A cell lysate can be inhibited by a known noncleavable, substrate-analogue peptide NS3 inhibitor, PepInh-1. (A) Whole (−)-tet UNS3-4A cell lysates were tested for their ability to cleave IQFS-1 substrate in a continuous protease assay following preincubation for 15 min at 30 °C with PepInh-1. Fluorescence released from duplicate reactions containing (−)-tet cell lysate only (closed circles), (−)-tet cell lysate containing 0.375 μM PepInh-1 (open triangles), (−)-tet cell lysate containing 5 μM PepInh-1 (closed triangles), or (+)-tet cell lysate only (closed squares) was plotted against time (RFU, $\lambda_{ex} = 320$ nm; $\lambda_{em} = 420$ nm). (B) Titration of NS3-4A activity in (−)-tet UNS3-4A cell lysate using PepInh-1. The residual protease activity of (−)-tet UNS3-4A cell lysate preincubated with PepInh-1 at varying concentrations was plotted as a percentage of the activity of (−)-tet cell lysate containing no PepInh-1 (100%).

cleavage of the substrate occurred with lysate from cells in which NS3-4A expression was repressed. These findings indicated that cleavage of IQFS-1 was mediated by the NS3-4A protease expressed in the (−)-tet cells; however, since the release of fluorescence from the IQFS substrate occurs following cleavage between any of the bonds in the peptide sequence between the donor and acceptor, we sought to verify that cleavage of the IQFS occurred after the cysteine residue in the P1 position, consistent with the known proteolytic specificity of NS3-4A (*10*, *40*−*42*).

Whole protease assay reactions containing UNS3-4A cell lysates were analyzed by RP-HPLC to resolve the IQFS-1 cleavage products, which were detected following elution using a fluorescence detector (Figure 2C). Control protease assay reactions in which no cell lysate was added or in which purified recombinant NS3-4A protease was included were analyzed at the same time to indicate the elution times of fluorescent peptides corresponding to uncleaved IQFS substrate [Abz-DDIVPCSMSY(NO_2)T-NH_2] and recombinant NS3-4A cleaved IQFS substrate fragment (Abz-DDIVPC-OH), respectively.

Figure 2C shows representative RP-HPLC chromatograms of the resolution of IQFS-1 digestion products generated by (−)-tet UNS3-4A cell lysate, (+)-tet UNS3-4A cell lysate, purified recombinant NS3-4A and from reactions containing neither recombinant NS3-4A or UNS3-4A cell lysate (IQFS-1 only). Uncleaved IQFS-1 is eluted at 36 min and is observed in every chromatogram. Chromatograms derived from reactions containing recombinant NS3-4A protease contain an additional peak, corresponding to the cleaved IQFS-1 peptide fragment, which is eluted at 31 min. This elution profile indicates that cleavage of the IQFS by NS3-4A only occurs at one position in the peptide, as expected, since the IQFS contains only one cysteine residue. Comparison with the chromatogram from (−)-tet cell lysate-containing reactions shows that the same elution profile is also produced in these samples, with a single cleavage product generated having the same retention time as that of the cleavage product generated by recombinant NS3-4A (31 min). Consistent with the fluorescence emission data, the Abz-DDIVPC-OH cleavage product is not detected in the reactions containing (+)-tet cell lysate. However, it is noted that a small peak corresponding to a fluorescent product eluted earlier than that generated by NS3-4A protease is detected in assay reactions from both (+)- and (−)-tet cell lysates, representing a cleavage product that is most likely generated by a cellular protease.

Inhibition of Protease Activity in (−)-tet UNS3-4A Cell Lysate Using a Substrate-Analogue NS3-4A Peptide Inhibitor. Having demonstrated that protease activity attributable to HCV NS3-4A could be detected in (−)-tet UNS3-4A cell lysate, we then sought to determine whether this activity could be inhibited by a known NS3-4A inhibitor (*43*). Inhibition of HCV NS3-4A by substrate-analogue inhibitors derived from the sequences of junctions within the viral polyprotein has been reported previously (*43*, *44*). Optimization of these peptidyl inhibitors by improving binding efficiencies and the incorporation of amino acid analogues and chiral isomers has resulted in the generation of potent active site directed inhibitors of NS3-4A, such as Ac-Asp-D-Glu-Leu-Ile-Cha-Cys-Pro-Cha-Asp-Leu-NH_2 (Ac/PepInh-1), which acts as a tight binding, noncleavable substrate-analogue inhibitor (*43*).

A peptidyl analogue of this peptide inhibitor (PepInh-1) was synthesized by adding a fluorescein group at the N-terminus (for the purpose of detection in another application) and tested for its ability to inhibit the protease activity of (−)-tet UNS3-4A cell lysate. UNS3-4A cell lysates were preincubated with protease assay constituents and PepInh-1 for 15 min at 30 °C, and then the residual protease activity was measured by addition of 0.1 mM IQFS-1. The amount of fluorescence detected from (−)-tet cell lysate-containing reactions decreased with increasing concentration of PepInh-1, indicating that PepInh-1 inhibits NS3-4A protease activity in a dose-dependent manner (Figure 3A).

Plotting the residual protease activity of (−)-tet cell lysate reactions containing varying concentrations of peptide inhibitor as a percentage of activity in the absence of inhibitor (expressed as 100%) results in a titration curve typical of a tight-binding inhibitor (Figure 3B). From this curve, the overall inhibition constant (K_i) of PepInh-1 against NS3-4A activity in (−)-tet cell lysate was calculated by curve fitting

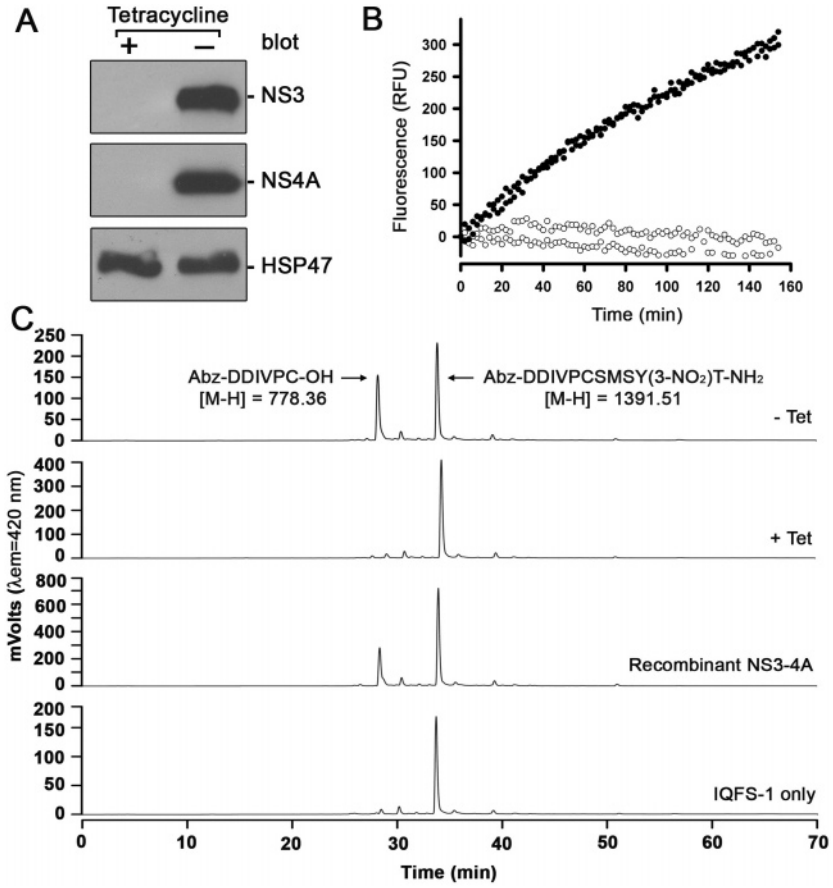

FIGURE 4: Specific detection of NS3-4A protease activity in membrane fractions isolated from (−)-tet UNS3-4A cells. (A) Western blot analysis of membrane fractions isolated from UNS3-4A cells. Equal amounts of (+)- and (−)-tet UNS3-4A cell membrane fractions were resolved by SDS−PAGE on either 10% or 15% gels and then analyzed by Western blotting using either NS3-, NS4A-, or HSP47-specific monoclonal antibodies. (B) Continuous on-line assay showing protease activity in membrane fractions isolated from (+)-tet (open circles) and (−)-tet (closed circles) UNS3-4A cells, measured as increase in fluorescence (RFU, λ_{ex} = 320 nm; λ_{em} = 420 nm) over time. (C) RP-HPLC analysis of fluorescent peptide products within protease assay reactions containing (from top to bottom) (−)-tet UNS3-4A membrane fraction, (+)-tet UNS3-4A membrane fraction, recombinant NS3-4A, and IQFS-1 only. Peaks corresponding to cleaved IQFS-1 (Abz-DDIVPC-OH) and uncleaved IQFS-1 [Abz-DDIVPCSMSY(3-NO_2)T-NH_2], as well as their observed molecular masses, are indicated. Fluorescent peptides were detected following elution using a fluorescence detector set at excitation and emission wavelengths of 320 and 420 nm, respectively.

using the Enzfitter program to be 124 ± 90 nM (mean K_i calculated from two independent experiments).

Detection of NS3-4A Protease Activity from the Isolated Membrane Fractions of UNS3-4A Cells. The HCV replicase complex, which includes the NS3-4A protease, associates with cellular ER-derived membranes (*22*, *45*, *46*). This was recently confirmed to be the site of viral replication by Hardy et al. and Lai et al. (*29*, *30*), who demonstrated that HCV NS5B RNA polymerase activity can be detected in membrane fractions isolated from HCV replicon-containing cell lines. Following similar protocols to those used in these studies, we isolated membrane fractions from UNS3-4A cells grown in the absence or presence of tetracycline and tested them for NS3-4A-specific protease activity in a continuous assay using IQFS-1 substrate. Figure 4A shows Western blot analysis of SDS−PAGE resolved membrane fractions isolated from (+)- and (−)-tet UNS3-4A cells using either NS3-, NS4A-, or cellular protein HSP47-specific antibodies. As expected, strong signals corresponding to both NS3 and NS4A are seen in (−)-tet membrane fractions but not in (+)-tet cell membrane fractions, indicating that we had successfully purified the NS3-4A-containing fraction from the cells. We further analyzed the (+)- and (−)-tet membrane fractions using an antibody specific for the cellular protein colligin (HSP47), a collagen-specific stress glycoprotein which resides in the ER (*47*). A band corresponding to HSP47 was detected in both (+)- and (−)-tet cell samples, indicating that this fraction corresponds to the cellular membrane fraction.

A representative plot of fluorescence released from protease assay reactions containing either (+)-tet or (−)-tet UNS3-4A cell membrane fractions is shown in Figure 4B. A significant linear increase in fluorescence was emitted from (−)-tet cell membrane reactions over time, whereas no increase in fluorescence was observed in reactions containing (+)-tet cell membrane extracts, indicating that, as was observed for UNS3-4A whole cell lysates, membrane fractions from UNS3-4A cells induced to express NS3-4A [(−)-tet] mediated proteolytic cleavage of the IQFS-1 substrate. In contrast, membrane fractions from UNS3-4A cells in which NS3-4A expression was repressed [(+)-tet] did not exhibit any detectable protease activity.

Membrane fraction-containing protease assay reactions were analyzed by RP-HPLC to confirm that the cleavage products generated were consistent with those generated by recombinant purified NS3-4A protease (Figure 4C). We note that the retention times of both uncleaved and cleaved IQFS-1 vary slightly from those presented in Figure 2C, due to a

different column being used to analyze these samples. An IQFS-1 cleavage product with the same retention time as that generated by purified recombinant NS3-4A was detected in (−)-tet cell membrane-containing assay reactions (28 min). Using MALDI-TOF MS analysis, we have confirmed that the molecular mass of this cleavage product generated by recombinant NS3-4A corresponds to that of the expected IQFS-1 N-terminal fragment (observed [M − H] = 778.36, calculated = 779.84). We have also confirmed the presence of the C-terminal IQFS-1 cleavage fragment (observed [M − H] = 628.06, calculated = 629.63) and the intact IQFS-1 peptide (observed [M − H] = 1391.63, calculated = 1392.45) in the same protease reaction sample. In contrast, no such cleavage product was detected in assay reactions containing (+)-tet cell membrane fractions. These data clearly indicate that the cleavage product generated by (−)-tet cellular membrane fractions results from NS3-4A-mediated proteolysis. Unlike the reactions containing (+)- and (−)-tet UNS3-4A whole cell lysate, no small fluorescent product (elution time 29 min; Figure 2C) was detected in reactions containing either (+)- and (−)-tet cell membrane fractions. These results demonstrate that NS3-4A protease expressed in UNS3-4A cells grown in the absence of tetracycline is efficiently isolated with cellular membrane fractions and that it remains enzymatically active throughout this process.

Determination of Kinetic Parameters for Membrane-Associated NS3-4A Protease. To determine kinetic constant parameters for membrane-associated NS3-4A enzyme, protease assays were performed in the presence of a range of concentrations of IQFS-1 (9−90 μM). The initial velocity values were then fitted to the Michaelis−Menten rate equation (Enzyme Kinetics Module, SigmaPlot) to determine the K_m and V_{max} values, which were found to be 51 ± 3 μM and 2.1 ± 0.3 μM/h, respectively (values represent the average determined from two independent experiments carried out in duplicate). A representative plot is shown in Figure 5A. The same kinetic analysis was also performed using recombinant purified NS3-4A and used to calculate K_m and V_{max} values of 60 ± 11 μM and 2.4 μM/h, respectively (Figure 5A, inset).

NS3-4A Protease Activity in Membrane Fractions Can Also Be Inhibited Using a Peptidyl Substrate-Analogue Inhibitor. As was found with whole lysates from (−)-tet UNS3-4A cells, preincubation of PepInh-1 with (−)-tet membrane fractions prior to addition of IQFS-1 resulted in a reduction of protease activity in a dose-dependent manner. Plotting the residual protease activity of (−)-tet cell membrane fractions following preincubation with PepInh-1 as a percentage of protease activity in the absence of inhibitor generates a titration curve of NS3-4A protease activity characteristic of a tight-binding inhibitor (Figure 5B). By fitting the data to an equation for equilibrium binding (*35*) using data from four independent experiments, a K_i value of 22 ± 6 nM for inhibition of membrane-associated NS3-4A by PepInh-1 was calculated. We also successfully used PepInh-1 as a titrating agent against recombinant purified NS3-4A, and a typical titration curve is shown in Figure 5B (inset). The K_i value obtained for recombinant NS3-4A was 15 ± 2 nM, corresponding to the average value derived from two independent experiments. In both cases, the calculated ratio of E_0/K_i (where E_0 is active enzyme concentration) was

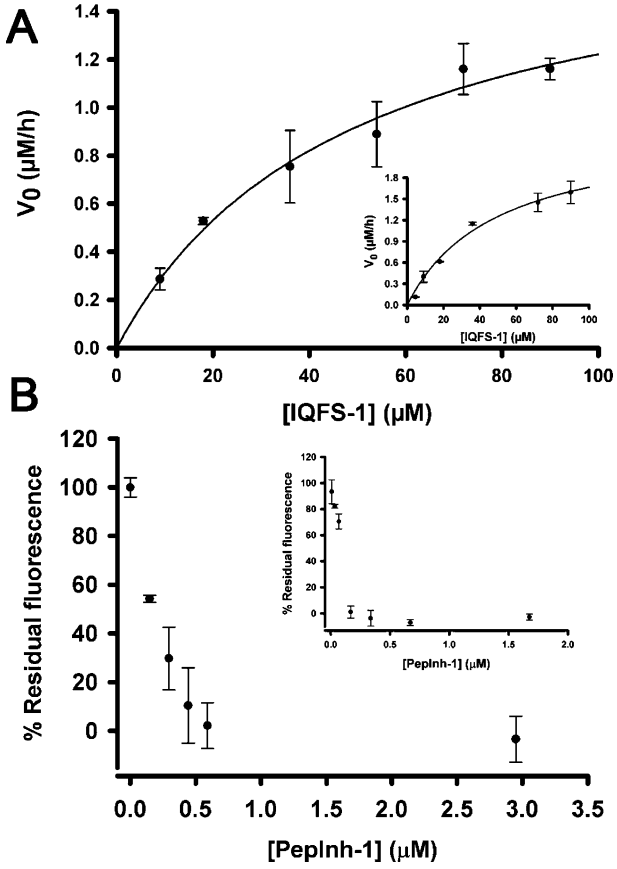

FIGURE 5: Enzymatic characterization of protease activity in (−)-tet UNS3-4A cell membrane fractions. Kinetic analysis of NS3-4A protease activity using IQFS-1 substrate concentrations in the range between 9 and 90 μM in (A) (−)-tet UNS3-4A cell membrane fractions and (inset) recombinant purified NS3-4A. Titration of NS3-4A protease activity using PepInh-1. The residual protease activity following preincubation with varying concentrations of PepInh-1 in (B) (−)-tet UNS3-4A cell membrane fraction and (inset) recombinant purified NS3-4A is plotted as a percentage of the activity of protease in the absence of PepInh-1 (100%).

greater than 2, confirming the validity of the K_i value obtained using PepInh-1 as a tight-binding titrant of NS3-4A (*35*). In addition, the E_0 value estimated from the titration curve generated against recombinant NS3-4A was approximately 60% of the value calculated by amino acid analysis, indicating that our recombinant enzyme preparation contains approximately 60% active enzyme (E_0 = 0.07 ± 0.02 μM).

Calculation of k_{cat} for Membrane-Associated NS3-4A Protease. Since PepInh-1 was a valid titration agent for both membrane-associated and recombinant NS3-4A protease, it was possible to obtain an estimate of active enzyme concentration E_0 for both forms of the enzyme (Figure 5B). Using this estimation, the kinetic constants k_{cat} and k_{cat}/K_m for membrane-associated NS3-4A were calculated to be 0.39 min^{-1} and 128 M^{-1} s^{-1}, respectively. Very similar kinetic constant values of 0.38 min^{-1} (k_{cat}) and 106 M^{-1} s^{-1} (k_{cat}/K_m) were determined for purified recombinant NS3-4A (values represent the average derived from two independent experiments).

DISCUSSION

We have developed an assay that allows the detection of virus-specific protease activity in mammalian cells which

Table 1: Comparison of Kinetic Constant Parameters for Membrane-Associated and Recombinant, Purified NS3-4A[a]

	K_m (μM)	V_{max} (μM/h)	k_{cat} (min^{-1})	k_{cat}/K_m ($M^{-1}\,s^{-1}$)	K_i (nM)
UNS3-4A MF	51 ± 3	2.1 ± 0.3	0.39 ± 0.07	128 ± 15	22 ± 6
E. coli NS3-4A	60 ± 11	2.4 ± 0.1	0.38 ± 0.07	106 ± 16	15 ± 2

[a] The average kinetic constant values obtained for NS3-4A within UNS3-4A cell membrane fractions (MF) and recombinant NS3-4A purified from *E. coli* are shown.

express the HCV NS3-4A heterocomplex (*14*). Using a novel fluorogenic substrate, we were able to detect NS3-4A specific protease activity in both whole cell lysates and membrane fractions derived from this cell line. Our assay is rapid and simple to perform and uses a substrate that is cleaved specifically by the NS3-4A protease with no significant detectable background cleavage mediated by cellular proteases. Our results also demonstrate that, as expected, NS3-4A is associated with the intracellular membranes from (−)-tet UNS3-4A cells and can therefore be enriched from whole cell lysate by preparing membrane fractions, remaining enzymatically active throughout this process.

Having established that our assay was sufficiently specific and sensitive to detect the protease activity associated with NS3-4A expressed in human cells, we sought to determine the kinetic parameters of membrane-associated NS3-4A. K_m and V_{max} values of 51 ± 3 μM and 2.1 ± 0.3 μM/h were calculated for the protease associated with membrane fractions from UNS3-4A cells using IQFS-1. Similar analysis using bacterially expressed, recombinant purified NS3-4A yielded K_m and V_{max} values of 60 ± 11 μM and 2.4 μM/h, respectively, for this form of the enzyme (Table 1). The K_m values indicate that recognition of the IQFS-1 substrate is very similar between membrane-associated and recombinant purified NS3-4A. Our K_m values are also comparable with those previously reported using peptide substrates also derived from the 5A/5B junction with recombinant NS3-4A purified from either bacteria (10 μM) or insect cells (59 μM) (*34*, *48*).

We then tested a known substrate-analogue peptide inhibitor (*43*) of recombinant NS3 protease (PepInh-1) in our assay to ascertain if it was also effective against membrane-associated NS3-4A expressed in mammalian cells. Since the interactions of substrate-analogue NS3 inhibitors, such as PepInh-1, with NS3-4A are similar to those which occur between natural substrates and the protease complex (*43*, *49*), this may also indicate whether substrate recognition by NS3-4A protease is altered as a result of membrane attachment. The sequence of PepInh-1 also incorporates unnatural amino acids, which have previously been reported as revealing differences in the substrate recognition of NS3 proteases from different HCV strains (*50*).

Titration experiments using PepInh-1 revealed that it was a potent tight-binding inhibitor of the protease activity of both forms of the NS3-4A protease. The resulting K_i values obtained for membrane-associated NS3-4A (22 ± 6 nM) and recombinant NS3-4A (15 ± 2 nM) were in close agreement, indicating that there was essentially no difference in recognition of the PepInh-1 substrate analogue exhibited between the two different forms of the enzyme (Table 1). As with our findings with the recognition of IQFS-1, association of NS3-4A with intracellular membranes does not appear to influence the recognition or effectiveness of the substrate-analogue peptide inhibitor.

Although substrate recognition, as indicated by both K_m and K_i values, appears to be the same for both forms of the enzyme, it is possible that membrane attachment may alter the catalytic turnover of IQFS-1 exhibited by NS3-4A. Since the inhibition profile of PepInh-1 against membrane-associated NS3-4A was typical of that of a tight-binding inhibitor (Figure 5B), it served as a useful titration agent to estimate the active enzyme concentration (E_0) in the UNS3-4A cell membrane preparations, enabling us to calculate the steady-state kinetic parameters k_{cat} and k_{cat}/K_m for the enzyme expressed in human cells.

The k_{cat} values for IQFS-1 by membrane-associated NS3-4A (0.39 min^{-1}) and recombinant purified NS3-4A (0.38 min^{-1}) were essentially identical, indicating that there was no difference in the catalytic turnover of IQFS-1 between membrane-attached and recombinant enzyme. This would suggest that membrane attachment does not influence the steady-state specificity of NS3-4A, at least with regard to this peptide substrate. Generally, the k_{cat} values we obtained using IQFS-1 are lower than those previously reported using other peptide substrates based on the same HCV polyprotein junction, indicating that it is not as efficiently processed (*34*, *41*, *48*). However, IQFS-1 is nonetheless a valuable tool to assay the NS3-4A activity within mammalian cell extracts, since its overall efficiency of cleavage (k_{cat}/K_m) is sufficient to permit continuous detection of virus-specific proteolysis from within complex protein mixtures and is also resistant to degradation and processing by cellular proteases.

Our steady-state kinetic analysis using IQFS-1 and titration experiments using PepInh-1 indicate that there is no discernible difference in either substrate recognition or catalytic turnover between membrane-associated NS3-4A expressed in human cells and recombinant NS3-4A purified from *E. coli* (Table 1). This suggests that, at least with respect to these peptide substrates/substrate analogue inhibitors, association of the NS3-4A protease with intracellular membranes has little effect on either its substrate specificity or catalytic turnover. Since the naturally occurring form of the NS3-4A protease in infected cells is membrane-anchored through insertion of the hydrophobic NS4A N-terminal α-helix into the ER membrane, we anticipated that we would see a difference in the protease activity of membrane-associated NS3-4A as compared to recombinant, purified enzyme. We expected that membrane-anchored NS3-4A might represent the optimal conformation for enzymatic activity and that therefore this would be reflected in different steady-state kinetic parameters for this form of the enzyme compared with recombinant protein, in which the position of the hydrophobic NS4A N-terminal α-helix is unknown and could potentially interfere with the overall conformation of the heterocomplex. The effect that this NS4A domain may have on the conformation of the NS3-4A complex that may influence its substrate-binding or catalytic properties is hard to assess since in all the X-ray and NMR studies performed

on the NS3-4A protease complex so far, the N- and C-terminal domains of NS4A are absent from the resolved structures (*51*, *52*).

However, our results using IQFS-1 and PepInh-1 would suggest that membrane association does not alter the enzymatic properties of the NS3-4A protease, which naturally raises the question: why in infected cells is it important for the NS3-4A heterocomplex to be associated with the ER? As NS3-4A is a multifunctional enzyme, it is possible that membrane attachment may be important for regulating and/or coordinating the different enzymatic functions of NS3-4A, such as the ATPase or helicase activities. There have been several contradictory studies regarding whether there is functional interaction between the protease and helicase domains of NS3 and the role that NS4A plays in modulating the helicase activity of the heterocomplex (*34*, *53*−*56*). Hence, further investigation of whether the association of NS4A with intracellular membranes influences regulation of the different enzymatic activities of NS3-4A is required. Alternatively, association of the NS3-4A complex with the ER membrane may serve solely to localize the enzyme to the correct intracellular site for viral replication and ensure that NS3-4A is assembled into the HCV replicase complex. Appropriate intracellular targeting of the NS3-4A heterocomplex is necessary to bring the NS3 helicase domain and the viral NS5B polymerase, which is also associated with the same intracellular membranes (*19*), within close proximity to each other.

Our novel assay system permits the detection of NS3-4A protease activity in a physiologically relevant environment, as NS3-4A remains associated with intracellular membranes, the proposed site of viral replicase assembly and RNA replication. It is likely, therefore, that in the membrane-associated NS3-4A heterocomplex the interdomain contacts between the NS3 protease, helicase, and NS4A domains, all essential for HCV replication, will be preserved. We note, however, that the other components of the viral replicase complex, NS4B, NS5A, and NS5B, are not expressed in this cell line. Since several putative interactions between NS3-4A and the other HCV replicase proteins have been proposed (*6*, *24*, *27*, *57*), the conformation adopted by NS3-4A could be altered in their absence, and ultimately this may affect either the steady-state specificity or catalytic turnover rates observed.

We believe that this novel high-throughput fluorescence assay will be an important tool with which to further study the properties of the membrane-associated HCV NS3-4A protease heterocomplex. It may also be useful for evaluating novel, putative NS3-4A-directed inhibitors in a more physiologically relevant environment than current commonly used in vitro assays, since it is also possible that the binding and effectiveness of nonsubstrate analogue small molecule NS3 protease inhibitors may differ between membrane-associated and NS3-4A purified from *E. coli*. In addition, as our assay enables direct continuous monitoring of protease activity, the mechanism of inhibition of NS3-4A protease inhibitors can also be elucidated. We are currently using this assay to investigate the effectiveness of novel putative protein-based inhibitors of the NS3-4A serine protease complex (*58*) and to screen small molecule libraries for putative inhibitors of membrane-associated NS3-4A protease.

ACKNOWLEDGMENT

We thank Dr. Darius Moradpour (University of Freiburg) for kindly providing the UNS3-4A cell line and for helpful advice regarding their propagation. We are grateful to Dr. Alla Kusch (Ivanovsky Institute of Virology, Moscow) for the kind gift of the monoclonal anti-NS4A antibody, Dr. Paola Gallinari (IRBM, Rome) for the NS3-4A bacterial expression plasmid, and Derek Smith (Genome BC Proteomics Centre) for help and expertise with the MS analysis of the protease assay reactions. We also thank Martin Richer and Morgan Martin for helpful discussions and reading of the manuscript.

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BI047408J