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Short Communication

Single-cell resolution imaging of membrane-anchored hepatitis C virus NS3/4A protease activity

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Abstract

The study of host and viral membrane-associated proteases has been hampered due to a lack of *in vivo* assays. We report here the development of a cell-based fluorescence assay for detecting hepatitis C virus (HCV) NS3/4A juxtamembrane protease activity. Intracellular membrane-anchored protein substrates were engineered comprising: (1) an endoplasmic reticulum targeting domain, the HCV NS5A N-terminal amphipathic α-helix; (2) a NS3/4A-specific cleavage site; and (3) a red fluorescent reporter group, DsRed. The results of our immunofluorescence and Western blotting studies demonstrate that our membrane-bound fluorescent probe was cleaved specifically and efficiently by NS3/4A expressed in human cells.

Keywords: activity-based probe; cell-based assay; *Discosoma* red fluorescent protein; intracellular proteolysis; juxtamembrane protease; membrane-bound serine protease; viral protease.

Understanding membrane-associated proteolysis is an exciting and emerging field of research, which has many biologic and therapeutic consequences (Bauvois, 2004; Ehrmann and Clausen, 2004). Membrane-associated proteases can be classified as either intramembrane proteases (IPs) if they hydrolyse polypeptides buried in a lipid bilayer, or juxtamembrane proteases (JPs) if they cleave proteins in the aqueous environment adjacent to the lipid bilayer (Golde and Eckman, 2003; Wolfe and Kopan, 2004). IPs are usually multi-pass transmembrane proteins; the active-site catalytic residues are located in transmembrane domains and hydrolyse other transmembrane domains via a poorly understood mechanism. In contrast, JPs utilise a diverse set of mechanisms to associate themselves with biological membranes; single or multiple transmembrane domains, glycosylphosphatidyl inositol (GPI)-linked residues, and protein-protein interactions are common tethering mechanisms (Netzel-Arnett et al., 2003).

Many positive-sense RNA viruses replicate their genomic RNA within large multi-protein complexes tethered to intracellular biological membranes (Salonen et al., 2005). These 'replicase' complexes contain non-structural (NS) viral enzymes (proteases, helicases, and polymerases), which are essential for viral replication. Transmembrane domains target the viral NS proteins to the membrane, and protein-protein interactions between the NS proteins help to assemble the replicase into a large complex. The membrane composition and structure are altered by the presence of these viral proteins; however, experimentation on NS proteins *in vivo*, where the impact of this unique microenvironment is present, is limited.

For the hepatitis C virus (HCV), the replicase is located in a modified endoplasmic reticulum (ER)-derived membrane, referred to as the 'membranous web' (Egger et al., 2002; Mottola et al., 2002). The HCV JP NS protein 3 (NS3) does not associate with the membranous web by itself, but is tethered to this membrane via an interaction with another viral protein, NS4A. NS4A is predicted to be a type II membrane protein and it forms a non-covalent association with NS3; this association is strictly required for full NS3 protease activity and specificity. The impact of this association is significant, since the NS3/4A heterocomplex plays an essential role in the HCV life cycle.

The HCV genome is comprised of a single strand of positive-sense polycystronic RNA, which is translated into a continuous polyprotein. Individual proteins are produced by proteolytic processing of the polyprotein by viral and host proteases. The NS3 protease is involved in processing five of the nine polyprotein junctions and is therefore an important therapeutic target. In fact, a small-molecule inhibitor of the NS3 protease has been shown to effectively reduce viral RNA levels in HCV-infected patients, but unfortunately no effective, specific anti-HCV compound has been brought to market to date (Lamarre et al., 2003; Goudreau and Llinas-Brunet, 2005). The study of NS3/4A and other membrane-associated proteases has been hampered due to a lack of *in vivo* assays.

Classical methods for studying protease activity and specificity *in vivo* have relied on the isolation and detection of a known substrate of the protease, usually by immunoprecipitation or Western blotting. These methods depend on the availability of an antibody directed at the protease substrate. Original experiments on HCV NS3/4A cleavage site specificity were successfully accomplished using these techniques; it was found that the NS3/4A cleavage site is Cys↓Ser/Ala (positions P1↓P1′), and NS3/4A has a preference for substrates with a neg-

atively charged residue in the P6 position (Glu/Asp) (Schechter and Berger, 1967; Zhang et al., 1997; Kim et al., 2000). Even though these are extremely useful techniques, they are less effective for membrane proteases and substrates, as antibodies are more difficult to raise against integral membrane proteins.

Fluorescence-based *in vivo* assays allow for single-cell resolution and less disruption of the cells under study. Two common assays use activity-based probes (ABPs) or fluorescently tagged substrates. ABPs are targeted to an active protease via a chemical group that forms a covalent linkage with the catalytic residues of the enzyme. This chemical 'warhead' is linked to a fluorescent molecule for detection. The limitations of this system are the lack of cell-permeable probes and irreversible inactivation of the target protease (Baruch et al., 2004). Alternatively, the fluorescently tagged substrate assays detect a change in the wavelength and/or intensity of light emitted from a fluorescent substrate after cleavage, and include quenched substrates and fluorescence-resonance energy transfer (FRET)-based probes (Jean et al., 1995a,b; Jones et al., 2000; Tawa et al., 2001; Richer et al., 2004; Hamill and Jean, 2005). In this study, we demonstrate a new fluorescence-tagged substrate approach to specifically target membrane-associated proteases.

We present here the design and evaluation of a cell-based fluorescent membrane-bound protease assay for HCV NS3/4A. This assay design is based on a simple readout involving the change in localisation of a fluorescent protein reporter group. The reporter group is tethered to the membrane via a membrane-anchoring domain (Figure 1A). Between the tethering domain and the fluorescent reporter group, a protease-specific cleavage sequence is added. If the target enzyme is active and can interact with the assay substrate, the linker is hydrolysed, liberating the fluorescent reporter into the cytosol (Figure 1B). This change in fluorescence from a membrane-bound to a diffuse cytoplasmic pattern can be detected using fluorescence microscopy.

The reporter chosen for this study was a red fluorescent protein, DsRed-Express (hereafter referred to as DsRed). DsRed is a tetrameric fluorescent protein isolated from the reef coral species *Discosoma* (Matz et al., 1999). This reporter was chosen because it can be used in conjunction with a number of other fluorophores, namely the green-shifted Alexa Fluor-488, which was used to probe for NS3 protease in these experiments. DsRed is located throughout the cytoplasm when expressed in mammalian cells (Figure 2A–C).

To customise the *in vivo* cleavage assay for NS3/4A, a viral targeting domain was used, as well as a NS3/4A-specific cleavage linker sequence. The membrane-targeting domain from another HCV NS protein, NS5A, was used as the anchoring domain; it was cloned upstream of DsRed, creating the plasmid pTm-DsRed (Figure 1C). The NS5A anchoring domain is a stretch of 31 N-terminal NS5A residues that are predicted to form an amphipathic α-helix and has been shown to target NS5A or GFP to the membrane (Elazar et al., 2003; Moradpour et al., 2005). As shown in Figure 2D–F, the Tm-DsRed protein has a punctate perinuclear staining, indicating DsRed has been successfully tethered to the membrane in these cells.

The addition of an NS3-specific cleavage site corresponding to the NS4B/5A junction (Asp-Cys-Ser-Thr-Pro-Cys↓Ser-Gly-Ser) completed the protease probe (Figure 1C). The resulting plasmid, pTm-4B/5A-DsRed, was transfected into mammalian cells and produced a fusion protein, Tm-4B/5A-DsRed; the pattern of localisation for this probe (Figure 2G–I) remained unchanged compared to the negative control plasmid, pTm-DsRed (Figure 2D–F).

Plasmids pTm-DsRed and pTm-4B/5A-DsRed were transfected into human cell lines that express NS3/4A under the control of a tetracycline-regulated promoter (Wolk et al., 2000; Hamill and Jean, 2005). In the presence of tetracycline, transcription of NS3/4A is repressed in these cells; mRNA and protein are only produced upon removal of tetracycline. Transfection of pTm-4B/5A-DsRed in NS3/4A-expressing cells resulted in the liberation of DsRed from the membrane, thus changing its location in the cell from membrane-bound punctate perinuclear (Figure 2G-I) to diffuse cytoplasmic (Figure 3D–F). This indicates efficient processing of our fluorescent probe by NS3/4A. As expected, NS3/4A did not cleave the Tm-DsRed protein, which contains no cleavage site linker (Figure 3A–C). In addition, when the P1

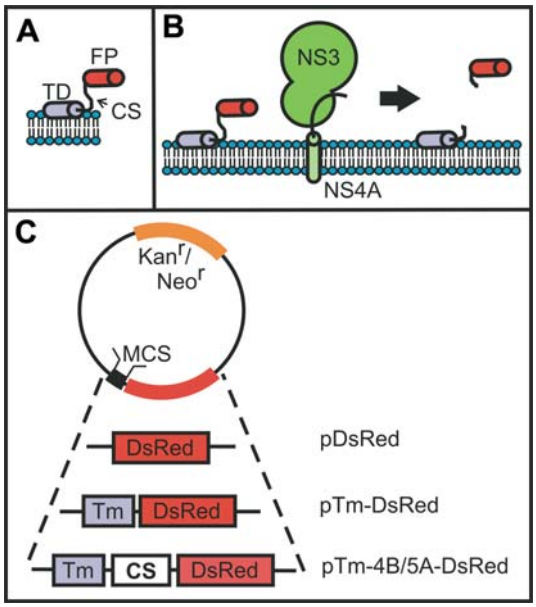

Figure 1 Design of a membrane-anchored fluorescent protease substrate.
(A) Intracellular membrane-anchored protein substrates were engineered comprising a targeting domain (TD), a protease-specific cleavage site (CS), and a fluorescent protein (FP) reporter group. (B) When targeted substrate is expressed with the HCV NS3/4A protease, cleavage of the CS releases the FP into the cytosol. (C) Description of plasmids used in this study. pDsRed is unmodified pDsRed-Express-N1 (Clontech, Mountain View, CA, USA). pTm-DsRed was created by inserting into the multiple cloning site (MCS) the HCV NS5A N-terminal amphipathic α-helix (Tm), which was PCR amplified from the Con1 strain subgenomic replicon (Moradpour et al., 2004) using primers that incorporated XhoI and EcoRI restriction enzyme sites for insertion into pDsRed. Overlapping oligonucleotides encoding the NS4B/5A cleavage site were annealed and inserted into EcoRI and AgeI double-digested pDsRed containing Tm, creating pTm-4B/5A-DsRed.

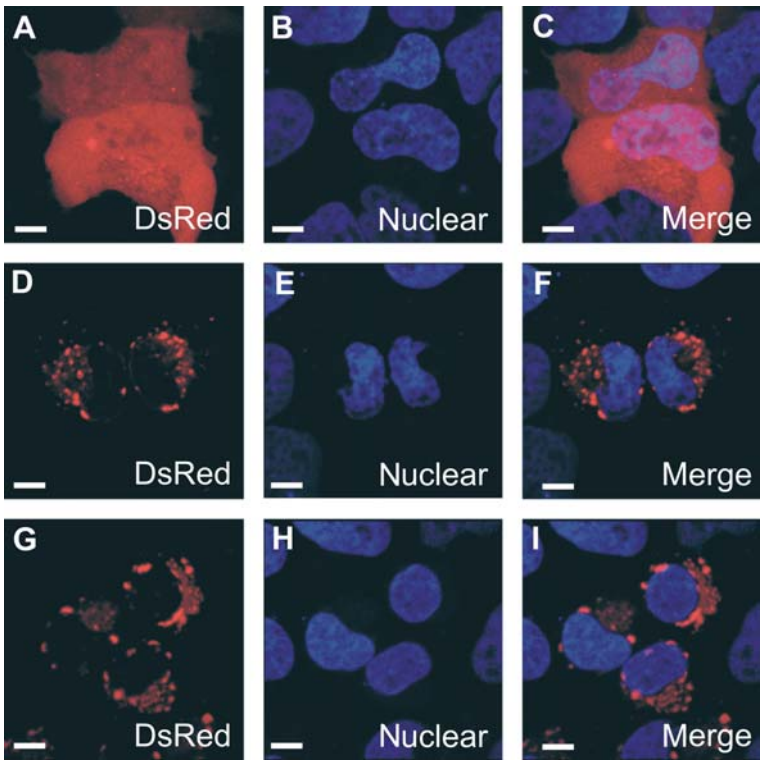

Figure 2 Tm-DsRed and Tm-4B/5A-DsRed are localised to perinuclear membranes.
Culturing of UNS3-4A human osteosarcoma cells in the presence of tetracycline was carried out as previously described (Hamill and Jean, 2005). Cells were grown on glass coverslips in 24-well plates and were transiently transfected with *Trans*IT transfection reagent according to the manufacturer's instructions (Mirus Bio Corporation, Madison, WI, USA). UNS3-4A cells were transfected with pDsRed (A–C), pTm-DsRed (D–F), or pTm-4B/5A-DsRed (G–I). At 24 h post-transfection, cells were fixed in 4% paraformaldehyde and the nuclei were stained with 4′,6-diamidino-2-phenylindole dihydrochloride (DAPI) according to the manufacturer's instructions (Molecular Probes, Eugene, OR, USA). Images were acquired using a Nikon Eclipse TE300 confocal microscope. The scale bar corresponds to 10 μm.

cysteine residue was changed to a serine residue [Tm-4B/5A(CP1S)-DsRed cleavage site: Asp-Cys-Ser-Thr-Pro-**Ser**↓Ser-Gly-Ser], no substrate cleavage was detectable using microscopy (Figure 3G–I).

For quantitation of the NS3/4A cleavage, Western blotting of whole cell lysates from cells expressing Tm-DsRed, Tm-4B/5A(CP1S)-DsRed, or Tm-4B/5A-DsRed in the presence and absence of tetracycline was performed using a polyclonal anti-DsRed antibody. Processed DsRed substrate can be distinguished from full-length unprocessed substrate; the processed DsRed reporter group no longer possesses the NS5A amphipathic α-helix and is therefore smaller than the unprocessed substrate (Figure 4, inset). The percentage of total substrate processed by NS3/4A was 57.4±4.9% of the Tm-4B/5A-DsRed probe, compared to background cleavage of 0.9–1.5±0.8% (Figure 4). The nearly absent background processing indicates that the probe is extremely stable in this cell line. This indicates an absence of host proteases able to efficiently cleave the constructs within the time frame of the experiment. Tm-4B/5A(CP1S)-DsRed exhibited 4.6±0.8% cleavage in the presence of NS3/4A, which was not significantly different from the cleavage of this substrate in the absence of NS3/4A (2.1±1.9%).

Other cleavage-site mutants and a mixed cleavage-site control were not processed by NS3/4A to any significant degree (Table 1). One of these mutants, in which the P1 and P6 positions were swapped, actually displayed a partial NS3/4A cleavage site in the N-terminus of the cleavage site linker; the NS5A/5B junction has Cys-Cys↓Ser in positions P2-P1↓P1′. The fact that only background levels of cleavage occurred with this substrate in the presence of NS3/4A highlights the specificity of NS3/4A for a longer, extended cleavage site and possibly for other cleavage site determinants, such as an acidic residue at P6.

Recently, three independent studies have demonstrated an important role of HCV NS3/4A protease in blocking the host anti-viral response. HCV has evolved an efficient strategy to block dsRNA-induced innate immune responses, by specifically cleaving and thereby inactivating two host proteins: Toll/IL-1 receptor-domain-containing adaptor inducing IFN-β (TRIF) (Li et al., 2005a) and mitochondrial antiviral signalling protein (MAVS) (Li et al., 2005b). TRIF is a signalling component downstream of the extracellular-dsRNA sensor, Toll-like receptor-3 (TLR-3), and MAVS is downstream of the intracellular-dsRNA sensor, retinoic acid inducible gene I (RIG-I). Both of these pathways lead to the induction of interferons that mediate an anti-viral state. The site that NS3 recognises in TRIF has remarkable homology with the viral NS4B/5A cleavage site, although an eight-residue polyproline track extends upstream from the P6 position in lieu of the acidic residue present in the HCV

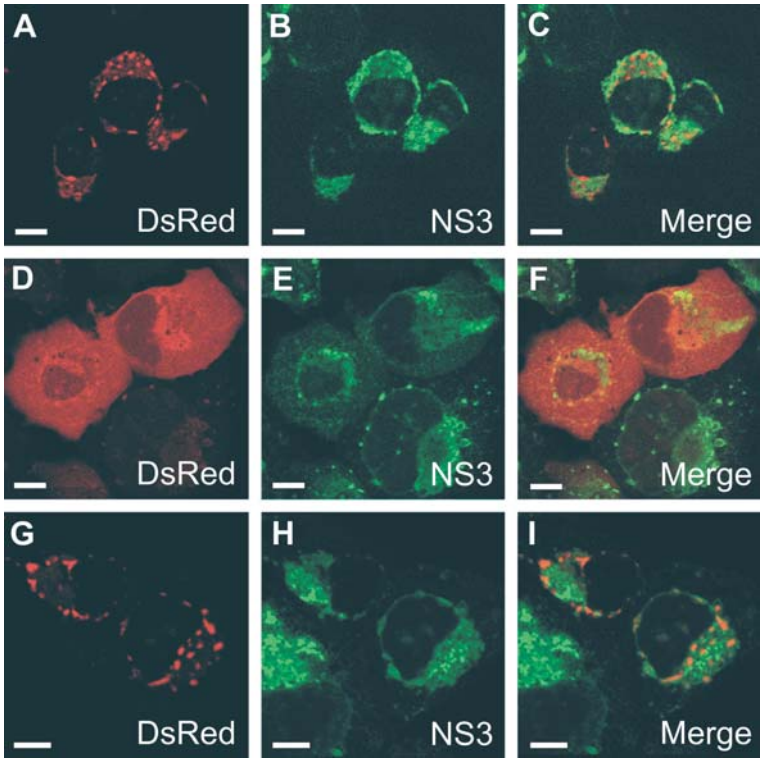

Figure 3 pTm-4B/5A-DsRed is processed by HCV NS3/4A.
To induce NS3/4A expression, UNS3-4A cells were grown in the absence of tetracycline for 24 h before transfection with pTm-DsRed (A–C), pTm-4B/5A-DsRed (D–F), or pTm-4B/5A(CP1S)-DsRed (G–I). At 24 h post-transfection without tetracycline, cells were fixed in 4% paraformaldehyde, permeabilised with 0.05% saponin, and stained with anti-NS3 mouse antibody (Novocastra Laboratories, Newcastle upon Tyne, UK) and secondary anti-mouse Alexa Fluor-488-conjugated antibody (Molecular Probes) as previously described (Hamill and Jean, 2005). pTm-4B/5A(CP1S)-DsRed was created by changing the P1 cysteine residue to a serine residue using site-directed mutagenesis (Quikchange, Stratagene, La Jolla, CA, USA). Images were acquired using a Zeiss Axiovert confocal microscope. The scale bar corresponds to 10 μm.

substrates (Ferreon et al., 2005). The site processed in MAVS is an unconventional Cys↓His. These new findings underline the importance of performing *in vivo* specificity assays for membrane-bound proteases.

A membrane-targeted assay is important owing to the significant role the membrane microenvironment plays for membrane-associated proteases. Not only do biological membranes control the location and trafficking of these proteases, but they can also modulate the microenvironment by changing protein and lipid composition, spatially and temporally; the best example being that of lipid rafts. HCV replication is thought to occur on lipid rafts and the impact of this environment of NS3/4A activity or specificity is unknown (Aizaki et al., 2004).

In conclusion, we have designed a protease substrate that is localised to the same intracellular membrane as the protease of interest, HCV NS3/4A. Using this substrate, membrane-associated NS3/4A protease activity was detected by fluorescence microscopy and quantified by Western blotting. This *in vivo* protease assay will help in the discovery, development, and evaluation of novel inhibitors of HCV NS3 protease, as well as provide physiologically relevant *in vivo* data on the protease activity.

The method of membrane-associated protease detection described here can easily be adapted to probe for other membrane-associated proteases by simply changing the cleavage sequence and the targeting domain. Of particular note is the potential application of this assay

Table 1 Tm-4B/5A-DsRed cleavage site variants are not cleaved by NS3/4A.

Description	Cleavage site sequence	Substrate cleaved (%)
Tm-4B/5A-DsRed	Asp-Cys-Ser-Thr-Pro-Cys↓Ser-Gly-Ser-Trp	57.4±4.9
No cysteines	Asp-**Ser**-Ser-Thr-Pro-**Ser**↓Ser-Gly-Ser-Trp	4.2±5.9
Swap P1 and P6	**Cys**-Cys-Ser-Thr-Pro-**Asp**↓Ser-Gly-Ser-Trp	1.0±0.6
Mixed non-prime site	**Cys**-**Pro**-Ser-Thr-**Cys**-**Asp**↓Ser-Gly-Ser-Trp	1.7±0.3
Mixed cleavage site	**Cys**-**Pro**-Ser-Thr-**Cys**-**Asp**↓**Trp**-**Ser**-Ser-**Gly**	1.5±1.3

All mutants were created by site-directed mutagenesis using Tm-4B/5A-DsRed as a template (Quikchange, Stratagene). Mutated residues are shown in bold font. Constructs were transfected into UNS3-4A cells and the percentage cleavage was quantified by Western blotting as described for Figure 4. Results are the average of at least two separate experiments.

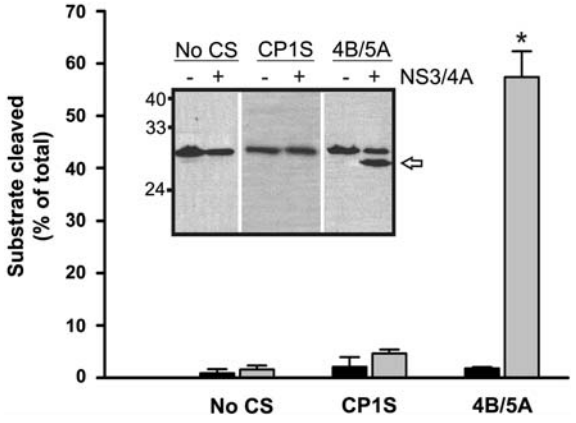

Figure 4 pTm-4B/5A-DsRed substrate cleavage can be quantified by Western blotting.
UNS3-4A cells were grown in the absence or presence of tetracycline in six-well plates, transfected, and harvested 24 h post-transfection by scraping and centrifugation (0.9 *g*, 10 s). The cell pellet was resuspended in hypotonic lysis buffer [10 mM Tris (pH 7.8), 10 mM NaCl, 1× EDTA-free complete protease inhibitor (Roche, Laval, Canada)]. Whole cell lysates were run on a 15% polyacrylamide gel and Western blot analysis was performed using anti-DsRed polyclonal antibody according to the manufacturer's instructions (BD Biosciences, Palo Alto, CA, USA). Inset: Western blot film showing Tm-DsRed, Tm-4B/5A(CP1S)-DsRed, and Tm-4B/5A-DsRed with and without NS3/4A expression. The arrow indicates cleaved Tm-4B/5A-DsRed substrate. The chemiluminescent signal was quantified using a VersaDoc multiimager (Bio-Rad, Mississauga, Canada). The percentage of substrate cleaved was calculated by dividing the total signal (cleaved plus uncleaved) by the cleaved signal, thereby normalising the readout for each sample. The bar chart represents the average of three separate experiments. $^{*}p<0.0001$.

to probe for IP activity. In this case, the substrate design is even easier, as the tethering transmembrane domain would also act as the substrate. We have established a powerful, easy-to-use assay that provides a template for much JP and IP research to come.

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