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In-cell selectivity profiling of membrane-anchored and replicase-associated hepatitis C virus NS3-4A protease reveals a common, stringent substrate recognition profile

Morgan M. Martin, Stephanie A. Condotta, Jeremy Fenn, Andrea D. Olmstead and François Jean*

Department of Microbiology and Immunology, University of British Columbia, 2350 Health Sciences Mall, Vancouver, BC V6T 1Z3, Canada

*Corresponding author
e-mail: fjean@mail.ubc.ca

Abstract

The need to identify anti-*Flaviviridae* agents has resulted in intensive biochemical study of recombinant nonstructural (NS) viral proteases; however, experimentation on viral protease-associated replication complexes in host cells is extremely challenging and therefore limited. It remains to be determined if membrane anchoring and/or association to replicase-membrane complexes of proteases, such as hepatitis C virus (HCV) NS3-4A, plays a regulatory role in the substrate selectivity of the protease. In this study, we examined trans-endoproteolytic cleavage activities of membrane-anchored and replicase-associated NS3-4A using an internally consistent set of membrane-anchored protein substrates mimicking all known HCV NS3-4A polyprotein cleavage sequences. Interestingly, we detected cleavage of substrates encoding for the NS4B/NS5A and NS5A/NS5B junctions, but not for the NS3/NS4A and NS4A/NS4B substrates. This stringent substrate recognition profile was also observed for the replicase-associated NS3-4A and is not genotype-specific. Our study also reveals that ER-anchoring of the substrate is critical for its cleavage by NS3-4A. Importantly, we demonstrate that in HCV-infected cells, the NS4B/NS5A substrate was cleaved efficiently. The unique ability of our membrane-anchored substrates to detect NS3-4A activity alone, in replication complexes, or within the course of infection, shows them to be powerful tools for drug discovery and for the study of HCV biology.

Keywords: cell-based assay; *Flaviviridae* protease; hepatitis C virus; induced-fit protease; membrane-anchored protease; viral protease.

Introduction

The hepatitis C virus (HCV) was identified in 1989 as the major causative agent of transfusion-associated non-A, non-B hepatitis (Choo et al., 1989). Much success has been achieved in understanding the mechanism by which this virus infects and replicates (Tang and Grise, 2009), information essential for the creation of vaccines and therapeutics (Pereira and Jacobson, 2009; Shimakami et al., 2009). HCV is a single-stranded positive-sense RNA virus belonging to the family *Flaviviridae*. Replication takes place in the cytoplasm of liver cells (Sansonno et al., 1995); the endoplasmic reticulum (ER) is restructured by viral proteins, creating a mitochondria-associated 'membranous web' that houses the large macromolecular protein assemblies responsible for copying the viral RNA (Moradpour et al., 2003). These 'replicase' complexes contain non-structural (NS) viral enzymes (proteases, helicases, and polymerases), the activities as well as the maturation of which are promising drug targets.

The HCV genome is a polycistronic RNA that is translated into one continuous polypeptide. The 10 proteins contained in this polyprotein (C-E1-E2-P7-NS2-NS3-NS4A-NS4B-NS5A-NS5B) are released by specific proteolytic cleavages mediated by both host and viral proteases (Dubuisson, 2007; Welbourn and Pause, 2007). One of the viral proteases, nonstructural protein 3 (NS3), is involved in the release of itself as well as NS4A, NS4B, NS5A, and NS5B from the polyprotein. This protease has been shown to be essential for HCV replication in chimpanzees and has been the target of intensive study and inhibitor screening (Kolykhalov et al., 1994; Chen and Njoroge, 2009; Raney et al., 2010).

NS3 is a bifunctional enzyme, containing both helicase and protease activities in distinct domains. The protease domain ($NS3^{pro}$) is a chymotrypsin-like serine protease that acts in concert with its cofactor, NS4A (Morikawa et al., 2011). NS4A integrates into the NS3 protease fold (Kim et al., 1996, 1998), stabilizing and targeting NS3 to the ER by virtue of the N-terminal hydrophobic region in NS4A (Wolk et al., 2000). Through transient expression of HCV NS precursors in mammalian cells, NS4A has been shown to increase NS3 proteolytic activity for the NS4A/NS4B and NS5A/NS5B junctions and to be essential for the cleavage of the NS4B/NS5A junction (Bartenschlager et al., 1994; Failla et al., 1994; Lin et al., 1994; Tanji et al., 1995). The region of NS4A required for this activation has been mapped to 13 central amino acids; a synthetic peptide corresponding to this region (pep4A) activates NS3 *in vitro* (Bartenschlager et al., 1995b; Steinkuhler et al., 1996b; Tomei et al., 1996; Richer et al., 2004).

The NS3-NS4A heterocomplex (referred to hereafter as NS3-4A) cleavage site conforms to a consensus pattern of D/E-X-X-X-X-C/T↓S/A (Grakoui et al., 1993). The *trans* cleavage sites (NS4A/NS4B, NS4B/NS5A, and NS5A/

NS5B) maintain a cysteine residue in the P1 position. In contrast, the NS3/NS4A junction has a threonine in the P1 position and is thought to only be cleaved in *cis* (Tomei et al., 1993; Bartenschlager et al., 1994). One group has reported *trans* cleavage of the NS3/NS4A junction by $NS3^{pro}$ once the NS3 protease domain was removed from the precursor polyprotein substrate (Lin et al., 1994); therefore, *trans* cleavage may be possible for NS3/NS4A peptide substrates under certain conditions.

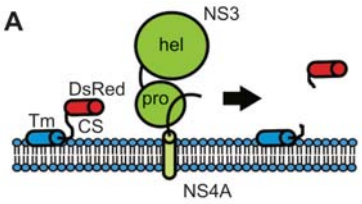
Many groups have analyzed, to some extent, the cleavage efficiency of NS3-4A towards the *trans* cleavage junctions *in vitro* (Steinkuhler et al., 1996a,b; Kakiuchi et al., 1997; Landro et al., 1997; Zhang et al., 1997; Gallinari et al., 1998; Sali et al., 1998; Taremi et al., 1998; Sardana et al., 1999). The general consensus of studies using $NS3^{pro}$ purified from *E. coli* in combination with pep4A was a ranking of the three cleavage sites based on the performance constant k_{cat}/K_m as follows: NS5A/NS5B>NS4A/NS4B>NS4B/NS5A (Steinkuhler et al., 1996b; Landro et al., 1997; Sardana et al., 1999). Recently, Romano et al. (2011) reported that this profile results from the differential ability of $NS3^{pro}$ to form electrostatic interactions with a peptide substrate *in vitro*. In contrast, utilizing NS3-4A purified from insect cells, Zhang et al. (1997) reported an alternative ranking of NS5A/NS5B>NS4B/NS5A>NS4A/NS4B. This discrepancy has not been clarified, and to our knowledge, no comparison of peptide substrate cleavage efficiency has been reported for NS3-4A *in vivo*. Also, the impact of other replicase components on proteolytic activity and selectivity has not been previously addressed.

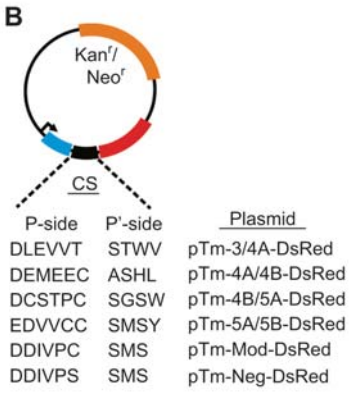
In this study, we report comparison of the cleavage efficiencies of all known HCV NS3-4A cleavage sequences in a cell-based system; we found that NS4B/NS5A and NS5A/NS5B were both processed efficiently in *trans*, in contrast to NS3/NS4A and NS4A/NS4B, which were not processed significantly above background. In addition, we found that other HCV proteins or replicase components do not change NS3-4A substrate selectivity or activity.

Results

Comparison of cleavage efficiency by NS3-4A

Previously, we constructed, expressed, and showed specific NS3-4A processing of a membrane-bound intracellular substrate based on the NS4B/NS5A junction (Tm-4B/5A-DsRed) (Martin and Jean, 2006). In order to compare NS3-4A cleavage in cells, we created intracellular protein substrates corresponding to the other natural HCV junctions (Figure 1). These tripartite substrates have a fluorescent reporter group, DsRed-Express, tethered to the ER via the HCV NS5A α-helix (Tm) (Brass et al., 2002; Elazar et al., 2003; Penin et al., 2004) (Figure 1A). Bridging Tm and DsRed-Express is a short sequence of amino acids, proteolysis of which releases DsRed-Express into the cytoplasm (Figure 1B). We aimed to use a cleavage sequence that was long enough to observe cleavage site differences but short enough to reduce the possibility of recognition by a host protease. A length of





P-side	P'-side	Plasmid
DLEVVT	STWV	pTm-3/4A-DsRed
DEMEEC	ASHL	pTm-4A/4B-DsRed
DCSTPC	SGSW	pTm-4B/5A-DsRed
EDVVCC	SMSY	pTm-5A/5B-DsRed
DDIVPC	SMS	pTm-Mod-DsRed
DDIVPS	SMS	pTm-Neg-DsRed

Figure 1 Membrane-anchored substrate design and cleavage site sequences.
(A) Intracellular membrane-anchored protein substrates were engineered comprising a targeting domain (Tm), a protease-specific cleavage site (CS), and the fluorescent reporter protein DsRed-Express (DsRed). When targeted substrate is expressed with the HCV NS3-4A protease, proteolysis of the CS releases DsRed into the cytosol. (B) Name and sequence of viral substrate plasmids used in this study. Amino acids P6-P4′ are displayed with a gap between P1 and P1′.

10 amino acids for the cleavage site was chosen based on *in vitro* studies that demonstrated that 10 residues are sufficient to measure differences between substrates (Steinkuhler et al., 1996b; Zhang et al., 1997). Substrate residues P6 to P4′ were introduced between Tm and DsRed-Express for the *trans* cleavage sites NS4A/NS4B and NS5A/NS5B. As a negative control, the sequence for the *cis* site NS3/NS4A was also introduced (Figure 1B).

The DNA plasmids encoding these substrates were transiently transfected into a tetracycline-regulated NS3-4A-expressing cell line, UNS3-4A (Wolk et al., 2000; Hamill and Jean, 2005). Protease activity was detected using confocal microscopy to visualize the release of DsRed-Express from the ER membrane: changing the localization in the cell from membrane-bound punctate perinuclear to diffusely cytoplasmic and nuclear. Efficient cleavage was apparent for the NS4B/NS5A as previously reported (Figure 2G–I) (Martin and Jean, 2006) as well as for the NS5A/NS5B junction (Figure 2J–L).

A modified NS5A/NS5B junction (Mod), first described by Kakiuchi et al. (1995), has been used extensively as an *in vitro* substrate (Shimizu et al., 1996; Vishnuvardhan et al., 1997; Gallinari et al., 1999; Hamill and Jean, 2005). This NS5A/NS5B junction has been altered by mutation of the P2 cysteine to a proline in order to prevent disulphide linkages from forming between the P1 and P2 cysteines (Kakiuchi et al., 1995). The Tm-Mod-DsRed substrate containing

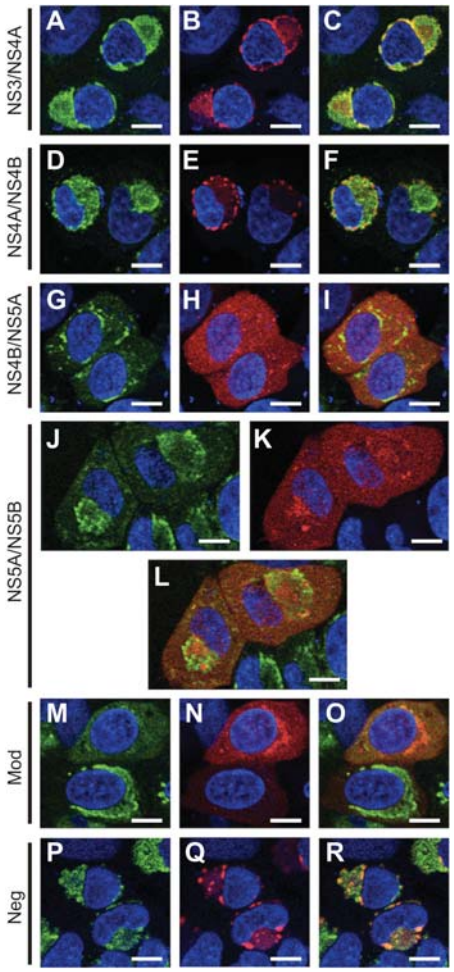


Figure 2 Efficient NS3-4A *trans* processing of Tm-4B/5A-DsRed, Tm-5A/5B-DsRed, and Tm-Mod-DsRed detected by confocal microscopy.
UNS3-4A cells were transfected with pTm-3/4A-DsRed (A–C), pTm-4A/4B-DsRed (D–F), pTm-4B/5A-DsRed (G–I), pTm-5A/5B-DsRed (J–L), pTm-Mod-DsRed (M–O), or pTm-Neg-DsRed (P–R). Cells were stained with anti-NS3 primary and Alexa 488 secondary antibodies (A, D, G, J, M, and P) and imaged with a confocal microscope. DsRed signal is shown in B, E, H, K, N, and Q. Merged image shown in C, F, I, L, O, and R. Cell nuclei were stained with DAPI (shown in blue). Scale bar corresponds to 10 μm.

this sequence was cleaved when expressed with NS3-4A (Figure 2M–O). As a control for specific NS3-4A cleavage, the P1 cysteine was replaced by a serine in this construct (pTm-Neg-DsRed), and removal of this residue was sufficient to abolish cleavage (Figure 2P–R).

NS3-4A was unable to cleave the NS3/NS4A junction in *trans* as expected (Figure 2A–C); however, no processing of the NS4A/NS4B junction was observed (Figure 2D–F).

Microscopy is a powerful technique for imaging single-cell phenomena, but in this case its sensitivity is limited due to the difficulty in detecting a small amount of diffuse, soluble DsRed-Express in conjunction with a very large amount of punctuate membrane-bound DsRed-Express. In order to increase the sensitivity of the assay and also to quantitatively compare the amount of substrate cleaved between populations of cells, we chose to analyze whole-cell lysates by Western blotting using an anti-DsRed antibody.

Cleavage of our substrates by NS3-4A resulted in detection of a ~29 kDa band corresponding to released DsRed-Express, which is easily resolved from the ~34 kDa intact substrate (Figure 3A). The percentage of substrate cleaved was calculated by dividing the integrated signal intensity of the cleaved band by the total integrated signal intensity of the cleaved and uncleaved bands. Virtually no cleavage of the substrates was seen when NS3-4A was not expressed (average <1%, Figure 3B), indicating that these intracellular substrates were stable for at least the duration of the experiment (24 h).

No significant differences were seen between the NS4B/NS5A, NS5A/NS5B, or Mod junctions (Figure 3B); all three were cleaved between 44% and 62% (NS4B/NS5A 62.3±6.4%, NS5A/NS5B 49.4±13.6%, Mod 44.2±16.0%). Although the NS3/NS4A processing was very slight (2.2±1.0%), this cleavage was found to be significant compared to the no-protease control (0.5±1.0%). However, when com-

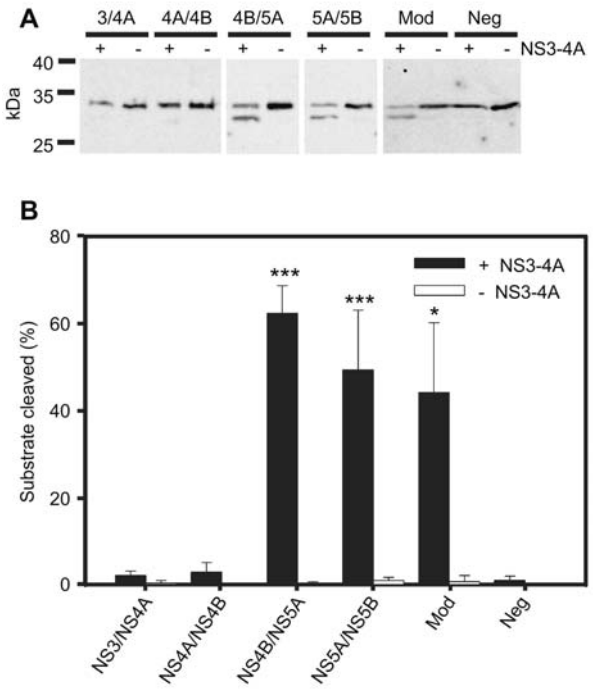


Figure 3 Efficient NS3-4A *trans* processing of Tm-4B/5A-DsRed, Tm-5A/5B-DsRed, and Tm-Mod-DsRed quantified by Western blotting.
(A) UNS3-4A cells were transfected with pTm-3/4A-DsRed, pTm-4A/4B-DsRed, pTm-4B/5A-DsRed, pTm-5A/5B-DsRed, pTm-Mod-DsRed, or pTm-Neg-DsRed. Whole cell lysates were probed by Western blotting using an anti-DsRed antibody. Cleaved substrate migrates faster than intact substrate due to loss of targeting domain (Tm) and non-prime-side cleavage site residues (~5 kDa). (B) Quantitation of Western blotting results showing percentage of substrate cleaved. Results shown are the average of at least two independent experiments. *$p<0.05$; ***$p<0.0005$.

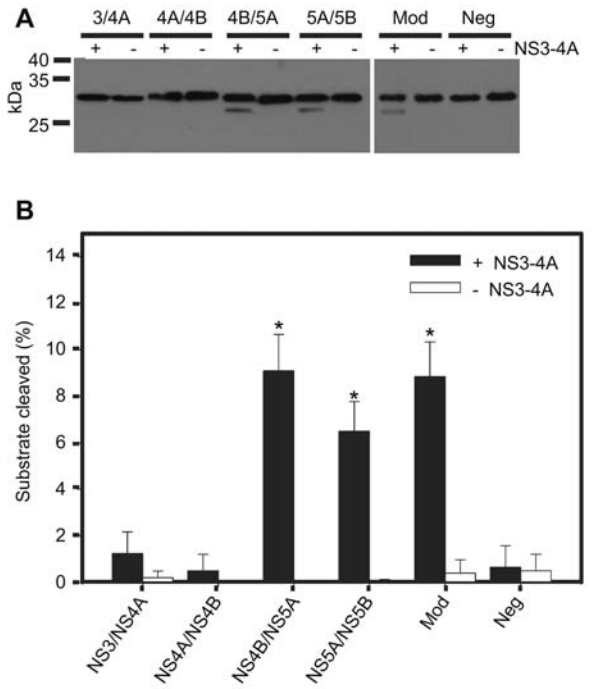


Figure 4 Replicase-associated NS3-4A displays equivalent substrate selectivity profile.
(A) UHCV-32 cells were transfected with pTm-3/4A-DsRed, pTm-4A/4B-DsRed, pTm-4B/5A-DsRed, pTm-5A/5B-DsRed, pTm-Mod-DsRed, or pTm-Neg-DsRed. Whole cell lysates were probed by Western blotting using an anti-DsRed antibody. (B) Quantification of Western blotting results showing the percentage of substrate cleaved. Results shown are the average of two independent experiments. *$p<0.05$.

pared to the negative control substrate with protease present, Tm-Neg-DsRed (1.1±1.0%), the cleavage is not significant and most likely represents a small increase in non-specific substrate degradation in NS3-4A-expressing cells. Surprisingly, the NS4A/NS4B (3.0±2.2%) junction was also not cleaved significantly more than the negative control. Occasionally, a slightly larger (~31 kDa) degradation product was detected in the Tm-4A/4B-DsRed sample, usually upon overexposure of the Western blot. The appearance of this higher product was not dependent on NS3-4A expression (data not shown).

Comparison of cleavage efficiency by NS3-4A in cells expressing HCV polyprotein

To investigate the possibility that substrate cleavage selectivity could be skewed due to NS3-4A expression in isolation from the other HCV proteins, we used the UHCV-32 cell line (Moradpour et al., 1998). These cells inducibly express the entire HCV polyprotein and allow analysis of replicase-associated NS3-4A (Figure 4). Although cleavage of all substrates was much reduced (~5–7-fold) in this cell-line, the same trends were seen confirming approximate equal cleavages of NS4B/NS5A, NS5A/NS5B, and Mod (9.1±1.6%, 6.5±1.3%, and 8.8±1.5%, respectively) as well as negligible cleavage of NS3/NS4A, NS4A/4B, and Neg (1.2±0.9%, 0.5±0.7%, and 0.6±0.9%, respectively) (Figure 4B).

Attempts at improving substrate design

We hypothesized that improving substrate design might allow for detection of cleavage of Tm-4A/4B-DsRed. We first created a soluble substrate of the same design that would be cleaved by membrane-bound NS3-4A. Three mutations (I8D, I12E, and F19D) known to release the NS5A amphipathic α-helix from the membrane (Elazar et al., 2003) were introduced into pTm-4A/4B-DsRed and pTm-4B/5A-DsRed (Figure 5A). The resulting plasmids denoted 'pTmSol-4A/4B-DsRed' and 'pTmSol-4B/5A-DsRed' were transfected into UNS3-4A cells and they showed the expected cytoplasmic localization by fluorescence microscopy (Figure 5B: TmSol-4B/5A-DsRed). Interestingly, in the absence of the ER-anchoring of the substrates, neither TmSol-4B/5A-DsRed (Figure 5C) nor TmSol-4A/4B-DsRed (data not shown) was processed by membrane-anchored NS3-4A, proving membrane association is an essential part of substrate recognition for our substrates.

Next, we hypothesized that replacing the NS5A amphipathic α-helix with the NS4A N-terminal hydrophobic domain (Figure 5D) (Brass et al., 2008) should target the substrate to the same lipid micro-environment as the protease and, therefore, we should observe a greater degree of substrate cleavage. The resulting plasmids denoted 'p4ATm-4A/4B-DsRed' and 'p4ATm-4B/5A-DsRed' were transfected into UNS3-4A cells. The p4ATm-4B/5A-DsRed (Figure 5E) and p4ATm-4A/4B-DsRed (data not shown) constructs local-

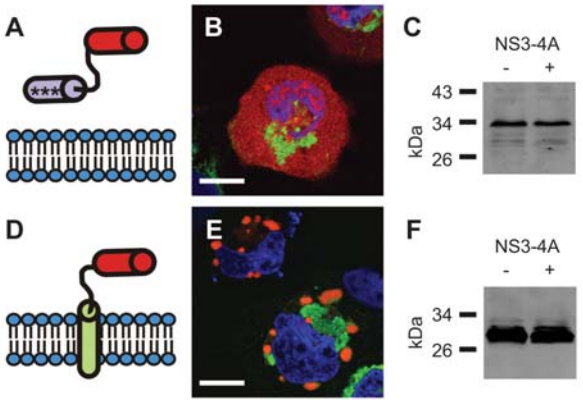


Figure 5 Altered substrate targeting eliminates NS3-4A cleavage. NS5A amphipathic α-helix targeting domain was (A) mutated to be soluble (SolTm) or (D) swapped for NS4A N-terminal hydrophobic domain (4ATm) in pTm-4B/5A-DsRed. UNS3-4A cells were transfected with (B) pSolTm-4B/5A-DsRed or (E) p4ATm-4B/5A-DsRed. Cells were probed with anti-NS3 primary and Alexa 488 secondary antibodies and imaged with a confocal microscope. NS3 signal is shown in green, DsRed signal is shown in red, and DAPI-stained nuclei are shown in blue. Scale bar corresponds to 10 μm. Whole cell lysates of (C) pSolTm-4B/5A-DsRed or (F) p4ATm-4B/5A-DsRed transfected cells were analyzed by Western blotting using anti-DsRed antibody.

ized perinuclearly as expected. However, neither of these constructs was endoproteolytically cleaved by NS3-4A as detected by fluorescence microscopy or Western blotting (p4ATm-4B/5A-DsRed: Figure 5E–F; p4ATm-4A/4B-DsRed: data not shown).

Comparison of viral cleavage sequences by genotype 1a and 2a NS3-4A

Both UNS3-4A and UHCV-32 are strain H, genotype 1b. Therefore, we next investigated whether cleavage site selectivity was a genotype-specific characteristic. NS3-4A from the Con1 strain (genotype 1a), and the JFH1 strain (genotype 2a), were subcloned into eukaryotic expression vectors such that NS3-4A would be expressed by the cytomegalovirus (CMV) promoter. Transfection of Huh-7 cells with both protease and substrate plasmids showed cleavage sequence preferences consistent with our previous findings (Figure 6). For both Con1 and JFH1 NS3-4A heterocomplexes, Tm-3/4A-DsRed and Tm-4A/4B-DsRed were not substantially cleaved, in contrast to the NS4B/NS5A and NS5A/NS5B junctions.

Comparison of host cleavage sequences by NS3-4A

The NS3-4A protease has been implicated in disrupting innate immunity dsRNA-sensor signaling cascades that lead to the induction of interferons. NS3-4A is thought to specifically cleave two host proteins: Toll/IL-1 receptor-domain-containing adaptor-inducing IFN-β (TRIF) and mitochondrial antiviral signaling protein (MAVS; also known as CARDIF, IPS-1, and VISA) (Li et al., 2005a,b; Cheng et al., 2006; Baril et al., 2009). TRIF is downstream from the extracellular/endosomal-dsRNA sensor, Toll-like receptor-3, and MAVS is downstream from the intracellular-dsRNA sensor, retinoic acid inducible gene I.

We aimed to determine the NS3-4A cleavage efficiencies of TRIF and MAVS and therefore constructed substrates with decapeptide cleavage sequences corresponding to the NS3-4A recognition sites (Figure 7A). UNS3-4A cells were transfected with pTm-TRIF-DsRed and pTm-MAVS-DsRed, and cleavage was measured for both constructs by Western blotting (Figure 7B). The TRIF substrate was significantly cleaved (35.1±1.2%) (Figure 7C), although less efficiently than the viral cleavage sites NS4B/NS5A and NS5A/NS5B (Figure 3B). The MAVS substrate was not significantly cleaved above background (11.7±6.4%) (Figure 7C).

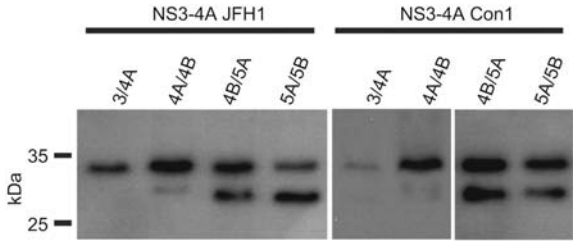


Figure 6 Substrate selectivity of NS3-4A genotype 1a and 2a. Huh-7 cells were double-transfected with pCMV-NS3-4A_Con1 (genotype 1a) or pCMV-NS3-4A_JFH1 (genotype 2a) and substrate plasmids: pTm-3/4A-DsRed, pTm-4A/4B-DsRed, pTm-4B/5A-DsRed, and pTm-5A/5B-DsRed. Whole cell lysates were probed by Western blotting using an anti-DsRed antibody.

Tm-4B/5A-DsRed is processed by NS3-4A during HCV infection

Since our substrates proved useful for probing in-cell NS3-4A activity alone and in complex with other replicase proteins, we hoped they would be recognized by NS3-4A during the course of HCV infection. Huh-7.5.1 cells were infected with JFH1 HCV (Wakita, 2009) and transfected with the Tm-4B/5A-DsRed substrate. We observed effective and significant processing of this substrate (38.4±11.6%, $p<0.05$) by NS3-4A during HCV infection (Figure 8).

Discussion

In this study, we have for the first time compared the relative intracellular cleavage efficiencies of HCV NS3-4A towards decapeptide substrate sequences corresponding to both viral and host substrates. We demonstrate no significant difference

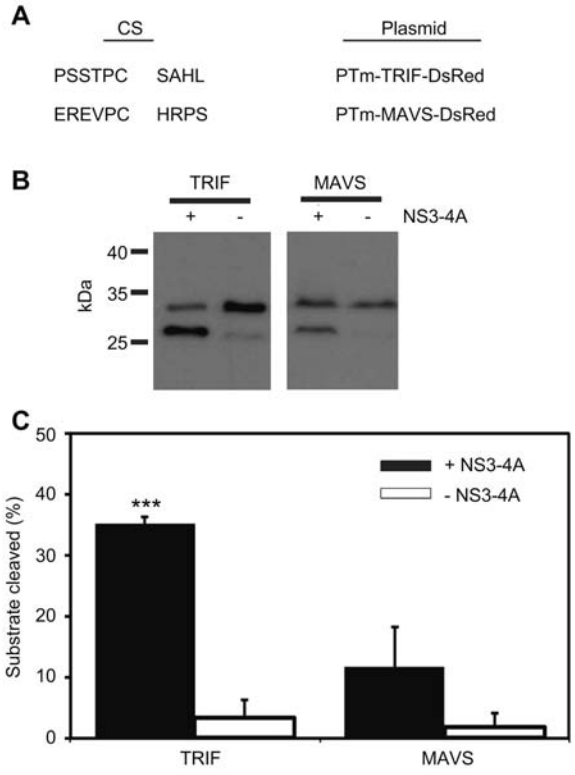


Figure 7 NS3-4A cleavage of TRIF and MAVS sequences. (A) Cleavage sequences (CS) P6-P4′ engineered into substrate plasmids. Gap indicates position of NS3-4A cleavage. (B) UNS3-4A cells were transfected with pTm-TRIF-DsRed and pTm-MAVS-DsRed. Whole cell lysates were analyzed by Western blotting using anti-DsRed antibody. (C) Quantification of Western blotting results showing the percentage of substrate cleaved. Results shown are the average of three independent experiments. ***$p<0.0005$.

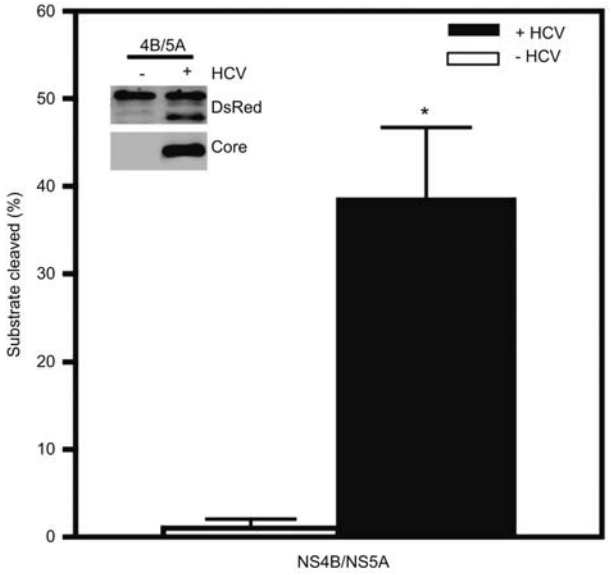


Figure 8 pTm-4B/5A-DsRed can be efficiently cleaved by NS3-4A during HCV infection.
Huh-7.5.1 cells were infected with HCV and then transfected with pTm-4B/5A-DsRed. Whole cell lysates were analyzed by Western blotting using anti-DsRed antibody. Results shown are the average of two independent experiments. *$p<0.05$.

between the NS4B/NS5A, NS5A/NS5B, and Mod junctions, all of which are cleaved efficiently. Cell-based assay screens using these junctions could easily be altered to use the NS4B/NS5A junction without loss of signal and to acquire the advantage of also screening for allosteric inhibitors of NS3-4A activation. As well, these data validate using a cell-based system only expressing NS3-4A for an initial or secondary drug screen, as the protease cleavage site selectivity is not changed within the replicase complex, and therefore small molecules targeted to the active site should bind the same when employed against replicating HCV.

Our data confirm the absence of *trans* cleavage at the NS3/NS4A junction (Tomei et al., 1993; Bartenschlager et al., 1994) and suggest that the cleavage of this junction observed by Lin et al. (1994) was mediated by protein folding or cleavage at a nearby alternative site (Hou et al., 2009).

Surprisingly, we show no detectable *trans* cleavage of the NS4A/NS4B sequence. This observation seems to hold true irrespective of NS3-4A genotype (1a, 1b, 2a) or whether NS3-4A is in replication complexes. Although it remains possible that efficient *trans* cleavage of the NS4A/NS4B site is mediated by determinants outside of the decapeptide cleavage sequence, such as substrate protein folding, we believe that poor NS4A/NS4B cleavage may reflect a biologically relevant substrate preference. In fact, detection of an NS4A-NS4B precursor by many groups supports this idea (Bartenschlager et al., 1994; Lin et al., 1994; Tanji et al., 1994; Bartenschlager et al., 1995a). Poor NS4A/NS4B cleavage could be important early in infection when protein concentrations are very low. If the NS4A/NS4B junction were to be processed in *trans* by a neighboring NS3-4A, the protease and polyprotein substrate could diffuse in the membrane, making maturation of the NS proteins a very inefficient process. The function of this cleavage order may also be temporally important for protein folding and proper insertion of transmembrane sequences. In addition, Konan et al. (2003) have shown that the NS4A-NS4B precursor, but not mature NS4A or NS4B, can reduce anterograde traffic from the ER to the Golgi, a process that may be pivotal for HCV to establish replication in infected cells.

It is also possible that in addition to cleavage of NS4A/NS4B in *trans*, cleavage may also occur in *cis*. Notably, previous work on the impact of NS junction mutations on polyprotein processing revealed certain mutations that differentially affected the *cis* junction (NS3/NS4A) as compared to the *trans* junctions (NS4B/NS5A, NS5A/NS5B) (Bartenschlager et al., 1995a). Introduction of these mutations into NS4A/NS4B showed a profile in-between the *cis* and *trans* cleavage site groupings. In particular, the ability to mutate the P1 cysteine in NS4A/NS4B to a threonine and not affect cleavage of this junction indicates the possibility of uni-molecular hydrolysis.

Recently, our group reported using membrane-targeted substrates to profile substrate selectivity of the West Nile virus NS2B-NS3 protease, indicating that this substrate design should prove useful for other membrane-associated proteases as well (Condotta et al., 2010). However, our unsuccessful attempt to improve substrate design in this study highlights the challenges in constructing intracellular substrates and underlines the importance of the targeting domain in such substrates. Our data suggest that multiple targeting domains should be investigated when developing such substrates for other proteases.

Ferreon et al. (2005) used a pep4A-NS3^{pro} fusion protein ('single-chain NS3') to compare TRIF peptide substrate cleavage to viral substrate cleavage *in vitro*. TRIF was processed less efficiently, about half as well as NS4B/NS5A. Our data confirm this fact inside cells.

Although we could show NS3-4A-mediated TRIF substrate cleavage, we could not demonstrate significant cleavage of the MAVS substrate. The MAVS sequence differs considerably from the viral junctions and TRIF; it has strongly basic arginines at the P5 and P2′ positions as well as an unconventional histidine in P1′. However, Jones et al. (2010) recently used a full-length MAVS fluorescent substrate to monitor HCV infection; therefore, NS3-4A cleavage of MAVS may depend on determinants outside of the P6-P4′ sequence that are able to compensate for the non-consensus cleavage site. It also has been recently suggested that dimerization of MAVS, mediated by the MAVS transmembrane domain, could be important for NS3-4A cleavage (Baril et al., 2009).

Importantly, the NS4B/NS5A substrate was cleaved efficiently in HCV-infected cells, providing a read-out of active NS3-4A during viral infection. The unique ability of our substrates to detect NS3-4A activity alone, in replication complexes, or within the course of HCV infection, allows them to be powerful tools for the study of HCV biology and for drug discovery.

Materials and methods

Construction of plasmid DNA

Plasmids were constructed by standard methods and verified by sequencing. The plasmid construction strategy is detailed below. Plasmid and primer sequences are available upon request.

i. pTm-3/4A-DsRed, pTm-4A/4B-DsRed, pTm-4B/5A-DsRed, pTm-5A/5B-DsRed, pTm-Mod-DsRed, pTm-Neg-DsRed. Overlapping oligonucleotides encoding the 10 amino-acid cleavage sites were annealed and ligated into EcoRI and AgeI double-digested pTm-DsRed. pTm-DsRed and pTm-4B/5A-DsRed have been described previously (Martin and Jean, 2006). pTm-DsRed was created by inserting DNA coding for the HCV NS5A 32 N-terminal amino-acid residues, which correspond to an amphipathic α-helix, referred to here as 'Tm', upstream of the DsRed-Express gene in pDsRed-Express-N1 (Clontech, Mountain View, CA, USA).
ii. pTmSol-4A/4B-DsRed and pTmSol-4B/5A-DsRed. Three mutations (I8D, I12E, and F19D) shown to release the NS5A amphipathic α-helix from the membrane (Elazar et al., 2003) were introduced into pTm-4A/4B-DsRed and pTm-4B/5A-DsRed by site-directed mutagenesis (QuikChange, Stratagene, La Jolla, CA, USA).
iii. p4ATm-4A/4B-DsRed and p4ATm-4B/5A-DsRed. The NS5A amphipathic α-helix sequence (^{1}SGSWLRDVWD WICTV-LTDFKTWLQSKLLPRLP^{32}) was replaced with the NS4A N-terminal hydrophobic domain (^{1}STWVLVGGVLAALAA-YCLST^{20}) in Tm-4A/4B-DsRed and Tm-4B/5A-DsRed substrates with three rounds of site-directed mutagenesis.
iv. pCMV-NS3-4A_Con1 and pCMV-NS3-4A_JFH1. NS3-4A was PCR amplified from pCon1/FL(I) (Blight et al., 2002) and pUC-vJFH1 (Wakita et al., 2005) using primers containing HindIII and XhoI restriction sites. Double-digested NS3-4A was ligated into double-digested pCMV-Tag 5A (Stratagene, La Jolla, CA, USA). pCMV-Tag 5A encodes for a C-terminal myc tag; the PCR primers used here were designed to encode a stop codon after the NS4A gene, preventing the myc tag from being translated.
v. pTm-TRIF-DsRed and pTm-MAVS-DsRed. Mutants were created by site-directed mutagenesis using pTm-4B/5A-DsRed as a template.

Cell culture

Reagents were purchased from Invitrogen (Burlington, ON, Canada). UNS3-4A and UHCV-32 cell lines were propagated as previously described (Moradpour et al., 1998; Hamill and Jean, 2005). Huh-7 cells were propagated in DMEM supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 100 μM non-essential amino acids, 50 units/ml penicillin, and 50 μg/ml streptomycin. Huh-7.5.1 cells were propagated in Huh-7 medium with the addition of 10 mM HEPES.

Fluorescence and immunofluorescence microscopy

Microscopy was done as previously described (Hamill and Jean, 2005; Martin and Jean, 2006). Coverslips were mounted with Vectashield mounting solution containing 4′,6-diamidino-2-phenylindole (DAPI, Vector Laboratories, Burlingame, CA, USA). Images were acquired using a Zeiss Axiovert confocal microscope (Toronto, ON, Canada) and assembled using Adobe Photoshop and Corel Draw.

Transfections and Western blotting

Transfections and Western blotting were done as previously described (Martin and Jean, 2006). Six well plates were seeded with 5×10^4 cells and grown for 2–3 days until >50% confluent. Cells were washed three times with PBS, and either tetracycline-replete media or media lacking tetracycline was added. Twenty-four hours post-tetracycline removal, cells were transfected with 2 μg per well of DNA plasmid using TransIT-LT1 transfection reagent. Twenty-four hours post-transfection, cells were washed with PBS, harvested by scraping, pelleted (900 g, 1 min), and frozen for Western blot analysis. Cell pellets were resuspended in hypotonic lysis buffer [10 mM Tris (pH 7.8), 10 mM NaCl, 1×complete EDTA-free protease inhibitors (Roche, Laval, QC, CA)]. SDS-protein loading dye was added, and samples were incubated at 95°C for 10 min. Cell lysates were resolved on a 15% SDS-polyacrylamide gel for 2.5 h at 110 V and transferred to a nitrocellulose membrane. The membrane was probed with polyclonal anti-DsRed (1:16 000 Lot#400467, 1:4000 Lot#5110235, 1:1000 Lot#7040094, Clontech, Mountain View, CA, USA) and goat-HRP anti-rabbit secondary antibody (1:1000, Amersham Biosciences, Piscataway, NJ, USA). Quantitation of the enhanced chemi-luminescent signal was performed on a VersaDoc MultiImager (Bio-Rad, Mississauga, ON, Canada) for a greater dynamic range than film (Martin and Bronstein, 1994). The percentage of substrate cleaved was calculated by dividing the cleaved signal by the total signal (cleaved plus uncleaved), thereby normalizing the readout for each sample.

For pCMV-NS3-4A_Con1 and pCMV-NS3-4A_JFH1 double transfections with substrate plasmids, methodology was the same as above with the following exceptions: Huh-7 cells were transfected with 2 μg each protease and substrate plasmid (4 μg total DNA) and were harvested 24 h post-transfection.

Statistical analysis

Statistical significance was calculated based on a two-tailed Student's *t*-test.

Hepatitis C virus preparation and infection

JFH1 cDNA (pJFH1) was used to generate HCV RNA and viral stocks as described (Zhong et al., 2005). Twenty-four well plates were seeded with 4×10^4 Huh-7.5.1 cells and grown overnight. Hepatitis C virus was added for a multiplicity of infection of 0.1 focus forming units per cell. Three days post-infection, cells were transfected with 600 ng of substrate constructs. Cells were harvested and processed as above, except that Western blots were simultaneously probed for DsRed and HCV core antigen (1:1000 ab2740, Abcam, Cambridge, MA, USA). Secondary detection was done with 1:10 000 IRDye 800CW donkey anti-rabbit and IRDye 680 donkey anti-mouse (LI-COR Biosciences, Lincoln, NE, USA). Imaging and quantitation were done with the LI-COR Odyssey Imaging System.

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