

FUNCTIONAL ANALYSIS OF HEPATITIS C VIRUS
NON-STRUCTURAL PROTEIN (NS) 3 PROTEASE AND VIRAL COFACTOR NS4A

by

MORGAN MACKENSIE MARTIN

B.Sc. The University of British Columbia, 2002

A THESIS SUBMITTED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR
THE DEGREE OF

DOCTOR OF PHILOSOPHY

in

THE FACULTY OF GRADUATE STUDIES

(Microbiology)

THE UNIVERSITY OF BRITISH COLUMBIA

(Vancouver)

August, 2008

© Morgan Mackensie Martin, 2008

Abstract

The hepatitis C virus (HCV) was identified in 1989 as the major causative agent of transfusion-associated non-A, non-B hepatitis and today represents a worldwide health crisis with prevalence estimates of 2.2%. HCV-specific therapeutics have never been more urgently needed. One of the validated drug targets is the non-structural (NS) protein 3 (NS3) membrane-bound protease. The major aim of this thesis was characterization of NS3 allosteric activation by its viral cofactor, NS4A. We hypothesized that there would be specific residues that dominate the interaction between NS3 and NS4A, and further hypothesized that binding and activation may be separate events mediated by different residues.

This thesis details the development of novel cell-based assays for detection of NS3-4A protease activity and heterocomplex formation. The protease assay substrate was a membrane-targeted intracellular protein, which upon proteolysis released a red fluorescent protein (FP) reporter, DsRed-Express, into the cytoplasm; this change was detected by microscopy or quantified by Western blotting. The complex formation assay detected fluorescence resonance energy transfer (FRET) between yellow and cyan FP-tagged NS3 and NS4A, respectively.

Our data shows binding can be functionally separated from activation. We identified two NS4A residues (I25 and I29) important for NS3 binding and two NS4A residues (V23 and I25) important for NS3 activation. Therefore the binding-pockets of these residues are prime targets for small-molecule therapeutic development.

In addition, I have compared the NS3-4A substrate sequence cleavage efficiencies *in vivo*. I have been able to show that the activation-dependent NS4B/NS5A junction is processed efficiently and the NS4A/NS4B junction is not. I have also shown NS3-4A substrate specificity is not modulated by replicase components; however the specific activity of this enzyme is increased.

The strength of this thesis work stems from the novel and creative development of cell-based assays that can easily be modified to study other membrane-associated proteases. *In vitro* assays fall short in that they do not take into account the unique micro-environment in which these proteases are found.

Table of Contents

Abstract.....	ii
Table of Contents.....	iii
List of Tables.....	v
List of Figures.....	vi
List of Illustrations.....	viii
Abbreviations.....	ix
Acknowledgements.....	x
Co-Authorship Statement.....	xii
Chapter 1 - Introduction.....	1
Hepatitis C virus history and epidemiology.....	1
Disease progression and treatment.....	2
Experimental systems to study HCV.....	4
HCV lifecycle.....	4
NS3.....	9
NS4A.....	12
NS3-4A heterocomplex.....	12
NS3-4A immune evasion.....	14
NS3-4A protease inhibitors.....	14
Aims.....	18
Hypothesis.....	18
Thesis overview.....	18
References.....	19
Chapter 2.....	37
References.....	45
Chapter 3.....	49
Introduction.....	49
Results.....	51
Discussion.....	60
Materials and Methods.....	63
References.....	66
Chapter 4.....	73
Introduction.....	73
Results.....	75
Discussion.....	81
Materials and Methods.....	84
References.....	87
Chapter 5 - Conclusion.....	92
Implications for NS3-4A drug discovery.....	93
Implications for polyprotein processing models.....	95
Implications for other <i>Flaviviridae</i>	97
Future directions.....	99
References.....	102
Appendix I – Chapter 2.....	105
Time course.....	105
Non-tethered substrate.....	105
Targeting domain swapping.....	106

Appendix II – Chapter 3	109
Figure 3.4	109
NS4A/NS4B cleavage.....	110
MAVS cleavage.....	114
Appendix III – Chapter 4.....	115
Fluorescence Polarization.....	119

List of Tables

Chapter 2	
Table 2.1 Tm-4B/5A-DsRed cleavage site variants are not cleaved by NS3-4A.....	44
Chapter 5	
Table 5.1 Select membrane associated proteases	94
Appendix II	
Table A2.1 Single point substitutions are able to rescue NS4A/NS4B cleavage	112

List of Figures

Chapter 1	
Figure 1.1 Estimated HCV Prevalence	1
Figure 1.2 Phylogram for HCV genotypes.	3
Figure 1.3 Crystal structure of NS3 ^{pro}	10
Figure 1.4 NMR stereoview of NS3 ^{pro}	11
Figure 1.5 Catalytic triad orientations	11
Figure 1.6 NS4A sequence	12
Figure 1.7 Crystal Structure of NS3 ^{pro} -pep4A	13
Figure 1.8 Select NS3-4A inhibitors.....	16
Chapter 2	
Figure 2.1 Tm-DsRed and Tm-4B/5A-DsRed are localized to perinuclear membranes.....	40
Figure 2.2 pTm-4B/5A-DsRed is processed by HCV NS3-4A.	42
Figure 2.3 pTm-4B/5A-DsRed substrate cleavage can be quantitated by Western blotting. ...	43
Chapter 3	
Figure 3.1 Efficient <i>trans</i> processing of Tm-4B/5A-DsRed, Tm-5A/5B-DsRed and Tm-Mod-DsRed by NS3-4A as detected by confocal microscopy.	54
Figure 3.2 Efficient <i>trans</i> processing of Tm-4B/5A-DsRed, Tm-5A/5B-DsRed and Tm-Mod-DsRed by NS3-4A as detected by Western blotting.....	55
Figure 3.3 Expression of NS3-4A in HCV polyprotein does not change substrate selectivity.	57
Figure 3.4 NS3-4A expression is at least 100-fold more in UNS3-4A cells as compared to UHCV-32 cells.....	58
Figure 3.5 Genotype 1a strain Con1 NS3-4A displays substrate selectivity alike genotype 1b NS3-4A.	58
Figure 3.6 NS3-4A cleavage of TRIF and MAVS sequences.	59
Chapter 4	
Figure 4.1 NS3-4A(V23A) and NS3-4A(I25A) are not proteolytically active	77
Figure 4.2 NS3-YFP and NS4A-CFP form an active heterocomplex.	78
Figure 4.3 I29 and I25 are important for NS3-YFP and NS4A-CFP to undergo FRET.....	79
Figure 4.4 NS4A(I29A)-CFP and NS4A(I25A)-CFP show binding defects.....	80
Figure 4.5 Model of I29, I25, and V23 binding pockets.....	82
Chapter 5	
Figure 5.1 Phylogram for selected <i>Flaviviridae</i> genotypes.....	97
Figure 5.2 Comparison of genus <i>Flavivirus</i> with genus <i>Hepacivirus</i>	98
Appendix I	
Figure A1.1 Localization of 4ATm-4A/4B-DsRed	106
Figure A1.2 NS3/4A causes reorganization of mitochondria.	107
Appendix II	
Figure A2.1 Alteration of UHCV-32 image reveals NS3.....	109
Figure 2.2 NS3-4A expression is about 8-fold more in UNS3-4A cell membrane fractions as compared to UHCV-32 cell membrane fractions.	110
Appendix III	
Figure A3.1 Alanine scanning of NS4A residues 21 to 54 identifies multiple residues involved in NS3 activation and inhibition	116
Figure A3.2 Alanine scanning of NS4A residues by FRET microscopy	118

Figure A3.3 Titration of NS3 ^{pro} against 15 fmoles of pep4AK-fluorescein.....	120
Figure A3.4 Z'-factor determination for NS3 ^{pro} and pep4AK-fluorescein.....	122

List of Illustrations

Chapter 1	
Illustration 1.1 HCV Entry.....	5
Illustration 1.2 Translation and polyprotein processing.	6
Illustration 1.3 HCV replicase complex.....	7
Illustration 1.4 Commonly used assays for detection of NS3-4A protease activity	15
Chapter 2	
Illustration 2.1 Design of a membrane-anchored fluorescent protease substrate	39
Chapter 3	
Illustration 3.1 Membrane-anchored substrate design and cleavage site sequences.	52
Chapter 4	
Illustration 4.1 DNA constructs used in this study	76
Chapter 5	
Illustration 5.1 Polyprotein processing steps	96
Appendix II	
Illustration A2.1 Sequence of pTm-4A/4B-DsRed mutants	111
Illustration A2.2 Schematic representation of the amino acid side chains of the NS3/4A cleavage sites	113
Appendix III	
Illustration A3.1 Mutants that have been analyzed for NS3 activation (A) and binding (B). .	115
Illustration A3.2 Fluorescence Polarization.....	119

Abbreviations

4',6-diamidino-2-phenylindole, dihydrochloride (DAPI), activity-based probe (ABP), alternate reading frame protein (ARF), cleavage site (CS), cluster of differentiation 81 (CD81), cyan fluorescent protein (CFP), dengue virus (DENV), double stranded (ds), DsRed-Express (DsRed), Dulbecco modified Eagle medium (DMEM), encephalitis virus (EV), endoplasmic reticulum (ER), envelope protein (E), fluorescent protein (FP), fluorescence resonance energy transfer (FRET), frameshift protein (F), glycosylphosphatidyl inositol (GPI), green fluorescent protein (GFP), hepatitis C virus (HCV), high performance liquid chromatography (HPLC), human immunodeficiency virus (HIV), internal ribosome entry site (IRES), intramembrane protease (IP), isopropyl-beta-D-thiogalactopyranoside (IPTG), Japanese encephalitis virus (JEV), juxtamembrane protease (JP), low-density lipoprotein receptor (LDL-R), major histocompatibility complex class I (MHC-I), mitochondrial antiviral signaling protein (MAVS), multiple cloning site (MCS), non-structural (NS), NS3 protease domain (NS3^{pro}), NS3 helicase domain (NS3^{hel}), NS5A N-terminal amphipathic alpha helix (Tm), nuclear magnetic resonance (NMR), peptide corresponding to central NS4A domain (pep4A), phosphate buffered saline (PBS), protein data bank (PDB), retinoic acid inducible gene I (RIG-I), scavenger receptor class B (SR-B1), secreted alkaline phosphatase (SEAP), signal peptidase (SP), signal peptide peptidase (SPP), TANK-binding kinase-1 (TBK-1), toll-like receptor-3 (TLR-3), toll/IL-1 receptor-domain-containing adaptor inducing IFN-beta (TRIF), West Nile virus (WNV), yellow fever virus (YFV), yellow fluorescent protein (YFP)

Acknowledgements

This work is supported by a Canadian Institutes of Health Research (CIHR) Initiative on Hepatitis C scholarship and grants EOP38153/EOP67273 (to F. Jean).

M.M. Martin gratefully acknowledges support from a CIHR-University of British Columbia (UBC) Training Program for Translational Research in Infectious Diseases (TRID) Doctoral Award, a CIHR Doctoral award, a CIHR-UBC TRID Top-up Award, a National Sciences and Engineering Research Council of Canada (NSERC) Postgraduate Scholarship (PGS-A) and a Michael Smith Foundation for Health Research (MSFHR) Trainee Award.

Dr. Darius Moradpour (University of Freiburg) kindly provided the tetracycline-regulated cell lines (UNS3-4A and UHCV-32). pCon1/FL(I) was supplied by Apath LLC (Saint Louis, Missouri, USA). pETNS3FL and pETNS3/4A plasmids were generously provided by Dr. Paola Gallinari (IRBM, Rome, IT).

I would like to acknowledge my supervisor, Dr. François Jean. Thank-you for allowing me to pursue such interesting research in a supportive and creative environment. Thank-you for being energetic when I was tired. And thank-you for being patient and helping me to become a better researcher.

I would like to thank committee members Drs Brett Finlay, Bob Hancock, and Chris Overall for constructive criticism and creative suggestions throughout this thesis work.

I would like to thank all the undergraduate students that helped me in the laboratory. Amanda for your undying optimism and dedication. Peter for always making me laugh and working through the pain (though you didn't have to). Anna for caring so much. Melanie for gracefully working in the cold with the slowest computer ever. Jason for the paper cranes and the smiles. Catharine for ton beau dépannage et ta bonne disposition. Emily for your perseverance. Dave for not giving up on polarization and putting up with me while I was writing my thesis.

I would like to thank all the other members (current and past) of the Jean lab team. Martine for her good and caring heart. Addy, Martin and Pam for all our fun days in Wesbrook. I would like to thank all the members of the department of Microbiology & Immunology for creating a welcoming and supportive environment for students.

I would like to thank my husband Ian for his continued support (emotional and technical). I think he should receive a masters. 😊 I would like to thank all of my friends (many of whom I have met while in the Jean laboratory) for all the fantastic memories and for forcing some work-life balance on me.

Co-Authorship Statement

For chapters 2 and 3, M.M. Martin performed all the experiments and prepared all of the figures. M.M. Martin designed the experiments and wrote the manuscript with advice from F. Jean.

For chapter 4, M.M. Martin subcloned the DNA constructs, created approximately 50% of the site-directed NS4A mutants, optimized the FRET experiments, performed experiments for figures 4.2 and 4.3A, and generated figure 4.5. M.M. Martin had much creative input on the direction of the project and will be responsible for writing the manuscript. P. Wang carried out experiments for the FRET and colocalization studies (Figures 4.3B and 4.4) and analysed the images for publication. A. Chau improved the design of the system used and carried out experiments for the activation studies. D. Moscher carried out screening and final experiments for the activation studies (Figure 4.1). C. Verreault optimized conditions for the activation studies. A. Jonas created some site-directed mutants for the FRET studies. M.M. Martin supervised and helped P. Wang, A. Chau, D. Moscher, C. Verreault, and A. Jonas with their laboratory work. F. Jean gave advice for experimental design and direction and will also critically review the manuscript.

Chapter 1 - Introduction

Hepatitis C virus history and epidemiology

By the mid 1970s diagnostic assays became available for the hepatitis B virus. This allowed screening of donated blood and halted transfer of viral hepatitis B through blood transfusions. However, it became apparent that another agent (or agents) continued to be transferred that caused hepatitis and liver disease, referred to as “non-A, non-B hepatitis” (121). A virus associated with this type of disease was identified by electron microscopy (27) and named the hepatitis C virus (HCV). In 1989, the Chiron Corporation published identification of HCV as a 10 Kb positive-sense RNA virus (24). The Chiron Corporation also developed an assay to detect anti-HCV antibodies and HCV was shown to be associated with the vast majority of parenterally-acquired and transfusion-associated non-A, non-B hepatitis (71).

Almost two decades have passed and hepatitis C is still a major health concern. Worldwide an estimated 2.2% of people are infected, corresponding to more than 130 million people (125) (Figure 1.1). In the developing world, new infections are mainly due to non-sterile medical procedures and unscreened blood-products (2). In developed countries, donated blood is screened for HCV; however injection drug needle sharing remains an important source of new infections in young adults (2).



Figure 1.1 Estimated HCV Prevalence

Data based on blood screening and mathematical modeling of infection rates. Figure from Alter, M.J., 2007 and the global burden of hepatitis C working group, 2004 (2, 125).

In 2002, the estimated HCV prevalence in Canada was 0.8% (51). However, in British Columbia the prevalence (1.5%) is almost double the national average, mainly due to a concentration of injection drug users in Vancouver, of which two out of three are HCV infected (30, 42, 134).

Disease progression and treatment

The current treatment for HCV infected patients is combination drug therapy with pegylated interferon- α and ribavirin. These drugs are broad-spectrum immune modulators that are used to treat various viral infections, including hepatitis B and D (17, 41, 64). Interferon- α is a cytokine of the innate immune response that induces a cellular anti-viral state by activation of immune cells as well as inducing changes in non-immune cells that ward off infection (127). It appears to initially block virus production or release (102). Ribavirin is a nucleoside analog and the mechanism by which it inhibits HCV is not clear (33). The success of this therapy is largely dependent on the genotype of HCV the patient is infected with, but on average it is unsuccessful at viral clearance in the majority of cases.

HCV isolates are currently grouped into six genotype categories that differ by as much as 50% at the protein level (Figure 1.2). These categories are diagnostically useful because different genotypes exhibit different responses to interferon-based therapies. Genotype 1 and 4 are harder to treat and require treatment schedules that are double (48 weeks versus 24 weeks) that of genotypes 2, 3 and 5 (62, 103, 108). Genotype 6 falls between these two groupings and the exact treatment schedule for this genotype is not yet optimized (103). Genotypes 1, 2 and 3 are most common in North America, Europe and Japan and the most research has been done on these genotypes. Genotype 4 accounts for 20% of all HCV infections worldwide, and is most prevalent in Africa and the Middle East. Genotype 5 is found most prevalent in South Africa and genotype 6 is a minor component of South East Asian infections.

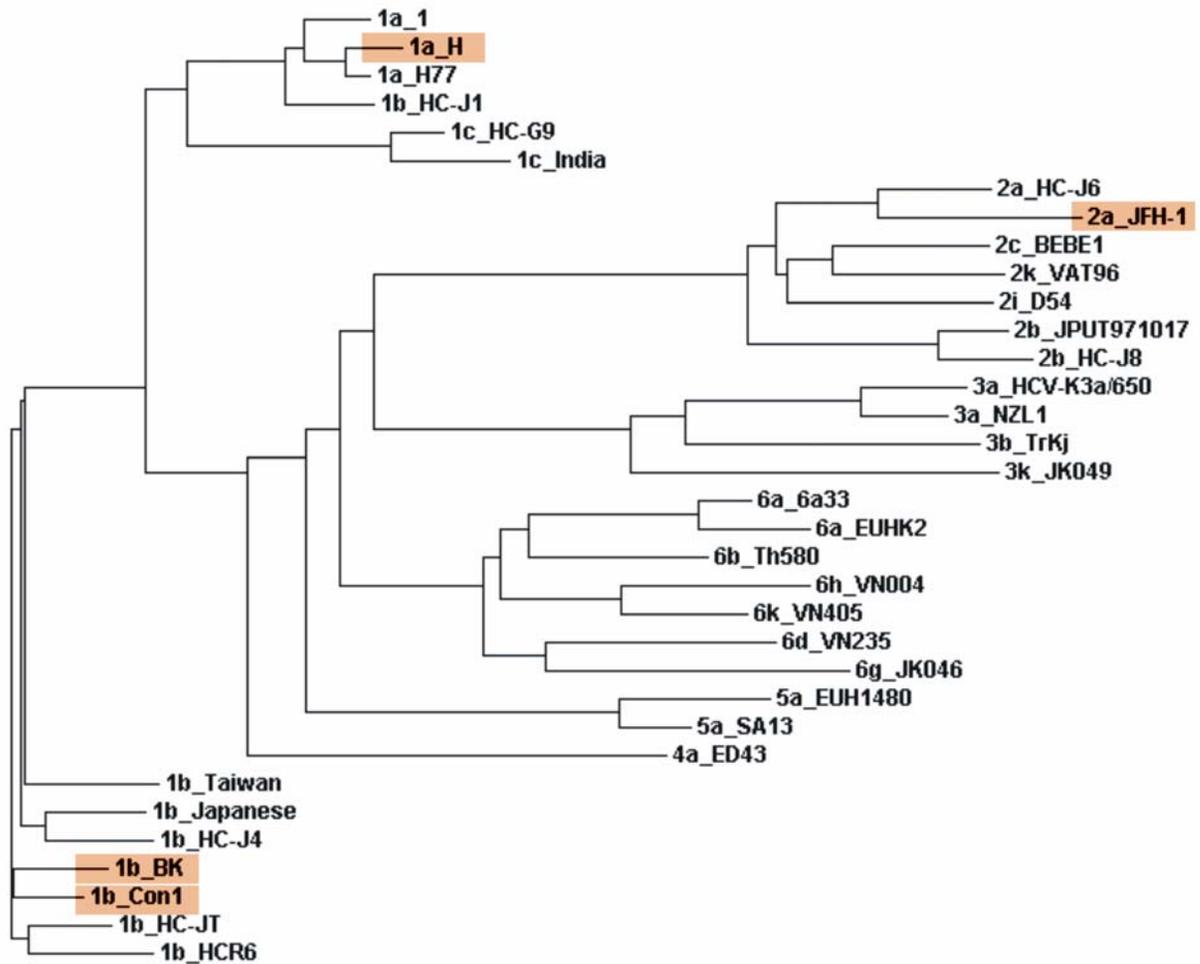


Figure 1.2 Phylogram for HCV genotypes.

Multiple sequence alignment and neighbour-joining phylogram was generated by comparing non-structural protein 3/4A (NS3-4A) sequences from all genotypes listed in NCBI Taxonomy database (accessed April 9, 2008) using Clustal W (12, 74, 112, 132). Excluded were non-classified HCV isolates. Nomenclature shown corresponds to "genotype"_"strain". Highlighted are strains relevant to this thesis.

HCV polymerase has a very high error frequency, approximately 1 base substitution every 10 Kb. When a patient is infected with the HCV virus, it replicates and forms a population of mutants termed a quasispecies. Due to the large variability in HCV sequence it has been difficult to categorize HCV isolates into strains and some researchers have suggested if enough isolates were sequenced, we would not observe genotype groupings, but a continuum of HCV isolates. The quasispecies model is important for HCV, as it predicts rapid viral mutation to evade antivirals and the immune response.

Experimental systems to study HCV

Historically, one of the major hurdles in HCV research and drug development has been a lack of experimental systems to study the virus and test inhibitors. For unknown reasons, all isolates of HCV except one (JFH-1) will not replicate in cells in culture. The JFH-1 virus (genotype 2a) was isolated from a patient with fulminant hepatitis in 2005 (129). This tool has greatly improved researcher's ability to study viral entry, viral assembly and release and to test inhibitors of all viral life stages.

Prior to the discovery of JFH-1, the most important development for studying HCV RNA replication has been the replicon system. The original replicon system was derived from a genotype 1b clone (Con1); it expressed only the non-structural (NS) proteins and self-replicated its RNA (88). This system has been extremely important for understanding NS protein interactions, identifying interacting host proteins and screening for replication inhibitors.

Chimpanzees are used as an animal model for viral infection and unfortunately good small animal models for HCV do not exist. A mouse liver-transplant model allows some work to be done. In this model, severe combined immunodeficiency mice are transplanted with human liver tissue (resulting in a chimeric mouse-human liver) and then infected with HCV (97).

HCV lifecycle

HCV is a hepatotropic virus; infection of other cell types has been reported but remains controversial. HCV enters liver cells by clathrin-mediated endocytosis after the HCV glycoproteins, a heterocomplex of envelope 1 (E1) and E2, bind to cell surface receptors (Illustration 1.1) (55). Many cell surface receptors have been implicated in HCV entry, although four appear to be most important: cluster of differentiation 81 (CD81), scavenger receptor class B (SR-B1), Claudin 1, and low-density lipoprotein receptor (LDL-R). (1, 10, 39, 107). CD81 and SR-B1 are thought to aid entry by specific interaction with E1 or E2 (135). Claudin 1 is a tight junction protein that is essential for HCV entry but is required at a late entry step, after initial virus binding (19, 39). Interestingly, the LDL-R is regarded as the main virus receptor, although the mechanism by which it binds virus is somewhat unique. HCV virus-like particles are bound by LDL-R after they associate with LDL and other lipoproteins in serum, possibly through an affinity of E2 for lipid moieties (136).

After initial binding and endocytosis, low pH conditions in the endosome trigger fusion between the viral envelope and the endosomal membrane, releasing the icosahedral nucleocapsid into the cytoplasm. The destabilized nucleocapsid releases the positive-sense HCV single-stranded (ss) RNA genome into the cytoplasm.

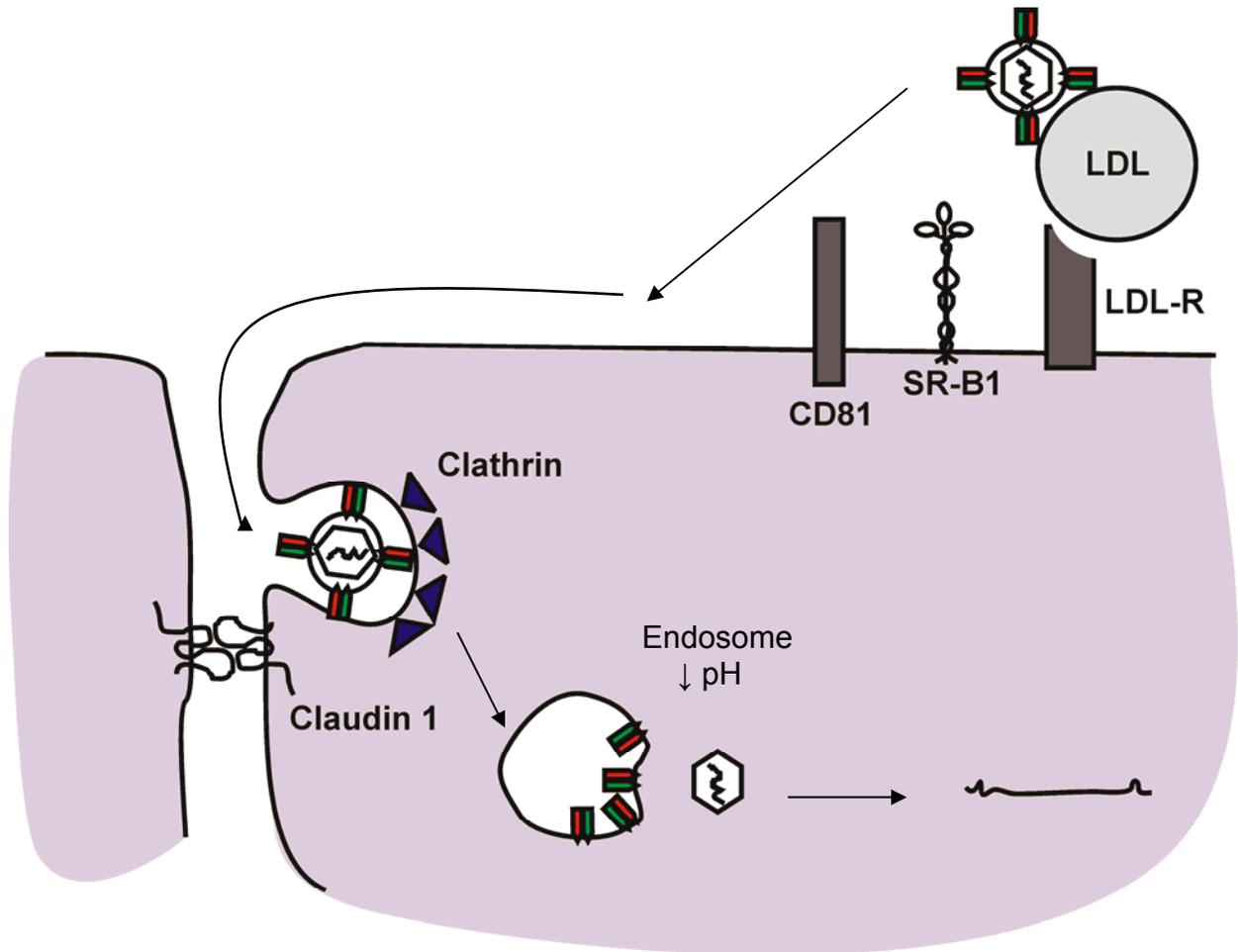


Illustration 1.1 HCV Entry

HCV binds to LDL in serum and is bound by LDL-R on hepatocytes. CD81 and/or SR-B1 function as co-receptors, mediating clathrin-coated pit endocytosis. Claudin 1 is required for a late entry step that is not well characterized. Reduction of pH in the endosome triggers viral and host membrane fusion and the release of the nucleocapsid into the cytoplasm. The viral RNA is released from the capsid.

The genome is recognized and translated by host ribosomes via its 5' internal ribosome entry site (IRES). The genome is polycistronic; the single open reading frame encodes for ten proteins as one long polyprotein which is co- and post-translationally cleaved by both host and viral proteases (Illustration 1.2) (13, 34, 131). At the N-terminus are the structural proteins which are liberated by a host signal peptidase (SP); SP cleaves the C/E1 and E1/E2 junctions very

quickly, however, cleavage of E2/p7 and p7/NS2 are delayed and results in an E2-p7-NS2 precursor (20, 80). The NS junctions are cleaved by the viral proteases NS2 and NS3. NS2 forms a cysteine protease dimer that cleaves at the NS2/NS3 junction in *cis* (89). NS3 processes the remaining cleavage sites and requires activation by NS4A for cleavage of the NS4B/NS5A junction (9, 40, 81, 122).

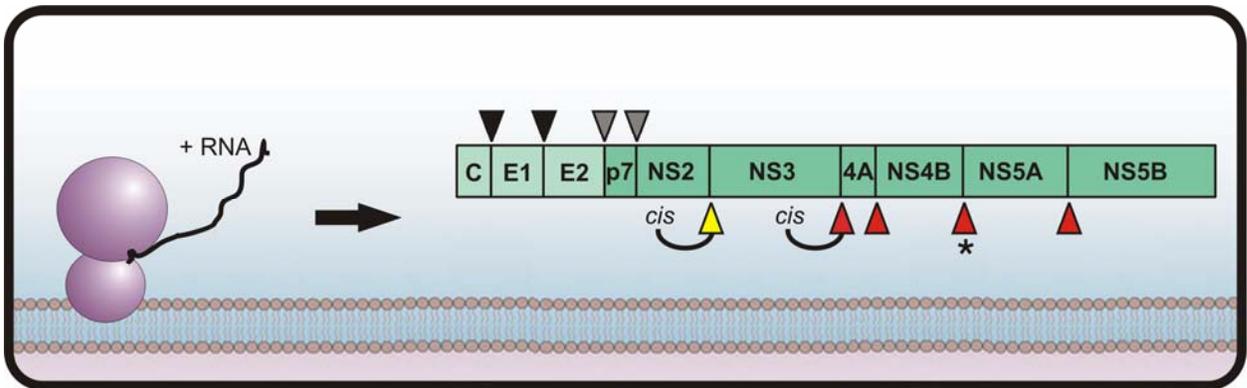


Illustration 1.2 Translation and polyprotein processing.

HCV viral RNA is translated into a long polyprotein. Proteins are produced via cleavage by host (upper arrows) and viral (lower arrows) cleavage. Upper arrows show sites of signal peptidase cleavage, black indicates efficient co-translational cleavage, and grey denotes inefficient post-translational cleavage. Yellow arrow shows site of NS2 *cis* cleavage. Red arrows show sites of NS3 cleavage in *cis* (NS3/NS4A) and *trans* (NS4A/NS4B, NS4B/NS5A, and NS5A/NS5B). * NS4B/NS5A junction requires NS4A activation of NS3 for cleavage at this site.

After polyprotein processing, the NS proteins, exclusive of NS2, form a replication complex (“replicase”) on the ER membrane (Illustration 1.3) (36). The replicase complex is held together by weak protein-protein interactions; the membrane targeting of each protein is essential for complex formation (47). Each replicase protein except for NS3 contains a membrane anchor domain; NS4A and NS4B have N-terminal hydrophobic and transmembrane domains, respectively (38, 133), NS5A membrane association is due to an N-terminal amphipathic alpha helix (18, 37), and NS5B has a C-terminal transmembrane domain (98). NS3 is anchored to the ER membrane by virtue of its interaction with NS4A (133).

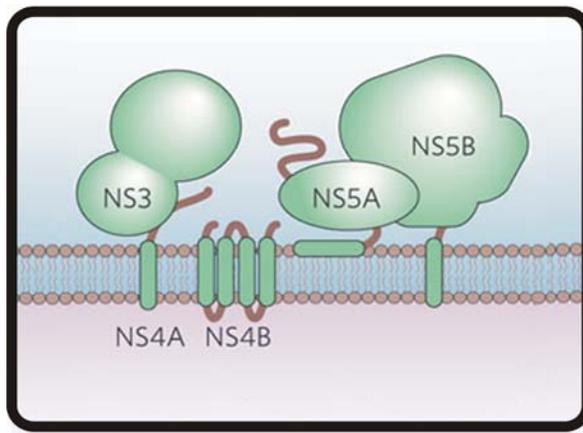


Illustration 1.3 HCV replicase complex

Topology of the HCV NS proteins contained in the replicase complex. Adapted by permission from Macmillan Publishers Ltd: Nature **436**: 933-8, copyright (2005), reference (86).

The stoichiometry of the components and whether host proteins are included within the replicase is not known, however, NS3 helicase and NS5A appear to form functional homodimers (22, 65, 99, 124). In addition, how protein function is modulated within the replicase is not known. NS3 is a bifunctional enzyme; it contains both a serine protease domain and a helicase domain (NS3^{pro} and NS3^{hel}, respectively). It is known that NS3^{hel} domain is able to modulate NS5B polymerase function, and vice versa (60, 106, 141).

The replicase is responsible for reproduction of the viral RNA; exactly how these NS and host proteins work together to accomplish this goal is still being elucidated. After or during translation, NS5A, an RNA-binding protein, is hypothesized to bind to the template RNA, providing a link to replication (56). NS5B, the RNA-dependent RNA polymerase, synthesizes a negative strand intermediate and then transcribes new genomes to be used for translation and virus production (11). During RNA replication, the NS3^{hel} and host cell helicases unwind secondary structure in ssRNA and uncouple template-transcript double-stranded (ds) RNA (46, 59, 72).

NS4B doesn't seem to interact directly with the RNA, instead it induces structural rearrangements in the ER membrane leading to formation of a mitochondria-associated 'membranous web' structure, the nodules of which are the location of the replicase (36, 63, 92, 100). The N-terminal transmembrane domain of NS4B is essential for this process and proper insertion of this domain relies upon NS3-mediated proteolysis (92). This rearrangement may

help the virus to sequester dsRNA from host detection, concentrate viral proteins in localized ‘factories’ and also prevent mitochondrial-mediated apoptosis (32, 54, 78).

Once enough structural proteins and new genomes have accumulated, assembly can occur on lipid rafts. The C protein requires processing from the host intramembrane protease signal peptide peptidase (SPP) in order to associate with these lipid droplets (93). The positive-sense RNA genome associates with C and the nucleocapsids bud into the ER, obtaining their double membrane and glycoproteins, E1 and E2. The viruses are then released through the secretory pathway by exocytosis. The small protein, p7, forms an ion channel in the ER and is essential for viral assembly and release. It has been shown that p7 can bind to C specifically, and interruption of this interaction will halt viral replication; however the reason for this interaction and the exact role the channel may play is not understood (48, 101, 120). NS2 is also essential for viral assembly and release, independent of its protease function (61). NS5A has also been shown to interact with C and other host proteins in a phosphorylation dependent manner to facilitate viral assembly (4, 123).

Recently, an alternative reading frame protein (ARF) or frameshift protein (F) has been described (130, 138). This protein is translated in the +1 frame in the C protein region (7), and almost all chronically infected patients have antibodies to ARF/F (25). Although the protein itself does not seem to be essential for HCV replication, stop codons introduced in the +1 frame are selected against, suggesting a conserved RNA element may be important (96).

NS3

The NS3 protein comprises an N-terminal serine protease domain and a C-terminal helicase domain, separated by a semi-flexible linker region (50, 111). There is no evidence that the two domains are separated during the HCV life cycle and in fact there appears to be regulation between the protease and helicase. NS3^{pro} increases helicase and NTPase activities (45, 49, 141), and NS3^{hel} mutations have been shown to alter inhibition by NS3^{pro} active site and serpin-based inhibitors (29, 95).

NS3^{hel} is a DExD/H box family helicase that is able to unwind both dsRNA and dsDNA. It functions as an oligomer and unwinds RNA by an ATP-coupled inchworm mechanism (35, 76, 117). NS3^{hel} has been shown to interfere with protein kinase A function (3, 14), a regulator of cellular metabolism, as well as to bind to histones, interfering with histone-DNA interactions (15, 16).

NS3^{pro} also has been found to interact with or modulate host proteins. NS3^{pro} is able to bind the tumor suppressor p53 (32, 58); during infection sequestration of p53 in the cytoplasm could interfere with apoptosis and lead to hepatocellular carcinoma. NS3^{pro} has been shown to increase activity of the cell secretory pathway via an interaction with ELKS δ and ELKS α (52). In addition, NS3^{pro} is able to disrupt immunoproteasome function through an interaction with LMP7 (low-molecular-mass protein 7), a process which may reduce presentation of HCV-derived peptides on major histocompatibility complex class I (MHC-I). The diverse roles NS3 plays in viral-induced cellular modulation and viral replication make it a very interesting therapeutic target.

The first crystal structure of NS3^{pro} was obtained in 1996, and it revealed a chymotrypsin-like fold with two β -barrels and a structural zinc molecule (Figure 1.3) (90). The crystal structure revealed an unusually shallow, extended substrate binding cleft which explains the biochemical observations that peptide substrates require a minimum of ten residues to act as a productive substrate (119, 142). The most striking feature of this crystal structure was that the catalytic triad (H57, D81, and S139) was not aligned; the aspartic acid residue D81 was oriented away from the active site. This was surprising as NS3 had been shown to cleave peptide and polyprotein precursor substrates without NS4A (9, 40, 81, 82, 118, 119, 122).

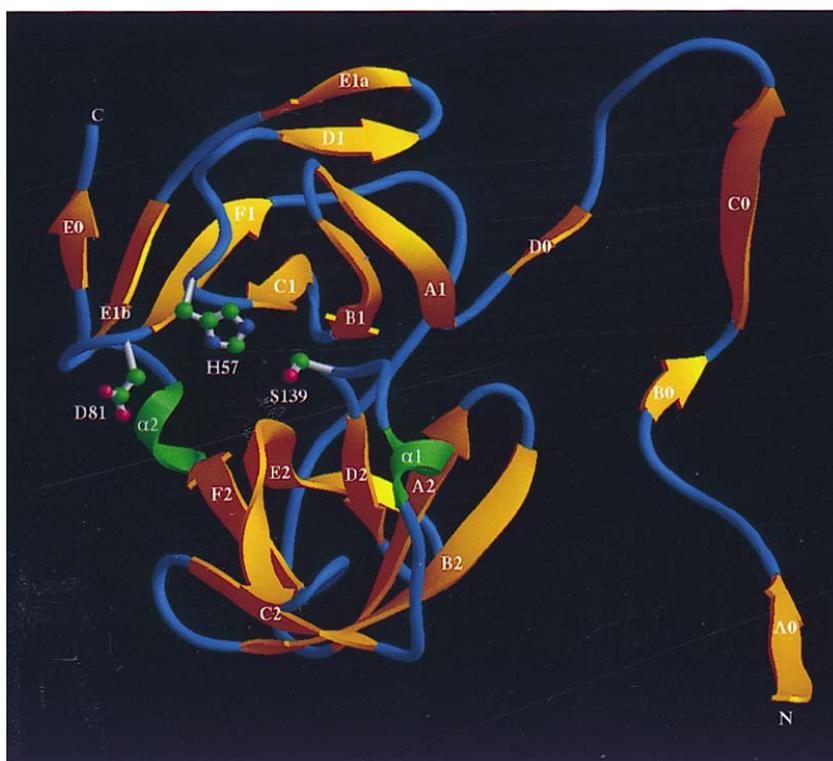


Figure 1.3¹ Crystal structure of NS3^{pro}

β-strands are shown in orange, α-helices are shown in green, and coils are blue. Catalytic residues are shown in ball and stick (H57, D81, and S139). Structural zinc molecule is not shown. (90)

Three years later, a nuclear magnetic resonance (NMR) structure was published that explained this apparent paradox (6). The N-terminal β-barrel showed multiple conformations in solution, as compared to the C-terminal β-barrel, the structure of which did not change significantly (Figure 1.4). The D81, located on the N-terminal β-barrel showed two conformations: the misalignment seen in the crystal structure and a proper active alignment (Figure 1.5). The misaligned conformation was predominant. It was proposed that in solution NS3 structure is flexible and this explained how the NS3 without NS4A showed activity towards substrates, even though it was shown in the crystal structure to be inactive. Stabilization of the active conformer of NS3 is facilitated through an interaction with NS4A.

¹ Reprinted from Cell, 87, Love RA, *et al.*, The crystal structure of hepatitis C virus NS3 proteinase reveals a trypsin-like fold and a structural zinc binding site, 331-42, copyright (1996), with permission from Elsevier.

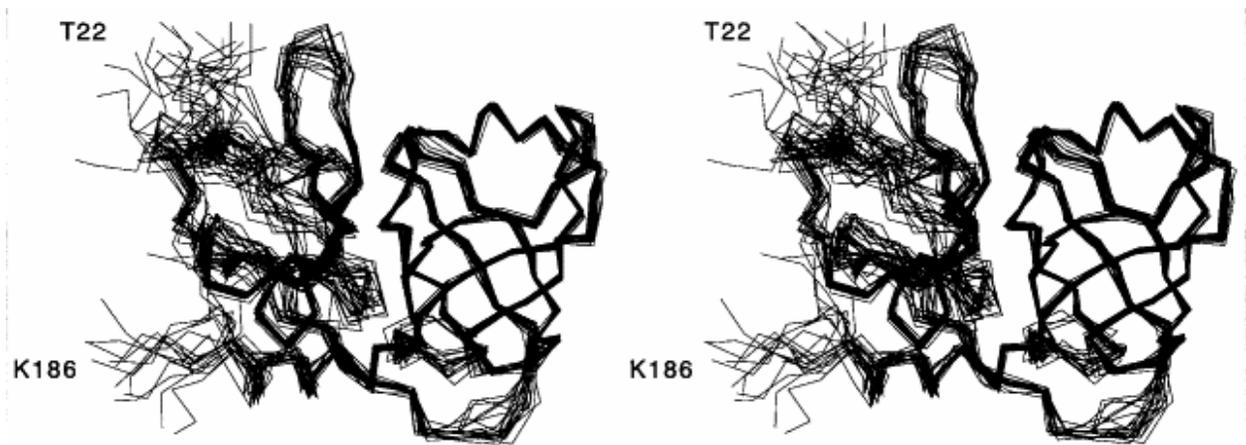


Figure 1.4² NMR stereoview of NS3^{pro}

Shown are the 20 minimal energy structures. T22 and K186 indicate the first and last residue detected by NMR, respectively (6).

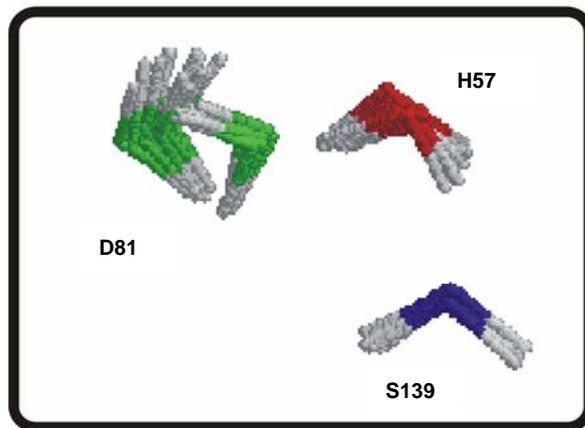


Figure 1.5 Catalytic triad orientations

NMR backbone structure of catalytic residues showing two alternate conformations for D81: a favored misalignment and a proper alignment. Structural data from 1BT7 in protein data bank (PDB), originally from Barbato et al. (6). Image created with RasMol (114).

² Reprinted from Journal of Molecular Biology, 289, Barbato G, *et al.*, The solution structure of the N-terminal proteinase domain of the hepatitis C virus (HCV) NS3 protein provides new insights into its activation and catalytic mechanism, 371-84, copyright (1999), with permission from Elsevier.

NS4A

NS4A is a small 54-residue protein with diverse functions. It is important for regulating the phosphorylation state of NS5A (68), and is able to inhibit translation through interaction with eukaryotic elongation factor 1a (43, 70). When expressed alone, the NS4A protein has been shown to localize to mitochondria and induce mitochondrial-associated oxidative stress, a precursor to cell damage and cancer (56, 115).

NS4A has three predicted ‘domains’ (Figure 1.6): 1) an N-terminal transmembrane domain, 2) an interaction domain with which it forms a heterodimer with NS3, and 3) a 20-residue stretch of hydrophilic amino acids, the end of which has the ability to fold into an α -helix when interacting with basic residues (85). A synthetic peptide corresponding to the central domain, “pep4A” (GSVVIVGRIILSGR), has been used to activate NS3 *in vitro* and this peptide cofactor elicits about 70 percent of the activation of the protease activity as compared to the full length cofactor (83). Exactly how NS4A activates NS3 has been a major focus of study.

Domain I	Domain II	Domain III
ST <u>WVLVGGVLAALAAAYCL</u> TTG	<i>SVVIVGRIILSGR</i> PAIV	PDRELLYQEFDEMEEC

Figure 1.6 NS4A sequence

Protein sequence of NS4A genotype 1b. Predicted transmembrane domain is underlined. Central residues that interact with NS3 are italicized.

NS3-4A heterocomplex

NS4A functions to localize, stabilize, and activate NS3. NS4A enhances NS3 cleavage efficiency at all junctions and is strictly required for the NS4B/NS5A junction to be cleaved by NS3. The crystal structure of NS3 bound to the pep4A has given insight into the mechanism of activation (Figure 1.7) (67). The NS4A central region integrates into the N-terminal NS3 β -barrel and the N-terminal of NS3 folds over NS4A. These changes result in the 6-strand N-terminal β -barrel becoming an eight-strand β -barrel. Most importantly, the folding of the NS3 N-terminus moves the D1/E1 strands further toward the active site (Figure 1.3 and 1.7) and the catalytic aspartic acid (D81) residue is stabilized in an active conformation. Whether NS4A binds to NS3 only in the active conformation, and then stabilizes this structure, or NS4A is able to induce NS3 conformational change by binding is not known.

Using deletion mutants, it was found that the first twenty-two residues of NS3 are required for a stable NS3-4A interaction, so the folding over of NS3 N-terminus appears to 'lock' the NS4A into place. The lack of deep pockets and the extended area of contact in the NS3-NS4A interaction suggest that this binding results from many weak interactions.

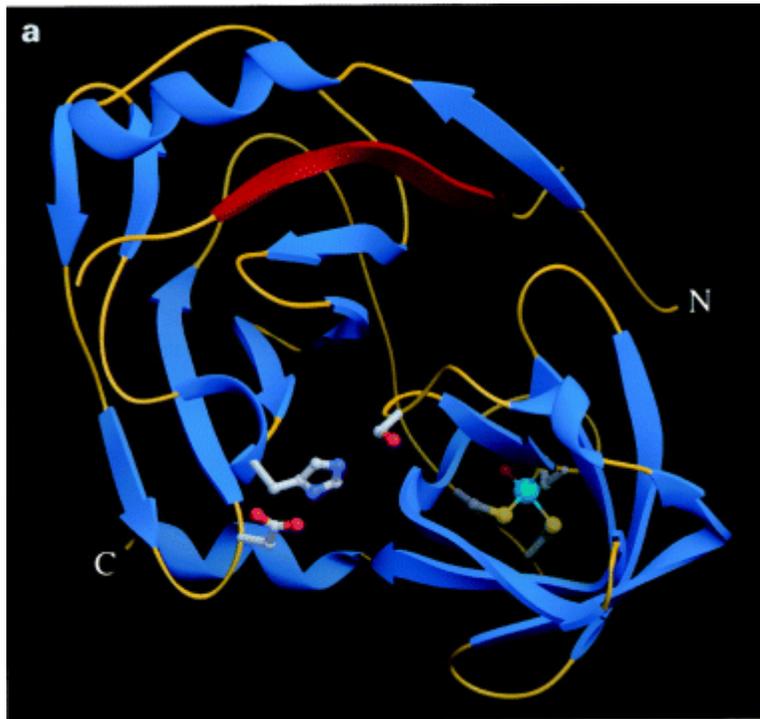


Figure 1.7³ Crystal Structure of NS3^{pr}-pep4A

Crystal structure is shown with all β -strands and α -helices in blue. NS4A is shown in red. Catalytic triad is shown with ball-and-stick modeling. Structural Zinc is colored sky blue with coordinating amino acid side chains shown with ball-and-stick modeling. (8)

Although this model seems simple, biochemical data indicates this N-terminal rearrangement may not account for the entire activation mechanism. NS4A mutants have been reported that still bind to NS3 but do not activate (116). These residues do not seem to interact directly with the NS3 N-terminal strand. Due to the extended contact area between NS4A and NS3, many investigators had deemed this interaction non-drugable (31).

³ Reprinted with permission Wiley-Blackwell Publishing Ltd. This figure was modified by Bartenschlager *et al.* (8) from Kim *et al.* (67). Reprinted from Cell, 87, Kim *et al.*, Crystal structure of the hepatitis C virus NS3 protease domain complexed with a synthetic NS4A cofactor peptide, 343-55, Copyright (1996), with permission from Elsevier.

NS3-4A immune evasion

NS3-4A has been implicated in immune evasion due to proteolysis of two host proteins involved in innate immune signaling: toll/IL-1 receptor-domain-containing adaptor inducing IFN-beta (TRIF) and mitochondrial antiviral signaling protein (MAVS; also known as CARDIF, IPS-1, and VISA) (21, 77, 78). TRIF and MAVS are downstream signaling molecules of the dsRNA-sensors toll-like receptor-3 (TLR-3) and retinoic acid inducible gene I (RIG-I), respectively. The TLR-3 pathway recognizes dsRNA found extracellularly and in endosomes, whereas RIG-I is an intracellular dsRNA sensor. Both of these signaling cascades induce an antiviral state in the host cell and neighboring cells, reducing viral replication and spread.

A small fraction of NS3-4A has been reported to be localized to mitochondria (63, 104); MAVS is found in the outer mitochondrial membrane and this colocalization may allow for cleavage of MAVS by NS3-4A (54, 84). However for TRIF, a cytoplasmic protein that is recruited to endosomal membranes, the model for cleavage is less clear. Recently, NS3 alone has been found to associate with TANK-binding kinase-1 (TBK-1) (105), the direct binding partner of TRIF during dsRNA signaling. It may be this interaction that allows for TRIF cleavage. Interestingly, this could be the selective pressure that keeps NS3 a soluble and active protease in equilibrium with the NS3-4A heterocomplex.

NS3-4A protease inhibitors

Protease inhibitors are some of the most successful anti-viral therapeutics. In the case of the human immunodeficiency virus (HIV) protease, research has led to the development of many protease inhibitors currently on the market. Elucidating the substrate specificity and the catalytic mechanism led to several peptidomimetic transition state analogues (75), and determination of the crystal structure allowed more drugs to be designed that disrupted protease structure (5).

NS3 is a prime target because it is essential for viral replication and helps evade the immune response by cleavage of TRIF and MAVS. NS3 protease activity is required to release the NS4A, NS4B, NS5A, and NS5B proteins from the polyprotein. Thus, inhibiting NS3 would prevent the maturation of the non-structural proteins necessary for viral replication and inhibit progression of infection. Also, inhibition of NS3 would allow for dsRNA-induced signaling cascades and boost the immune response against HCV. In 2000, it was shown that chimpanzees

infected with a HCV strain mutated to encode a catalytically-inactive NS3 did not develop an infection (69). Unfortunately, NS3-4A has proved to be a very difficult target.

Before inhibitors can be screened, a functional assay must be developed (Illustration 1.4). Protease assays that have been used *in vitro* primarily rely upon recombinant, purified NS3 cleavage of a peptide substrate and detection of that cleavage by high performance liquid chromatography (HPLC), fluorescence or enzymatic means. These systems almost exclusively use NS3^{pro} and pep4A. The limitation to this type of *in vitro* screening is that it is assumed that NS3^{pro}/pep4A has the same structure and properties of NS3-4A at the ER membrane and in complex with other NS proteins.

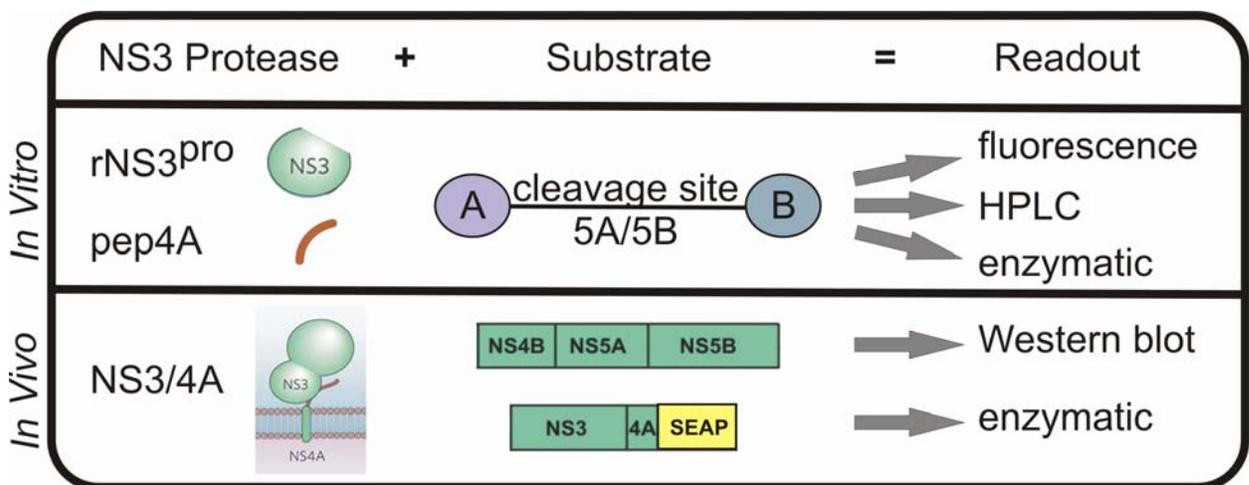


Illustration 1.4 Commonly used assays for detection of NS3-4A protease activity

In vitro assays rely mainly upon the use of recombinant NS3 protease domain (rNS3^{pro}). Activity is detected by addition of a peptide substrate usually corresponding to the NS5A-NS5B junction. The readout can be fluorometric or enzymatic, or the fragments can be detected by high performance liquid chromatography (HPLC). The most reported cell-based assays express NS3-4A in conjunction with, or fused to, a substrate. The NS3-4A activity is measured by Western blot detection of cleavage products or enzymatically (e.g. secreted alkaline phosphatase (SEAP)).

Common *in vivo* protease assays are designed such that NS3-4A is expressed as part of or at the same time as, a polyprotein precursor substrate. Cleavage is then detected by radiolabeled immunoprecipitation or Western blotting of cleavage products. This type of assay is low throughput and not quantitative. In one noteworthy assay, secreted alkaline phosphatase (SEAP) is fused to the end of NS4A, such that the activity of NS3-4A will release SEAP into the cytoplasm (23). SEAP is then trafficked out of the cell into the media and can be measured as a reflection of NS3-4A activity.

Thus far a few protease inhibitors have been identified by biopharmaceutical companies that show promise for treatment of patients (Figure 1.8), and many more are just beginning clinical trials. Of the inhibitors furthest along in the drug development process, BILN 2061, VX-950 and SCH503034 are active site inhibitors and ACH-806 is a NS4A antagonist.

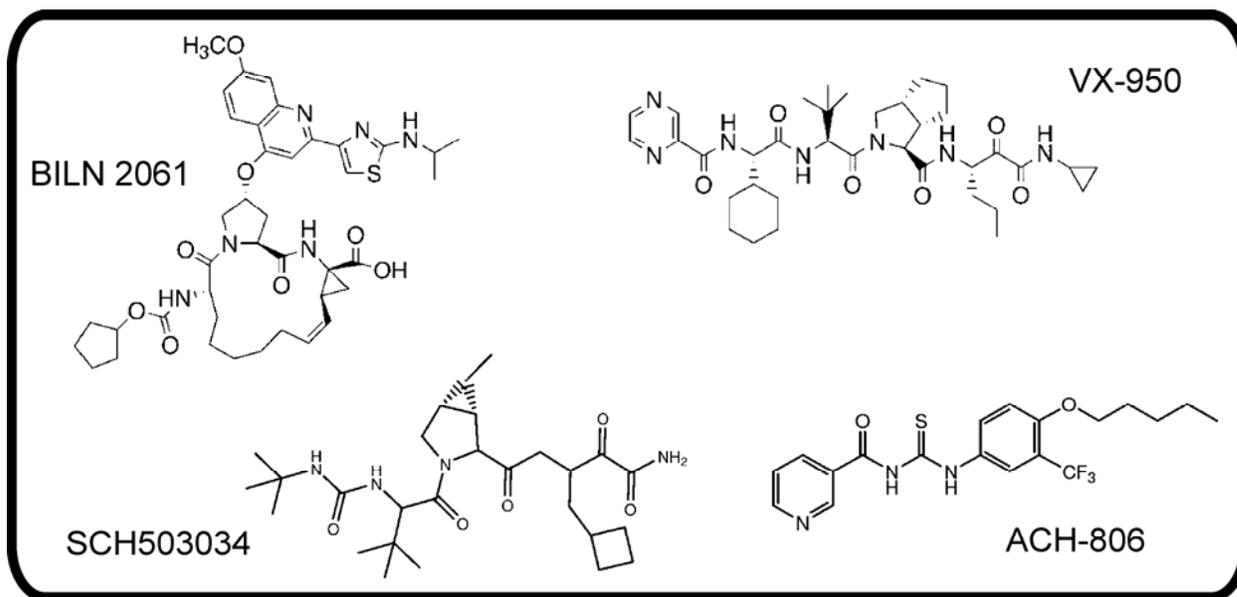


Figure 1.8 Select NS3-4A inhibitors

BILN 2061 Boehringer-Ingelheim developed a reversible macrocyclic inhibitor from structure-based studies on hexapeptide product inhibitors (73, 87, 126). This compound was the first specific HCV NS3 inhibitor reported and advanced to phase I exploratory clinical trials. The clinical trials were stopped 2 days into the study due to data from concurrent animal studies showing cardiac toxicity of BILN 2061 after four months at high doses (53). Nevertheless, the 48 hour clinical trials were very important, as they showed an immediate response in viral load reduction (2-3 log infectious units/mL) and acted as a proof of concept for development of NS3 protease inhibitors (53, 73).

VX-950 Vertex Pharmaceuticals have developed an α-ketoamide inhibitor called telaprevir (VX-950) (79). This reversible inhibitor is based on the covalent serine trap mechanism. Vertex has just completed successful phase 1b trials where patients with genotype 1 HCV were treated for 2 weeks with VX-950 or combination VX-950 and interferon-α (44). The

best results were in combination-treated patients, as interferon- α seemed to control breakout of resistant strains (66).

SCH503034 Schering-Plough has developed a reversible tripeptide-based inhibitor, SCH503034 (128). This compound was optimized through crystallographic studies, fitting P1 and P2 elements into S1 and S2 subsites (94, 110). SCH503034 has successfully completed phase-Ib clinical trials in genotype 1 HCV conventional therapy non-responders (113).

ACH-806 Achillion Pharmaceuticals have discovered an acylthiourea small molecule that is a NS4A-antagonist; it binds to NS4A and disrupts proper replicase complex formation (57). Compensating resistance mutations in NS3 appear in the region known to bind NS4A (139), and this compound acts synergistically with other active site NS3 inhibitors (137). It has successfully completed phase 1b clinical trials (109).

Resistance mutations often confer resistance to drugs with the same target therefore alternative target sites should be investigated. Four point mutations have been identified in replicon systems that confer resistance to BILN 2061: R155Q, A156T, and D168A/V (26, 91). A156T also confers resistance to VX-950 and SCH503034 (140). Shockingly, Cubero *et al.* (28) report a high prevalence (~1%) of the A156T mutation in the HCV quasispecies of a treatment-naive patient. Although this mutation decreases viral fitness by reducing NS3 proteolysis efficiency, second site mutations have been found to compensate (140). The possibility that all active-site NS3 inhibitors can be inactivated by one point mutation in NS3 is an ominous finding for HCV treatment. This underlines the ability of HCV to quickly develop resistance and highlights the need for development of inhibitors to multiple target sites including NS3 allosteric sites.

Aims

The aims of this thesis work were to investigate the interaction between HCV NS3 and NS4A in order to better understand the mechanism of activation and the role of this protease in the virus life cycle. Further, it was hoped to identify binding and activation hot spots in order to target these areas with small molecule drugs that would disrupt or inactivate the NS3-4A complex.

Hypothesis

It was hypothesized that there would be a handful of NS4A residues that dominate the interaction between NS3 and NS4A, and that binding and activation could be separate events mediated by different residues.

Thesis overview

This thesis details the development of a cell-based assay for detection of NS3-4A protease activity (Chapter 2). This assay was utilized to compare the *in vivo* cleavage efficiency of NS3-4A to its natural cleavage site junctions (Chapter 3) and also to determine NS4A residues important for NS3 activation (Chapter 4). In addition, a fluorescence-resonance energy transfer (FRET) assay was developed to measure NS3 and NS4A complex association, and this assay was used to determine NS4A residues important for NS3 binding (Chapter 4).

References

1. **Agnello, V., G. Abel, M. Elfahal, G. B. Knight, and Q. X. Zhang.** 1999. Hepatitis C virus and other flaviviridae viruses enter cells via low density lipoprotein receptor. *Proc. Natl. Acad. Sci. U. S. A.* **96**:12766-12771.
2. **Alter, M. J.** 2007. Epidemiology of hepatitis C virus infection. *World J. Gastroenterol.* **13**:2436-2441.
3. **Aoubala, M., J. Holt, R. A. Clegg, D. J. Rowlands, and M. Harris.** 2001. The inhibition of cAMP-dependent protein kinase by full-length hepatitis C virus NS3/4A complex is due to ATP hydrolysis. *J. Gen. Virol.* **82**:1637-1646.
4. **Appel, N., M. Zayas, S. Miller, J. Krijnse-Locker, T. Schaller, P. Friebe, S. Kallis, U. Engel, and R. Bartenschlager.** 2008. Essential role of domain III of nonstructural protein 5A for hepatitis C virus infectious particle assembly. *PLoS Pathog.* **4**:e1000035.
5. **Bannwarth, L. and M. Reboud-Ravaux.** 2007. An alternative strategy for inhibiting multidrug-resistant mutants of the dimeric HIV-1 protease by targeting the subunit interface. *Biochem. Soc. Trans.* **35**:551-554.
6. **Barbato, G., D. O. Cicero, M. C. Nardi, C. Steinkuhler, R. Cortese, R. De Francesco, and R. Bazzo.** 1999. The solution structure of the N-terminal proteinase domain of the hepatitis C virus (HCV) NS3 protein provides new insights into its activation and catalytic mechanism. *J. Mol. Biol.* **289**:371-384.
7. **Baril, M. and L. Brakier-Gingras.** 2005. Translation of the F protein of hepatitis C virus is initiated at a non-AUG codon in a +1 reading frame relative to the polyprotein. *Nucleic Acids Res.* **33**:1474-1486.
8. **Bartenschlager, R.** 1999. The NS3/4A proteinase of the hepatitis C virus: unravelling structure and function of an unusual enzyme and a prime target for antiviral therapy. *J. Viral Hepat.* **6**:165-181.

9. **Bartenschlager, R., L. Ahlborn-Laake, J. Mous, and H. Jacobsen.** 1994. Kinetic and structural analyses of hepatitis C virus polyprotein processing. *J. Virol.* **68**:5045-5055.
10. **Bartosch, B., A. Vitelli, C. Granier, C. Goujon, J. Dubuisson, S. Pascale, E. Scarselli, R. Cortese, A. Nicosia, and F. L. Cosset.** 2003. Cell entry of hepatitis C virus requires a set of co-receptors that include the CD81 tetraspanin and the SR-B1 scavenger receptor. *J. Biol. Chem.* **278**:41624-41630.
11. **Behrens, S. E., L. Tomei, and R. De Francesco.** 1996. Identification and properties of the RNA-dependent RNA polymerase of hepatitis C virus. *EMBO J.* **15**:12-22.
12. **Benson, D. A., I. Karsch-Mizrachi, D. J. Lipman, J. Ostell, B. A. Rapp, and D. L. Wheeler.** 2000. GenBank. *Nucleic Acids Res.* **28**:15-18.
13. **Bianchi, E. and A. Pessi.** 2002. Inhibiting viral proteases: challenges and opportunities. *Biopolymers* **66**:101-114.
14. **Borowski, P., M. Heiland, H. Feucht, and R. Laufs.** 1999. Characterisation of non-structural protein 3 of hepatitis C virus as modulator of protein phosphorylation mediated by PKA and PKC: evidences for action on the level of substrate and enzyme. *Arch. Virol.* **144**:687-701.
15. **Borowski, P., M. Heiland, K. Oehlmann, B. Becker, L. Kornetzky, H. Feucht, and R. Laufs.** 1996. Non-structural protein 3 of hepatitis C virus inhibits phosphorylation mediated by cAMP-dependent protein kinase. *Eur. J. Biochem.* **237**:611-618.
16. **Borowski, P., R. Kuhl, R. Laufs, J. Schulze zur Wiesch, and M. Heiland.** 1999. Identification and characterization of a histone binding site of the non-structural protein 3 of hepatitis C virus. *J. Clin. Virol.* **13**:61-69.
17. **Bosch, M. E., A. J. Sanchez, F. S. Rojas, and C. B. Ojeda.** 2007. Ribavirin: analytical determinations since the origin until today. *J. Pharm. Biomed. Anal.* **45**:185-193.
18. **Brass, V., E. Bieck, R. Montserret, B. Wolk, J. A. Hellings, H. E. Blum, F. Penin, and D. Moradpour.** 2002. An amino-terminal amphipathic alpha-helix mediates membrane association of the hepatitis C virus nonstructural protein 5A. *J. Biol. Chem.* **277**:8130-8139.

19. **Brazzoli, M., A. Bianchi, S. Filippini, A. Weiner, Q. Zhu, M. Pizza, and S. Crotta.** 2008. CD81 is a central regulator of cellular events required for hepatitis C virus infection of human hepatocytes. *J. Virol.* **82**:8316-8329.
20. **Carrere-Kremer, S., C. Montpellier, L. Lorenzo, B. Brulin, L. Cocquerel, S. Belouzard, F. Penin, and J. Dubuisson.** 2004. Regulation of hepatitis C virus polyprotein processing by signal peptidase involves structural determinants at the p7 sequence junctions. *J. Biol. Chem.* **279**:41384-41392.
21. **Cheng, G., J. Zhong, and F. V. Chisari.** 2006. Inhibition of dsRNA-induced signaling in hepatitis C virus-infected cells by NS3 protease-dependent and -independent mechanisms. *Proc. Natl. Acad. Sci. U. S. A.* **103**:8499-8504.
22. **Cho, H. S., N. C. Ha, L. W. Kang, K. M. Chung, S. H. Back, S. K. Jang, and B. H. Oh.** 1998. Crystal structure of RNA helicase from genotype 1b hepatitis C virus. A feasible mechanism of unwinding duplex RNA. *J. Biol. Chem.* **273**:15045-15052.
23. **Cho, Y. G., S. H. Yang, and Y. C. Sung.** 1998. *In vivo* assay for hepatitis C viral serine protease activity using a secreted protein. *J. Virol. Methods.* **72**:109-115.
24. **Choo, Q. L., G. Kuo, A. J. Weiner, L. R. Overby, D. W. Bradley, and M. Houghton.** 1989. Isolation of a cDNA clone derived from a blood-borne non-A, non-B viral hepatitis genome. *Science* **244**:359-362.
25. **Cohen, M., L. Bachmatov, Z. Ben-Ari, Y. Rotman, R. Tur-Kaspa, and R. Zemel.** 2007. Development of specific antibodies to an ARF protein in treated patients with chronic HCV infection. *Dig. Dis. Sci.* **52**:2427-2432.
26. **Courcambeck, J., M. Bouzidi, R. Perbost, B. Jouirou, N. Amrani, P. Cacoub, G. Pepe, J. M. Sabatier, and P. Halfon.** 2006. Resistance of hepatitis C virus to NS3-4A protease inhibitors: mechanisms of drug resistance induced by R155Q, A156T, D168A and D168V mutations. *Antivir Ther.* **11**:847-855.
27. **Coursaget, P., P. Maupas, P. Levin, and F. Barin.** 1979. Virus-like particles associated with non-A, non-B hepatitis. *Lancet* **2**:92-93.

28. **Cubero, M., J. I. Esteban, T. Otero, S. Sauleda, M. Bes, R. Esteban, J. Guardia, and J. Quer.** 2008. Naturally occurring NS3-protease-inhibitor resistant mutant A156T in the liver of an untreated chronic hepatitis C patient. *Virology* **370**:237-245.
29. **Dahl, G., A. Sandstrom, E. Akerblom, and U. H. Danielson.** 2007. Effects on protease inhibition by modifying of helicase residues in hepatitis C virus nonstructural protein 3. *FEBS J.* **274**:5979-5986.
30. **Daly, P.** 2000. Hepatitis C and the British Columbia experience with hepatitis A vaccination. *J. Viral Hepat.* **7 Suppl 1**:23-25.
31. **De Francesco, R., A. Pessi, and C. Steinkuhler.** 1999. Mechanisms of hepatitis C virus NS3 proteinase inhibitors. *J. Viral Hepat.* **6 Suppl 1**:23-30.
32. **Deng, L., M. Nagano-Fujii, M. Tanaka, Y. Nomura-Takigawa, M. Ikeda, N. Kato, K. Sada, and H. Hotta.** 2006. NS3 protein of Hepatitis C virus associates with the tumour suppressor p53 and inhibits its function in an NS3 sequence-dependent manner. *J. Gen. Virol.* **87**:1703-1713.
33. **Dixit, N. M. and A. S. Perelson.** 2006. The metabolism, pharmacokinetics and mechanisms of antiviral activity of ribavirin against hepatitis C virus. *Cell Mol. Life Sci.* **63**:832-842.
34. **Dubuisson, J.** 2007. Hepatitis C virus proteins. *World J. Gastroenterol.* **13**:2406-2415.
35. **Dumont, S., W. Cheng, V. Serebrov, R. K. Beran, I. Tinoco Jr, A. M. Pyle, and C. Bustamante.** 2006. RNA translocation and unwinding mechanism of HCV NS3 helicase and its coordination by ATP. *Nature* **439**:105-108.
36. **Egger, D., B. Wolk, R. Gosert, L. Bianchi, H. E. Blum, D. Moradpour, and K. Bienz.** 2002. Expression of hepatitis C virus proteins induces distinct membrane alterations including a candidate viral replication complex. *J. Virol.* **76**:5974-5984.
37. **Elazar, M., K. H. Cheong, P. Liu, H. B. Greenberg, C. M. Rice, and J. S. Glenn.** 2003. Amphipathic helix-dependent localization of NS5A mediates hepatitis C virus RNA replication. *J. Virol.* **77**:6055-6061.

38. **Elazar, M., P. Liu, C. M. Rice, and J. S. Glenn.** 2004. An N-terminal amphipathic helix in hepatitis C virus (HCV) NS4B mediates membrane association, correct localization of replication complex proteins, and HCV RNA replication. *J. Virol.* **78**:11393-11400.
39. **Evans, M. J., T. von Hahn, D. M. Tscherne, A. J. Syder, M. Panis, B. Wolk, T. Hatziioannou, J. A. McKeating, P. D. Bieniasz, and C. M. Rice.** 2007. Claudin-1 is a hepatitis C virus co-receptor required for a late step in entry. *Nature* **446**:801-805.
40. **Failla, C., L. Tomei, and R. De Francesco.** 1994. Both NS3 and NS4A are required for proteolytic processing of hepatitis C virus nonstructural proteins. *J. Virol.* **68**:3753-3760.
41. **Farci, P., L. Chessa, C. Balestrieri, G. Serra, and M. E. Lai.** 2007. Treatment of chronic hepatitis D. *J. Viral Hepat.* **14 Suppl 1**:58-63.
42. **Fischer, B., K. Kalousek, J. Rehm, J. Powis, M. Krajden, and J. Reimer.** 2006. Hepatitis C, illicit drug use and public health: does Canada really have a viable plan? *Can. J. Public Health* **97**:485-488.
43. **Florese, R. H., M. Nagano-Fujii, Y. Iwanaga, R. Hidajat, and H. Hotta.** 2002. Inhibition of protein synthesis by the nonstructural proteins NS4A and NS4B of hepatitis C virus. *Virus Res.* **90**:119-131.
44. **Forestier, N., H. W. Reesink, C. J. Weegink, L. McNair, T. L. Kieffer, H. M. Chu, S. Purdy, P. L. Jansen, and S. Zeuzem.** 2007. Antiviral activity of telaprevir (VX-950) and peginterferon alfa-2a in patients with hepatitis C. *Hepatology* **46**:640-648.
45. **Frick, D. N., R. S. Rypma, A. M. Lam, and B. Gu.** 2004. The nonstructural protein 3 protease/helicase requires an intact protease domain to unwind duplex RNA efficiently. *J. Biol. Chem.* **279**:1269-1280.
46. **Goh, P. Y., Y. J. Tan, S. P. Lim, Y. H. Tan, S. G. Lim, F. Fuller-Pace, and W. Hong.** 2004. Cellular RNA helicase p68 relocalization and interaction with the hepatitis C virus (HCV) NS5B protein and the potential role of p68 in HCV RNA replication. *J. Virol.* **78**:5288-5298.

47. **Gosert, R., W. Jendrszczok, J. M. Berke, V. Brass, H. E. Blum, and D. Moradpour.** 2005. Characterization of nonstructural protein membrane anchor deletion mutants expressed in the context of the hepatitis C virus polyprotein. *J. Virol.* **79**:7911-7917.
48. **Griffin, S. D., L. P. Beales, D. S. Clarke, O. Worsfold, S. D. Evans, J. Jaeger, M. P. Harris, and D. J. Rowlands.** 2003. The p7 protein of hepatitis C virus forms an ion channel that is blocked by the antiviral drug, Amantadine. *FEBS Lett.* **535**:34-38.
49. **Gu, B., C. M. Pruss, A. T. Gates, and S. S. Khandekar.** 2005. The RNA-unwinding activity of hepatitis C virus non-structural protein 3 (NS3) is positively modulated by its protease domain. *Protein Pept. Lett.* **12**:315-321.
50. **Hahm, B., D. S. Han, S. H. Back, O. K. Song, M. J. Cho, C. J. Kim, K. Shimotohno, and S. K. Jang.** 1995. NS3-4A of hepatitis C virus is a chymotrypsin-like protease. *J. Virol.* **69**:2534-2539.
51. **Health Canada.** Web page <http://www.hc-sc.gc.ca/dc-ma/hep/index-eng.php>. Last updated Apr 25, 2007. Accessed Jun 10, 2008.
52. **Hidajat, R., M. Nagano-Fujii, L. Deng, M. Tanaka, Y. Takigawa, S. Kitazawa, and H. Hotta.** 2005. Hepatitis C virus NS3 protein interacts with ELKS- $\{\delta\}$ and ELKS- $\{\alpha\}$, members of a novel protein family involved in intracellular transport and secretory pathways. *J. Gen. Virol.* **86**:2197-2208.
53. **Hinrichsen, H., Y. Benhamou, H. Wedemeyer, M. Reiser, R. E. Sentjens, J. L. Calleja, X. Forns, A. Erhardt, J. Cronlein, R. L. Chaves, C. L. Yong, G. Nehmiz, and G. G. Steinmann.** 2004. Short-term antiviral efficacy of BILN 2061, a hepatitis C virus serine protease inhibitor, in hepatitis C genotype 1 patients. *Gastroenterology* **127**:1347-1355.
54. **Hiscott, J., J. Lacoste, and R. Lin.** 2006. Recruitment of an interferon molecular signaling complex to the mitochondrial membrane: disruption by hepatitis C virus NS3-4A protease. *Biochem. Pharmacol.* **72**:1477-1484.

55. **Hsu, M., J. Zhang, M. Flint, C. Logvinoff, C. Cheng-Mayer, C. M. Rice, and J. A. McKeating.** 2003. Hepatitis C virus glycoproteins mediate pH-dependent cell entry of pseudotyped retroviral particles. *Proc. Natl. Acad. Sci. U. S. A.* **100**:7271-7276.
56. **Huang, L., J. Hwang, S. D. Sharma, M. R. Hargittai, Y. Chen, J. J. Arnold, K. D. Raney, and C. E. Cameron.** 2005. Hepatitis C virus nonstructural protein 5A (NS5A) is an RNA-binding protein. *J. Biol. Chem.* **280**:36417-36428.
57. **Huang, M., Y. Sun, W. Yang, X. Hou, J. Fabrycki, X. Nie, A. Sanchez, Y. Zhao, A. Phadke, and M. Deshpande.** 2007. A Potent Inhibitor of HCV Replication with a Novel Mechanism of Action. *J. Hepatol.* **46**:S221-S221.
58. **Ishido, S. and H. Hotta.** 1998. Complex formation of the nonstructural protein 3 of hepatitis C virus with the p53 tumor suppressor. *FEBS Lett.* **438**:258-262.
59. **Isken, O., M. Baroth, C. W. Grassmann, S. Weinlich, D. H. Ostareck, A. Ostareck-Lederer, and S. E. Behrens.** 2007. Nuclear factors are involved in hepatitis C virus RNA replication. *RNA* **13**:1675-1692.
60. **Jennings, T. A., Y. Chen, D. Sikora, M. K. Harrison, B. Sikora, L. Huang, E. Jankowsky, M. E. Fairman, C. E. Cameron, and K. D. Raney.** 2008. RNA unwinding activity of the hepatitis C virus NS3 helicase is modulated by the NS5B polymerase. *Biochemistry* **47**:1126-1135.
61. **Jones, C. T., C. L. Murray, D. K. Eastman, J. Tassello, and C. M. Rice.** 2007. Hepatitis C virus p7 and NS2 proteins are essential for production of infectious virus. *J. Virol.* **81**:8374-8383.
62. **Kamal, S. M.** 2007. Improving outcome in patients with hepatitis C virus genotype 4. *Am. J. Gastroenterol.* **102**:2582-2588.
63. **Kasprzak, A., J. Seidel, W. Biczysko, J. Wysocki, R. Spachacz, and M. Zabel.** 2005. Intracellular localization of NS3 and C proteins in chronic hepatitis C. *Liver Int.* **25**:896-903.
64. **Keeffe, E. B. and P. Marcellin.** 2007. New and emerging treatment of chronic hepatitis B. *Clin. Gastroenterol. Hepatol.* **5**:285-294.

65. **Khu, Y. L., E. Koh, S. P. Lim, Y. H. Tan, S. Brenner, S. G. Lim, W. J. Hong, and P. Y. Goh.** 2001. Mutations that affect dimer formation and helicase activity of the hepatitis C virus helicase. *J. Virol.* **75**:205-214.
66. **Kieffer, T. L., C. Sarrazin, J. S. Miller, M. W. Welker, N. Forestier, H. W. Reesink, A. D. Kwong, and S. Zeuzem.** 2007. Telaprevir and pegylated interferon-alpha-2a inhibit wild-type and resistant genotype 1 hepatitis C virus replication in patients. *Hepatology* **46**:631-639.
67. **Kim, J. L., K. A. Morgenstern, C. Lin, T. Fox, M. D. Dwyer, J. A. Landro, S. P. Chambers, W. Markland, C. A. Lepre, E. T. O'Malley, S. L. Harbeson, C. M. Rice, M. A. Murcko, P. R. Caron, and J. A. Thomson.** 1996. Crystal structure of the hepatitis C virus NS3 protease domain complexed with a synthetic NS4A cofactor peptide. *Cell* **87**:343-355.
68. **Koch, J. O. and R. Bartenschlager.** 1999. Modulation of hepatitis C virus NS5A hyperphosphorylation by nonstructural proteins NS3, NS4A, and NS4B. *J. Virol.* **73**:7138-7146.
69. **Kolykhalov, A. A., K. Mihalik, S. M. Feinstone, and C. M. Rice.** 2000. Hepatitis C virus-encoded enzymatic activities and conserved RNA elements in the 3' nontranslated region are essential for virus replication *in vivo*. *J. Virol.* **74**:2046-2051.
70. **Kou, Y. H., S. M. Chou, Y. M. Wang, Y. T. Chang, S. Y. Huang, M. Y. Jung, Y. H. Huang, M. R. Chen, M. F. Chang, and S. C. Chang.** 2006. Hepatitis C virus NS4A inhibits cap-dependent and the viral IRES-mediated translation through interacting with eukaryotic elongation factor 1A. *J. Biomed. Sci.* **13**:861-874.
71. **Kuo, G., Q. L. Choo, H. J. Alter, G. L. Gitnick, A. G. Redeker, R. H. Purcell, T. Miyamura, J. L. Dienstag, M. J. Alter, and C. E. Stevens.** 1989. An assay for circulating antibodies to a major etiologic virus of human non-A, non-B hepatitis. *Science* **244**:362-364.
72. **Kyono, K., M. Miyashiro, and I. Taguchi.** 2002. Human eukaryotic initiation factor 4AII associates with hepatitis C virus NS5B protein *in vitro*. *Biochem. Biophys. Res. Commun.* **292**:659-666.
73. **Lamarre, D., P. C. Anderson, M. Bailey, P. Beaulieu, G. Bolger, P. Bonneau, M. Bos, D. R. Cameron, M. Cartier, M. G. Cordingley, A. M. Faucher, N. Goudreau, S. H. Kawai, G.**

Kukolj, L. Lagace, S. R. LaPlante, H. Narjes, M. A. Poupart, J. Rancourt, R. E. Sentjens, R. St George, B. Simoneau, G. Steinmann, D. Thibeault, Y. S. Tsantrizos, S. M. Weldon, C. L. Yong, and M. Llinas-Brunet. 2003. An NS3 protease inhibitor with antiviral effects in humans infected with hepatitis C virus. *Nature* **426**:186-189.

74. **Larkin, M. A., G. Blackshields, N. P. Brown, R. Chenna, P. A. McGettigan, H. McWilliam, F. Valentin, I. M. Wallace, A. Wilm, R. Lopez, J. D. Thompson, T. J. Gibson, and D. G. Higgins.** 2007. Clustal W and Clustal X version 2.0. *Bioinformatics* **23**:2947-2948.

75. **Lebon, F. and M. Ledecq.** 2000. Approaches to the design of effective HIV-1 protease inhibitors. *Curr. Med. Chem.* **7**:455-477.

76. **Levin, M. K. and S. S. Patel.** 1999. The helicase from hepatitis C virus is active as an oligomer. *J. Biol. Chem.* **274**:31839-31846.

77. **Li, K., E. Foy, J. C. Ferreon, M. Nakamura, A. C. Ferreon, M. Ikeda, S. C. Ray, M. Gale Jr, and S. M. Lemon.** 2005. Immune evasion by hepatitis C virus NS3/4A protease-mediated cleavage of the Toll-like receptor 3 adaptor protein TRIF. *Proc. Natl. Acad. Sci. U. S. A.* **102**:2992-2997.

78. **Li, X. D., L. Sun, R. B. Seth, G. Pineda, and Z. J. Chen.** 2005. Hepatitis C virus protease NS3/4A cleaves mitochondrial antiviral signaling protein off the mitochondria to evade innate immunity. *Proc. Natl. Acad. Sci. U. S. A.* **102**:17717-17722.

79. **Lin, C., A. D. Kwong, and R. B. Perni.** 2006. Discovery and development of VX-950, a novel, covalent, and reversible inhibitor of hepatitis C virus NS3.4A serine protease. *Infect. Disord. Drug Targets* **6**:3-16.

80. **Lin, C., B. D. Lindenbach, B. M. Pragai, D. W. McCourt, and C. M. Rice.** 1994. Processing in the hepatitis C virus E2-NS2 region: identification of p7 and two distinct E2-specific products with different C termini. *J. Virol.* **68**:5063-5073.

81. **Lin, C., B. M. Pragai, A. Grakoui, J. Xu, and C. M. Rice.** 1994. Hepatitis C virus NS3 serine proteinase: trans-cleavage requirements and processing kinetics. *J. Virol.* **68**:8147-8157.

82. **Lin, C. and C. M. Rice.** 1995. The hepatitis C virus NS3 serine proteinase and NS4A cofactor: establishment of a cell-free trans-processing assay. *Proc. Natl. Acad. Sci. U. S. A.* **92**:7622-7626.
83. **Lin, C., J. A. Thomson, and C. M. Rice.** 1995. A central region in the hepatitis C virus NS4A protein allows formation of an active NS3-NS4A serine proteinase complex *in vivo* and *in vitro*. *J. Virol.* **69**:4373-4380.
84. **Lin, R., J. Lacoste, P. Nakhaei, Q. Sun, L. Yang, S. Paz, P. Wilkinson, I. Julkunen, D. Vitour, E. Meurs, and J. Hiscott.** 2006. Dissociation of a MAVS/IPS-1/VISA/Cardif-IKKepsilon molecular complex from the mitochondrial outer membrane by hepatitis C virus NS3-4A proteolytic cleavage. *J. Virol.* **80**:6072-6083.
85. **Lindenbach, B. D., B. M. Pragai, R. Montserret, R. K. Beran, A. M. Pyle, F. Penin, and C. M. Rice.** 2007. The C terminus of hepatitis C virus NS4A encodes an electrostatic switch that regulates NS5A hyperphosphorylation and viral replication. *J. Virol.* **81**:8905-8918.
86. **Lindenbach, B. D. and C. M. Rice.** 2005. Unravelling hepatitis C virus replication from genome to function. *Nature* **436**:933-938.
87. **Llinas-Brunet, M., M. D. Bailey, G. Bolger, C. Brochu, A. M. Faucher, J. M. Ferland, M. Garneau, E. Ghio, V. Gorys, C. Grand-Maitre, T. Halmos, N. Lapeyre-Paquette, F. Liard, M. Poirier, M. Rheume, Y. S. Tsantrizos, and D. Lamarre.** 2004. Structure-activity study on a novel series of macrocyclic inhibitors of the hepatitis C virus NS3 protease leading to the discovery of BILN 2061. *J. Med. Chem.* **47**:1605-1608.
88. **Lohmann, V., F. Korner, J. Koch, U. Herian, L. Theilmann, and R. Bartenschlager.** 1999. Replication of subgenomic hepatitis C virus RNAs in a hepatoma cell line. *Science* **285**:110-113.
89. **Lorenz, I. C., J. Marcotrigiano, T. G. Dentzer, and C. M. Rice.** 2006. Structure of the catalytic domain of the hepatitis C virus NS2-3 protease. *Nature* **442**:831-835.

90. **Love, R. A., H. E. Parge, J. A. Wickersham, Z. Hostomsky, N. Habuka, E. W. Moomaw, T. Adachi, and Z. Hostomska.** 1996. The crystal structure of hepatitis C virus NS3 proteinase reveals a trypsin-like fold and a structural zinc binding site. *Cell* **87**:331-342.
91. **Lu, L., T. J. Pilot-Matias, K. D. Stewart, J. T. Randolph, R. Pithawalla, W. He, P. P. Huang, L. L. Klein, H. Mo, and A. Molla.** 2004. Mutations conferring resistance to a potent hepatitis C virus serine protease inhibitor *in vitro*. *Antimicrob. Agents Chemother.* **48**:2260-2266.
92. **Lundin, M., H. Lindstrom, C. Gronwall, and M. A. Persson.** 2006. Dual topology of the processed hepatitis C virus protein NS4B is influenced by the NS5A protein. *J. Gen. Virol.* **87**:3263-3272.
93. **Ma, H. C., Y. Y. Ku, Y. C. Hsieh, and S. Y. Lo.** 2007. Characterization of the cleavage of signal peptide at the C-terminus of hepatitis C virus core protein by signal peptide peptidase. *J. Biomed. Sci.* **14**:31-41.
94. **Madison, V., A. J. Prongay, Z. Guo, N. Yao, J. Pichardo, T. Fischmann, C. Strickland, J. Myers Jr, P. C. Weber, B. M. Beyer, R. Ingram, Z. Hong, W. W. Prorise, L. Ramanathan, S. S. Taremi, T. Yarosh-Tomaine, R. Zhang, M. Senior, R. S. Yang, B. Malcolm, A. Arasappan, F. Bennett, S. L. Bogen, K. Chen, E. Jao, Y. T. Liu, R. G. Lovey, A. K. Saksena, S. Venkatraman, V. Girijavallabhan, and F. G. Njoroge.** 2008. Key steps in the structure-based optimization of the hepatitis C virus NS3/4A protease inhibitor SCH503034. *J. Synchrotron Radiat.* **15**:204-207.
95. **Martin, F., N. Dimasi, C. Volpari, C. Perrera, S. Di Marco, M. Brunetti, C. Steinkuhler, R. De Francesco, and M. Sollazzo.** 1998. Design of selective eglin inhibitors of HCV NS3 proteinase. *Biochemistry* **37**:11459-11468.
96. **McMullan, L. K., A. Grakoui, M. J. Evans, K. Mihalik, M. Puig, A. D. Branch, S. M. Feinstone, and C. M. Rice.** 2007. Evidence for a functional RNA element in the hepatitis C virus core gene. *Proc. Natl. Acad. Sci. U. S. A.* **104**:2879-2884.

97. **Mercer, D. F., D. E. Schiller, J. F. Elliott, D. N. Douglas, C. Hao, A. Rinfret, W. R. Addison, K. P. Fischer, T. A. Churchill, J. R. Lakey, D. L. Tyrrell, and N. M. Kneteman.** 2001. Hepatitis C virus replication in mice with chimeric human livers. *Nat. Med.* **7**:927-933.
98. **Moradpour, D., V. Brass, E. Bieck, P. Friebe, R. Gosert, H. E. Blum, R. Bartenschlager, F. Penin, and V. Lohmann.** 2004. Membrane association of the RNA-dependent RNA polymerase is essential for hepatitis C virus RNA replication. *J. Virol.* **78**:13278-13284.
99. **Moradpour, D., V. Brass, and F. Penin.** 2005. Function follows form: the structure of the N-terminal domain of HCV NS5A. *Hepatology* **42**:732-735.
100. **Mottola, G., G. Cardinali, A. Ceccacci, C. Trozzi, L. Bartholomew, M. R. Torrisi, E. Pedrazzini, S. Bonatti, and G. Migliaccio.** 2002. Hepatitis C virus nonstructural proteins are localized in a modified endoplasmic reticulum of cells expressing viral subgenomic replicons. *Virology* **293**:31-43.
101. **Murray, C. L., C. T. Jones, J. Tassello, and C. M. Rice.** 2007. Alanine scanning of the hepatitis C virus core protein reveals numerous residues essential for production of infectious virus. *J. Virol.* **81**:10220-10231.
102. **Neumann, A. U., N. P. Lam, H. Dahari, D. R. Gretch, T. E. Wiley, T. J. Layden, and A. S. Perelson.** 1998. Hepatitis C viral dynamics *in vivo* and the antiviral efficacy of interferon-alpha therapy. *Science* **282**:103-107.
103. **Nguyen, M. H. and E. B. Keeffe.** 2005. Prevalence and treatment of hepatitis C virus genotypes 4, 5, and 6. *Clin. Gastroenterol. Hepatol.* **3**:S97-S101.
104. **Nomura-Takigawa, Y., M. Nagano-Fujii, L. Deng, S. Kitazawa, S. Ishido, K. Sada, and H. Hotta.** 2006. Non-structural protein 4A of Hepatitis C virus accumulates on mitochondria and renders the cells prone to undergoing mitochondria-mediated apoptosis. *J. Gen. Virol.* **87**:1935-1945.
105. **Otsuka, M., N. Kato, M. Moriyama, H. Taniguchi, Y. Wang, N. Dharel, T. Kawabe, and M. Omata.** 2005. Interaction between the HCV NS3 protein and the host TBK1 protein leads to inhibition of cellular antiviral responses. *Hepatology* **41**:1004-1012.

106. **Piccininni, S., A. Varaklioti, M. Nardelli, B. Dave, K. D. Raney, and J. E. McCarthy.** 2002. Modulation of the hepatitis C virus RNA-dependent RNA polymerase activity by the non-structural (NS) 3 helicase and the NS4B membrane protein. *J. Biol. Chem.* **277**:45670-45679.
107. **Pileri, P., Y. Uematsu, S. Campagnoli, G. Galli, F. Falugi, R. Petracca, A. J. Weiner, M. Houghton, D. Rosa, G. Grandi, and S. Abrignani.** 1998. Binding of hepatitis C virus to CD81. *Science* **282**:938-941.
108. **Poordad, F., K. R. Reddy, and P. Martin.** 2008. Rapid virologic response: a new milestone in the management of chronic hepatitis C. *Clin. Infect. Dis.* **46**:78-84.
109. **Pottage, J., E. Lawitz, D. Mazur, D. L. Wyles, H. Vargas, R. Ghalib, R. Gugliotti, M. Donohue, and H. Robison.** 2007. Short-Term Antiviral Activity and Safety of ACH-806 (GS-9132), an NS4A Antagonist, in HCV Genotype 1 Infected Individuals. *J. Hepatol.* **46**:S294-S295.
110. **Prongay, A. J., Z. Guo, N. Yao, J. Pichardo, T. Fischmann, C. Strickland, J. Myers Jr, P. C. Weber, B. M. Beyer, R. Ingram, Z. Hong, W. W. Prorise, L. Ramanathan, S. S. Taremi, T. Yarosh-Tomaine, R. Zhang, M. Senior, R. S. Yang, B. Malcolm, A. Arasappan, F. Bennett, S. L. Bogen, K. Chen, E. Jao, Y. T. Liu, R. G. Lovey, A. K. Saksena, S. Venkatraman, V. Girijavallabhan, F. G. Njoroge, and V. Madison.** 2007. Discovery of the HCV NS3/4A protease inhibitor (1R,5S)-N-[3-amino-1-(cyclobutylmethyl)-2,3-dioxopropyl]-3-[2(S)-[[[(1,1-dimethylethyl)amino]carbonyl]amino]-3,3-dimethyl-1-oxobutyl]-6,6-dimethyl-3-azabicyclo[3.1.0]hexan-2(S)-carboxamide (Sch 503034) II. Key steps in structure-based optimization. *J. Med. Chem.* **50**:2310-2318.
111. **Rosales-Leon, L., G. Ortega-Lule, and B. Ruiz-Ordaz.** 2007. Analysis of the domain interactions between the protease and helicase of NS3 in dengue and hepatitis C virus. *J. Mol. Graph. Model.* **25**:585-594.
112. **Saitou, N. and M. Nei.** 1987. The neighbor-joining method: a new method for reconstructing phylogenetic trees. *Mol. Biol. Evol.* **4**:406-425.

113. **Sarrazin, C., R. Rouzier, F. Wagner, N. Forestier, D. Larrey, S. K. Gupta, M. Hussain, A. Shah, D. Cutler, J. Zhang, and S. Zeuzem.** 2007. SCH 503034, a novel hepatitis C virus protease inhibitor, plus pegylated interferon alpha-2b for genotype 1 nonresponders. *Gastroenterology* **132**:1270-1278.
114. **Sayle, R. A. and E. J. Milner-White.** 1995. RASMOL: biomolecular graphics for all. *Trends Biochem. Sci.* **20**:374.
115. **Selimovic, D. and M. Hassan.** 2008. Inhibition of hepatitis C virus (HCV) core protein-induced cell growth by non-structural protein 4A (NS4A) is mediated by mitochondrial dysregulation. *Bosn J. Basic Med. Sci.* **8**:4-11.
116. **Shimizu, Y., K. Yamaji, Y. Masuho, T. Yokota, H. Inoue, K. Sudo, S. Satoh, and K. Shimotohno.** 1996. Identification of the sequence on NS4A required for enhanced cleavage of the NS5A/5B site by hepatitis C virus NS3 protease. *J. Virol.* **70**:127-132.
117. **Sikora, B. T., Y. Chen, C. F. Lichti, M. K. Harrison, T. A. Jennings, Y. Tang, A. J. Tackett, J. B. Jordan, J. Sakon, C. E. Cameron, and K. D. Raney.** 2008. Hepatitis C Virus NS3 helicase forms oligomeric structures that exhibit optimal DNA unwinding activity *in vitro*. *J. Biol. Chem.*
118. **Steinkuhler, C., L. Tomei, and R. De Francesco.** 1996. *In vitro* activity of hepatitis C virus protease NS3 purified from recombinant Baculovirus-infected Sf9 cells. *J. Biol. Chem.* **271**:6367-6373.
119. **Steinkuhler, C., A. Urbani, L. Tomei, G. Biasiol, M. Sardana, E. Bianchi, A. Pessi, and R. De Francesco.** 1996. Activity of purified hepatitis C virus protease NS3 on peptide substrates. *J. Virol.* **70**:6694-6700.
120. **Steinmann, E., F. Penin, S. Kallis, A. H. Patel, R. Bartenschlager, and T. Pietschmann.** 2007. Hepatitis C virus p7 protein is crucial for assembly and release of infectious virions. *PLoS Pathog.* **3**:e103.

121. **Tabor, E., J. H. Hoofnagle, L. A. Smallwood, J. A. Drucker, G. C. Pineda-Tamondong, L. Y. Ni, T. J. Greenwalt, L. F. Barker, and R. J. Gerety.** 1979. Studies of donors who transmit posttransfusion hepatitis. *Transfusion* **19**:725-731.
122. **Tanji, Y., M. Hijikata, S. Satoh, T. Kaneko, and K. Shimotohno.** 1995. Hepatitis C virus-encoded nonstructural protein NS4A has versatile functions in viral protein processing. *J. Virol.* **69**:1575-1581.
123. **Tellinghuisen, T. L., K. L. Foss, and J. Treadaway.** 2008. Regulation of hepatitis C virion production via phosphorylation of the NS5A protein. *PLoS Pathog.* **4**:e1000032.
124. **Tellinghuisen, T. L., J. Marcotrigiano, and C. M. Rice.** 2005. Structure of the zinc-binding domain of an essential component of the hepatitis C virus replicase. *Nature* **435**:374-379.
125. **The Global Burden Of Hepatitis C Working Group.** 2004. Global burden of disease (GBD) for hepatitis C. *J. Clin. Pharmacol.* **44**:20-29.
126. **Tsantrizos, Y. S.** 2004. The design of a potent inhibitor of the hepatitis C virus NS3 protease: BILN 2061--from the NMR tube to the clinic. *Biopolymers* **76**:309-323.
127. **Unterholzner, L. and A. G. Bowie.** 2008. The interplay between viruses and innate immune signaling: recent insights and therapeutic opportunities. *Biochem. Pharmacol.* **75**:589-602.

128. Venkatraman, S., S. L. Bogen, A. Arasappan, F. Bennett, K. Chen, E. Jao, Y. T. Liu, R. Lovey, S. Hendrata, Y. Huang, W. Pan, T. Parekh, P. Pinto, V. Popov, R. Pike, S. Ruan, B. Santhanam, B. Vibulbhan, W. Wu, W. Yang, J. Kong, X. Liang, J. Wong, R. Liu, N. Butkiewicz, R. Chase, A. Hart, S. Agrawal, P. Ingravallo, J. Pichardo, R. Kong, B. Baroudy, B. Malcolm, Z. Guo, A. Prongay, V. Madison, L. Broske, X. Cui, K. C. Cheng, Y. Hsieh, J. M. Brisson, D. Prelusky, W. Korfmacher, R. White, S. Bogdanowich-Knipp, A. Pavlovsky, P. Bradley, A. K. Saksena, A. Ganguly, J. Piwinski, V. Girijavallabhan, and F. G. Njoroge. 2006. Discovery of (1R,5S)-N-[3-amino-1-(cyclobutylmethyl)-2,3-dioxopropyl]-3-[2(S)-[[[(1,1-dimethylethyl)amino]carbonyl]amino]-3,3-dimethyl-1-oxobutyl]-6,6-dimethyl-3-azabicyclo[3.1.0]hexan-2(S)-carboxamide (SCH 503034), a selective, potent, orally bioavailable hepatitis C virus NS3 protease inhibitor: a potential therapeutic agent for the treatment of hepatitis C infection. *J. Med. Chem.* **49**:6074-6086.
129. Wakita, T., T. Pietschmann, T. Kato, T. Date, M. Miyamoto, Z. Zhao, K. Murthy, A. Habermann, H. G. Krausslich, M. Mizokami, R. Bartenschlager, and T. J. Liang. 2005. Production of infectious hepatitis C virus in tissue culture from a cloned viral genome. *Nat. Med.* **11**:791-796.
130. Walewski, J. L., T. R. Keller, D. D. Stump, and A. D. Branch. 2001. Evidence for a new hepatitis C virus antigen encoded in an overlapping reading frame. *RNA* **7**:710-721.
131. Welbourn, S. and A. Pause. 2007. The hepatitis C virus NS2/3 protease. *Curr. Issues Mol. Biol.* **9**:63-69.
132. Wheeler, D. L., C. Chappey, A. E. Lash, D. D. Leipe, T. L. Madden, G. D. Schuler, T. A. Tatusova, and B. A. Rapp. 2000. Database resources of the National Center for Biotechnology Information. *Nucleic Acids Res.* **28**:10-14.
133. Wolk, B., D. Sansonno, H. G. Krausslich, F. Dammacco, C. M. Rice, H. E. Blum, and D. Moradpour. 2000. Subcellular localization, stability, and trans-cleavage competence of the hepatitis C virus NS3-NS4A complex expressed in tetracycline-regulated cell lines. *J. Virol.* **74**:2293-2304.

134. **Wu, H. X., J. Wu, T. Wong, T. Donaldson, K. Dinner, A. Andonov, J. Ip Chan, B. Moffat, B. Baptiste, J. Furseth, D. Poliquin, G. Bolesnikov, A. Giulivi, and S. Paton.** 2006. Enhanced surveillance of newly acquired hepatitis C virus infection in Canada, 1998 to 2004. *Scand. J. Infect. Dis.* **38**:482-489.
135. **Wunschmann, S., J. D. Medh, D. Klinzmann, W. N. Schmidt, and J. T. Stapleton.** 2000. Characterization of hepatitis C virus (HCV) and HCV E2 interactions with CD81 and the low-density lipoprotein receptor. *J. Virol.* **74**:10055-10062.
136. **Wunschmann, S., H. M. Muller, C. S. Stipp, M. E. Hemler, and J. T. Stapleton.** 2006. *In vivo* interaction between hepatitis C virus (HCV) envelope glycoprotein E2 and serum lipoproteins (LPs) results in enhanced cellular binding of both HCV E2 and LPs. *J. Infect. Dis.* **194**:1058-1067.
137. **Wyles, D. L., K. A. Kaihara, and R. T. Schooley.** 2008. Synergy of an HCV NS4A antagonist in combination with HCV protease and polymerase inhibitors. *Antimicrob. Agents Chemother.*
138. **Xu, Z., J. Choi, T. S. Yen, W. Lu, A. Strohecker, S. Govindarajan, D. Chien, M. J. Selby, and J. Ou.** 2001. Synthesis of a novel hepatitis C virus protein by ribosomal frameshift. *EMBO J.* **20**:3840-3848.
139. **Yang, W., Y. Zhao, J. Fabrycki, X. Hou, X. Nie, A. Sanchez, A. Phadke, M. Deshpande, A. Agarwal, and M. Huang.** 2008. Selection of Replicon Variants Resistant to ACH-806, a Novel Hepatitis C Virus Inhibitor with No Cross-Resistance to NS3 Protease and NS5B Polymerase Inhibitors. *Antimicrob. Agents Chemother.*
140. **Yi, M., X. Tong, A. Skelton, R. Chase, T. Chen, A. Prongay, S. L. Bogen, A. K. Saksena, F. G. Njoroge, R. L. Veselenak, R. B. Pyles, N. Bourne, B. A. Malcolm, and S. M. Lemon.** 2006. Mutations conferring resistance to SCH6, a novel hepatitis C virus NS3/4A protease inhibitor. Reduced RNA replication fitness and partial rescue by second-site mutations. *J. Biol. Chem.* **281**:8205-8215.

141. **Zhang, C., Z. Cai, Y. C. Kim, R. Kumar, F. Yuan, P. Y. Shi, C. Kao, and G. Luo.** 2005. Stimulation of hepatitis C virus (HCV) nonstructural protein 3 (NS3) helicase activity by the NS3 protease domain and by HCV RNA-dependent RNA polymerase. *J. Virol.* **79**:8687-8697.
142. **Zhang, R., J. Durkin, W. T. Windsor, C. McNemar, L. Ramanathan, and H. V. Le.** 1997. Probing the substrate specificity of hepatitis C virus NS3 serine protease by using synthetic peptides. *J. Virol.* **71**:6208-6213.

Chapter 2

Single-cell resolution imaging of membrane-anchored hepatitis C virus NS3-4A protease activity⁴

Understanding membrane-associated proteolysis is an exciting emerging field of research, which has many biologic and therapeutic consequences (3, 5). Membrane-associated proteases can be classified as either intramembrane proteases (IPs), if they hydrolyze polypeptides buried in a lipid bilayer, or juxtamembrane proteases (JPs), if they cleave proteins in the aqueous environment adjacent to the lipid bilayer (8, 27). IPs are usually multi-pass transmembrane proteins; the active site catalytic residues are located in transmembrane domains and hydrolyze other transmembrane domains via a poorly understood mechanism. In contrast, JPs utilize a diverse set of mechanisms to associate themselves with biological membranes; single or multiple transmembrane domains, glycosylphosphatidyl inositol (GPI)-linked residues, or protein-protein interactions are common tethering mechanisms (22).

Many positive-sense RNA viruses replicate their genomic RNA within large multi-protein complexes tethered to intracellular biological membranes (24). 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 assemble the replicase into a large complex. The membrane composition and structure is altered by the presence of these viral proteins; however, experimentation on NS proteins *in vivo*, where the impact of this unique micro-environment 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’ (4, 21). 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

⁴ A version of this chapter has been published. Martin, M.M. and F. Jean. 2006. Single-cell resolution imaging of membrane-anchored hepatitis C virus NS3/4A protease activity. *Biol. Chem.* 387:1075-80.

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 polycistronic 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 (9, 15). 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 negatively-charged residue in the P6 position (Glu/Asp) (14, 25, 29). 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 enzyme catalytic residues. This chemical "warhead" is linked to a fluorescent molecule for detection. Limitations to this system are the lack of cell-permeable probes and irreversible inactivation of the target protease (2). 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 (10-13, 18, 23, 26). In this study, we demonstrate a new fluorescent-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; change in localization of a fluorescent protein reporter group. The reporter group is tethered to the membrane via a membrane-anchoring domain (Illustration 2.1A). 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 will be hydrolyzed, liberating the fluorescent reporter into the cytosol (Illustration 2.1B). This change in fluorescence pattern from membrane-bound to diffusely cytoplasmic can be detected using fluorescence microscopy.

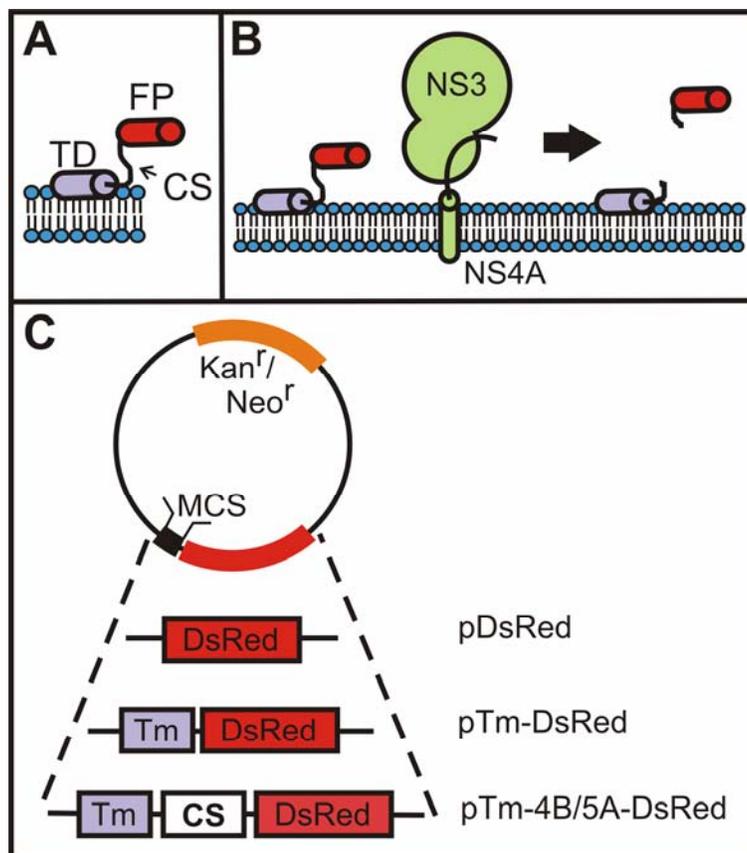


Illustration 2.1 Design of a membrane-anchored fluorescent protease substrate

(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 alpha-helix (Tm). Overlapping oligonucleotides encoding for the NS4B/5A cleavage site were annealed and inserted into pDsRed containing Tm, creating pTm-4B/5A-DsRed.

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* (19). 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 (Illustration 2.1C).

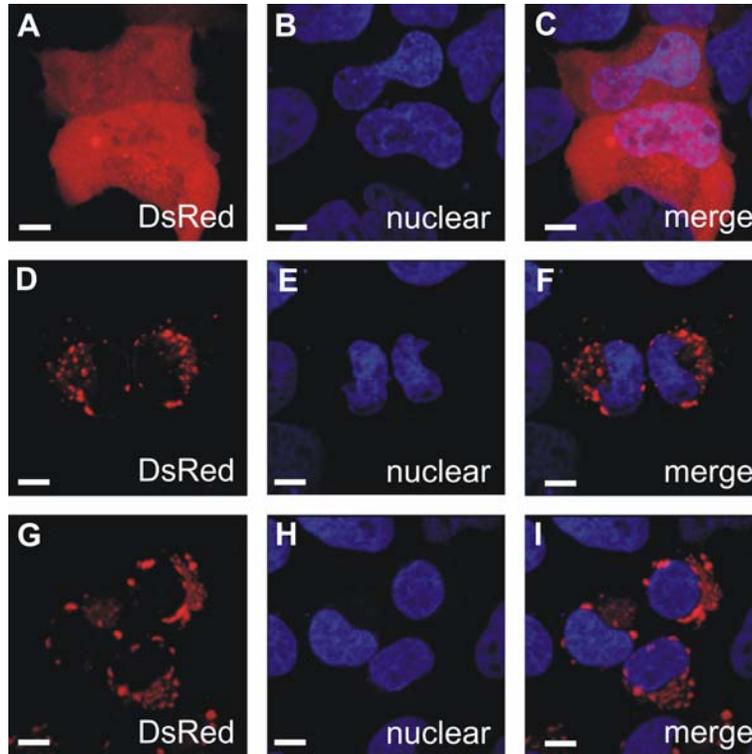


Figure 2.1 Tm-DsRed and Tm-4B/5A-DsRed are localized to perinuclear membranes

Culturing of UNS3-4A human osteosarcoma cells in the presence of tetracycline was done as previously described (10). Cells were grown on glass coverslips in 24-well plates and were transiently transfected with *TransIT* transfection reagent according to 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). 24 hours 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. Bar corresponds to 10 μ m.

In order to customize 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 (Illustration 2.1C). The NS5A anchoring domain is a stretch of 31 N-terminal NS5A residues that are predicted to form an amphipathic

alpha-helix and has been shown to target NS5A or GFP to the membrane (6, 20). As shown in Figure 2.1D-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 a NS3-specific cleavage site corresponding to the NS4B/NS5A junction (Asp-Cys-Ser-Thr-Pro-Cys↓Ser-Gly-Ser) completed the protease probe (Illustration 2.1C). The resulting plasmid, pTm-4B/5A-DsRed, was transfected into mammalian cells and produced a fusion protein, Tm-4B/5A-DsRed; the pattern of localization for this probe (Figure 2.1G-I) remained unchanged compared to the negative control plasmid, pTm-DsRed (Figure 2.1D-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 (10, 28). 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; changing its location in the cell from membrane-bound punctate perinuclear (Figure 2.1G-I) to diffuse cytoplasmic (Figure 2.2D-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 2.2A-C). In addition, when the P1 cysteine residue was changed to a serine residue (Tm-4B/5A(CP1S)-DsRed cleavage site: Asp-Cys-Ser-Thr-Pro-Ser↓Ser-Gly-Ser) substrate cleavage was undetectable using microscopy (Figure 2.2G-I).

To attain quantitation of the NS3-4A cleavage, Western blotting of whole cell lysate 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 alpha helix and therefore is smaller than the unprocessed substrate (Figure 2.3, inset). The percentage of the total substrate found to be processed by NS3-4A was $57.4 \pm 4.9\%$ of the Tm-4B/5A-DsRed probe, compared to the background cleavage of $0.9-1.5 \pm 0.8\%$ (Figure 2.3). The nearly absent background processing indicated 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 $4.6 \pm 0.8\%$ cleavage in the presence of NS3-4A, which was not significantly different from the cleavage of this substrate in the absence of NS3-4A ($2.1 \pm 1.9\%$).

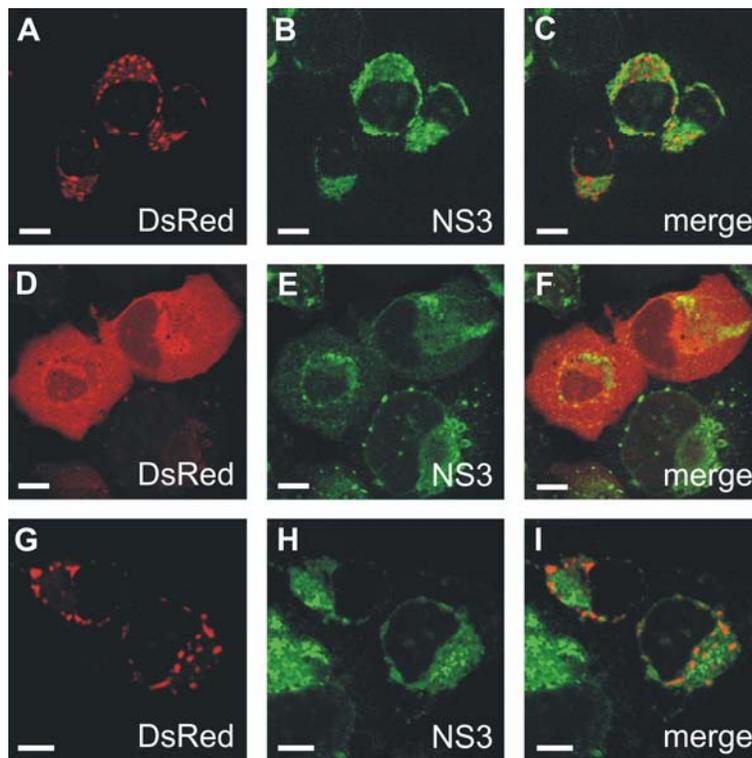


Figure 2.2 pTm-4B/5A-DsRed is processed by HCV NS3-4A.

To induce NS3-4A expression, UNS3-4A cells were grown in the absence of tetracycline for 24 hours before transfection with pTm-DsRed (A-C), pTm-4B/5A-DsRed (D-F), or pTm-4B/5A(CP1S)-DsRed (G-I). 24 hours post transfection without tetracycline, cells were fixed in 4% paraformaldehyde, permeabilized with 0.05% saponin, and stained with anti-NS3 mouse antibody (Novocastra Laboratories, Newcastle upon Tyne, UK) and secondary anti-mouse Alexa Fluor-488 (Molecular Probes, Eugene, OR, USA) as previously described (10). pTm-4B/5A(CP1S)-DsRed was created by changing the P1 cysteine residue to a serine residue using site-directed mutagenesis (Quikchange, Stratagene). Images were acquired using a Zeiss Axiovert confocal microscope. Bar corresponds to 10 μm .

Other cleavage site mutants and a mixed cleavage site control were not processed by NS3-4A to any significant degree (Table 2.1). One of these mutants, where the P1 and P6 position were swapped, actually displayed a partial NS3-4A cleavage site in the N-terminus of the cleavage site linker; the NS5A/NS5B 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 in P6.

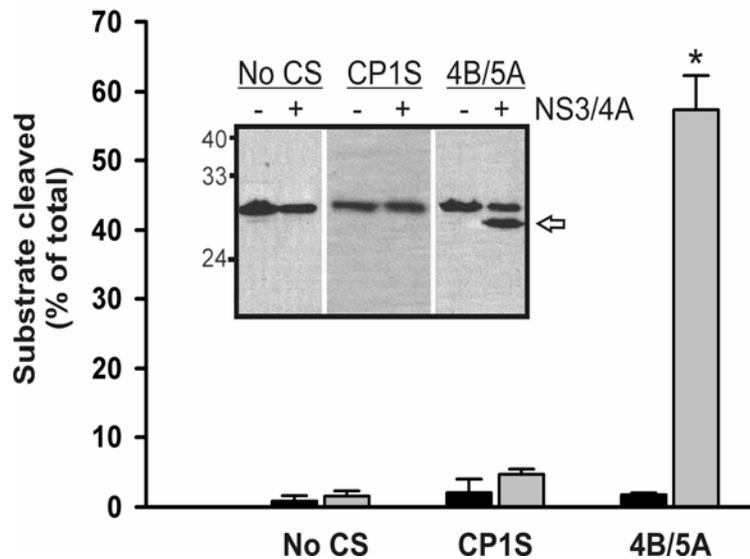


Figure 2.3 pTm-4B/5A-DsRed substrate cleavage can be quantitated by Western blotting.

UN3-4A cells were grown in the absence or presence of tetracycline in 6-well plates, transfected, and harvested 24 hours post transfection by scraping and centrifugation (0.9 xg, 10 sec). The cell pellet was resuspended in hypotonic lysis buffer (10 mM Tris [pH 7.8], 10 mM NaCl, 1 x EDTA-free complete protease inhibitor (Roche)). Whole cell lysates were run on a 15% polyacrylamide gel and Western blot analysis was performed using anti-DsRed polyclonal antibody according to manufacturer's instructions (BD Biosciences, Palo Alto, CA, USA). Inset is the 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 quantitated using a VersaDoc multiimager (BioRad). Percent of substrate cleaved was calculated by dividing the total signal (cleaved plus uncleaved) by the cleaved signal, thereby normalizing the readout for each sample. The bar chart represents the average of three separate experiments. * $p < 0.0001$

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-beta (TRIF) and mitochondrial antiviral signaling protein (MAVS) (16, 17). TRIF is a signaling 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 which mediate an anti-viral state. The site that NS3 recognizes in TRIF has remarkable homology with the viral NS4B/NS5A cleavage site, although an 8-residue polyproline track extends upstream from the P6 position in lieu of the acidic residue present in the HCV substrates (7). 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.

Description ^a	Cleavage Site Sequence	Percent of Substrate Cleaved (%) ^b
Tm-4B/5A-DsRed	Asp-Cys-Ser-Thr-Pro-Cys ↓ Ser-Gly-Ser-Trp	57.4 ± 4.9
CP1S	Asp-Cys-Ser-Thr-Pro- Ser ↓ Ser-Gly-Ser-Trp	4.6 ± 0.8
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

Table 2.1 Tm-4B/5A-DsRed cleavage site variants are not cleaved by NS3-4A.

^aAll mutants were created using site-directed mutagenesis using Tm-4B/5A-DsRed as a template (Quikchange, Stratagene). ^bConstructs were transfected into UNS3-4A cells and the cleavage percentage quantitated by Western blotting as described in Figure 4. Shown is the average of two separate experiments.

A membrane-targeted assay is important due 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 also can 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 (1).

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

References

1. **Aizaki, H., K. J. Lee, V. M. Sung, H. Ishiko, and M. M. Lai.** 2004. Characterization of the hepatitis C virus RNA replication complex associated with lipid rafts. *Virology* **324**:450-461.
2. **Baruch, A., D. A. Jeffery, and M. Bogyo.** 2004. Enzyme activity--it's all about image. *Trends Cell Biol.* **14**:29-35.
3. **Bauvois, B.** 2004. Transmembrane proteases in cell growth and invasion: new contributors to angiogenesis? *Oncogene* **23**:317-329.
4. **Egger, D., B. Wolk, R. Gosert, L. Bianchi, H. E. Blum, D. Moradpour, and K. Bienz.** 2002. Expression of hepatitis C virus proteins induces distinct membrane alterations including a candidate viral replication complex. *J. Virol.* **76**:5974-5984.
5. **Ehrmann, M. and T. Clausen.** 2004. Proteolysis as a regulatory mechanism. *Annu. Rev. Genet.* **38**:709-724.
6. **Elazar, M., K. H. Cheong, P. Liu, H. B. Greenberg, C. M. Rice, and J. S. Glenn.** 2003. Amphipathic helix-dependent localization of NS5A mediates hepatitis C virus RNA replication. *J. Virol.* **77**:6055-6061.
7. **Ferreon, J. C., A. C. Ferreon, K. Li, and S. M. Lemon.** 2005. Molecular determinants of TRIF proteolysis mediated by the hepatitis C virus NS3-4A protease. *J. Biol. Chem.* **280**:20483-20492.
8. **Golde, T. E. and C. B. Eckman.** 2003. Physiologic and pathologic events mediated by intramembranous and juxtamembranous proteolysis. *Sci. STKE* **2003**:RE4.
9. **Goudreau, N. and M. Llinas-Brunet.** 2005. The therapeutic potential of NS3 protease inhibitors in HCV infection. *Expert Opin. Investig. Drugs* **14**:1129-1144.
10. **Hamill, P. and F. Jean.** 2005. Enzymatic characterization of membrane-associated hepatitis C virus NS3/4A heterocomplex serine protease activity expressed in human cells. *Biochemistry* **44**:6586-6596.

11. **Jean, F., A. Basak, J. DiMaio, N. G. Seidah, and C. Lazure.** 1995. An internally quenched fluorogenic substrate of prohormone convertase 1 and furin leads to a potent prohormone convertase inhibitor. *Biochem. J.* **307 (Pt 3):**689-695.
12. **Jean, F., A. Boudreault, A. Basak, N. G. Seidah, and C. Lazure.** 1995. Fluorescent peptidyl substrates as an aid in studying the substrate specificity of human prohormone convertase PC1 and human furin and designing a potent irreversible inhibitor. *J. Biol. Chem.* **270:**19225-19231.
13. **Jones, J., R. Heim, E. Hare, J. Stack, and B. A. Pollok.** 2000. Development and application of a GFP-FRET intracellular caspase assay for drug screening. *J. Biomol. Screen.* **5:**307-318.
14. **Kim, S. Y., K. W. Park, Y. J. Lee, S. H. Back, J. H. Goo, O. K. Park, S. K. Jang, and W. J. Park.** 2000. *In vivo* determination of substrate specificity of hepatitis C virus NS3 protease: genetic assay for site-specific proteolysis. *Anal. Biochem.* **284:**42-48.
15. **Lamarre, D., P. C. Anderson, M. Bailey, P. Beaulieu, G. Bolger, P. Bonneau, M. Bos, D. R. Cameron, M. Cartier, M. G. Cordingley, A. M. Faucher, N. Goudreau, S. H. Kawai, G. Kukolj, L. Lagace, S. R. LaPlante, H. Narjes, M. A. Poupert, J. Rancourt, R. E. Sentjens, R. St George, B. Simoneau, G. Steinmann, D. Thibeault, Y. S. Tsantrizos, S. M. Weldon, C. L. Yong, and M. Llinas-Brunet.** 2003. An NS3 protease inhibitor with antiviral effects in humans infected with hepatitis C virus. *Nature* **426:**186-189.
16. **Li, K., E. Foy, J. C. Ferreon, M. Nakamura, A. C. Ferreon, M. Ikeda, S. C. Ray, M. Gale Jr, and S. M. Lemon.** 2005. Immune evasion by hepatitis C virus NS3/4A protease-mediated cleavage of the Toll-like receptor 3 adaptor protein TRIF. *Proc. Natl. Acad. Sci. U. S. A.* **102:**2992-2997.
17. **Li, X. D., L. Sun, R. B. Seth, G. Pineda, and Z. J. Chen.** 2005. Hepatitis C virus protease NS3/4A cleaves mitochondrial antiviral signaling protein off the mitochondria to evade innate immunity. *Proc. Natl. Acad. Sci. U. S. A.* **102:**17717-17722.
18. **Matayoshi, E. D., G. T. Wang, G. A. Krafft, and J. Erickson.** 1990. Novel fluorogenic substrates for assaying retroviral proteases by resonance energy transfer. *Science* **247:**954-8.

19. **Matz, M. V., A. F. Fradkov, Y. A. Labas, A. P. Savitsky, A. G. Zairaisky, M. L. Markelov, and S. A. Lukyanov.** 1999. Fluorescent proteins from nonbioluminescent Anthozoa species. *Nat. Biotechnol.* **17**:969-973.
20. **Moradpour, D., V. Brass, and F. Penin.** 2005. Function follows form: the structure of the N-terminal domain of HCV NS5A. *Hepatology* **42**:732-735.
21. **Mottola, G., G. Cardinali, A. Ceccacci, C. Trozzi, L. Bartholomew, M. R. Torrisi, E. Pedrazzini, S. Bonatti, and G. Migliaccio.** 2002. Hepatitis C virus nonstructural proteins are localized in a modified endoplasmic reticulum of cells expressing viral subgenomic replicons. *Virology* **293**:31-43.
22. **Netzel-Arnett, S., J. D. Hooper, R. Szabo, E. L. Madison, J. P. Quigley, T. H. Bugge, and T. M. Antalis.** 2003. Membrane anchored serine proteases: a rapidly expanding group of cell surface proteolytic enzymes with potential roles in cancer. *Cancer Metastasis Rev.* **22**:237-258.
23. **Richer, M. J., L. Juliano, C. Hashimoto, and F. Jean.** 2004. Serpin mechanism of hepatitis C virus nonstructural 3 (NS3) protease inhibition: induced fit as a mechanism for narrow specificity. *J. Biol. Chem.* **279**:10222-10227.
24. **Salonen, A., T. Ahola, and L. Kaariainen.** 2005. Viral RNA replication in association with cellular membranes. *Curr. Top. Microbiol. Immunol.* **285**:139-173.
25. **Schechter, I. and A. Berger.** 1967. On the size of the active site in proteases. I. Papain. *Biochem. Biophys. Res. Commun.* **27**:157-162.
26. **Tawa, P., J. Tam, R. Cassady, D. W. Nicholson, and S. Xanthoudakis.** 2001. Quantitative analysis of fluorescent caspase substrate cleavage in intact cells and identification of novel inhibitors of apoptosis. *Cell Death Differ.* **8**:30-37.
27. **Wolfe, M. S. and R. Kopan.** 2004. Intramembrane proteolysis: theme and variations. *Science* **305**:1119-1123.

28. **Wolk, B., D. Sansonno, H. G. Krausslich, F. Dammacco, C. M. Rice, H. E. Blum, and D. Moradpour.** 2000. Subcellular localization, stability, and trans-cleavage competence of the hepatitis C virus NS3-NS4A complex expressed in tetracycline-regulated cell lines. *J. Virol.* **74**:2293-2304.
29. **Zhang, R., J. Durkin, W. T. Windsor, C. McNemar, L. Ramanathan, and H. V. Le.** 1997. Probing the substrate specificity of hepatitis C virus NS3 serine protease by using synthetic peptides. *J. Virol.* **71**:6208-6213.

Chapter 3

Analysis of hepatitis C virus NS3-4A *trans* cleavage efficiency *in vivo*⁵

Introduction

The hepatitis C virus (HCV) was identified in 1989 as the major causative agent of transfusion-associated non-A, non-B hepatitis (9). By 2004, the prevalence of HCV had increased to an estimated 2.2% worldwide (54) and HCV-specific therapeutics have never been more urgently needed. Much success has been achieved in understanding the mechanism by which this virus infects and replicates, information essential for the creation of vaccines and therapeutics (12, 37).

HCV is a single-stranded positive-sense RNA virus belonging to the family *Flaviviridae* that replicates in the cytoplasm of liver cells (45). HCV modifies the endoplasmic reticulum (ER) to create a mitochondria-associated ‘membranous web’; the web nodules contain assemblies of large macromolecular protein complexes that copy the viral RNA (13, 41). These ‘replicase’ complexes contain non-structural (NS) viral enzymes (proteases, helicases, polymerases), and the activities as well as the maturation of these NS proteins are drug targets.

The HCV genome is a polycistronic RNA which is translated into one continuous polypeptide chain. 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 (5, 11, 60). One of these proteases, non-structural 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 drug screening (10, 29, 55).

NS3 is a bifunctional enzyme; it is comprised of both a helicase domain and a protease domain. The protease domain (NS3^{pro}) is a chymotrypsin-like serine protease that acts in concert with its cofactor, NS4A. NS4A integrates into the NS3 protease fold, stabilizing and targeting

⁵ A version of this chapter will be submitted for publication. Martin, M.M. and F. Jean. Analysis of hepatitis C virus NS3-4A *trans* cleavage efficiency *in vivo*.

NS3 to the ER (26, 61). 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 NS4B/NS5A junction to be cleaved (1, 15, 34, 52). The region of NS4A required for this activation has been mapped to 13 central amino acids; a synthetic peptide corresponding to this region (pep4A) can activate NS3 *in vivo* (3, 50, 52, 57).

The NS3-NS4A heterocomplex (referred to hereafter as NS3-4A) cleavage sites conform to a consensus pattern of D/E-X-X-X-X-C/T↓S/A (19). 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* (1, 47, 56). However, Lin and colleagues did observe *trans* cleavage of the NS3/NS4A junction by NS3^{pro} when the NS3 protease domain was removed from the precursor polyprotein substrate (34). Therefore *trans* cleavage may be possible for NS3/4A 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* (17, 24, 31, 44, 46, 49, 50, 53, 62). 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 (31, 46, 50). In contrast, utilizing NS3-4A purified from insect (Sf9) cells, Zhang *et al.* reported an alternative ranking of NS5A/NS5B > NS4B/NS5A > NS4A/NS4B (62). 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.

Recently, the NS3-4A protease has been implicated in disrupting innate immunity dsRNA-sensor signaling cascades that lead to the induction of interferons. NS3-4A specifically cleaves two host proteins: toll/IL-1 receptor-domain-containing adaptor inducing IFN-beta (TRIF) and mitochondrial antiviral signaling protein (MAVS; also known as CARDIF, IPS-1, and VISA) (8, 32, 33). TRIF is 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).

This realization has made NS3-4A a more attractive drug target as inhibition of NS3-4A would not only stop viral replication but also boost the immune response against HCV. Ferreon *et al.* (16) have used a pep4A-NS3^{pro} fusion protein (“single-chain NS3”) to compare TRIF peptide substrate cleavage to viral substrate cleavage *in vitro*. They found that TRIF was processed less efficiently than viral substrates, about half as well as NS4B/NS5A. However, no comparison of TRIF or MAVS cleavage sequences has been done *in vivo*.

In this study, we report comparison of all known HCV NS3-4A cleavage sequences in a cell-based system and find 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 other HCV proteins or replicase components do not change NS3-4A substrate selectivity but may promote proteolysis. We also show NS3-4A-specific proteolysis of TRIF and MAVS peptide cleavage sequences and that the prime-side residues of MAVS decrease proteolysis by NS3-4A.

Results

In order to compare NS3-4A cleavage sequences in cells, we created intracellular fluorescent substrates corresponding to the natural HCV junctions (Illustration 3.1). These substrates were designed such that NS3-4A proteolysis would release a reporter group, DsRed-Express, localized to the ER into the cytoplasm. In this way cleavage could be detected via microscopy by observing the change in DsRed-Express localization in the cell. We selected the HCV NS5A N-terminal amphipathic alpha-helix (referred to as “Tm”) to tether the substrate, as it has been shown to localize GFP, tagged at either end, to the ER (7, 14, 42). Substrate residues P6 to P4’ were introduced between Tm and DsRed-Express. A length of 10-amino acids for the cleavage site was chosen due to *in vitro* studies on substrate length that showed 10 residues are sufficient to detect cleavage (50, 62). In addition, by keeping the substrate length as short as possible, we reasoned the chance of the linker being specifically recognized by a host protease would be reduced. Successful expression and specific NS3-4A processing of the Tm-4B/5A-DsRed substrate has been reported previously (39).

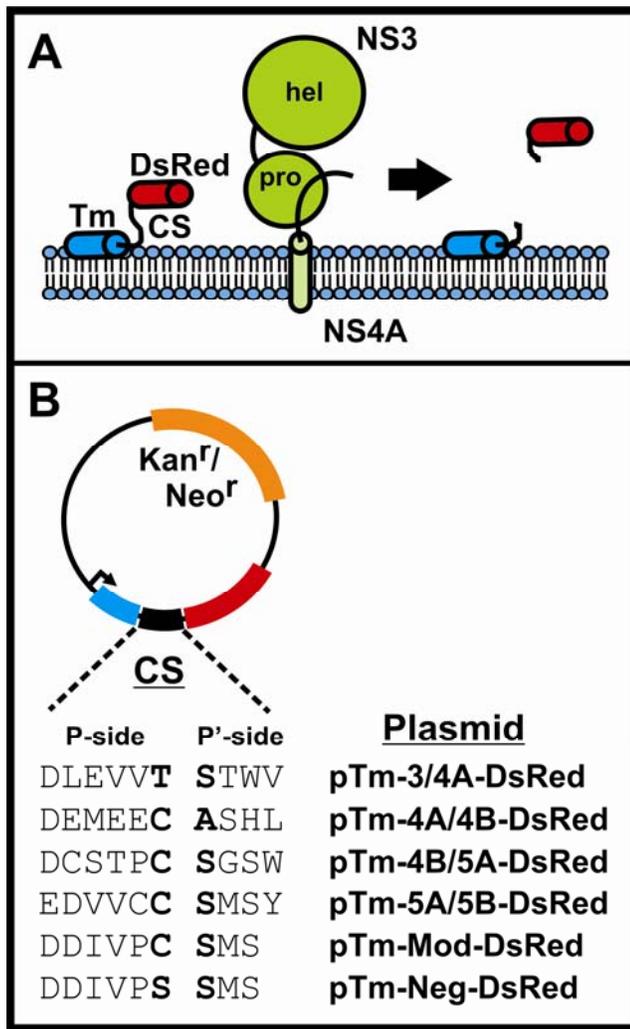


Illustration 3.1 Membrane-anchored substrate design and cleavage site sequences.

(A) Intracellular membrane-anchored protein substrates were engineered comprising a targeting domain (Tm), a protease-specific cleavage site (CS), and the fluorescent protein DsRed-Express (DsRed) reporter group. 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'.

The DNA plasmids encoding these substrates were transiently transfected into a tetracycline-regulated NS3-4A-expressing cell line, UNS3-4A (21, 61). NS3 protease activity was detected using confocal microscopy to visualize the release of DsRed-Express from the membrane: changing the localization in the cell from membrane-bound punctate perinuclear to diffusely cytoplasmic and nuclear.

By confocal microscopy efficient cleavage was apparent for NS5A/NS5B as expected based on the *in vitro* kinetic ranking of the substrates (Figure 3.1J-L), as well as for NS4B/NS5A

(Figure 3.1G-I) as previously reported (39). No cleavage was detectable for the NS3/NS4A or NS4A/NS4B junction (Figure 3.1A-C, D-F).

A modified NS5A/NS5B junction (Mod), first described by Kakiuchi *et al.* (23, 25), has been used extensively as an *in vitro* substrate (18, 21, 48, 58). 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 (23). The Tm-Mod-DsRed substrate containing this sequence was cleaved when expressed with NS3-4A (Figure 3.1M-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 3.1P-R).

Microscopy is a powerful technique for imaging single cell phenomena, but for this assay it is limited by its inability to detect a small amount of diffuse, soluble DsRed-Express in conjunction with a very large amount of punctuate DsRed-Express. In order to increase the sensitivity of the assay and also 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.

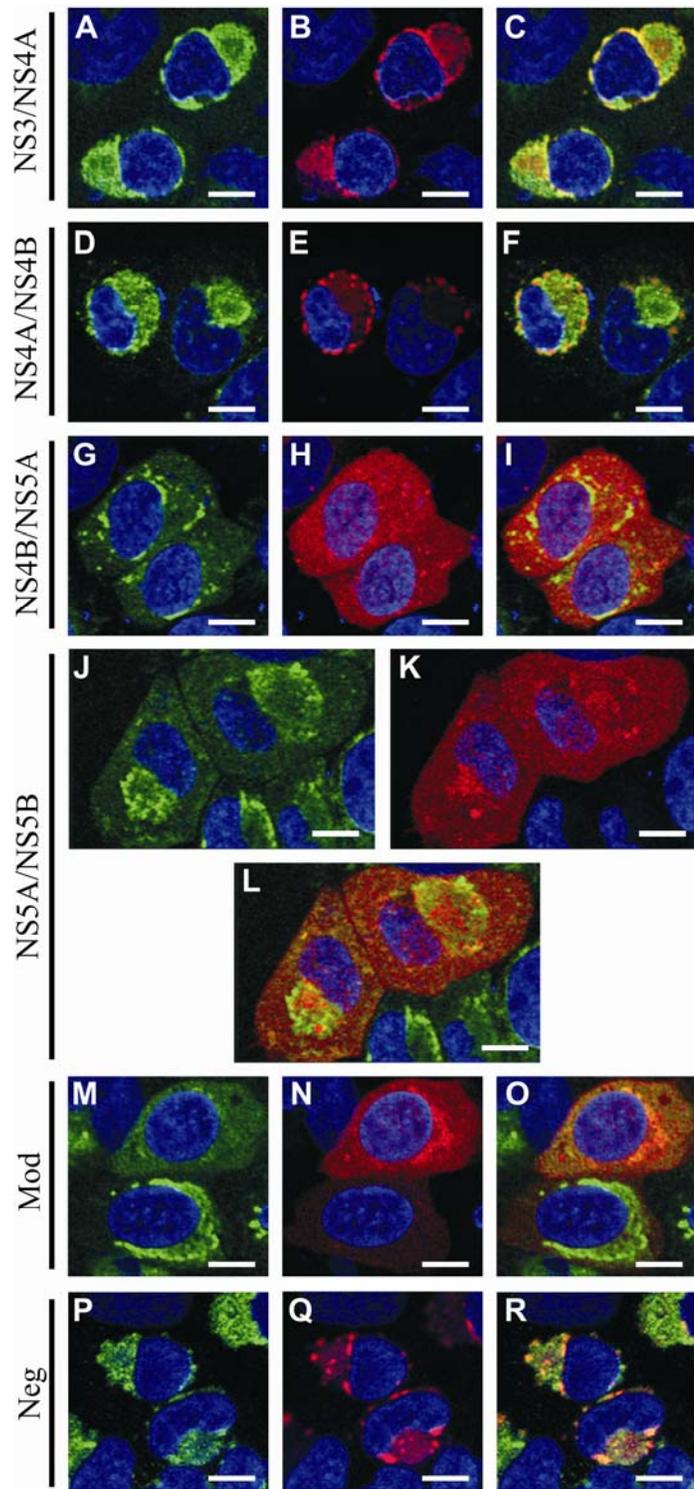


Figure 3.1 Efficient *trans* processing of Tm-4B/5A-DsRed, Tm-5A/5B-DsRed and Tm-Mod-DsRed by NS3-4A as detected by confocal microscopy.

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-Express signal is shown in B,E,H,K,N, and Q. Cell nuclei were stained with DAPI (shown in blue). Scale bar corresponds to 10 μ m.

Cleavage 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 3.2A). Band intensities were electronically captured on a charge-coupled device (CCD) and corrected for background. The percentage of substrate cleaved was calculated by dividing the signal intensity of the cleaved band by the total signal intensity of the cleaved and uncleaved bands. The percentage of substrate cleaved in this system may represent a balance between the affinity of the enzyme for its substrate (K_m), the rate at which the substrate is cleaved (k_{cat}), and the inhibition of the enzyme by its products (K_i). Since all substrates are presented in a similar way, comparison of the percentage cleaved allows for a relative measure of junction cleavage efficiency.

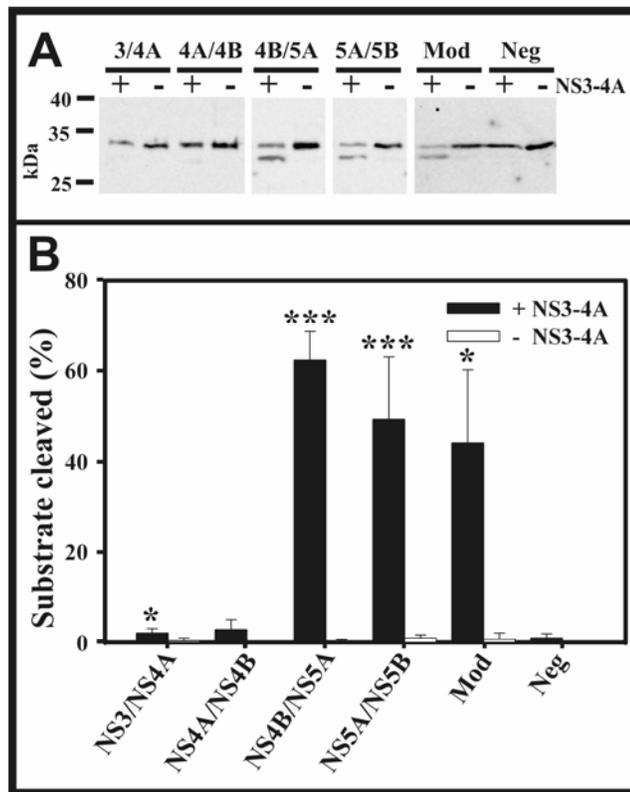


Figure 3.2 Efficient *trans* processing of Tm-4B/5A-DsRed, Tm-5A/5B-DsRed and Tm-Mod-DsRed by NS3-4A as detected by Western blotting

UN3-4A 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. A) 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) Quantification of Western blotting results showing the percentage of substrate cleaved. Results shown are the average of at least two independent experiments. * $p < 0.02$, *** $p < 0.0005$

Surprisingly, no significant differences were seen between the NS4B/NS5A, NS5A/NS5B or Mod junctions (Figure 3.2B); all three were cleaved between 44 and 63% (NS4A/NS4B $62.3 \pm 6.4\%$, NS4B/NS5A $49.4 \pm 13.6\%$, Mod $44.2 \pm 16.0\%$). Although the NS3/NS4A processing was very slight ($2.2 \pm 1.0\%$), this substrate was cleaved significantly more than when no protease was present ($0.5 \pm 1.0\%$). However, this cleavage was not meaningful as both the NS3/NS4A and NS4A/NS4B ($3.0 \pm 2.2\%$) junctions were not cleaved significantly more than the negative control, Tm-Neg-DsRed ($1.1 \pm 1.0\%$). Virtually no cleavage of the substrates was seen when NS3-4A was not expressed (average $<1\%$), indicating these intracellular substrates are stable for at least the duration of the experiment (24 hrs). Occasionally a slightly larger (~ 31 kDa) degradation product would be detected in the Tm-NS4A/NS4B-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).

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 (40). These cells express the entire HCV polyprotein under control of tetracycline, and allow analysis of replicase-associated NS3-4A proteolytic activity (Figure 3.3). Although cleavage of all substrates was much reduced (~ 5 to 7-fold) in this cell-line, the same trends were seen confirming approximate equal cleavages of NS4B/NS5A, NS5A/NS5B, and Mod, ($9.1 \pm 1.6\%$, $6.5 \pm 1.3\%$, and $8.8 \pm 1.5\%$, respectively) as well as negligible cleavage of NS3/NS4A, NS4A/4B, or Neg ($1.2 \pm 0.9\%$, $0.5 \pm 0.7\%$, and $0.6 \pm 0.9\%$, respectively).

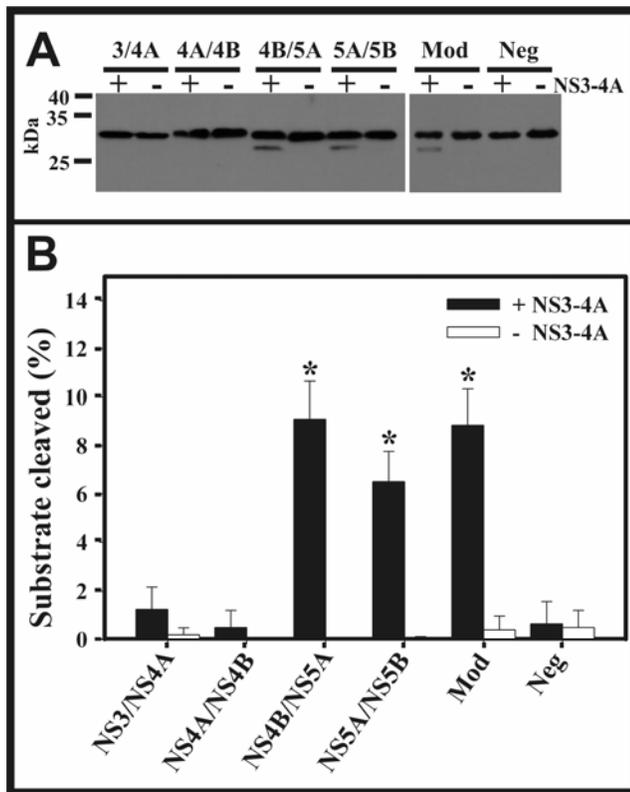


Figure 3.3 Expression of NS3-4A in HCV polyprotein does not change substrate selectivity.

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. A) 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. * $p < 0.02$

Due to the large difference in cleavage levels between UNS3-4A and UHCV-32 cells, we sought to compare levels of NS3 expression in these two cell lines in order to determine if the reduction in activity was completely due to reduced NS3-4A levels in UHCV-32 cells. UNS3-4A and UHCV-32 cells were grown without tetracycline for 48 hours, enumerated, and resolved on SDS-PAGE. NS3 was detected by Western blot analysis using an anti-NS3 antibody (Figure 3.4). Only a small amount of NS3 was detected in UHCV-32 cell lysates and the signal intensity of this band was too dim to be reliably quantified (beneath the linear range). However, this data showed that there is at least 100-fold reduction in NS3 levels in UHCV-32 cells.

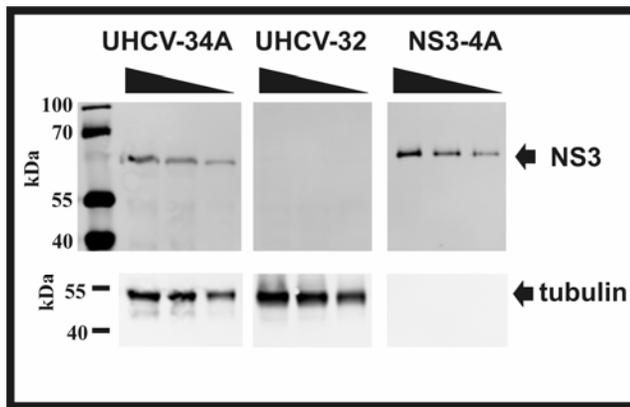


Figure 3.4 NS3-4A expression is at least 100-fold more in UNS3-4A cells as compared to UHCV-32 cells. Titration of UHCV-34A and UHCV-32 whole cell lysates and detection of NS3-4A by Western blotting using an anti-NS3 monoclonal antibody. *E. coli* expressed and purified NS3 is used as a positive control. Simultaneous probing for tubulin using an anti-tubulin polyclonal antibody, allows for normalization of the NS3 signal.

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 JFH1 strain (genotype 2a) and the Con1 strain (genotype 1a) were subcloned into eukaryotic expression vectors such that NS3-4A would be expressed by the CMV promoter. Double transfection of Huh7 cells with both protease and substrate plasmids showed results consistent with our previous findings (Figure 3.5). 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.

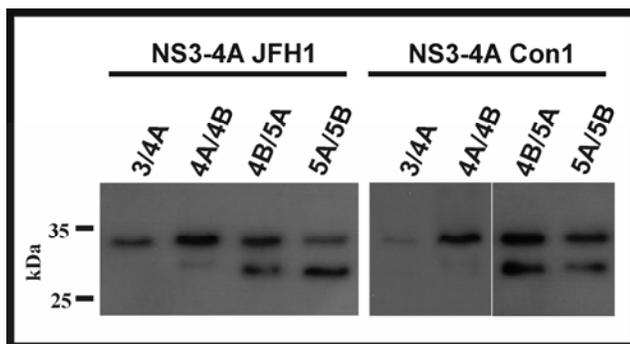


Figure 3.5 Genotype 1a strain Con1 NS3-4A displays substrate selectivity alike genotype 1b NS3-4A. Huh7 cells were double transfected with pCMV-NS3-4A_Con1 (genotype 1a) and substrate plasmids: pTm-3/4A-DsRed, pTm-4A/4B-DsRed, pTm-4B/5A-DsRed, and pTm-5A/5B-DsRed. Whole cell lysates were probed by Western blotting using an anti-DsRed antibody.

NS3-4A aids in HCV immune evasion by processing of TRIF and MAVS, therefore we aimed to determine the cleavage efficiencies of these substrates. The site that NS3 recognizes in TRIF is very similar to the viral NS4B/NS5A cleavage site (Figure 3.6A), in that both have the same P4-P1' residues. In contrast, the MAVS site is divergent from viral junction sequences. It has the strongly basic amino acid arginine in the P5 and P2' position as well as an unconventional histidine in the P1' position; the natural HCV junctions contain no basic residues (lysine or arginine), and serine or alanine is found in the P1' position of all HCV junctions.

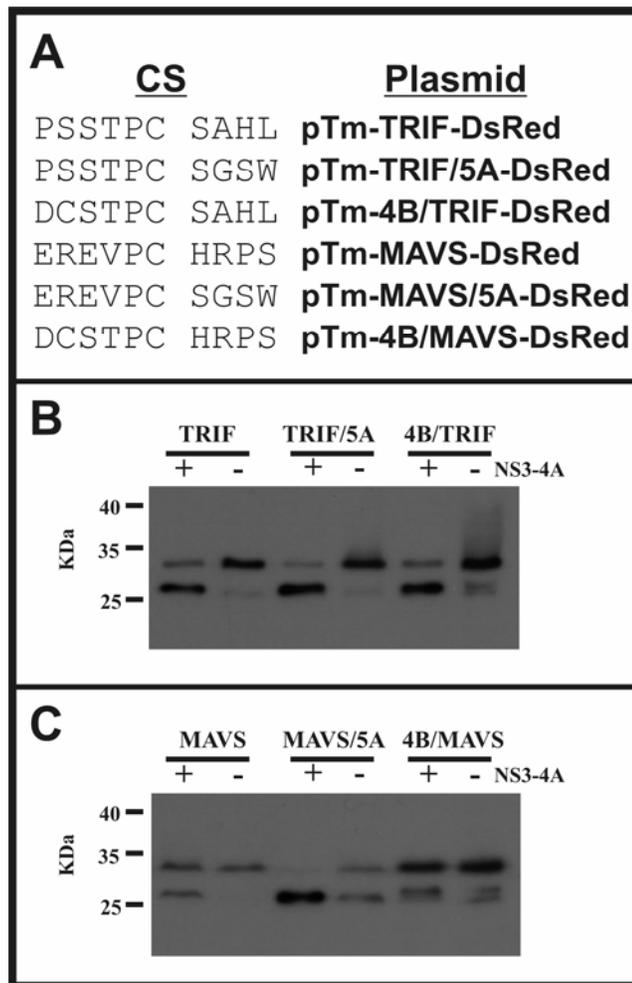


Figure 3.6 NS3-4A cleavage of TRIF and MAVS sequences.

A) Cleavage sequences (CS) P6-P4' engineered into substrate plasmids. Gap indicates position of NS3-4A cleavage (between P1 and P1'). B) UNS3-4A cells were transfected with pTm-TRIF-DsRed and chimeric constructs pTm-TRIF/5A-DsRed and pTm-4B/TRIF-DsRed; whole cell lysates were analyzed by western blotting using anti-DsRed antibody. C) UNS3-4A cells were transfected with pTm-MAVS-DsRed and chimeric constructs pTm-MAVS/5A-DsRed and pTm-4B/MAVS-DsRed; whole cell lysates were analyzed by western blotting using anti-DsRed antibody.

UNS3-4A cells were transfected with pTm-TRIF-DsRed and pTm-MAVS-DsRed, but unfortunately both sequences displayed instability at or nearby the NS3-4A cleavage site, generating background cleavage in the absence of NS3-4A (Figure 3.6B-C). However, specific cleavage by NS3-4A was still observed. The TRIF cleavage sequence showed NS3-4A specific cleavage that seemed unchanged when chimeric cleavage sites were constructed with either the P- or P'-side of TRIF paired with the complementary portion of the NS4B/NS5A junction (Figure 3.6A-B). The chimeric MAVS substrate including the MAVS non-prime side showed greatly improved cleavage, but also higher background levels. Introduction of the prime side of the MAVS cleavage sequence into NS4B/NS5A resulted in no NS3-4A specific cleavage at the P1-P1' position, however, a slightly higher product was observed.

Discussion

We have compared for the first time HCV NS3-4A decapeptide substrate cleavage efficiencies intracellularly and report no significant difference between the NS4B/NS5A, NS5A/NS5B and Mod junctions. We have also shown in this system that the NS3/NS4A and NS4A/NS4B junctions are not efficiently processed by NS3-4A. These findings are significantly different from the *in vitro* profiling of NS3-4A, performed mostly with NS3 protease domain in conjunction with a truncated peptide NS4A (31, 46, 50). This work highlights the impact that working with full-length protease and cofactor in a physiological setting can have on the measured enzyme selectivity. Thus, it will be very important to re-examine the protease selectivity of other membrane associated proteases in such an assay system.

Our data confirms inefficient *trans* cleavage of the NS3/NS4A junction, and suggests that cleavage of this junction observed by Lin *et al.* (34) was mediated by protein folding or perhaps cleavage at a nearby alternative site. We show NS4A/NS4B was not processed efficiently by any of the three genotypes tested (1a, 1b, 2a). Also, NS3-4A in association with other NS proteins was unable to process this junction to any significant degree. It would be interesting to map the substrate determinants responsible for this poor *trans* cleavage as the P2, P3, and P1'-P4' residues are all divergent from NS4B/NS5A and NS5A/NS5B.

Although it remains possible that in the context of the polyprotein efficient *trans* cleavage of the NS4A/NS4B site is mediated by determinants outside of the cleavage sequence,

including protein folding, we believe poor NS4A/NS4B cleavage may reflect a biologically relevant substrate preference. In fact, detection of a NS4A-NS4B precursor by many groups supports this idea (1, 2, 34, 51).

Poor NS4A/NS4B cleavage could be important early in infection when protein concentrations are very low. When the RNA is first translated into a polyprotein, NS3 associates with NS4A and undergoes self-cleavage co-translationally (15). NS3 would remain complexed with NS4A-NS4B-NS5A-NS5B until translation is complete and then processing of the junctions occurs in an order that allows NS3 to remain with the substrate (NS5A/NS5B, then NS4B/NS5A, then NS4A/NS4B) (36, 51). Early on, if the NS4A/NS4B junction were to be processed in *trans* by a neighboring NS3-4A, the protease and 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.* have shown the NS4A-NS4B precursor to slow anterograde traffic from the ER to the Golgi (30), a process which may be pivotal for HCV to establish replication in infected cells.

It is possible that in addition to cleavage of NS4A/NS4B *in trans* (3), 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). Introduction of these mutations into NS4A/NS4B showed a profile in between the *cis* and *trans* cleavage site groupings (2, 28, 51). In particular, the ability to mutate the P1 cysteine residue in NS4A/NS4B to a threonine and not affect cleavage of this junction (2, 28), indicates a possibility of uni-molecular hydrolysis.

Also, we show no modulation of protease selectivity by other HCV NS proteins or replicase components, but that the protease activity within the replicase may be increased. The level of NS3-4A cleavage in UNS3-4A cells as compared to in the UHCV-32 cells drops 5 to 7-fold, however, the protease level drops at least 100-fold, suggesting that NS3-4A in the context of the replicase is more active.

We have also shown the processing of cleavage sequences corresponding to innate immune signaling molecules, TRIF and MAVS. Unfortunately, due to background cleavage of these constructs direct comparison with viral sites was not possible. Interestingly, the chimera

substrate Tm-4B/MAVS-DsRed was not processed between the P1-P1' residues (cysteine-histidine). The detection of a slightly larger cleavage product from Tm-4B/MAVS-DsRed most likely corresponds to alternate cleavage at the P5-P4 position (cysteine-serine). The additional P4-P1 residues still attached to the DsRed product would explain the observed increase size (predicted molecular mass increase of 388 Da).

This indicates that MAVS P'-side residues are not well tolerated by NS3-4A and consequently the P-side of the MAVS cleavage site must compensate in some way. The most striking difference about the P-side of MAVS is the positively charged arginine residue in P5. Intriguingly, Kim *et. al.* (27) have reported P4-P1' library screening of *in vivo* NS3-4A substrate specificity; twenty-five percent (17/69) of the preferred sequences isolated contained a positively charged amino acid (arginine>lysine) in the P2-P4 position (including one sequence with arginine in P2 and P4). This preference was greater than that for negatively charged residues, of which only nine percent (6/69) of the sequences isolated contained an aspartic acid or glutamic acid.

NS3-4A may have an unrecognized substrate preference for positively charged residues and we suggest it is this preference that compensates for MAVS prime-side residues. In fact, the aspartic acid at position 168 (D168) in NS3 is modeled form part of the S4 binding pocket and could interact with positively charged substrate side chains (4, 35). Interestingly, D168 is mutated to a neutral glutamine in HCV genotype 3. Considering that polymorphisms in the MAVS cleavage sequence are rare, it may be possible that mutations in NS3 account for some of genotype 3's responsiveness to interferon and ribavirin treatment because of a reduction in the ability to disrupt interferon pathways through MAVS (20, 22).

This study has direct application to NS3-4A drug discovery. Since inside cells NS4B/NS5A is cleaved just as efficiently as NS5A/NS5B or Mod, cell-based assay screens using the NS5A/NS5B or Mod junctions could easily be altered to use the NS4B/NS5A junction without loss of signal and acquire the advantage of also screening for allosteric inhibitors of NS3-4A activation. As well, this data validates using a cell-based system only expressing NS3-4A for an initial or secondary drug screen, as the protease 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.

Materials and Methods

Construction of plasmid DNA

Plasmids were constructed by standard methods and verified by sequencing. Description of 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 (39). pTm-DsRed was created by inserting DNA coding for the HCV NS5A 31 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) *pCMV-NS3-4A_Con1* and *pCMV-NS3-4A_JFH1*. NS3-4A was PCR amplified from pCon1/FL(I) (6), and pUC-vJFH1 (59) 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.

(iii) *pTm-TRIF-DsRed*, *pTm-TRIF/5A-DsRed*, *pTm-4B/TRIF-DsRed*, *pTm-MAVS-DsRed*, *pTm-MAVS/5A-DsRed*, *pTm-4B/MAVS-DsRed*. All mutants were created by site-directed mutagenesis using pTm-4B/5A-DsRed as a template (Quikchange, Stratagene).

Cell culture

UNS3-4A and UHCV-32 cell lines (21, 40, 61) were propagated in Dulbecco modified Eagle medium (DMEM, Gibco, Invitrogen) supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 500 μ g/mL geneticin, 50 units/mL penicillin, 50 μ g/mL streptomycin, 1 μ g/mL puromycin, and 1 μ g/mL tetracycline. Huh7 cells were propagated in DMEM supplemented with

10% fetal bovine serum, 2 mM L-glutamine, 100 μ M non-essential amino acids, 50 units/mL penicillin, and 50 μ g/mL streptomycin.

All cells were grown at 37°C in 5% CO₂.

Fluorescence and immunofluorescence microscopy

Microscopy was done as previously described (39). 5 X 10⁴ cells were seeded in 24-well plates on top of glass coverslips (13 mm diameter, 1.5 thickness) and grown for 1 day (>50% confluent). Cells were washed 3 times with PBS and either tetracycline-replete media or media lacking tetracycline was added. 24 hrs post tetracycline removal, cells were transfected with 1 μ g/well of DNA plasmid using TransIT-LT1 transfection reagent (Mirus Bio, Madison, WI, USA). 24 hrs post transfection cells were washed with PBS and fixed in 3.8% formaldehyde for 30 min. Immunofluorescence staining was done using 1:100 anti-NS3 (Novocastra Laboratories, Newcastle upon Tyne, UK) and 1:100 Alexa Fluor-488-conjugated anti-mouse antibody (Molecular Probes, Invitrogen). Coverslips were mounted with Vectasheild mounting solution containing 4',6-diamidino-2-phenylindole (DAPI) (Vector Laboratories, Burlingame, CA, USA). Images were acquired using a Zeiss Axiovert confocal microscope.

Transfections and Western blotting

Transfections and Western blotting was done as previously described (39). 6-well plates were seeded with 5 X 10⁴ cells and grown for 2-3 days until greater than 50% confluent. Cells were washed 3 times with PBS and either tetracycline-replete media or media lacking tetracycline was added. 24 hrs post tetracycline removal, cells were transfected with 2 μ g per well of DNA plasmid using TransIT-LT1 transfection reagent (Mirus Bio, Madison, WI, USA). 24 hrs 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, 1X complete EDTA-free protease inhibitors (Roche, Laval, 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 hours at 110 V and transferred to a nitrocellulose membrane. The membrane was probed with polyclonal anti-DsRed (1:16,000 Lot#400467, 1:4,000 Lot#5110235, 1:1,000 Lot#7040094, Clontech, Mountain View, CA, USA) and goat-HRP anti-rabbit secondary antibody (1:1,000, Amersham Biosciences,

Piscataway, NJ, USA). Quantitation of the enhanced chemiluminescent signal was performed on a VersaDoc multiimager (Bio-Rad, Mississauga, CA) for a greater dynamic range than film (38). 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: Huh7 cells were seeded 5×10^4 cells per well in 6-well plates and grown to greater than 50% confluency. These cells were transfected with 2 μg each protease and substrate plasmid (4 μg total DNA) and were harvested 24 hrs post transfection.

Western blotting for NS3 was done using 1:1000 monoclonal anti-NS3 mouse antibody (Novocastra Laboratories, Newcastle upon Tyne, UK) and 1:1000 goat-HRP anti-mouse secondary antibody (Amersham Biosciences).

Statistical significance was calculated based on two-tailed Student T-test.

Expression and purification of full-length NS3

Purification was done as previously reported with some minor modifications (17). pETNS3FL (17) was transformed into BL21(DE3)pLysS competent *E. coli* (Promega, Madison, WI, USA). A single transformed bacterial colony was grown at 37°C to an absorbance at 600 nm of 0.8 in Modified M9 Medium [5 g glucose per liter, 1 g ammonium sulfate per liter, 100 mM potassium phosphate (pH 7), 5 μM biotin, 7 μM thiamine, 0.5% Casamino Acids, 0.5 mM MgSO_4 , 0.5 mM CaCl_2 , 13 μM FeSO_4 , 100 $\mu\text{g}/\text{ml}$ ampicillin, and 50 $\mu\text{g}/\text{ml}$ kanamycin]. ZnCl_2 and isopropyl-beta-D-thiogalactopyranoside (IPTG) was then added for a final concentration of 100 μM and 600 μM , respectively, and induction of the NS3 continued at 31°C for 22 hrs. Protein was purified by nickel-affinity chromatography using an Äktapurifier FPLC (Amersham Biosciences). Elution was done by stepwise reduction in pH in 20 mM sodium phosphate buffer, 500 mM NaCl: pH 7.3, pH 6.0, pH 5.5, and pH 4.5. The pH 7.3 wash buffer contained 10 mM imidazole to displace weakly bound proteins. Purity and activity of eluted NS3 was assessed by coomassie blue stain of an SDS-gel and a previously described *in vitro* protease assay, respectively (21, 43). Protein concentration was determined by amino acid analysis (Advanced Protein Technology Centre, University of Toronto, CA).

References

1. **Bartenschlager, R., L. Ahlborn-Laake, J. Mous, and H. Jacobsen.** 1994. Kinetic and structural analyses of hepatitis C virus polyprotein processing. *J. Virol.* **68**:5045-5055.
2. **Bartenschlager, R., L. Ahlborn-Laake, K. Yasargil, J. Mous, and H. Jacobsen.** 1995. Substrate determinants for cleavage in cis and in trans by the hepatitis C virus NS3 proteinase. *J. Virol.* **69**:198-205.
3. **Bartenschlager, R., V. Lohmann, T. Wilkinson, and J. O. Koch.** 1995. Complex formation between the NS3 serine-type proteinase of the hepatitis C virus and NS4A and its importance for polyprotein maturation. *J. Virol.* **69**:7519-7528.
4. **Beyer, B. M., R. Zhang, Z. Hong, V. Madison, and B. A. Malcolm.** 2001. Effect of naturally occurring active site mutations on hepatitis C virus NS3 protease specificity. *Proteins* **43**:82-88.
5. **Bianchi, E. and A. Pessi.** 2002. Inhibiting viral proteases: challenges and opportunities. *Biopolymers* **66**:101-114.
6. **Blight, K. J., J. A. McKeating, and C. M. Rice.** 2002. Highly permissive cell lines for subgenomic and genomic hepatitis C virus RNA replication. *J. Virol.* **76**:13001-13014.
7. **Brass, V., E. Bieck, R. Montserret, B. Wolk, J. A. Hellings, H. E. Blum, F. Penin, and D. Moradpour.** 2002. An amino-terminal amphipathic alpha-helix mediates membrane association of the hepatitis C virus nonstructural protein 5A. *J. Biol. Chem.* **277**:8130-8139.
8. **Cheng, G., J. Zhong, and F. V. Chisari.** 2006. Inhibition of dsRNA-induced signaling in hepatitis C virus-infected cells by NS3 protease-dependent and -independent mechanisms. *Proc. Natl. Acad. Sci. U. S. A.* **103**:8499-8504.
9. **Choo, Q. L., G. Kuo, A. J. Weiner, L. R. Overby, D. W. Bradley, and M. Houghton.** 1989. Isolation of a cDNA clone derived from a blood-borne non-A, non-B viral hepatitis genome. *Science* **244**:359-362.

10. **De Francesco, R. and A. Carfi.** 2007. Advances in the development of new therapeutic agents targeting the NS3-4A serine protease or the NS5B RNA-dependent RNA polymerase of the hepatitis C virus. *Adv. Drug Deliv. Rev.* **59**:1242-1262.
11. **Dubuisson, J.** 2007. Hepatitis C virus proteins. *World J. Gastroenterol.* **13**:2406-2415.
12. **Dustin, L. B. and C. M. Rice.** 2007. Flying under the radar: the immunobiology of hepatitis C. *Annu. Rev. Immunol.* **25**:71-99.
13. **Egger, D., B. Wolk, R. Gosert, L. Bianchi, H. E. Blum, D. Moradpour, and K. Bienz.** 2002. Expression of hepatitis C virus proteins induces distinct membrane alterations including a candidate viral replication complex. *J. Virol.* **76**:5974-5984.
14. **Elazar, M., K. H. Cheong, P. Liu, H. B. Greenberg, C. M. Rice, and J. S. Glenn.** 2003. Amphipathic helix-dependent localization of NS5A mediates hepatitis C virus RNA replication. *J. Virol.* **77**:6055-6061.
15. **Failla, C., L. Tomei, and R. De Francesco.** 1994. Both NS3 and NS4A are required for proteolytic processing of hepatitis C virus nonstructural proteins. *J. Virol.* **68**:3753-3760.
16. **Ferreon, J. C., A. C. Ferreon, K. Li, and S. M. Lemon.** 2005. Molecular determinants of TRIF proteolysis mediated by the hepatitis C virus NS3/4A protease. *J. Biol. Chem.* **280**:20483-20492.
17. **Gallinari, P., D. Brennan, C. Nardi, M. Brunetti, L. Tomei, C. Steinkuhler, and R. De Francesco.** 1998. Multiple enzymatic activities associated with recombinant NS3 protein of hepatitis C virus. *J. Virol.* **72**:6758-6769.
18. **Gallinari, P., C. Paolini, D. Brennan, C. Nardi, C. Steinkuhler, and R. De Francesco.** 1999. Modulation of hepatitis C virus NS3 protease and helicase activities through the interaction with NS4A. *Biochemistry* **38**:5620-5632.
19. **Grakoui, A., D. W. McCourt, C. Wychowski, S. M. Feinstone, and C. M. Rice.** 1993. Characterization of the hepatitis C virus-encoded serine proteinase: determination of proteinase-dependent polyprotein cleavage sites. *J. Virol.* **67**:2832-2843.

20. **Hadziyannis, S. J. and J. S. Koskinas.** 2004. Differences in epidemiology, liver disease and treatment response among HCV genotypes. *Hepatol. Res.* **29**:129-135.
21. **Hamill, P. and F. Jean.** 2005. Enzymatic characterization of membrane-associated hepatitis C virus NS3-4A heterocomplex serine protease activity expressed in human cells. *Biochemistry* **44**:6586-6596.
22. **Huang, K. H., J. Bruneau, N. Shoukry, and N. F. Bernard.** 2008. Spontaneous resolution of hepatitis C virus infection is not due to a mutation at Cys-508 of MAVS/VISA/IPS-1/CARDIF. *J. Clin. Virol.*
23. **Kakiuchi, N., M. Hijikata, Y. Komoda, Y. Tanji, Y. Hirowatari, and K. Shimotohno.** 1995. Bacterial expression and analysis of cleavage activity of HCV serine proteinase using recombinant and synthetic substrate. *Biochem. Biophys. Res. Commun.* **210**:1059-1065.
24. **Kakiuchi, N., Y. Komoda, M. Hijikata, and K. Shimotohno.** 1997. Cleavage activity of hepatitis C virus serine proteinase. *J. Biochem.* **122**:749-755.
25. **Kakiuchi, N., S. Nishikawa, M. Hattori, and K. Shimotohno.** 1999. A high throughput assay of the hepatitis C virus nonstructural protein 3 serine proteinase. *J. Virol. Methods* **80**:77-84.
26. **Kim, J. L., K. A. Morgenstern, C. Lin, T. Fox, M. D. Dwyer, J. A. Landro, S. P. Chambers, W. Markland, C. A. Lepre, E. T. O'Malley, S. L. Harbeson, C. M. Rice, M. A. Murcko, P. R. Caron, and J. A. Thomson.** 1996. Crystal structure of the hepatitis C virus NS3 protease domain complexed with a synthetic NS4A cofactor peptide. *Cell* **87**:343-355.
27. **Kim, S. Y., K. W. Park, Y. J. Lee, S. H. Back, J. H. Goo, O. K. Park, S. K. Jang, and W. J. Park.** 2000. *In vivo* determination of substrate specificity of hepatitis C virus NS3 protease: genetic assay for site-specific proteolysis. *Anal. Biochem.* **284**:42-48.
28. **Kolykhalov, A. A., E. V. Agapov, and C. M. Rice.** 1994. Specificity of the hepatitis C virus NS3 serine protease: effects of substitutions at the 3/4A, 4A/4B, 4B/5A, and 5A/5B cleavage sites on polyprotein processing. *J. Virol.* **68**:7525-7533.

29. **Kolykhalov, A. A., K. Mihalik, S. M. Feinstone, and C. M. Rice.** 2000. Hepatitis C virus-encoded enzymatic activities and conserved RNA elements in the 3' nontranslated region are essential for virus replication *in vivo*. *J. Virol.* **74**:2046-2051.
30. **Konan, K. V., T. H. Giddings Jr, M. Ikeda, K. Li, S. M. Lemon, and K. Kirkegaard.** 2003. Nonstructural protein precursor NS4A/B from hepatitis C virus alters function and ultrastructure of host secretory apparatus. *J. Virol.* **77**:7843-7855.
31. **Landro, J. A., S. A. Raybuck, Y. P. Luong, E. T. O'Malley, S. L. Harbeson, K. A. Morgenstern, G. Rao, and D. J. Livingston.** 1997. Mechanistic role of an NS4A peptide cofactor with the truncated NS3 protease of hepatitis C virus: elucidation of the NS4A stimulatory effect via kinetic analysis and inhibitor mapping. *Biochemistry* **36**:9340-9348.
32. **Li, K., E. Foy, J. C. Ferreon, M. Nakamura, A. C. Ferreon, M. Ikeda, S. C. Ray, M. Gale Jr, and S. M. Lemon.** 2005. Immune evasion by hepatitis C virus NS3/4A protease-mediated cleavage of the Toll-like receptor 3 adaptor protein TRIF. *Proc. Natl. Acad. Sci. U. S. A.* **102**:2992-2997.
33. **Li, X. D., L. Sun, R. B. Seth, G. Pineda, and Z. J. Chen.** 2005. Hepatitis C virus protease NS3/4A cleaves mitochondrial antiviral signaling protein off the mitochondria to evade innate immunity. *Proc. Natl. Acad. Sci. U. S. A.* **102**:17717-17722.
34. **Lin, C., B. M. Pragai, A. Grakoui, J. Xu, and C. M. Rice.** 1994. Hepatitis C virus NS3 serine proteinase: trans-cleavage requirements and processing kinetics. *J. Virol.* **68**:8147-8157.
35. **Liu, R., K. Abid, J. Pichardo, V. Paziienza, P. Ingravallo, R. Kong, S. Agrawal, S. Bogen, A. Saksena, K. C. Cheng, A. Prongay, F. G. Njoroge, B. M. Baroudy, and F. Negro.** 2007. *In vitro* antiviral activity of SCH446211 (SCH6), a novel inhibitor of the hepatitis C virus NS3 serine protease. *J. Antimicrob. Chemother.* **59**:51-58.
36. **Lohmann, V., J. O. Koch, and R. Bartenschlager.** 1996. Processing pathways of the hepatitis C virus proteins. *J. Hepatol.* **24**:11-19.

37. **Manns, M. P., G. R. Foster, J. K. Rockstroh, S. Zeuzem, F. Zoulim, and M. Houghton.** 2007. The way forward in HCV treatment--finding the right path. *Nat. Rev. Drug Discov.* **6**:991-1000.
38. **Martin, C. S. and I. Bronstein.** 1994. Imaging of chemiluminescent signals with cooled CCD camera systems. *J. Biolumin. Chemilumin.* **9**:145-153.
39. **Martin, M. M. and F. Jean.** 2006. Single-cell resolution imaging of membrane-anchored hepatitis C virus NS3/4A protease activity. *Biol. Chem.* **387**:1075-1080.
40. **Moradpour, D., P. Kary, C. M. Rice, and H. E. Blum.** 1998. Continuous human cell lines inducibly expressing hepatitis C virus structural and nonstructural proteins. *Hepatology* **28**:192-201.
41. **Mottola, G., G. Cardinali, A. Ceccacci, C. Trozzi, L. Bartholomew, M. R. Torrisi, E. Pedrazzini, S. Bonatti, and G. Migliaccio.** 2002. Hepatitis C virus nonstructural proteins are localized in a modified endoplasmic reticulum of cells expressing viral subgenomic replicons. *Virology* **293**:31-43.
42. **Penin, F., V. Brass, N. Appel, S. Ramboarina, R. Montserret, D. Ficheux, H. E. Blum, R. Bartenschlager, and D. Moradpour.** 2004. Structure and function of the membrane anchor domain of hepatitis C virus nonstructural protein 5A. *J. Biol. Chem.* **279**:40835-40843.
43. **Richer, M. J., L. Juliano, C. Hashimoto, and F. Jean.** 2004. Serpin mechanism of hepatitis C virus nonstructural 3 (NS3) protease inhibition: induced fit as a mechanism for narrow specificity. *J. Biol. Chem.* **279**:10222-10227.
44. **Sali, D. L., R. Ingram, M. Wendel, D. Gupta, C. McNemar, A. Tsarbopoulos, J. W. Chen, Z. Hong, R. Chase, C. Risano, R. Zhang, N. Yao, A. D. Kwong, L. Ramanathan, H. V. Le, and P. C. Weber.** 1998. Serine protease of hepatitis C virus expressed in insect cells as the NS3/4A complex. *Biochemistry* **37**:3392-3401.
45. **Sansonno, D., A. R. Iacobelli, V. Cornacchiulo, M. Distasi, and F. Dammacco.** 1995. Immunohistochemical detection of hepatitis C virus-related proteins in liver tissue. *Clin. Exp. Rheumatol.* **13 Suppl 13**:S29-32.

46. **Sardana, V. V., J. T. Blue, J. Zugay-Murphy, M. K. Sardana, and L. C. Kuo.** 1999. An uniquely purified HCV NS3 protease and NS4A(21-34) peptide form a highly active serine protease complex in peptide hydrolysis. *Protein Expr. Purif.* **16**:440-447.
47. **Schechter, I. and A. Berger.** 1967. On the size of the active site in proteases. I. Papain. *Biochem. Biophys. Res. Commun.* **27**:157-162.
48. **Shimizu, Y., K. Yamaji, Y. Masuho, T. Yokota, H. Inoue, K. Sudo, S. Satoh, and K. Shimotohno.** 1996. Identification of the sequence on NS4A required for enhanced cleavage of the NS5A/5B site by hepatitis C virus NS3 protease. *J. Virol.* **70**:127-132.
49. **Steinkuhler, C., L. Tomei, and R. De Francesco.** 1996. *In vitro* activity of hepatitis C virus protease NS3 purified from recombinant Baculovirus-infected Sf9 cells. *J. Biol. Chem.* **271**:6367-6373.
50. **Steinkuhler, C., A. Urbani, L. Tomei, G. Biasiol, M. Sardana, E. Bianchi, A. Pessi, and R. De Francesco.** 1996. Activity of purified hepatitis C virus protease NS3 on peptide substrates. *J. Virol.* **70**:6694-6700.
51. **Tanji, Y., M. Hijikata, Y. Hirowatari, and K. Shimotohno.** 1994. Hepatitis C virus polyprotein processing: kinetics and mutagenic analysis of serine proteinase-dependent cleavage. *J. Virol.* **68**:8418-8422.
52. **Tanji, Y., M. Hijikata, S. Satoh, T. Kaneko, and K. Shimotohno.** 1995. Hepatitis C virus-encoded nonstructural protein NS4A has versatile functions in viral protein processing. *J. Virol.* **69**:1575-1581.
53. **Taremi, S. S., B. Beyer, M. Maher, N. Yao, W. Prosise, P. C. Weber, and B. A. Malcolm.** 1998. Construction, expression, and characterization of a novel fully activated recombinant single-chain hepatitis C virus protease. *Protein Sci.* **7**:2143-2149.
54. **The Global Burden Of Hepatitis C Working Group.** 2004. Global burden of disease (GBD) for hepatitis C. *J. Clin. Pharmacol.* **44**:20-29.

55. **Thomson, J. A. and R. B. Perni.** 2006. Hepatitis C virus NS3-4A protease inhibitors: countering viral subversion *in vitro* and showing promise in the clinic. *Curr. Opin. Drug Discov. Devel.* **9**:606-617.
56. **Tomei, L., C. Failla, E. Santolini, R. De Francesco, and N. La Monica.** 1993. NS3 is a serine protease required for processing of hepatitis C virus polyprotein. *J. Virol.* **67**:4017-4026.
57. **Tomei, L., C. Failla, R. L. Vitale, E. Bianchi, and R. De Francesco.** 1996. A central hydrophobic domain of the hepatitis C virus NS4A protein is necessary and sufficient for the activation of the NS3 protease. *J. Gen. Virol.* **77 (Pt 5)**:1065-1070.
58. **Vishnuvardhan, D., N. Kakiuchi, P. T. Urvil, K. Shimotohno, P. K. Kumar, and S. Nishikawa.** 1997. Expression of highly active recombinant NS3 protease domain of hepatitis C virus in *E. coli*. *FEBS Lett.* **402**:209-212.
59. **Wakita, T., T. Pietschmann, T. Kato, T. Date, M. Miyamoto, Z. Zhao, K. Murthy, A. Habermann, H. G. Krausslich, M. Mizokami, R. Bartenschlager, and T. J. Liang.** 2005. Production of infectious hepatitis C virus in tissue culture from a cloned viral genome. *Nat. Med.* **11**:791-796.
60. **Welbourn, S. and A. Pause.** 2007. The hepatitis C virus NS2/3 protease. *Curr. Issues Mol. Biol.* **9**:63-69.
61. **Wolk, B., D. Sansonno, H. G. Krausslich, F. Dammacco, C. M. Rice, H. E. Blum, and D. Moradpour.** 2000. Subcellular localization, stability, and trans-cleavage competence of the hepatitis C virus NS3-NS4A complex expressed in tetracycline-regulated cell lines. *J. Virol.* **74**:2293-2304.
62. **Zhang, R., J. Durkin, W. T. Windsor, C. McNemar, L. Ramanathan, and H. V. Le.** 1997. Probing the substrate specificity of hepatitis C virus NS3 serine protease by using synthetic peptides. *J. Virol.* **71**:6208-6213.

Chapter 4

Dissecting HCV NS4A binding and activation of NS3 protease⁶

Introduction

The members of the virus family *Flaviviridae*, genus *flavivirus* includes many important human pathogens: yellow fever virus (YFV), West Nile virus (WNV), Dengue virus (DENV), and many encephalitis viruses (EVs). Although, vaccines are available for YFV, Japanese EV (JEV) and Tick-borne EV, their limited use has allowed these viruses continue to cause disease in humans (4, 16, 17). YFV, DENV, and JEV are of extreme importance for human health as together they cause an estimated 50,250,000 infections per year worldwide (15).

In 1989, a new genus, *hepacivirus*, was added to the *Flaviviridae* family with the identification of the Hepatitis C virus (HCV) (10). This virus shares remarkable similarity to the *flaviviruses* although it neither uses a vector for transmission nor is encephalitic, as it mainly infects liver tissue. Another significant difference is that HCV establishes a chronic infection in the vast majority of patients; currently it is estimated that around 2.2% of the worldwide population is chronically infected with HCV (1). Despite an urgent need, no specific therapeutics have been approved for human use for any member in this virus family despite decades of research to this end.

Viruses belonging to the *Flaviviridae* family have single-stranded positive-sense RNA genomes that are replicated via large macromolecular protein complexes ('replicase' complexes) tethered to intracellular membranes (29). Their proteins are encoded in one continuous open reading frame and they rely upon proteolysis to cleave the polyprotein precursor into individual proteins. Non-structural (NS) protein 3 (NS3) plays a major role in cleavage of this polyprotein and is therefore a very attractive drug target (5, 11, 22, 34).

⁶ A version of this chapter will be submitted for publication. Martin, M.M., Wang, P., Chau, A., Mosher, D., Verreault, C., Jonas, A., and F. Jean. Dissecting hepatitis C virus NS4A binding and activation of NS3 protease.

For HCV, there are NS3 active site inhibitors in clinical trials, however, many of these drugs are rendered ineffective by a single point mutation in or near the NS3 active site (12, 18, 30, 39). In addition to discovery and development of active site inhibitors for this protease, allosteric inhibitors should be developed that target structural sites and protein-protein interaction sites, in order to slow the emergence of drug resistant variants by treatment of patients with a multi-drug regimen (28).

HCV NS3 serine protease non-covalently associates with a viral cofactor, NS4A (homologue of *flavivirus* NS2B). NS4A is a small protein of only fifty-four amino acids. The central portion (residues 21-34) integrates into the NS3 fold, becoming a β -strand in the N-terminal eight-strand β -barrel (19). This interaction is essential for stabilization of the NS3 aspartic acid catalytic residue 81 (D81) in the proper alignment. A synthetic peptide corresponding to the central residues of NS4A (pep4A) has been used to activate NS3 *in vitro* (6, 33-35).

The N-terminal 20 residues of NS4A are predicted to form a membrane-spanning α -helix which allows NS4A to localize NS3 at the ER membrane (26). This localization is essential for NS3 integration into the replicase complex (14). The role of the C-terminal 20 residues (35-54) of NS4A is not fully understood. Residues 35 to 39 are predicted to form a structural kink and the remainder is able to fold into an alpha-helix upon binding to a basic surface (24). This region is thought to interact with NS3 and/or NS5A, and may be important for the regulation of NS5A phosphorylation (20).

Analysis of NS4A residues important for NS3 activation has previously been done and has focused on residues 21 to 34, however the results have been confusing to interpret due to the inability of many assays to distinguish between binding and activation defects and also due to the varying of assay systems, amino acid substitutions, and protease substrates between studies. Butkiewicz *et al.* (9) and Shimizu *et al.* (32) used *in vitro* assay systems with pep4A and identified NS4A residues V24, I25, and I29 as being important for activation. However, Lin *et al.* (23) and Bartenschlager *et al.* (6) reported I25 mutants that did not affect NS3 cleavage. For residues V23, V26, G27, R28, L31, S32, and R34, the ability to affect activation of NS3 varied between reports (6, 9, 23, 32). Shimizu *et al.* (32) found that greater activation of NS3 could be achieved by using higher concentrations of these pep4A mutants, indicating that for the majority of these activation-impaired pep4A mutants decreased binding of pep4A to NS3 was probably

responsible for the decreased activity. The only mutant that was concentration independent was I29S, indicating this mutant either was unable bind NS3 at all or it was unable to activate once bound. Lin *et al.* (23) showed that *in vivo* NS4A(I29S) did not immunoprecipitate with NS3, suggesting that this mutant is deficient in binding. A clearer understanding of the binding and activation determinants of NS4A is needed to design inhibitors to this interaction.

Here we aimed to dissect NS4A binding and activation in cell-based systems. We were interested not only on the impact of residues located in the activating domain of NS3, but also in residues in the kink region and the acidic domain. We introduced single alanine point mutations in NS4A and measured their impact on activation and binding of NS3. We have utilized a previously described cell-based protease assay to assess activation and report the development and application of a novel fluorescent resonance energy transfer (FRET)-based assay to measure heterocomplex formation. We confirm that I29 is involved in NS3 binding, as is I25, and that a pocket of activation-essential residues exists around V23.

Results

In order to identify NS4A residues important for NS3 activation, NS3-4A was subcloned into a eukaryotic expression vector and single point mutations to alanine were made in the NS4A activation domain, kink region, and acidic domain (Illustration 4.1). A cell-based protease assay was used to measure *in vivo* protease activation (25). The substrate, Tm-4B/5A-DsRed, contains a decapeptide cleavage sequence (P6-P4') corresponding to the activation-dependent NS4B/NS5A junction. In this assay, a membrane tethered reporter (DsRed-Express) is released upon NS3-4A proteolysis; the change in localization of DsRed-Express or in the size of the substrate can be detected by fluorescent microscopy or Western blotting, respectively.

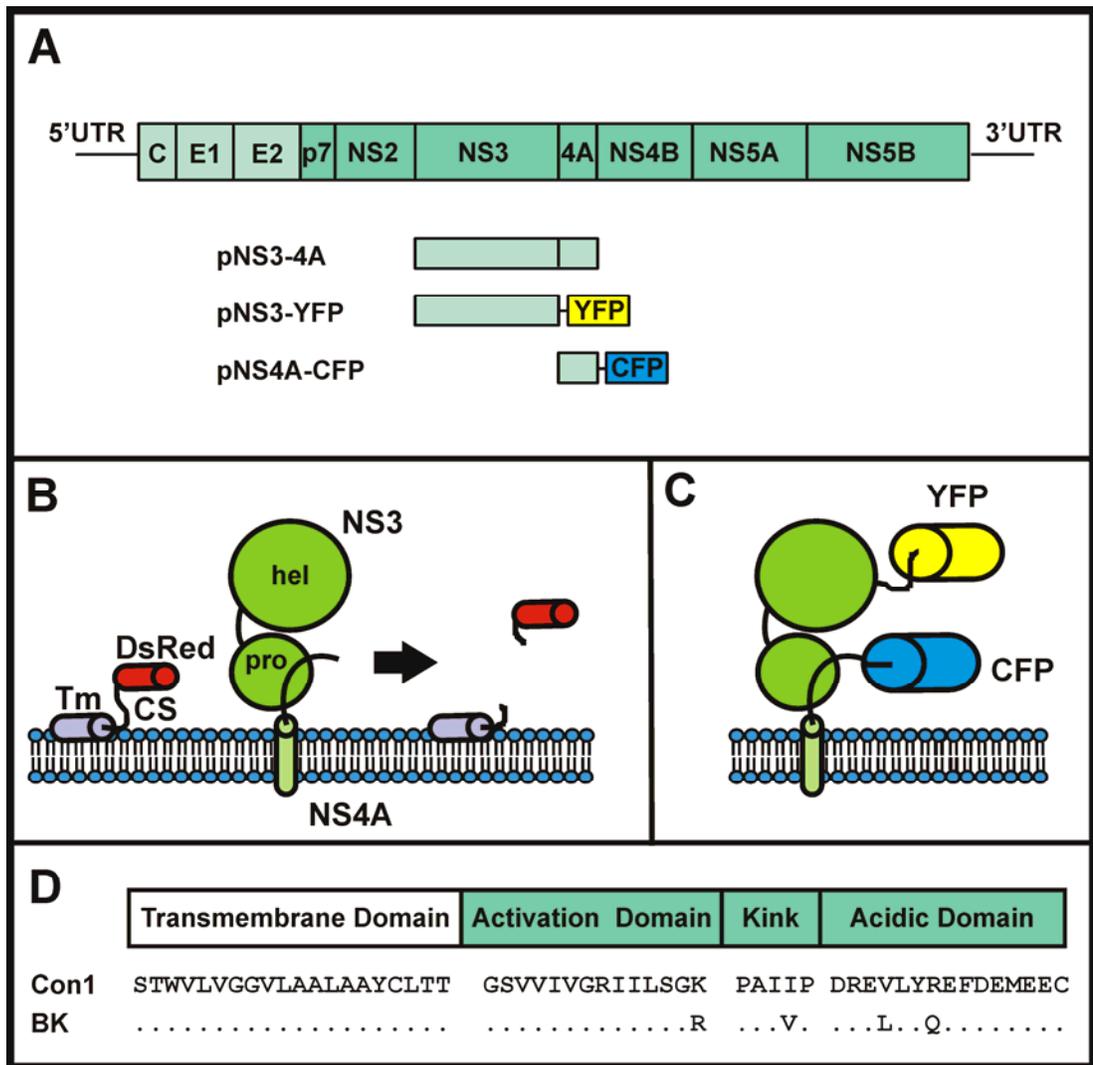


Illustration 4.1 DNA constructs used in this study

A) Illustration of HCV genome and NS3 and NS4A constructs. pNS3-4A is Con1 strain (genotype 1a). pNS3-CFP and pNS4A-YFP are BK strain (genotype 1b). B) Intracellular membrane-anchored protein substrates comprise a targeting domain (Tm), a NS4B/NS5A cleavage sequence (CS), and a DsRed-Express reporter group (DsRed). When the substrate is expressed with NS3-4A, cleavage of the CS releases DsRed into the cytosol. C) When NS3-YFP and NS4A-CFP fusion proteins are expressed together, they associate, bringing YFP and CFP into close proximity. D) Domains of NS4A defined as in Lindenbach *et al.* (24). In this study, green regions (activation domain, kink, and acidic domain) have been systematically alanine scanned in pNS3-4A and pNS4A-CFP.

NS3-4A wild-type and alanine mutant-expressing plasmids were titrated against a constant amount of substrate and protease activity was measured by comparing percentage of cleaved substrate after 24 hours (Figure 4.1). We observed two mutations, V23A and I25A, which dramatically reduced cleavage. For these two mutations, cleavage was not increased by expression of more protease, indicating a complete lack of activation or binding. The mutant NS3-4A(V24A) showed a reduction in protease cleavage as compared to the wild-type control, but cleavage could be increased with expression of more protease, indicating a partial activation or binding defect.

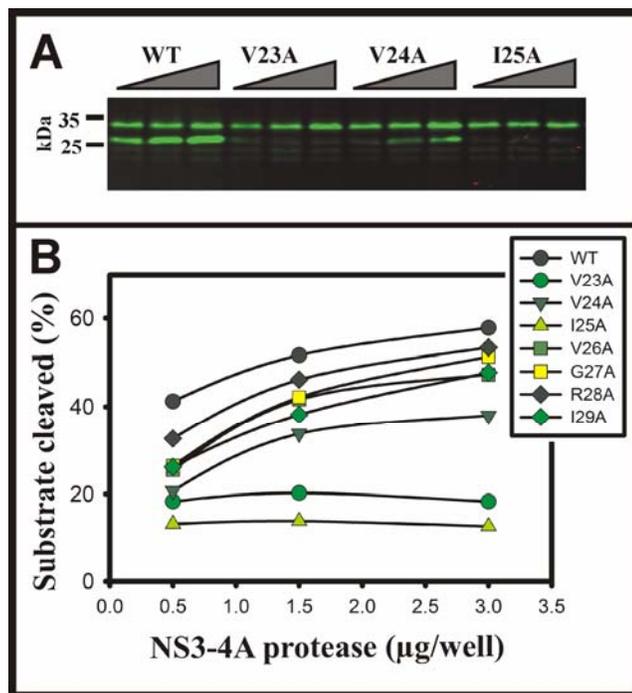


Figure 4.1 NS3-4A(V23A) and NS3-4A(I25A) are not proteolytically active

A) Titration of 0.5, 1.5, and 3.0 µg/well of NS3-4A against substrate (2 µg/well). Cleavage visualized by Western blotting for DsRed-Express. B) Quantitation of percent substrate cleaved. Average of 2 separate experiments. WT = wild type.

In order to determine if these activation defects were due to reductions in NS4A binding, a fluorescence resonance energy transfer (FRET) system was developed to measure NS3-NS4A complex formation. FRET is a powerful tool to investigate protein-protein interactions. The method depends upon the transfer of energy between a fluorescent donor and a fluorescent acceptor which are in close proximity (usually 10-100Å). The fluorophores are selected such that the emission wavelength of the donor group overlaps with the excitation wavelength of the

acceptor group; direct excitation of the donor group will result in transfer of energy to the acceptor fluorophore which will then emit fluorescence. A commonly used donor/acceptor pair is the green fluorescent protein variants, cyan fluorescent protein (CFP) and yellow fluorescent protein (YFP) (27).

NS3 was subcloned into the pYFP-N1 vector and NS4A was subcloned into the pECFP-N1 vector such that the YFP and CFP would be C-terminal to NS3 and NS4A (Illustration 4.1). To determine the impact of these large protein tags on NS3-4A association and activation, the fusion proteins were expressed with the activation dependent substrate, Tm-4B/5A-DsRed, and analyzed by microscopy (Figure 4.2). Tm-4B/5A-DsRed expressed alone was punctuate and perinuclear as previously reported (Figure 4.2A). NS3-YFP expressed with Tm-4B/5A-DsRed was located diffusely in the cytoplasm and nucleus was not able to process the substrate, as expected (Figure 4.2B). Triple transfection of NS3-YFP, NS4A-CFP, and Tm-4B5A-DsRed showed recruitment of NS3 to perinuclear membranes. The release of DsRed-Express into the cytoplasm proved that the fluorescent protein fusions of NS3 and NS4A form an active complex in which NS4A has properly activated NS3 (Figure 4.2C).

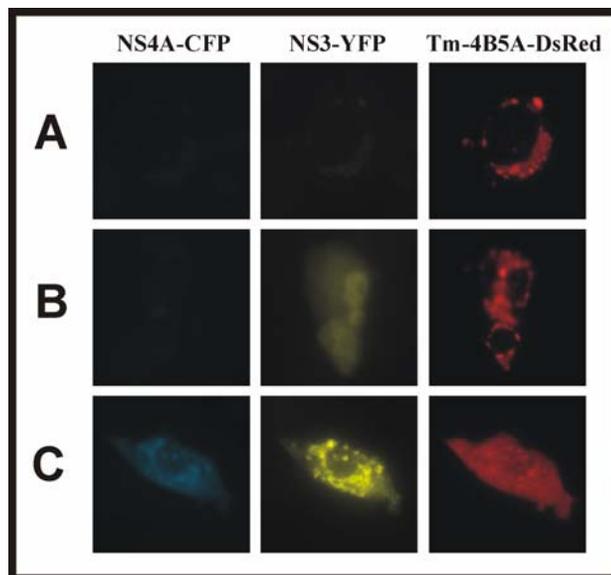


Figure 4.2 NS3-YFP and NS4A-CFP form an active heterocomplex.

Fluorescent microscope images of: A) Tm-4B5A-DsRed only, B) NS3-YFP and Tm-4B5A-DsRed, and C) NS3-YFP, NS4A-CFP, and Tm-4B5A-DsRed.

To measure FRET between NS3-YFP and NS4A-CFP, we chose to use the sensitized emission method of FRET detection in order to avoid problems with the acceptor photobleaching method (36). In sensitized emission, a FRET filter set is used that allows excitation of CFP and detection of emission from YFP. This FRET image (FRET^{raw}) must be corrected for CFP emission into the FRET emission filter (“bleedthrough”) and YFP emission due to YFP excitation via the CFP excitation filter (“crosstalk”) (Figure 4.3). This corrected, “calculated” FRET image (FRET^{cal}) is then divided by either the CFP or YFP image for a normalized FRET value that represents the amount of FRET transfer per fluorophore. This value can be compared between different mutants to show increased or decreased FRET due to a conformational change in the complex.

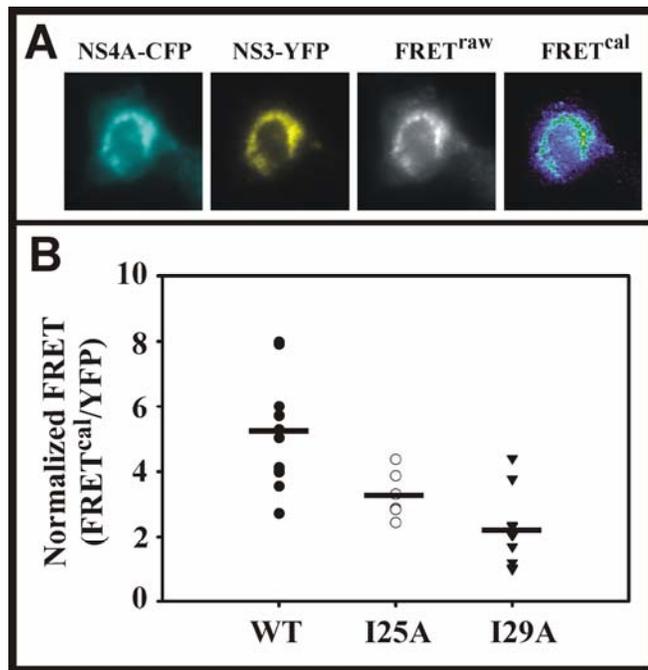


Figure 4.3 I29 and I25 are important for NS3-YFP and NS4A-CFP to undergo FRET

A) FRET microscopy showing raw FRET image (FRET^{raw}) and calculated FRET image (FRET^{cal}). B) Normalized FRET values. Each point represents one analyzed cell and the average value is shown by the line. I25A $P = 0.02$, I29A $P = 0.0002$. WT = wild type.

We chose to normalize to the YFP signal incase there was a difference in the amount of NS3 expressed as compared to NS4A. If more NS3-YFP was expressed than NS4A-CFP, the excess NS3-YFP would not localize to the membrane and would be located diffusely in the cytoplasm and nucleus, as observed in Figure 4.2B when NS3-YFP is expressed alone. In the

opposite case, where an excess of NS4A-CFP is expressed as compared to NS3-YFP, normalization to the YFP signal, normalizes the data to the number of heterocomplexes present.

Analysis of alanine point mutations in the activation domain, the kink region, and the acidic domain showed two mutations that were able to significantly change the FRET effect: NS4A(I25A)-CFP and NS4A(I29A)-CFP. The normalized FRET signal for wild-type NS4A-CFP was 5.2 ± 1.4 . In both cases, the FRET signal was decreased, indicating less FRET transfer between CFP and YFP. I29A showed a larger and more significant decrease to 2.2 ± 1.2 , as compared to I25A which reduced the signal to 3.3 ± 0.7 .

For both of these mutants, it was observed that they showed association defects (Figure 4.4). NS4A(I29A)-CFP was not able to recruit NS3-YFP and NS4A(I25A)-CFP was only able to partially localize NS3-YFP.

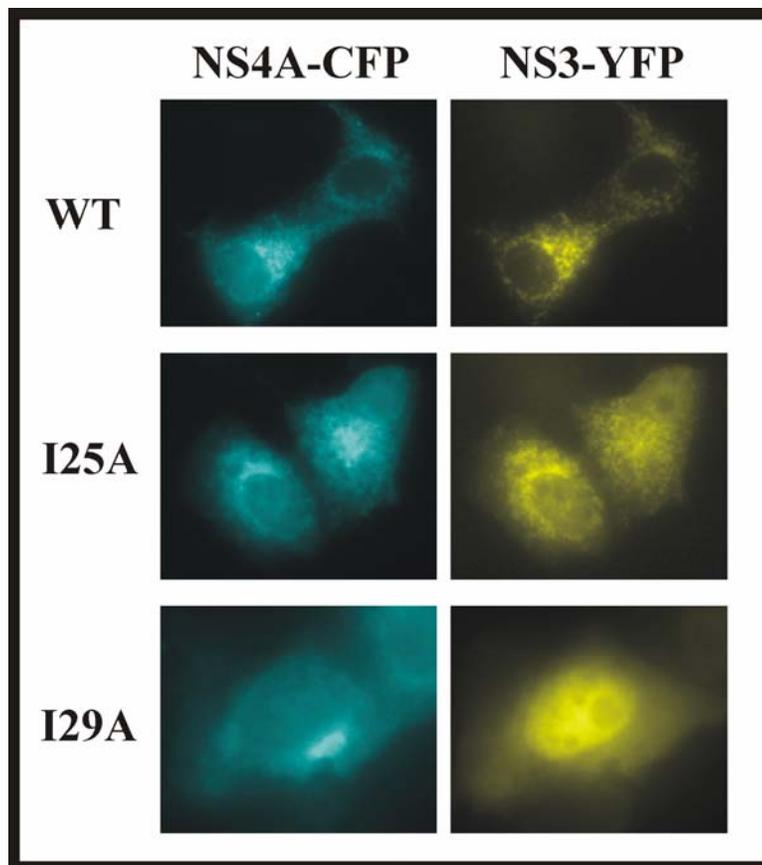


Figure 4.4 NS4A(I29A)-CFP and NS4A(I25A)-CFP show binding defects

Fluorescent microscopy images showing partial and full dissociation of NS3-YFP from I25A and I29A mutants, respectively. WT = wild type

Discussion

Alanine scanning of residues important for activation identified a patch of residues important for NS4A activation of NS3: V23, V24, and V25. Although V24 had been identified as important for NS3 activation prior to this study, the important impact of V23 was not fully appreciated because of inconsistent reports (9, 23). Also, our data clarifies the conflicting reports as to the importance of I25 as NS4A(I25A) was shown to induce a breakdown in complex formation or stability, as seen by partial co-localization of this mutant with NS3. This partial phenotype may be responsible for the varied response of I25 mutants in different experimental systems (6, 9, 23, 32). NS4A(V23A) and NS4A(V24A) however showed neither a change in association nor a decrease in FRET. These residues interact directly with the D1/E1 loop of the NS3 N-terminal β -barrel (Figure 4.5A). It can be hypothesized that these residues push the loop away from the NS3 N-terminus, stabilizing D81 in the active site.

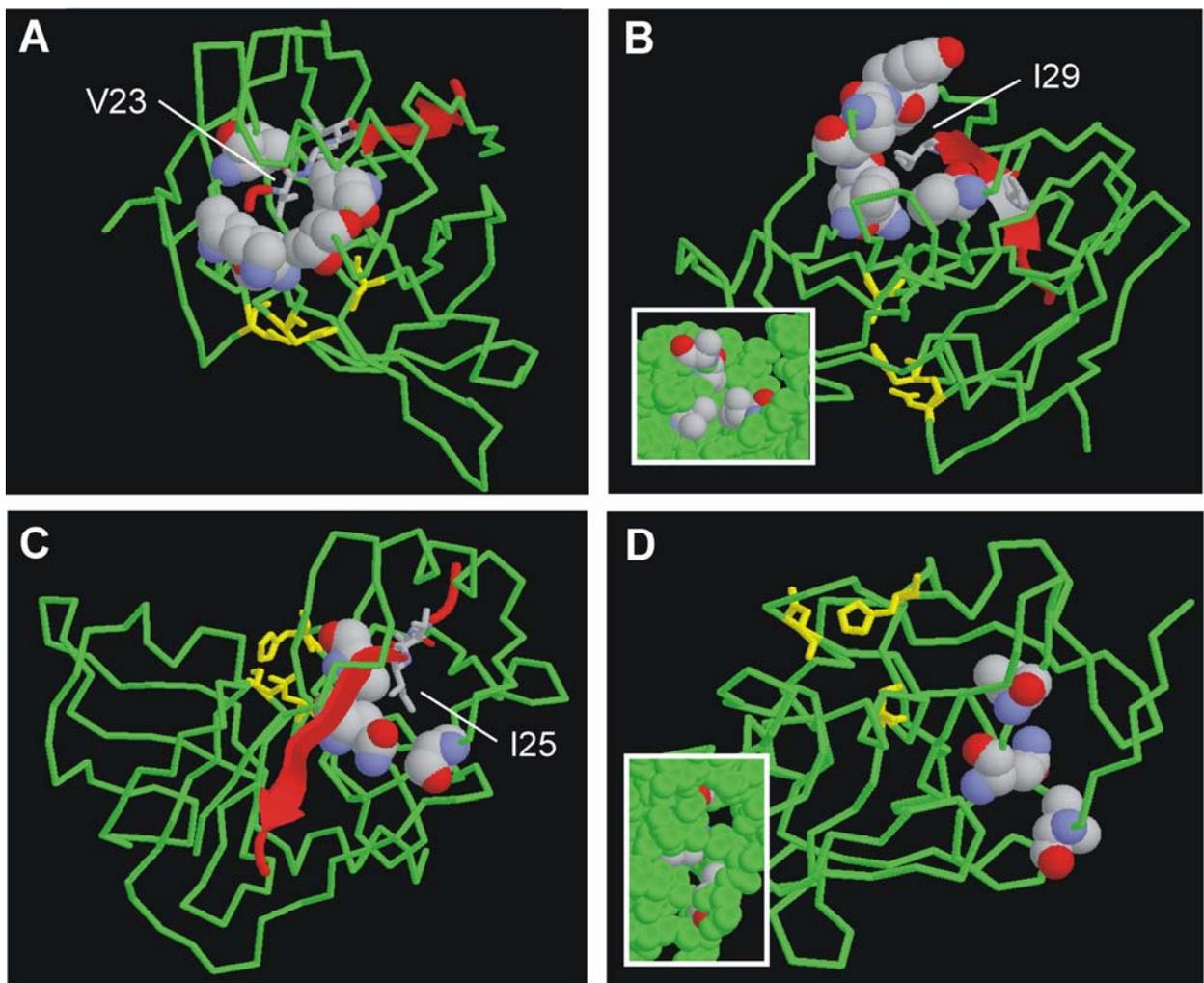


Figure 4.5 Model of I29, I25, and V23 binding pockets

Structure of NS3^{pro}-pep4A showing residues of NS3 that interact with V23 (A), I29 (B) and I25 (C) in space fill. Inset is the space fill display of the surface of NS3^{pro} without pep4A bound, showing access to the I29 binding pocket (C) and the buried and misformed nature of the I25 binding pocket (D). NS3-4A crystal structure from 1CU1 in PDB, originally from Yao *et al.* (38). NS3 alone NMR structural data from 1BT7 in PDB, originally from Barbato *et al.* (3). Catalytic residues are shown in yellow and pep4A is displayed as a red ribbon. Image created with RasMol (31).

Two single point mutations (I29A and I25A) were able to disrupt NS3 binding: I29A completely and I25A partially. We hypothesize that I29A is involved in initial recognition and I25A is involved in stable complex formation. I29 interacts with a pocket of hydrophobic residues in NS3 that is accessible in the NS3-alone structure (Figure 4.5B). In contrast, the I25 binding pocket is not accessible in this structure and also is malformed (Figure 4.5C,D). We hypothesize structural rearrangements from I29 binding may trigger a zipper-like incorporation of NS4A into the NS3 fold, anchored by I29 and I25. The binding of I29 and I25 forces V23 to sterically move the D1/E1 loop, which properly aligns the active site.

In general, it is surprising that a single, conservative mutation such as hydrophobic valine or isoleucine to a hydrophobic alanine can have such a dramatic effect on activation and/or binding, especially in a long stretch of hydrophobic residues. It is however encouraging for drug development and identification of the activating and binding contribution of NS4A in a cell based system is extremely significant as these areas may now be targeted to disrupt protease activation *in vivo*. The confirmation of I29 as the key residue for association is important as the I29 binding site, partially formed in NS3 alone, can be targeted for the development and discovery of small molecule inhibitors. We were concerned that the YFP and CFP tag on NS3 and NS4A would partially destabilize the complex so that the effects of mutations might exaggerated in the FRET system, however the detection of only two deleterious mutations is not consistent with this hypothesis.

The apparent contradiction between I29A not binding in the FRET assay but not showing an activation defect can be explained by the differences in the two systems. In the activation system, NS3-4A is expressed as a proprotein which folds and associates in *cis* prior to NS3/NS4A cleavage (37), bypassing the proposed I29 requirement for initial recognition. In the FRET system, the proteins are expressed as separate proteins and must associate in *trans*, thereby depending on I29-mediated initial recognition. It is also possible that the presence of substrate in the activation assays may change the conformation of NS3 allowing NS4A(I29A) to bind. NS3 has been shown to undergo conformational changes upon substrate binding by an “induced fit” mechanism (2, 7). Rearrangement upon substrate binding may stabilize NS3 in an activated conformation, exposing the I25 binding pocket and facilitating binding of NS4A(I29A).

The FRET system used to measure NS3 and NS4A complex association is extremely novel and can be used to characterize the mechanism of action of allosteric inhibitors, specifically NS4A-antagonists.

Materials and Methods

Construction of plasmid DNA

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

(i) *pTm-4B/5A-DsRed and pNS3-4A_Con1*. pTm-4B/5A-DsRed has been described previously (25). This plasmid encodes for the HCV NS5A 31 N-terminal amino acid residues, which correspond to an amphipathic α -helix, referred to here as “Tm”, followed by a sequence encoding for the NS4B/NS5A cleavage sequence (P6-P4’), all of which is upstream of the DsRed-Express gene in pDsRed-Express-N1 (Clontech, Mountain View, CA, USA). pNS3-4A encodes for NS3-4A from pCon1/FL(I) (8) expressed without any tag under control of CMV promoter.

(ii) *pNS3-CFP and pNS4A-YFP*. NS3 and NS4A were PCR amplified from pETNS34A (13) and subcloned into the pEYFP-N1 and pECFP-N1 vectors (Clontech) using AgeI and XhoI restriction endonucleases. This cloning resulted in YFP and CFP being C-terminal to NS3 and NS4A, respectively.

All point mutations were introduced using Quikchange site-directed mutagenesis (Quikchange, Stratagene). Constructs were verified by sequencing.

Cell culture

Huh7 cells were propagated in DMEM supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 100 μ M non-essential amino acids, 50 units/mL penicillin, and 50 μ g/mL streptomycin. Vero cells were propagated in DMEM supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 50 units/mL penicillin, and 50 μ g/mL streptomycin.

All cells were grown at 37°C in 5% CO₂.

Transfections and Western blotting

Transfections and Western blotting was done as previously described (25). 6-well plates were seeded with 5×10^4 Huh7 cells and grown for 2-3 days until greater than 50% confluent. Cells were transfected with 2 μg per well of pTm-4B/5A-DsRed DNA plasmid and various amounts of pNS3-4A using TransIT-LT1 transfection reagent (Mirus Bio, Madison, WI, USA); for titration experiments, 0.5, 1.5 and 3.0 μg of DNA was used. 24 hrs 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, 1X complete EDTA-free protease inhibitors (Roche, Laval, 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 hours at 110 V and transferred to a nitrocellulose membrane. The membrane was probed with polyclonal anti-DsRed (1:1,000, Clontech, Mountain View, CA, USA) and goat-680 anti-rabbit secondary antibody (1:4,000). Quantitation of the enhanced chemiluminescent signal was performed on an Odyssey imager (Licor). 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.

Statistical significance was calculated based on two-tailed Student T-test.

Fluorescence microscopy

Microscopy was done as previously described (25). 5×10^4 cells Vero cells were seeded in 24-well plates on top of glass coverslips (13 mm diameter, 1.5 thickness) and grown for 1 day (>50% confluent). Cells were transfected with 1 μg /well of DNA plasmid using TransIT-LT1 transfection reagent (Mirus Bio, Madison, WI, USA). 24 hrs post transfection cells were washed with PBS and fixed in 3.8% formaldehyde for 30 min. Images were acquired using a Discovery-1 fluorescent microscope.

FRET microscopy

Samples were prepared as for fluorescent imaging except cells were transfected with 1.25 μg NS4A-CFP, 1.5 μg NS3-YFP and 5 μl DNAfectin (Mirus Bio, Madison, WI, USA) per well. Cells were fixed 12-14 hours post transfection. Three sets of images were acquired using a Discovery-1 fluorescent microscope: a CFP image, a YFP image, and a FRET image. Bleedthrough and crosstalk values were determined by measuring the percentage of fluorescence that was detectable in the FRET filter set with single transfections of NS3-YFP or NS4A-CFP. The values were determined to be about 30% for CFP and 5% for YFP, however these values were checked during every experiment. The FRET^{cal} image was created using the following formula: $\text{FRET}^{\text{cal}} = \text{FRET}^{\text{raw}} - \text{CFP} * (\text{bleedthrough coefficient}) - \text{YFP} * (\text{crosstalk coefficient})$. The FRET^{cal} image was then divided by the YFP image to give a normalized FRET image. The value at the position of NS3-YFP and NS4A-CFP colocalization was averaged to give the normalized FRET value for that sample.

References

1. **Alter, M. J.** 2007. Epidemiology of hepatitis C virus infection. *World J. Gastroenterol.* **13**:2436-2441.
2. **Barbato, G., D. O. Cicero, F. Cordier, F. Narjes, B. Gerlach, S. Sambucini, S. Grzesiek, V. G. Matassa, R. De Francesco, and R. Bazzo.** 2000. Inhibitor binding induces active site stabilization of the HCV NS3 protein serine protease domain. *EMBO J.* **19**:1195-1206.
3. **Barbato, G., D. O. Cicero, M. C. Nardi, C. Steinkuhler, R. Cortese, R. De Francesco, and R. Bazzo.** 1999. The solution structure of the N-terminal proteinase domain of the hepatitis C virus (HCV) NS3 protein provides new insights into its activation and catalytic mechanism. *J. Mol. Biol.* **289**:371-384.
4. **Barrett, A. D. and S. Higgs.** 2007. Yellow fever: a disease that has yet to be conquered. *Annu. Rev. Entomol.* **52**:209-229.
5. **Bartenschlager, R., L. Ahlborn-Laake, J. Mous, and H. Jacobsen.** 1994. Kinetic and structural analyses of hepatitis C virus polyprotein processing. *J. Virol.* **68**:5045-5055.
6. **Bartenschlager, R., V. Lohmann, T. Wilkinson, and J. O. Koch.** 1995. Complex formation between the NS3 serine-type proteinase of the hepatitis C virus and NS4A and its importance for polyprotein maturation. *J. Virol.* **69**:7519-7528.
7. **Bianchi, E., S. Orru, F. Dal Piaz, R. Ingenito, A. Casbarra, G. Biasiol, U. Koch, P. Pucci, and A. Pessi.** 1999. Conformational changes in human hepatitis C virus NS3 protease upon binding of product-based inhibitors. *Biochemistry* **38**:13844-13852.
8. **Blight, K. J., J. A. McKeating, and C. M. Rice.** 2002. Highly permissive cell lines for subgenomic and genomic hepatitis C virus RNA replication. *J. Virol.* **76**:13001-13014.

9. **Butkiewicz, N. J., M. Wendel, R. Zhang, R. Jubin, J. Pichardo, E. B. Smith, A. M. Hart, R. Ingram, J. Durkin, P. W. Mui, M. G. Murray, L. Ramanathan, and B. Dasmahapatra.** 1996. Enhancement of hepatitis C virus NS3 proteinase activity by association with NS4A-specific synthetic peptides: identification of sequence and critical residues of NS4A for the cofactor activity. *Virology* **225**:328-338.
10. **Choo, Q. L., G. Kuo, A. J. Weiner, L. R. Overby, D. W. Bradley, and M. Houghton.** 1989. Isolation of a cDNA clone derived from a blood-borne non-A, non-B viral hepatitis genome. *Science* **244**:359-362.
11. **Failla, C., L. Tomei, and R. De Francesco.** 1994. Both NS3 and NS4A are required for proteolytic processing of hepatitis C virus nonstructural proteins. *J. Virol.* **68**:3753-3760.
12. **Forestier, N., H. W. Reesink, C. J. Weegink, L. McNair, T. L. Kieffer, H. M. Chu, S. Purdy, P. L. Jansen, and S. Zeuzem.** 2007. Antiviral activity of telaprevir (VX-950) and peginterferon alfa-2a in patients with hepatitis C. *Hepatology* **46**:640-648.
13. **Gallinari, P., C. Paolini, D. Brennan, C. Nardi, C. Steinkuhler, and R. De Francesco.** 1999. Modulation of hepatitis C virus NS3 protease and helicase activities through the interaction with NS4A. *Biochemistry* **38**:5620-5632.
14. **Gosert, R., W. Jendrschok, J. M. Berke, V. Brass, H. E. Blum, and D. Moradpour.** 2005. Characterization of nonstructural protein membrane anchor deletion mutants expressed in the context of the hepatitis C virus polyprotein. *J. Virol.* **79**:7911-7917.
15. **Gould, E. A. and T. Solomon.** 2008. Pathogenic flaviviruses. *Lancet* **371**:500-509.
16. **Gritsun, T. S., P. A. Nuttall, and E. A. Gould.** 2003. Tick-borne flaviviruses. *Adv. Virus Res.* **61**:317-371.
17. **Kaur, R. and S. Vрати.** 2003. Development of a recombinant vaccine against Japanese encephalitis. *J. Neurovirol.* **9**:421-431.
18. **Kieffer, T. L., C. Sarrazin, J. S. Miller, M. W. Welker, N. Forestier, H. W. Reesink, A. D. Kwong, and S. Zeuzem.** 2007. Telaprevir and pegylated interferon-alpha-2a inhibit wild-type and resistant genotype 1 hepatitis C virus replication in patients. *Hepatology* **46**:631-639.

19. **Kim, J. L., K. A. Morgenstern, C. Lin, T. Fox, M. D. Dwyer, J. A. Landro, S. P. Chambers, W. Markland, C. A. Lepre, E. T. O'Malley, S. L. Harbeson, C. M. Rice, M. A. Murcko, P. R. Caron, and J. A. Thomson.** 1996. Crystal structure of the hepatitis C virus NS3 protease domain complexed with a synthetic NS4A cofactor peptide. *Cell* **87**:343-355.
20. **Koch, J. O. and R. Bartenschlager.** 1999. Modulation of hepatitis C virus NS5A hyperphosphorylation by nonstructural proteins NS3, NS4A, and NS4B. *J. Virol.* **73**:7138-7146.
21. **Koch, J. O., V. Lohmann, U. Herian, and R. Bartenschlager.** 1996. *In vitro* studies on the activation of the hepatitis C virus NS3 proteinase by the NS4A cofactor. *Virology* **221**:54-66.
22. **Lin, C., B. M. Pragai, A. Grakoui, J. Xu, and C. M. Rice.** 1994. Hepatitis C virus NS3 serine proteinase: trans-cleavage requirements and processing kinetics. *J. Virol.* **68**:8147-8157.
23. **Lin, C. and C. M. Rice.** 1995. The hepatitis C virus NS3 serine proteinase and NS4A cofactor: establishment of a cell-free trans-processing assay. *Proc. Natl. Acad. Sci. U. S. A.* **92**:7622-7626.
24. **Lindenbach, B. D., B. M. Pragai, R. Montserret, R. K. Beran, A. M. Pyle, F. Penin, and C. M. Rice.** 2007. The C terminus of hepatitis C virus NS4A encodes an electrostatic switch that regulates NS5A hyperphosphorylation and viral replication. *J. Virol.* **81**:8905-8918.
25. **Martin, M. M. and F. Jean.** 2006. Single-cell resolution imaging of membrane-anchored hepatitis C virus NS3/4A protease activity. *Biol. Chem.* **387**:1075-1080.
26. **Mottola, G., G. Cardinali, A. Ceccacci, C. Trozzi, L. Bartholomew, M. R. Torrisi, E. Pedrazzini, S. Bonatti, and G. Migliaccio.** 2002. Hepatitis C virus nonstructural proteins are localized in a modified endoplasmic reticulum of cells expressing viral subgenomic replicons. *Virology* **293**:31-43.
27. **Pollok, B. A. and R. Heim.** 1999. Using GFP in FRET-based applications. *Trends Cell Biol.* **9**:57-60.

28. **Pottage, J., E. Lawitz, D. Mazur, D. L. Wyles, H. Vargas, R. Ghalib, R. Gugliotti, M. Donohue, and H. Robison.** 2007. Short-Term Antiviral Activity and Safety of ACH-806 (GS-9132), an NS4A Antagonist, in HCV Genotype 1 Infected Individuals. *J. Hepatol.* **46**:S294-S295.
29. **Salonen, A., T. Ahola, and L. Kaariainen.** 2005. Viral RNA replication in association with cellular membranes. *Curr. Top. Microbiol. Immunol.* **285**:139-173.
30. **Sarrazin, C., R. Rouzier, F. Wagner, N. Forestier, D. Larrey, S. K. Gupta, M. Hussain, A. Shah, D. Cutler, J. Zhang, and S. Zeuzem.** 2007. SCH 503034, a novel hepatitis C virus protease inhibitor, plus pegylated interferon alpha-2b for genotype 1 nonresponders. *Gastroenterology* **132**:1270-1278.
31. **Sayle, R. A. and E. J. Milner-White.** 1995. RASMOL: biomolecular graphics for all. *Trends Biochem. Sci.* **20**:374.
32. **Shimizu, Y., K. Yamaji, Y. Masuho, T. Yokota, H. Inoue, K. Sudo, S. Satoh, and K. Shimotohno.** 1996. Identification of the sequence on NS4A required for enhanced cleavage of the NS5A/5B site by hepatitis C virus NS3 protease. *J. Virol.* **70**:127-132.
33. **Steinkuhler, C., A. Urbani, L. Tomei, G. Biasiol, M. Sardana, E. Bianchi, A. Pessi, and R. De Francesco.** 1996. Activity of purified hepatitis C virus protease NS3 on peptide substrates. *J. Virol.* **70**:6694-6700.
34. **Tanji, Y., M. Hijikata, S. Satoh, T. Kaneko, and K. Shimotohno.** 1995. Hepatitis C virus-encoded nonstructural protein NS4A has versatile functions in viral protein processing. *J. Virol.* **69**:1575-1581.
35. **Tomei, L., C. Failla, R. L. Vitale, E. Bianchi, and R. De Francesco.** 1996. A central hydrophobic domain of the hepatitis C virus NS4A protein is necessary and sufficient for the activation of the NS3 protease. *J. Gen. Virol.* **77 (Pt 5)**:1065-1070.
36. **Valentin, G., C. Verheggen, T. Piolot, H. Neel, M. Coppey-Moisan, and E. Bertrand.** 2005. Photoconversion of YFP into a CFP-like species during acceptor photobleaching FRET experiments. *Nat. Methods* **2**:801.

37. **Wang, W., F. C. Lahser, M. Yi, J. Wright-Minogue, E. Xia, P. C. Weber, S. M. Lemon, and B. A. Malcolm.** 2004. Conserved C-terminal threonine of hepatitis C virus NS3 regulates autoproteolysis and prevents product inhibition. *J. Virol.* **78**:700-709.
38. **Yao, N., P. Reichert, S. S. Taremi, W. W. Prorise, and P. C. Weber.** 1999. Molecular views of viral polyprotein processing revealed by the crystal structure of the hepatitis C virus bifunctional protease-helicase. *Structure* **7**:1353-1363.
39. **Yi, M., X. Tong, A. Skelton, R. Chase, T. Chen, A. Prongay, S. L. Bogen, A. K. Saksena, F. G. Njoroge, R. L. Veselenak, R. B. Pyles, N. Bourne, B. A. Malcolm, and S. M. Lemon.** 2006. Mutations conferring resistance to SCH6, a novel hepatitis C virus NS3/4A protease inhibitor. Reduced RNA replication fitness and partial rescue by second-site mutations. *J. Biol. Chem.* **281**:8205-8215.

Chapter 5 - Conclusion

The aims of this thesis work were to investigate the interaction between NS3 and NS4A in order to better understand the mechanism of activation and the role of this protease in the virus life cycle. It was hypothesized that there would be a handful of residues that dominate the interaction between NS3 and NS4A, and that binding and activation could be separate events mediated by different residues.

These aims have been successfully addressed. Two NS4A residues (I25 and I29) were identified that are important for NS3 binding and three NS4A residues (V23, V24, and I25) were identified that are important for NS3 activation. This data supports the hypothesis that binding can be separated from activation and therefore the I29 binding-pocket has been identified as a prime target for small-molecule therapeutic development.

In addition, NS3-4A substrate sequence cleavage efficiencies were compared *in vivo*. The activation-dependent NS4B/NS5A junction is processed efficiently, whereas the NS4A/NS4B junction is not. This data helps explain HCV polyprotein processing early in replication. It was also shown NS3-4A substrate specificity is not modulated by other replicase components; however the specific activity of this enzyme is increased within the replicase complex.

The strength of this thesis work stems from the novel and creative development of cell-based assays for studying this membrane-bound protease. *In vitro* assays fall short in that they do not take into account the unique micro-environment in which this protease is found. As well, these *in vivo* assays can be modified to study other membrane-associated proteases.

Implications for NS3-4A drug discovery

Thus far, most NS3-4A drug discovery efforts have focused on active site inhibitors of NS3-4A. However, the quick development of resistance, often to multiple drugs with one point mutation, is a major problem that will limit the usefulness and impact of these valuable compounds. One method to delay resistance is to use a combination of therapeutics, each with different target proteins or mechanisms. This strategy has successfully been applied to HIV therapy and is able to prolong the therapeutic window for these drugs from a few months to many years (12).

The major aim of this thesis was characterization of NS4A allosteric activation of NS3. This and other HCV protein-protein interactions are now being recognized as important drug targets. The assays developed here will be extremely important for the identification and characterization of inhibitors to this interaction. The cell-based activity assay measures directly NS3 protease activity as a consequence of NS4A binding and activation and the FRET-interaction assay is able to distinguish between inhibitors that will displace NS4A and inhibitors that will bind to the mature heterocomplex. The true value of these assays to NS3-4A drug development is that they are cell-based assays that can measure activation and binding inside cells without laborious processing steps. Considering NS3-4A is a membrane-associated protease and the characteristics of this protease can change when expressed as a truncated protein *in vitro* (for example the decreased ability to process the NS4B/NS5A junction *in vitro*), it is extremely important to use mature and membrane-bound heterocomplex for drug validation.

At a structural level, two residues important for binding and an activation hot-spot in NS4A were identified. This will allow for rational design of NS4A-antagonists that can block the binding pocket or activating cascade in NS3. The identification of these key areas is a very big step forward in understanding of how this enzyme is activated by NS4A.

It has also been shown here, that NS3-4A expressed alone has the same substrate selectivity and specificity as when expressed with all other HCV proteins. This is very significant, as it validates the use of cell-based NS3-4A systems for drug discovery. The substrate selectivity observed was different than reported *in vitro* preferences, and this further argues for the use of cell-based systems for drug optimization studies.

Current NS3-4A protease assay platforms are missing the ability to screen for NS4A antagonists based on their substrate cleavage site choice (usually NS5A/NS5B or NS4A/NS4B). We have shown that contrary to initial *in vitro* reports, the NS4B/NS5A site is cleaved efficiently *in vivo*, and can be substituted in existing cell-based systems to increase lead drug hits.

The assay designs can be used as a template for other membrane-associated proteases, many of which are therapeutically relevant (Table 5.1). For these proteases, developing *in vivo* assays is essential as they likely will not have the same conformation, activity, or selectivity *in vivo*.

Disease	Membrane Protease
Alzheimer's	Beta-secretase Gamma-secretase
Cardiovascular, Cancer	Site-2 protease
Hepatitis C	Signal peptide peptidase
Malaria	Malaria SPP
Parasitic Infections	Rhomboid

Table 5.1 Select membrane associated proteases
signal peptide peptidase (SPP)

Implications for polyprotein processing models

The NS4A/NS4B junction has been shown to be processed very poorly in *trans*. Also, increased protease activity within the replicase complex has been demonstrated, and NS4A residues important for NS3 binding and activation have been identified. These findings can be incorporated into the polyprotein processing model (findings from this thesis shown in bold):

1) NS3 is translated as part of the polyprotein (Illustration 5.1A). 2) NS3 folds to incorporate the NS4A activation domain into the N-terminal β -barrel (19). **Residues important in this stage are NS4A I25 that allows for stable complex formation and residue V23 that activates NS3** (Illustration 5.1B). 3) NS3 cleaves itself out of the polyprotein in *cis* (2, 15, 18), and remains associated with NS4A-polyprotein (Illustration 5.1C). If NS3 becomes dissociated (non-covalent interaction in equilibrium), **NS4A I29 is important in mediating NS3 recognition of NS4A for re-binding** 4) Translation of the entire polyprotein is completed and NS3 processes the NS5A/NS5B junction first (11, 17), **probably due to protein folding not cleavage site sequence differences between NS4B/NS5A and NS5A/NS5B** (Illustration 5.1D). 5) The NS4B/NS5A junction is cleaved next, **due to the fact it is processed much more efficiently than the NS4A/NS4B cleavage site**. 6) NS4A/NS4B is cleaved last (2, 3, 10, 17), **due to poor cleavage efficiency of this junction**. This cleavage order allows NS3 to remain associated with its substrate. In addition, the NS4A-NS4B precursor is responsible for slowing trafficking of proteins through the secretory pathway, a process which may be very important for replication (8). 7) Once the N-terminus of NS4B is released, membranous web formation is triggered. 8) NS3-4A associates with other HCV NS and host proteins to form the replicase complex (7), **and is activated perhaps by stabilization of the interaction between NS3 and NS4A** (Illustration 5.1E).

Early in replication the NS3-4A cleavages are probably mediated uni-molecularly and as more protein accumulates NS3-4A can efficiently cleave NS4B/NS5A and NS5A/NS5B in *trans*, creating an alternative pathway where NS4B/NS5A is cleaved before NS5A/NS5B (2, 10, 17).

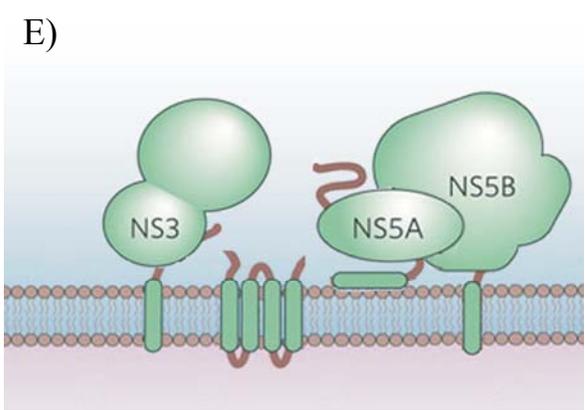
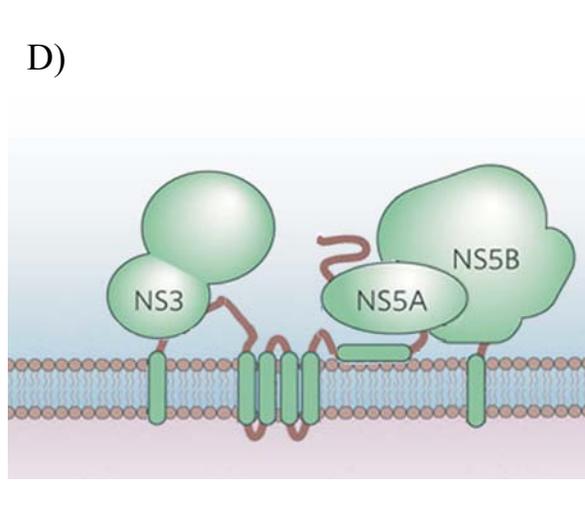
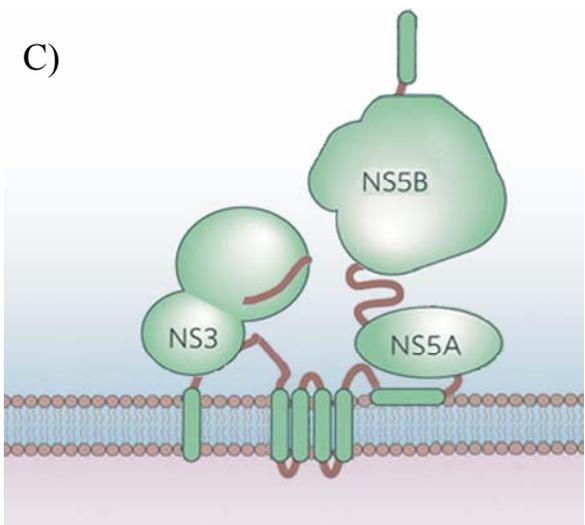
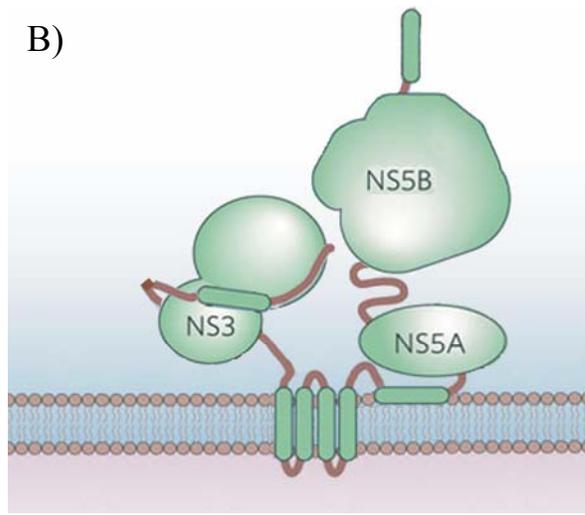
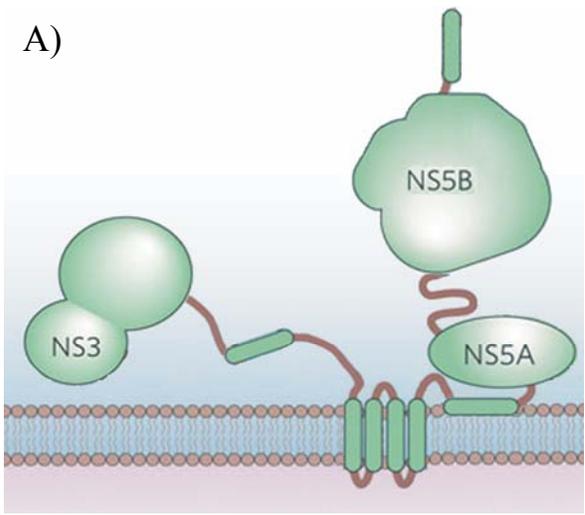


Illustration 5.1 Polyprotein processing steps
 See previous page for description of different stages.

Implications for other *Flaviviridae*

The members of the virus family *Flaviviridae*, genus *flavivirus* includes many important human pathogens: Yellow Fever virus (YFV), West Nile virus (WNV), Dengue virus (DENV), and encephalitis viruses (EVs) (Figure 5.1).

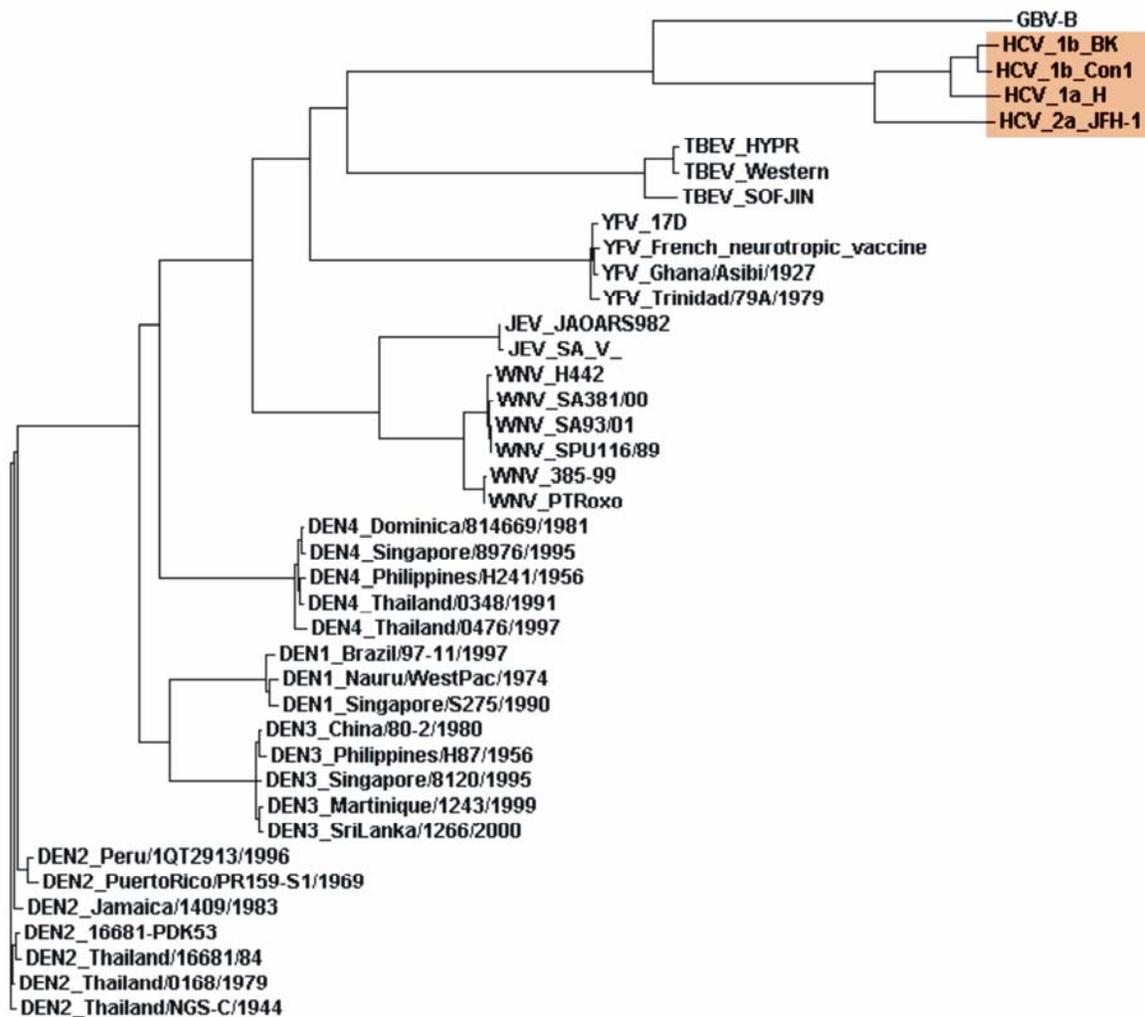


Figure 5.1 Phylogram for selected *Flaviviridae* genotypes.

Multiple sequence alignment and neighbour-joining phylogram was generated by comparing NS3-4A sequences (HCV and GBV) and NS2B-NS3 sequences (TBEV, YFV, JEV, WNV, DEN) from all genotypes listed in NCBI Taxonomy database (accessed April 9, 2008) using Clustal W (4, 9, 14, 20). Excluded were non-classified isolates and HCV strains not used in thesis work. Nomenclature shown corresponds to “species”_”genotype”_”strain” (HCV) and “species”_”strain” (non-HCV viruses). Highlighted are HCV strains relevant to this thesis. GBV = Hepatitis GB virus B, TBEV = Tick-borne encephalitis virus, YFV = Yellow fever virus, JEV = Japanese encephalitis virus, WNV = West Nile virus, DEN = Dengue virus.

There are major differences between NS3 from HCV and NS3 from YFV, WNV, DENV, and EVs. The latter are activated by NS2B, not NS4A, a protein that is much longer and less hydrophobic than its NS3 counterpart (Figure 5.2). NS2B has been shown to interact with NS3-NS2B substrates, forming part of the substrate binding cleft in WNV (1). NS3-2B recognizes dibasic cleavage sequences and is able to cleave short substrates unlike HCV NS3-4A (13, 16).

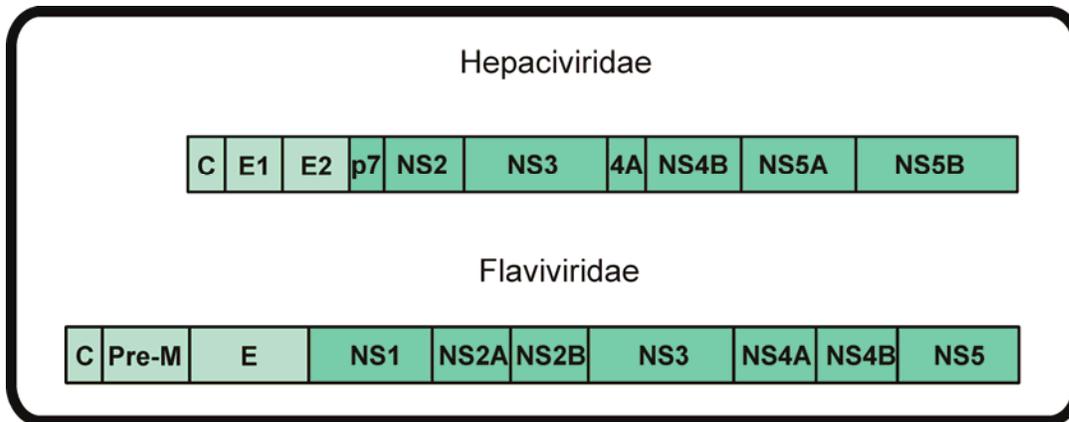


Figure 5.2 Comparison of genus Flavivirus with genus Hepacivirus

Despite these differences, striking similarities exist. The general fold of NS3-2B and NS3-4A is extremely similar (21). Both are comprised of two β -barrels; the active site residues aspartic acid and histidine are found in the N-terminal subdomain and serine is found in the C-terminal subdomain. Flexibility of NS3 exists in the absence of NS2B binding, destabilizing the catalytic triad.

The cell-based protease assay developed within this thesis can be applied to other *Flaviviridae* members such as WNV, DENV, and YFV simply by changing the cleavage site to encode for dibasic residues. The FRET assay may also work, however the introduction of the CFP and YFP is not straight forward into the full-length NS2B sequence; it contains hydrophobic sequences at both the N-terminus and C-terminus thought to mediate membrane association (5, 6).

Future directions

Chapter 2

It would be of great interest to develop the cell-based protease assay into a high-throughput assay for screening of inhibitors. There are multiple algorithms available to do such screening, a few of which should be evaluated for this system. UNS3-4A cells should be used for this purpose and transfected in 96-well plates with pTm-4B/5A-DsRed with or without inhibitor present. It may be important to incubate the inhibitors with the UNS3-4A cells starting with removal of the tetracycline that induces NS3-4A expression (24 hours pre-transfection). 24 hours post-transfection cells should be fixed and the nuclei stained with DAPI. On a high-throughput microscope such as the Cellomics system (Thermo Scientific) or the Discovery-1 system (Molecular Devices) the cells should be imaged in the blue channel (for DAPI) and also in the red channel (for DsRed). The challenge will be to detect the cells with diffuse cytoplasmic staining, and this is made more difficult because many cells will have punctate stain which will result in a short exposure time and practical invisibility of the cleaved DsRed. However, this dim, cleaved DsRed will be measurable by the microscope. I would first try to quantify the amount of DsRed co-localized with the DAPI stain. Only cells with cleaved substrate should have DsRed in the nucleus and therefore this measurement should correspond with NS3-4A cleavage. Alternatively, algorithms could calculate how much DsRed is in a radius around the nucleus, for example 2 microns, and a decrease in DsRed should correlate with NS3-4A cleavage. Lastly, the size of DsRed patches could be measured and increasing size should correlate with NS3-4A cleavage.

In addition to applying the cell-based protease assay developed here to other membrane-associated proteases, including other *Flaviviridae*, this assay can be used to discover host modulators of HCV NS3-4A proteolysis. A stable cell-line expressing pTm-4B/5A-DsRed and NS3-4A could be subjected to an RNA interference (RNAi) library in high throughput format. Detection of proteolysis could be automated by measuring size or intensity of red areas; an average high intensity and small area would indicate NS3-4A activity knock-down. This same high throughput assay could be used to screen for both NS3-4A active site and allosteric inhibitors in cells.

Chapter 3

The discovery that the NS4A/NS4B cleavage junction is poorly processed is very interesting. By introduction of single amino acid substitutions in the NS4A/NS4B sequence to one that was efficiently cleaved, such as NS4B/NS5A, the exact determinants of poor cleavage could be understood. This work has already been started.

The hypothesis that different genotypes of NS3-4A, specifically genotype 3, may process MAVS poorly due to absent negative charges, for example D168, in the substrate binding cleft deserves more attention. If true, it would partly explain why genotype 3 is more responsive to interferon treatment as it cannot disrupt innate antiviral signaling pathways as well as other genotypes. Introduction of a positively charged arginine in P5 of pTm-4A/4B-DsRed, could show whether this residue alone would be able to rescue NS4A/NS4B cleavage in genotype 1a, 1b or 2a. However, it would be best to acquire NS3-4A genotype 3 in a mammalian expression vector and test directly its ability to process pTm-MAVS-DsRed. Then the D168 negative charge could be introduced into NS3-4A genotype 3 to test if MAVS cleavage can be restored. These experiments would be clinically relevant. If D168 in the NS3-4A S4 pocket is important for viral interference in interferon signaling, a compound could be developed to bind and mask D168. This compound in combination with interferon treatment would greatly increase the number of patients that clear the virus. In fact, all three active site protease inhibitors that have made it to clinical trials (BILN 2061, VX-950, and SCH503054) extend from P1 to P4. This is very interesting, as it suggests that for an inhibitor to be successful, not only must it stop protease activity (P1) but also must block S4. These inhibitors are selected based on a block of NS3 proteolysis, however, it could be suggested that perhaps allowing proteolysis to continue, but blocking S4 would hinder NS3-4A cleavage of MAVS and therefore, promote viral clearance in combination with interferon.

Chapter 4

A natural extension of the work presented in Chapter 4, is to additionally identify NS3 residues that are essential for NS4A I25 and I29 binding. This can be done by mutagenesis of residues modeled to interact with I25 and I29 and detection of complex formation by co-localization microscopy and FRET. It would be interesting to test the model of how NS4A V23 activates NS3 also by mutagenesis. This process could be modeled in silico and additional surfaces on NS3 could be identified for inhibitor targeting.

In silico screening of inhibitors that fit into the I29 or I25 binding pockets could be done. Synthesis and testing of these allosteric inhibitors could provide new lead compounds for drug development.

References

1. **Aleshin, A. E., S. A. Shiryayev, A. Y. Strongin, and R. C. Liddington.** 2007. Structural evidence for regulation and specificity of flaviviral proteases and evolution of the Flaviviridae fold. *Protein Sci.* **16**:795-806.
2. **Bartenschlager, R., L. Ahlborn-Laake, J. Mous, and H. Jacobsen.** 1994. Kinetic and structural analyses of hepatitis C virus polyprotein processing. *J. Virol.* **68**:5045-5055.
3. **Bartenschlager, R., L. Ahlborn-Laake, K. Yasargil, J. Mous, and H. Jacobsen.** 1995. Substrate determinants for cleavage in cis and in trans by the hepatitis C virus NS3 proteinase. *J. Virol.* **69**:198-205.
4. **Benson, D. A., I. Karsch-Mizrachi, D. J. Lipman, J. Ostell, B. A. Rapp, and D. L. Wheeler.** 2000. GenBank. *Nucleic Acids Res.* **28**:15-18.
5. **Brinkworth, R. I., D. P. Fairlie, D. Leung, and P. R. Young.** 1999. Homology model of the dengue 2 virus NS3 protease: putative interactions with both substrate and NS2B cofactor. *J. Gen. Virol.* **80 (Pt 5)**:1167-1177.
6. **Droll, D. A., H. M. Krishna Murthy, and T. J. Chambers.** 2000. Yellow fever virus NS2B-NS3 protease: charged-to-alanine mutagenesis and deletion analysis define regions important for protease complex formation and function. *Virology* **275**:335-347.
7. **Egger, D., B. Wolk, R. Gosert, L. Bianchi, H. E. Blum, D. Moradpour, and K. Bienz.** 2002. Expression of hepatitis C virus proteins induces distinct membrane alterations including a candidate viral replication complex. *J. Virol.* **76**:5974-5984.
8. **Konan, K. V., T. H. Giddings Jr, M. Ikeda, K. Li, S. M. Lemon, and K. Kirkegaard.** 2003. Nonstructural protein precursor NS4A/B from hepatitis C virus alters function and ultrastructure of host secretory apparatus. *J. Virol.* **77**:7843-7855.
9. **Larkin, M. A., G. Blackshields, N. P. Brown, R. Chenna, P. A. McGettigan, H. McWilliam, F. Valentin, I. M. Wallace, A. Wilm, R. Lopez, J. D. Thompson, T. J. Gibson, and D. G. Higgins.** 2007. Clustal W and Clustal X version 2.0. *Bioinformatics* **23**:2947-2948.

10. **Lin, C., B. M. Pragai, A. Grakoui, J. Xu, and C. M. Rice.** 1994. Hepatitis C virus NS3 serine proteinase: trans-cleavage requirements and processing kinetics. *J. Virol.* **68**:8147-8157.
11. **Lohmann, V., J. O. Koch, and R. Bartenschlager.** 1996. Processing pathways of the hepatitis C virus proteins. *J. Hepatol.* **24**:11-19.
12. **Martinez-Cajas, J. L. and M. A. Wainberg.** 2008. Antiretroviral therapy : optimal sequencing of therapy to avoid resistance. *Drugs* **68**:43-72.
13. **Mueller, N. H., C. Yon, V. K. Ganesh, and R. Padmanabhan.** 2007. Characterization of the West Nile virus protease substrate specificity and inhibitors. *Int. J. Biochem. Cell Biol.* **39**:606-614.
14. **Saitou, N. and M. Nei.** 1987. The neighbor-joining method: a new method for reconstructing phylogenetic trees. *Mol. Biol. Evol.* **4**:406-425.
15. **Schechter, I. and A. Berger.** 1967. On the size of the active site in proteases. I. Papain. *Biochem. Biophys. Res. Commun.* **27**:157-162.
16. **Shiryaev, S. A., I. A. Kozlov, B. I. Ratnikov, J. W. Smith, M. Lebl, and A. Y. Strongin.** 2007. Cleavage preference distinguishes the two-component NS2B-NS3 serine proteinases of Dengue and West Nile viruses. *Biochem. J.* **401**:743-752.
17. **Tanji, Y., M. Hijikata, Y. Hirowatari, and K. Shimotohno.** 1994. Hepatitis C virus polyprotein processing: kinetics and mutagenic analysis of serine proteinase-dependent cleavage. *J. Virol.* **68**:8418-8422.
18. **Tomei, L., C. Failla, E. Santolini, R. De Francesco, and N. La Monica.** 1993. NS3 is a serine protease required for processing of hepatitis C virus polyprotein. *J. Virol.* **67**:4017-4026.
19. **Wang, W., F. C. Lahser, M. Yi, J. Wright-Minogue, E. Xia, P. C. Weber, S. M. Lemon, and B. A. Malcolm.** 2004. Conserved C-terminal threonine of hepatitis C virus NS3 regulates autoproteolysis and prevents product inhibition. *J. Virol.* **78**:700-709.

20. **Wheeler, D. L., C. Chappey, A. E. Lash, D. D. Leipe, T. L. Madden, G. D. Schuler, T. A. Tatusova, and B. A. Rapp.** 2000. Database resources of the National Center for Biotechnology Information. *Nucleic Acids Res.* **28**:10-14.
21. **Zhou, H., N. J. Singh, and K. S. Kim.** 2006. Homology modeling and molecular dynamics study of West Nile virus NS3 protease: a molecular basis for the catalytic activity increased by the NS2B cofactor. *Proteins* **65**:692-701.

Appendix I – Chapter 2

Time course

All experiments shown were stopped (cells fixed or harvested) at 24 hours post-transfection. Attempts at showing a time course of cleavage were challenging due to the threshold of detection and also due to continuous protein production and variation between cells. The earliest time point that DsRed can be detected is about 12 hours post-transfection. At this time, the level of DsRed produced is difficult to reproducibly detect by Western blotting and it is difficult to detect the cleaved substrate by fluorescence microscopy. These early time points however suggest that substrate is already cleaved to the approximate proportions that are seen at the 24 hour time point. This is not surprising as protease is present during translation of the substrate (NS3-4A production is induced 24 hours pre-transfection).

To show a real time course of cleavage, an alternate experimental set-up would be needed. Substrate should be transfected first (preferably a stable cell line would be made) and then 24 hours after protease should be expressed. This would allow detection of the early time points of cleavage. Problems may arise due to the degradation of DsRed substrate at the same time as NS3-4A proteolysis, depending on the time it takes NS3-4A to process the substrate. Also, new substrate would continue to be made, unless the stable cell line was one with controlled transcription.

Non-tethered substrate

We wondered whether a soluble substrate of the same design would be cleaved by membrane-bound NS3-4A. To investigate this, three mutations (I8D, I12E, and F19D) known to release the NS5A amphipathic alpha helix from the membrane (Elazar *et al.*, J Virol. 2003 77(10): p6055-61) were introduced into pTm-4A/4B-DsRed and pTm-4B/5A-DsRed. The solubilized Tm-4A/4B-DsRed and Tm-4B/5A-DsRed constructs showed cytoplasmic localization by fluorescence microscopy. These substrates were evaluated by Western blotting and neither substrate was processed efficiently proving membrane association is an essential part of the substrate design.

Targeting domain swapping

We wondered if the NS5A targeting domain could be changed to another targeting domain and still obtain the same results. We decided to replace the NS5A targeting sequence (¹SGSWLRDVWDWICTVLDFKTLWQSKLLPRLPEF³⁴) with the NS4A N-terminus transmembrane domain (¹STWVLVGGVLAALAAAYCLST²⁰) in the Tm-4A/4B-DsRed and Tm-4B/5A-DsRed substrates. After failed attempts at a PCR-ligation based approach (the NS4A transmembrane domain is so small, the recovery from gel or column purification is very poor), I chose to replace the domain using three rounds of site-directed mutagenesis. The resulting plasmids, denoted “p4ATm-4A/4B-DsRed” and “p4ATm-4B/5A-DsRed” were completed and transfected into the tetracycline-regulated NS3/4A-expressing cell line. Surprisingly, these constructs localize perinuclearly and to some type of cell cytoskeleton (Figure A1.1). The cytoskeleton staining appears to resemble intermediate filaments. Neither of these constructs are processed by NS3-4A as detected by fluorescence microscopy or Western blotting. The lack of cleavage underlines the importance of the targeting domain in such membrane-bound substrates and suggests that multiple targeting domains should be investigated when developing such substrates for other target proteases.

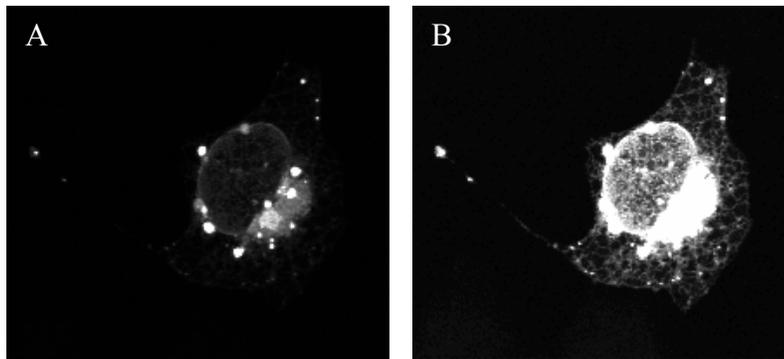


Figure A1.1 Localization of 4ATm-4A/4B-DsRed

Confocal microscope images of DsRed fluorescence showing regular (A) and overexposure (B) of localization of 4ATm-4A/4B-DsRed. In the overexposed image, DsRed localized to the cell cytoskeleton is visible.

The localization of the 4ATm substrates is an interesting result, as little is known about the mechanisms by which NS4A is sorted and targeted in the cell. NS4A has been shown to localize to the outer mitochondrial membrane when expressed alone in cells (Nomura-Takigawa *et al.*, J Gen Virol. 2006 87(7):1935-45) and when NS4A or NS3-4A are expressed in human cells, a redistribution of the mitochondria occurs, causing the mitochondria in the cell to

accumulate in a perinuclear region where NS3 and NS4A are co-localized (Figure A1.2) (Nomura-Takigawa *et al.*, J Gen Virol. 2006 87(7):1935-45).

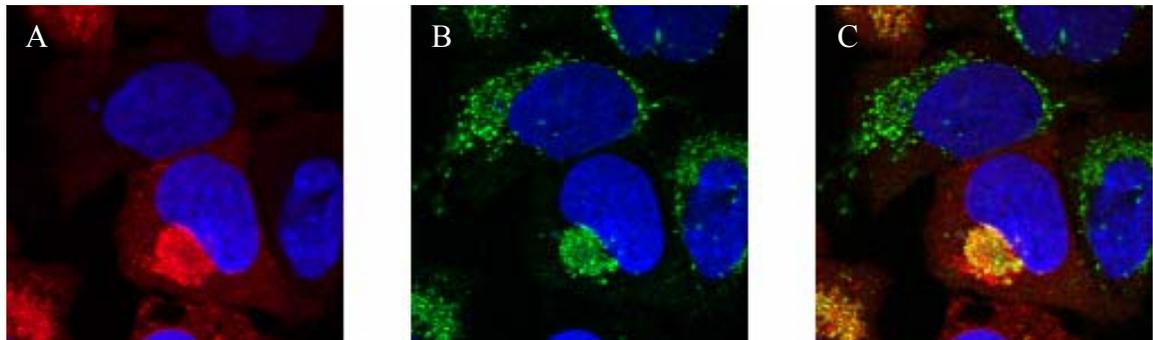


Figure A1.2 NS3/4A causes reorganization of mitochondria.

Immunofluorescence of NS3/4A expressing cells was performed using A) anti-NS3 antibody (red) and B) anti-mortalin antibody (green). C) overlay of images. DAPI nuclei stain shown in blue.

In HCV-infected cells, mitochondria have been shown to be enveloped in endoplasmic-reticulum-derived membrane and colocalized with the viral replication complex. Maintaining a tight association with mitochondria may be advantageous for HCV persistence: 1) HCV could sequester the energy produced for use in viral replication (helicase, polymerase), 2) HCV could evade a mitochondrial-mediated innate immune response via NS3-4A-mediated cleavage events, and 3) HCV could halt mitochondrial-mediated apoptosis. Importantly, it may be the ability of the NS4A hydrophobic domain to bind cytoskeleton that allows for the redistribution of the mitochondria.

The 4ATm-4B/5A-DsRed construct has been simplified by stepwise site-directed mutagenesis (work done by M. Martin and E. Thong). This new construct 4ATm-DsRed contains the NS4A hydrophobic domain (¹STWVLVGGVLAALAAAYCLST²⁰) and the DsRed protein (starting at position 30) separated by a glycine/serine-rich linker region (²¹SGSSGSGAT²⁹). This construct maintains the association with cytoskeleton.

The localization of 4ATm-DsRed should be better defined by co-localization microscopy with various cytoskeleton markers and cell organelle stains. The ability of this construct to reorganize mitochondria should be assessed. A number of experiments can be done with 4ATm-DsRed to investigate the determinants of cytoskeleton association or mitochondrial reorganization. Mutational analysis of various regions of 4ATm as well as altering the

aggregation state of the 4ATm should be done. Disruption of the alpha-helical structure can be accomplished by introduction of a proline residue in the 4ATm. Also, mutation of possible protein-protein interaction spots (N-terminal serine-threonine, C-terminal cysteine) should be done. The DsRed protein used in this construct is a tetrameric protein that is able to form high order oligomers when tethered to a membrane. It would be interesting to subclone the 4ATm into other dimeric or monomeric vectors (for example GFP, monomeric RFP, or a myc-tagged vector) to determine if localization is an aggregation-dependent process. The cloning plans for all of the above have been designed and so far 4ATm(Δ ST)-DsRed has been created in which the first two residues serine and threonine are mutated to two alanines.

Appendix II – Chapter 3

Figure 3.4

In figure 3.4, it is very difficult to detect the NS3 from UHCV-32 cells. A gamma-altered image is provided (Figure A2.1) to show that NS3 is indeed present.

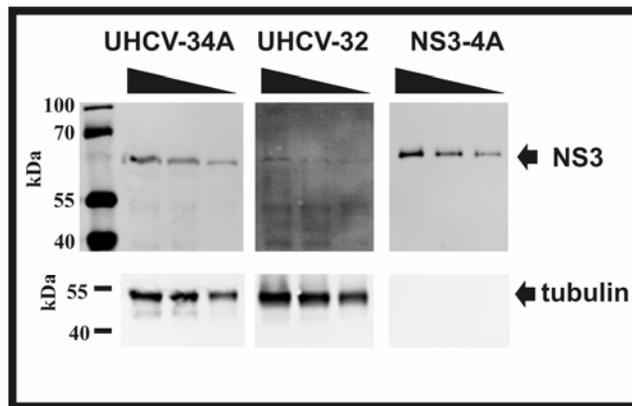


Figure A2.1 Alteration of UHCV-32 image reveals NS3.

Titration of UHCV-34A and UHCV-32 whole cell lysates and detection of NS3-4A by Western blotting using an anti-NS3 monoclonal antibody. *E. coli* expressed and purified NS3 is used as a positive control. Simultaneous probing for tubulin using an anti-tubulin polyclonal antibody, allows for normalization of the NS3 signal. The central UHCV-32 panel has been altered using the “curves” tool in Adobe Photoshop to reveal the NS3 band.

I attempted to increase the amount of NS3 loaded by making membrane preparations of UNS3-4A and UHCV-32 cells 48 hours post-induction. Membrane preparations are made by resuspending the cell pellet in ice cold hypotonic lysis buffer [10 mM Tris (pH 7.8), 10 mM NaCl, 1X complete EDTA-free protease inhibitors (Roche, Laval, CA)] for 20 min on ice followed by disruption using a 18-gauge needle (approximately 40 times through the syringe). Lysates were centrifuged at 900 g for 5 min at 4°C to remove the nuclei, and the supernatants were further centrifuged at 15,000 g for 20 min at 4°C to pellet the NS3-4A-containing cellular membranes. Membrane pellets were resuspended in hypotonic lysis buffer, boiled in SDS-PAGE loading dye and run on gel. The resulting Western blot showed robust detection of NS3 in both UNS3-4A and UHCV-32 cells; however the difference in NS3 expression was only ~8-fold (Figure A2.2).

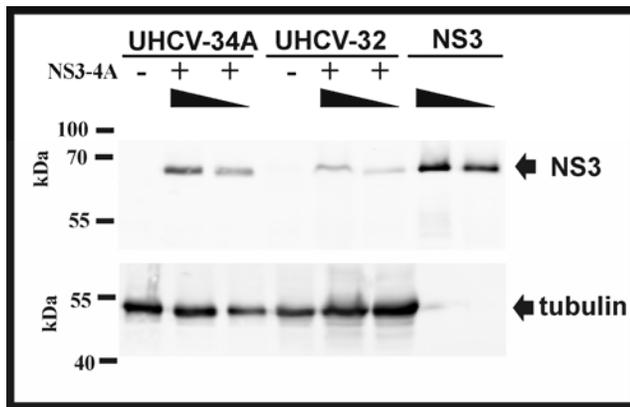


Figure 2.2 NS3-4A expression is about 8-fold more in UNS3-4A cell membrane fractions as compared to UHCV-32 cell membrane fractions.

Membrane fractions from 0.5 and 0.25×10^6 UHCV-34A cells and 1.5 and 0.75×10^6 UHCV-32 cells were resolved on SDS-PAGE. NS3 was detected by Western blotting using an anti-NS3 monoclonal primary antibody and IRDye 680 donkey anti-mouse secondary. *E. coli* expressed and purified NS3 used as a positive control. Tubulin was simultaneously detected with an anti-tubulin rabbit polyclonal primary antibody and IRDye 800 donkey anti-rabbit secondary.

This discrepancy was resolved when the nuclear fraction (usually discarded) was run on Western blot. NS3-4A was co-purifying with the nucleus only in the UNS3-4A cells and therefore membrane fractionation was enriching for NS3-4A preferentially in the UHCV-32 cells. This is not surprising given the localization of NS3-4A in these two cell lines; in UNS3-4A cells, NS3-4A is located perinuclearly and in UHCV-32 cells, NS3-4A is located throughout the cytoplasm in the membranous web. I would suggest that these experiments be redone, perhaps by lysing the cells and only discarding the cytoplasmic fraction. This would be accomplished by skipping the low speed centrifugation step in the above protocol.

NS4A/NS4B cleavage

In order to publish chapter 3, it may be necessary to investigate two aspects of the work in greater detail: the poor *trans* cleavage of the NS4A/NS4B junction and the TRIF/MAVS cleavage.

It would be of great interest to pinpoint which residues are essential for the reduced cleavage of NS4A/NS4B or which substitutions could rescue NS4A/NS4B cleavage. The latter has already been started. I have created a panel of constructs with single point mutations in

NS4A/NS4B (Illustration A2.1). These mutations correspond to either the NS4B/NS5A or the NS5A/NS5B junctions. All 15 possible substitutions have been made.

Initial attempts at quantifying cleavage efficiency of these constructs yielded surprising results. Virtually all substitutions tested increased cleavage of the NS4A/NS4B junction (Table A2.1).

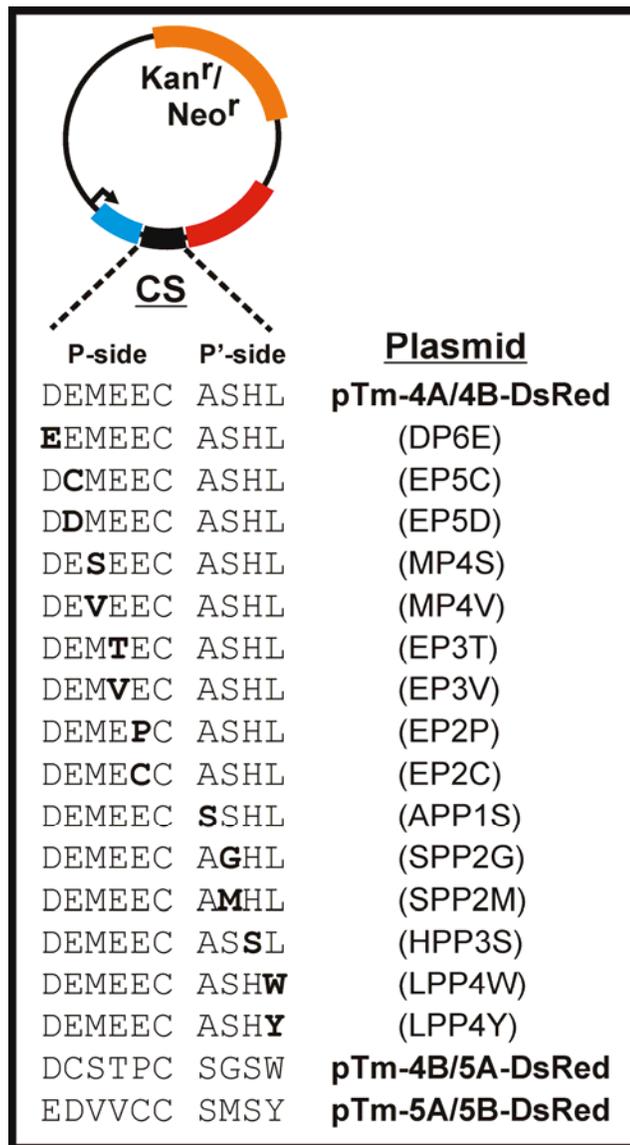


Illustration A2.1 Sequence of pTm-4A/4B-DsRed mutants
Mutations (bold) were introduced by site-directed mutagenesis.

NS3 Cleavage Sequence in Plasmid	% Cleaved
Tm-DE V E E EC ↓ ASHL-DsRed	21
Tm-DEM T E E EC ↓ ASHL-DsRed	66
Tm-DEM V E E EC ↓ ASHL-DsRed	30
Tm-DEME P C ↓ ASHL-DsRed	47
Tm-DEME C C ↓ ASHL-DsRed	25
Tm-DEME S EC ↓ S SHL-DsRed	22
Tm-DEME A EC ↓ A MHL-DsRed	20
Tm-DEME S EC ↓ AS S L-DsRed	10
Tm-DEME S EC ↓ ASH Y -DsRed	24

Table A2.1 Single point substitutions are able to rescue NS4A/NS4B cleavage

We were excited because this indicates a very strong selection pressure to maintain all NS4A/NS4B residues as poorly cleaved, as any substitution can increase cleavage efficiency. However, a closer inspection of the cleavage bands for these constructs revealed a doublet cleavage band: one upper band, presumably due to host proteolysis, and one lower band due to NS3-4A-specific cleavage. Both bands had been quantified together because they run very close. Therefore these experiments need to be repeated, running the gel for 1 hour longer (3.5 hours in total at 110 V) to resolve these two bands. Also it will be essential to compare with the negative control (no NS3-4A) to ensure that none of these constructs are processed by host proteases. In addition the EP6D and EP5D are fairly conservative substitutions (Illustration A2.2), and therefore we would expect them to serve as good negative controls and not see an increase in cleavage.

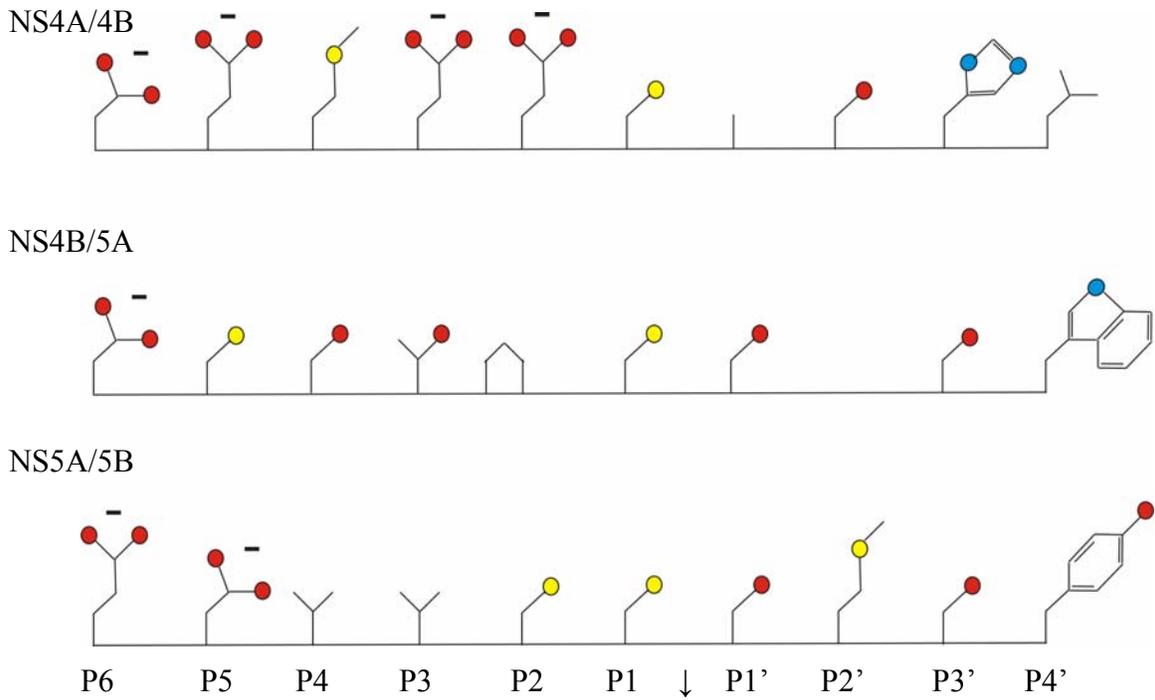


Illustration A2.2 Schematic representation of the amino acid side chains of the NS3/4A cleavage sites

The major differences for the NS4A/4B cleavage site are the Met in P4, the negative charges in P3 and P2, and all of the P' positions. Carbons are diagramed as the stick backbone, oxygen is red, sulfur is yellow, and nitrogen is blue. Charges of side chains at pH 7.0 are shown.

MAVS cleavage

The MAVS and MAVS chimera cleavage patterns are very interesting and lead to the hypothesis that the prime-side of MAVS is poorly cleaved and that the non-prime side may compensate with a positively charged P5 arginine, which may interact with the negatively charged NS3 S4 residue aspartic acid 168. This hypothesis should be tested. By removal of the negative charge of D168 (D168N, D168L) in the pNS3-4A_Con1 plasmid, we would expect a decrease in MAVS cleavage efficiency. Also, introduction of a P5 arginine into pTm-4A/4B-DsRed may be able to rescue cleavage of this junction. To test whether the prime-side is indeed poorly cleaved, point mutations corresponding to the MAVS prime-side residues (P1'H, P2'R, P3'P) can be introduced into the NS4B/NS5A substrate and the cleavage efficiency measured. The primers for these constructs have been designed and the cloning is underway.

The background cleavage of these constructs remains a very significant problem and should be investigated. Firstly, the silencing of NS3-4A in the tetracycline regulated cell line UNS3-4A should be confirmed. Since tetracycline is inactivated by light, it is possible that the powdered tetracycline stock is too old and contains lower amounts of active tetracycline. In addition, it may be possible to reduce the background cleavage of these constructs by using an alternate host cell line such as Huh7 and transfecting these cells with NS3-4A and the cleavage constructs as done in Chapter 4.

Appendix III – Chapter 4

All positions in the activation domain, kink, and acidic domain of NS4A have been mutated and tested in the activation assay (Illustration A3.1). All of these positions except for 9 remaining mutants have been analyzed for FRET (Illustration A3.1).

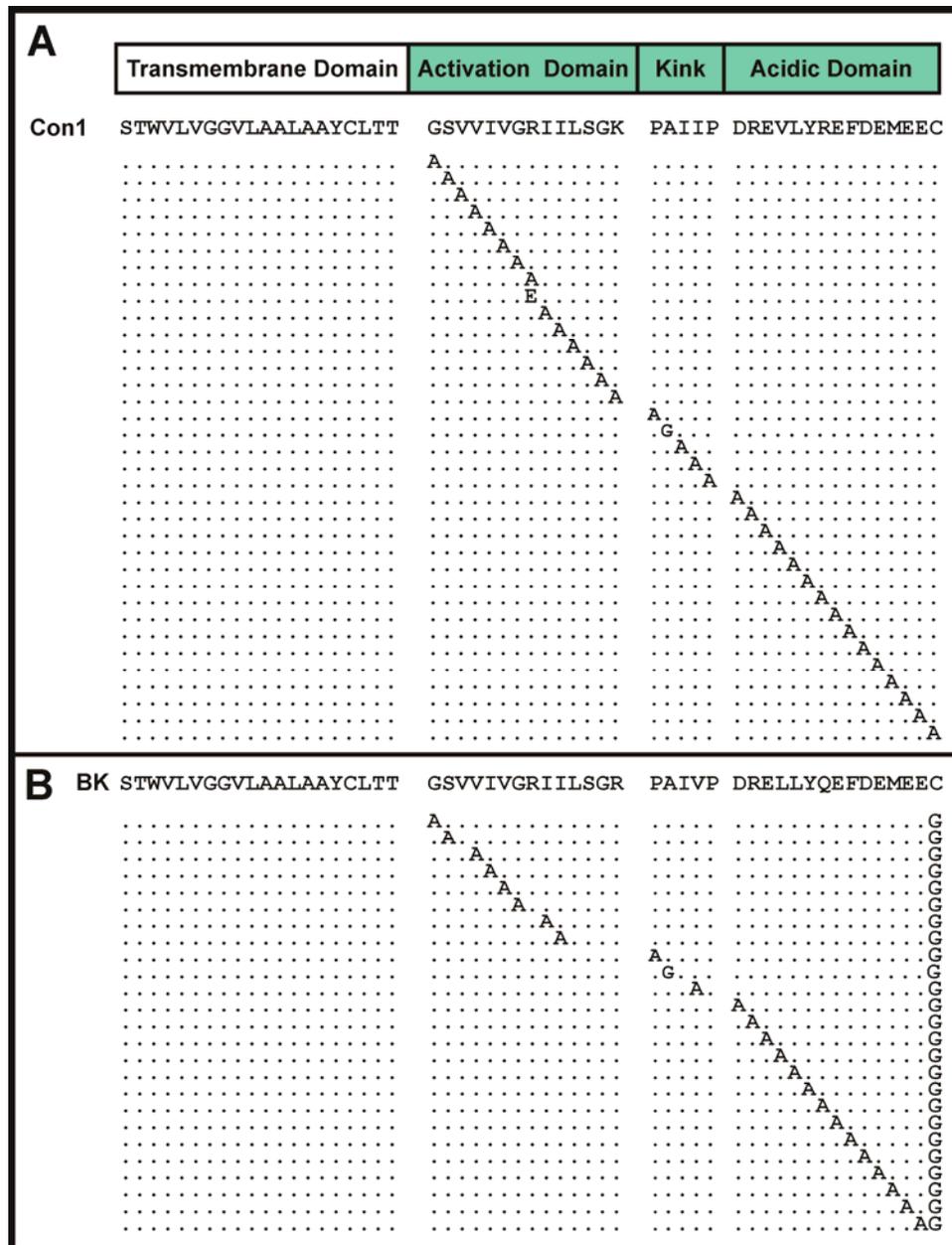


Illustration A3.1 Mutants that have been analyzed for NS3 activation (A) and binding (B)

The C54 residue in the NS4A-CFP construct (B) has been mutated to G in order to prevent NS3 cleavage of NS4A-CFP.

The titration of NS3-4A mutants shown in chapter 4 was done to ensure saturation of the protease signal for all of the mutants. This guaranteed that the plasmid DNA was not lower in concentration (for example due to partial degradation), and therefore would not give a false knock-down of activity. The results of the titration indicated that 2 µg of NS3-4A plasmid was sufficient to saturate the system and therefore the activity scanning was repeated using this set amount of protease plasmid (Figure A3.1, done by D. Moscher, A. Chau and M. Martin).

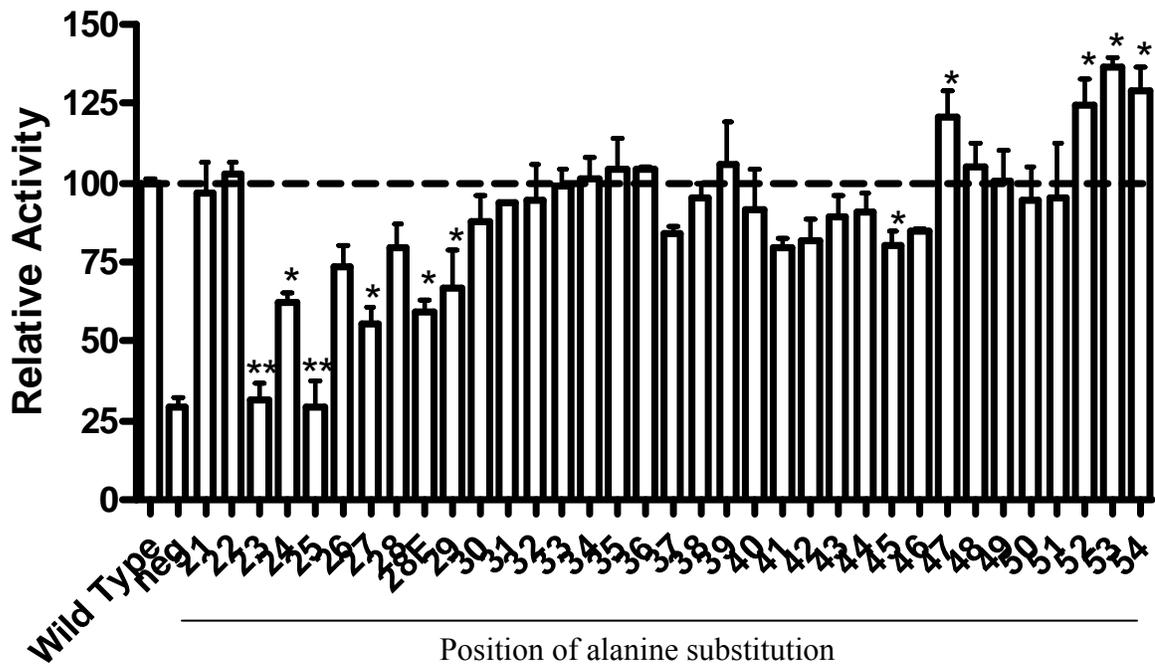


Figure A3.1 Alanine scanning of NS4A residues 21 to 54 identifies multiple residues involved in NS3 activation and inhibition

Huh7 cells were transfected with 2 µg each pTm-4B/5A-DsRed and pNS3-4A_Con1 wild type or alanine mutant. 24 hours post transfection cells were harvested and analysed by Western blotting. Samples were done in triplicate and normalized to the wild type control for that experiment. A negative control with no protease transfected (neg) was also done to estimate non-specific cleavage levels. Experiments were repeated at least three times and until the standard deviation between biological replicates was under 10%. ** no significant difference from the negative control (neg), *significantly different from wild-type NS3-4A.

In addition to the residues discovered by the initial titration of NS3-4A mutants presented in chapter 4 (V23, V24, I25) other mutations have been identified; G27A, R28E, I29A, and Y45A cause decreases in NS3 activity whereas E47A, E52A, E53A, and C54A cause a significant increase in NS3 activity. An increased NS3-4A activity suggests that under normal conditions E47, E52, E53 and C54 inhibit or control NS3-4A cleavage. This is not entirely

surprising as it has been shown that the last six residues (DEMEEC) of NS4A can inhibit NS3 through product inhibition; it was these six residues that formed the basis for design and development of the BILN 2061 inhibitor. This inhibition has however not been shown in the context of mature heterocomplex formation which makes this observation very novel. Either these negatively charged residues are inhibiting the enzyme in *cis* (NS4A folds back into the active site) or in *trans* (the C-terminus NS4A of one NS3-4A complex inhibits a nearby NS3-4A); it will be very difficult to distinguish between these two possibilities, but regardless of the mechanism of inhibition, it is most likely biologically relevant. NS3-4A dimerizes for helicase function and in the replicase complexes, there would be many molecules of NS3-4A concentrated together allowing for *trans* modulation. Creation of a triple mutant E52V, E53V, C54T is underway.

Analysis of the FRET effect for 25 of the 34 NS4A-CFP alanine substitution mutants has been completed (Figure A3.2, done by P. Wang). All the remaining 9 mutants have been created (done by M. Martin, A. Jonas, and P. Wang). In addition all the 34 alanine substitutions have been made in NS4A-YFP vector (done by M. Martin, A. Jonas, and P. Wang).

The FRET analysis shows two NS4A-CFP mutants with significant differences in FRET transfer: G21A (increased) and I29A (decreased). G21 is located at the interface between the lipid bilayer and the NS3 binding site. It is possible that increasing the size of the side chain at this position imposes a more rigid structure on the NS3-4A heterocomplex, which leads to greater FRET transfer.

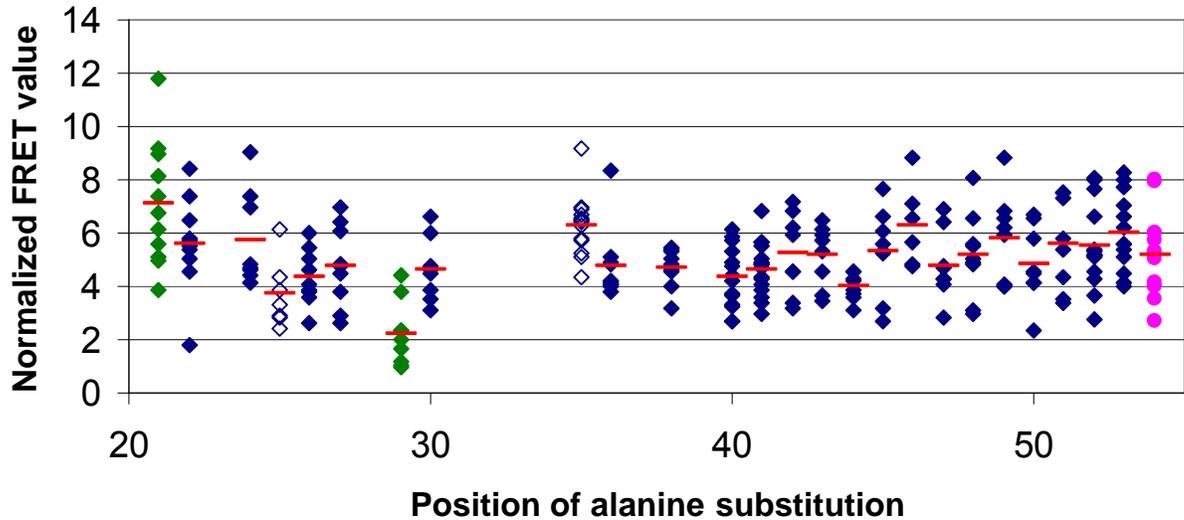


Figure A3.2 Alanine scanning of NS4A residues by FRET microscopy

Vero cells were transfected with 1.25 μg NS4A-CFP and 1.5 μg NS3-YFP with 5 μL DNAfectin per well. 14 hours post transfection cells were harvested and analysed by microscopy (see material and methods section, chapter 4). FRET signal was normalized to YFP intensity. Each point represents one cell. The pink circles represent wild-type NS4A-CFP and NS3-YFP. The average normalized FRET signal is shown by the red line in each lane. Solid blue diamonds indicate no significant difference from the wild-type, green diamonds indicate significantly different from wild-type (P value < 0.5), and hollow diamonds indicate almost significantly different from the wild-type (P value of just over 0.5).

It will be important to test the two theories regarding NS4A(I29A) activation and binding (discussed in chapter 4). First, it will be essential to ensure proper NS3-4A processing has taken place in the pNS3-4A(I29A) construct by Western blotting for NS3 in membrane fractions of transfected Huh7 cells. Enrichment of NS3-4A-containing membranes by membrane fractionation will probably be necessary to visualize NS3 due to poor antibody affinity to the Con1 genotype.

Ideally, in conjunction with showing proper *cis* processing of NS3-4A(I29A), we would also show the localization of NS3 by immunofluorescence in these cells. If NS3 is properly bound to NS4A it would show a perinuclear membrane-bound staining pattern. However, the lack of commercially available anti-NS3 antibodies that recognize the Con1 strain of HCV is a difficulty. If deemed essential, this experiment can be done by inserting by site-directed mutagenesis an epitope tag onto the N-terminus of NS3 and then detecting the tagged-NS3 by immunofluorescence.

To test the induced fit model the Tm-4B/5A-DsRed substrate should be transfected in conjunction with NS3-YFP and NS4A-CFP and if correct, there should be a redistribution of NS3-YFP from cytoplasmic and nuclear to perinuclear and punctate.

Fluorescence Polarization

I have applied an *in vitro* method, fluorescence polarization, to measure association between NS3 and NS4A. In fluorescence polarization, the smaller protein is fluorescently tagged and excited with polarized light. Polarized light is then emitted from the fluorophore and the amount of emitted light that is still aligned with the excitation light is directly proportional to the molecule's rotation rate in solution. The rotation rate decreases when the tagged molecule binds to an interacting protein because it increases in size (Illustration A3.2).

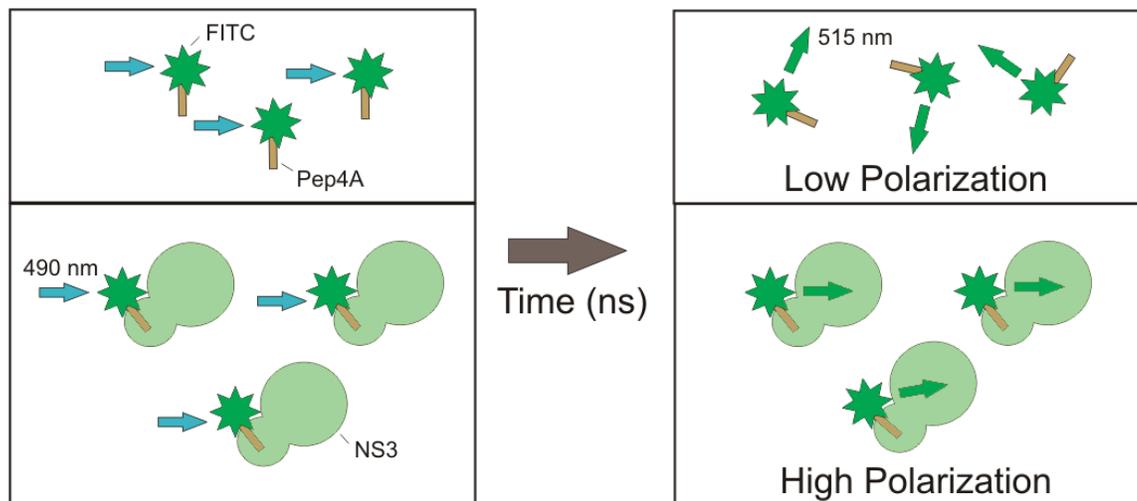


Illustration A3.2 Fluorescence Polarization

In fluorescence polarization, the smaller protein (the central domain of NS4A, “pep4A”) is fluorescently tagged and excited with polarized light. Polarized light is then emitted from the fluorophore and the amount of emitted light that is still aligned with the excitation light is directly proportional to the molecule's rotation rate in solution. The rotation rate decreases when the tagged molecule binds to an interacting protein because it increases in size.

I used NS3^{pro} and pep4A-fluorescein to optimize the assay conditions. The concentration of pep4AK-fluorescein, NaCl, DTT and detergent (Tween-20) were all optimized in order to obtain the best signal-to-noise ratio and most stable signal. To the same end, reaction temperature was optimized and addition of a stabilizing protein was evaluated. The optimized assay conditions were as follows done in black 384-well plates and a Victor³ polarimeter (Perkin

Elmer): 15 fmoles pep4AK (750 pM) in 50 mM Hepes (pH 7.3), 100 mM NaCl, 0.03% Tween-20, and 1 mM DTT in a final volume of 20 μ L. Incubation temperature was 26°C and readings stabilized after 2 hours.

NS3^{pro} was successfully titrated against pep4AK-fluorescein (Figure A3.3) a 1:1 stoichiometry of binding was observed as expected.

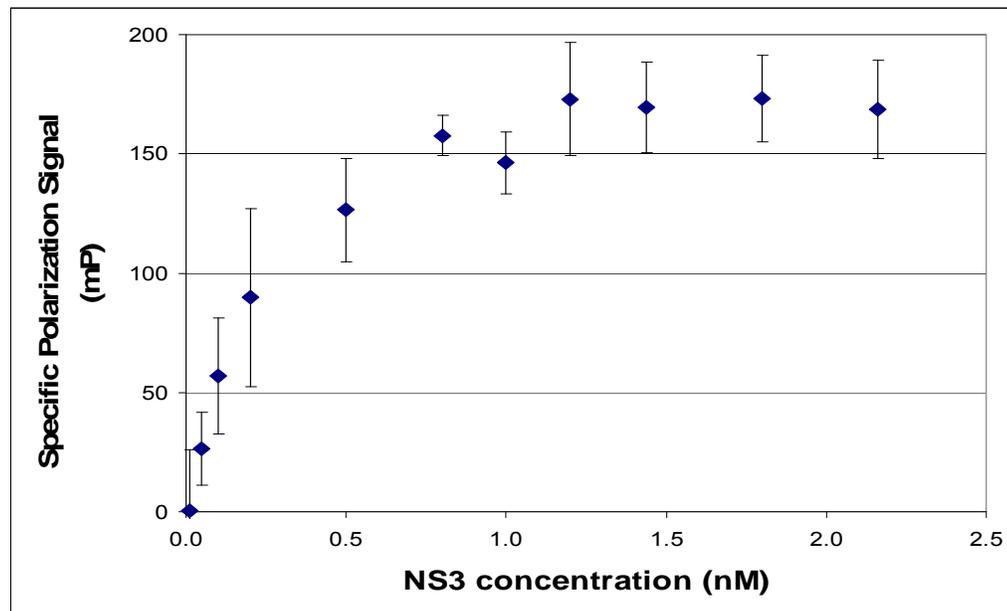


Figure A3.3 Titration of NS3^{pro} against 15 fmoles of pep4AK-fluorescein.

Varying amounts of NS3^{pro} were added to 15 fmoles pep4AK in 50 mM Hepes (pH 7.3), 100 mM NaCl, 0.03% Tween-20, and 1 mM DTT in a final volume of 20 μ L. Incubation temperature was 26°C and readings were taken every 15 minutes for at least 3 hours. The specific polarization signal is the average polarization value minus the average of the background polarization value at each time point. Each experiment was done in quadruplicate with the most deviating reading at each time point ignored in further calculations. This graph is the average of three time points (120, 135, and 150 min) from two experiments.

Evaluation of full-length NS3 in this system showed non-specific increases in polarization signal, most likely due to the high glycerol concentration in which this enzyme must be stored. Evaluation of the NS4A activation domain, kink, and acidic domain (“soluble 4A” or “s4A”) conjugated to fluorescein in this system showed a beginning polarization value that corresponds to the larger size of the s4A peptide in comparison to pep4A. The polarization value was very high, but determination of a K_d value should be possible. In addition, s4A is very hard to synthesize and only small amounts were available for use.

Testing of pep4A alanine mutants for binding to NS3^{pro} should also be done by fluorescence polarization. This data should complement the FRET assay as well as the activity assay, as it could detect very small differences in binding affinity. NS4A peptides were originally to be produced using the pPeptide expression and purification system developed by MoBiTec, however this process proved time consuming and synthesis of pep4AK-fluorescein is cost effective and fast.

K_d values are usually found for untagged variants by competition experiments with the fluorescent wild-type binder. My initial attempts at K_d determination by this method showed that with increasing concentrations of pep4AK-fluorescein, the peptide alone gives a high polarization reading, indicating that it may be aggregating. This is a common problem with hydrophobic peptides and might be remedied by addition of DMSO (5-10%) to the assay or by switching to polyethylene glycol (PEG)-conjugated peptides. DMSO or PEG may not fix the pep4AK aggregation problem, in which case each variant could be synthesized conjugated to fluorescein. The K_d can then be obtained by titrating Δ NS3 against a constant amount of the peptide.

This assay also has the potential to be a very useful high-throughput drug screening assay for allosteric inhibitors of NS3. To validate polarization assays for high-throughput screening, the Z' -factor is used. The Z' -factor is a measure of the statistical significance and takes into account the difference between the signal and background values, as well as the standard deviation between the positive and negative controls. An assay with a Z' -factor ≥ 0.5 is a robust assay that can be used for drug screening. Using the optimized conditions, the Z' -factor for this assay was consistently above 0.5 after 100 minutes of incubation (Figure A3.4). Using the peptide s4A in a high-throughput screening assay however would not be possible as Z' -factor of 0.5 will most likely not be reached with this larger peptide.

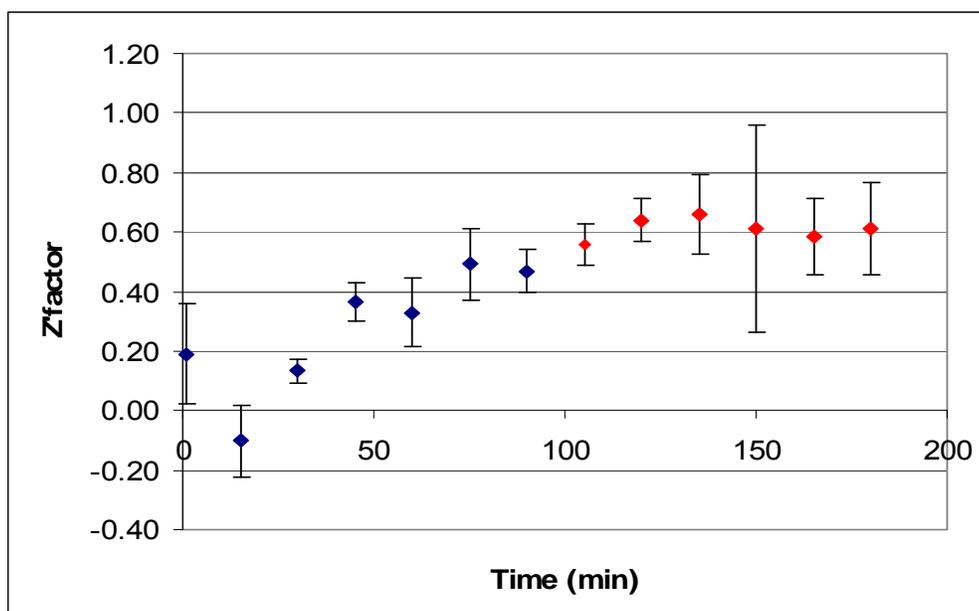


Figure A3.4 Z'-factor determination for NS3^{pro} and pep4AK-fluorescein.

2.5 μL NS3^{pro} was added to 15 fmoles pep4AK in 50 mM Hepes (pH 7.3), 100 mM NaCl, 0.03% Tween-20, 1 mM DTT in a final volume of 20 μL and incubated at 26°C. Readings were taken every 15 minutes for at least 3 hours.

The Z'-factor is calculated by the equation $Z' \text{-factor} = 1 - (3SD^+ + 3SD^-) / |\bar{\varnothing}^+ - \bar{\varnothing}^-|$ where “+” is the positive signal, “-” is the negative control, SD is standard deviation and $\bar{\varnothing}$ is the average polarization signal. The red points indicate a Z'-factor ≥ 0.5 . Each experiment was done in quintuplicate with the most deviating reading at each time point ignored in further calculations. This graph represents the average of three experiments.