Functional Characterisation of the Hepatitis C Virus NS2/3 and NS2 Proteins

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ABSTRACT

The hepatitis C virus (HCV) has infected over 170 million people worldwide and often leads to a chronic infection which can result in liver fibrosis, liver cirrhosis and hepatocellular carcinoma. In order to develop more specific therapies for this disease, thorough understanding of the virus and its proteins is essential. This thesis focuses on the characterisation of two of these HCV encoded proteins, NS2/3 and NS2. The HCV NS2/3 protease cleaves between NS2 and NS3 and we show that NS2/3 activity is absolutely required for genome replication. Indeed, mutation of any of the catalytic active site residues in NS2/3 to alanine results in the inability of NS2 replicons to replicate in both transient and stable replication assays. However this effect is not explained by impairment of the catalytic activities of NS3 as NS3 ATPase, helicase and protease activities are not significantly affected by the presence of uncleaved NS2 in several *in vitro* and cell based assays. Furthermore, while the mutant uncleaved NS2/3 protein is found to be rapidly degraded in cell systems in a proteasome-dependent manner, NS2 lysine mutagenesis that results in stabilization of uncleaved NS2/3 is insufficient to rescue replication of NS2/3 protease inactive replicons. This suggests that NS2/3 cannot functionally replace NS3 for viral replication and that cleavage to liberate NS3 is absolutely required. Furthermore, we show that cleaved NS2 is also rapidly degraded in transient and stable RNA replication systems and that this degradation is conserved amongst several genotypes, indicating that control of NS2 levels could be important for the viral life cycle. Interestingly, in contrast to uncleaved NS2/3, NS2 degradation does not appear to be dependent on ubiquitination or proteasomal degradation. Finally, several lysine residues in NS2 are identified to be involved in HCV infectivity in a JFH-1 infection system, indicating these are important amino acids/regions involved in viral assembly/release/infectivity.

RESUME

Plus de 170 millions de personnes sont infectées par le virus de l'hépatite C, une infection souvent chronique qui peut entraîner le développement de graves conséquences pour la santé. Afin de développer des traitements spécifiques contre l'hépatite C, il est essentiel de bien comprendre le fonctionnement moléculaire du virus et les rôles des protéines produites par celui-ci. Cette thèse est donc centrée sur la caractérisation fonctionnelle de deux de ces protéines, NS2/3 et NS2. NS2/3 est une protéase autocatalytique qui clive entre NS2 et NS3. Nous démontrons que l'activité de NS2/3 est essentielle pour la réplication de l'ARN génomique du virus. La mutagénèse dirigée inactivant le site catalytique de NS2/3 rend des réplicons contenant NS2 incapables de se répliquer dans des essais de réplication stables et transitoires. Cependant, cet effet sur la réplication génomique n'est pas le résultat d'une déficience au niveau des activités catalytiques de NS3. Les fonctions ATPase, hélicase et protéase de NS3 ne sont pas affectées de façon significative par la présence de NS2 lors d'essais in vitro et cellulaires. Toutefois, nous démontrons que la protéine NS2/3 inactive est rapidement dégradée par la voie du système ubiquitine-protéasome. Bien que la mutagénèse des lysines de NS2 résulte en une stabilisation des niveaux de NS2/3, ces mutations sont toutefois incapables de surmonter la déficience au niveau de la réplication des réplicons contenant une protéine NS2/3 inactive pour l'autoclivage. Ces résultats suggèrent donc que NS2/3 ne peut accomplir le rôle de NS3 lors de la réplication génomique virale. De plus, nos résultats démontrent que la protéine NS2 est aussi dégradée rapidement dans plusieurs systèmes de réplication de l'ARN du virus. Cette dégradation est observée chez plusieurs génotypes, suggérant que le contrôle des niveaux de NS2 pourrait être important pour le cycle de vie du virus. Cependant, les niveaux de NS2 ne dépendent pas de l'ubiquitination de cette protéine, ni de l'activité du protéasome. Finalement, les résultats de nos expériences utilisant le système d'infection JFH-1 impliquent plusieurs lysines de NS2 dans la production de virus infectieux, ce qui identifie des acides aminés ou des régions de NS2 qui seraient importants pour l'assemblage de particules virales infectieuses.

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CONTRIBUTION OF AUTHORS

CHAPTER 2: **Welbourn S**, Green R, Gamache I, Dandache S, Lohmann V, Bartenschlager R, Meerovitch K and Pause A. "Hepatitis C Virus NS2/3 Processing Is Required for NS3 Stability and Viral RNA Replication" *J. Biol. Chem.* 280 (2005) pp29604-29611

I generated or participated in the generation of the constructs shown in Figures 2.1A, 2.1C, 2.1D and the data shown in Figures 2.2, 2.3B, 2.4, 2.5, 2.6, 2.7 and 2.8. I also generated the purified mutant enzymes used in Table 2.1. I analysed the data, assembled the figures and wrote the paper. Robin Green contributed to the generation of the constructs shown in Figures 2.1A and 2.1D, performed initial optimizations for Figure 2.4 and helped generate the enzymes used in Table 2.1. Isabelle Gamache provided technical assistance and participated in Figures 2.3B, 2.6, 2.7 and 2.8. Serge Dandache participated in the cloning of the contructs shown in Figure 2.1A, generated the constructs shown in Figure 2.1B and provided Figure 2.3A. Volker Lohmann and Ralf Bartenshalager provided the WT and H952A mutant of the construct in Figure 2.1C and shared unpublished data. Karen Meerovitch performed the protease assay in Table 2.1.

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I performed all experiments shown in Figures 3.2, 3.3, 3.4, 3.5, 3.S1 and 3.S3, analysed data, assembled the figures and wrote the paper. Valerie Breton, together with myself, participated in the mutagenesis of the constructs used in Figures 3.2, 3.4, 3.6A-D and 3.S2. Vlastimil Jirasko provided the infectivity experiments in Figures 3.6A-D and 3.S2. Simon Reiss provided Figure 3.S4 and Francois Penin generated the JFH-1 NS2 model shown in Figure 3.6E.

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LIST OF ABBREVIATIONS

A	alanine
ALT	alanine aminotransferase
ARFP	alternative reading frame protein
Asp	aspartic acid
ATP	adenosine 5'-triphosphate
BVDV	bovine viral diarrhea virus
C-	carboxy
C	cysteine
Cd	cadmium
cDNA	complementary DNA
CIDE-B	cell death-inducing DFF45-like effector
CKII	casein kinase II
CMM	canine microsomal membranes
CRE	*cis*-acting replication element
Cys	cysteine
DMEM	Dulbecco's Modified Eagle's Medium
DMSO	dimethyl sulphoxide
DNA	deoxyribonucleic acid
ds	double-stranded
DTT	dithiothreitol
E	envelope
E	glutamic acid
E6AP	E6-associated protein
EDTA	ethylenediaminetetraacetic acid
EGTA	ethylene glycol tetraacetic acid
EMCV	encephalomyocarditis virus
ER	endoplasmic reticulum
ET	adapted wild type replicon
F	frameshift

GBV	GB-virus
Glu	glutamic acid
GTP	guanosine-5'-triphosphate
H	histidine
HAV	hepatitis A virus
HBV	hepatitis B virus
HCC	hepatocellular carcinoma
HCV	hepatitis C virus
HCVcc	hepatitis C virus cell culture system
HCVpp	hepatitis C virus pseudoparticle system
His	histidine
HIV	human immunodeficiency virus
hPLC1	human homolog 1 of protein linking intergrin-associated protein and cytoskeleton
HRP	horseradish peroxidase
HSP-90	heat shock protein 90
IDU	injection drug user
IFN	interferon
IP	immunoprecipitation
IPS-1	interferon-beta promoter stimulator 1
IRES	internal ribosomal entry site
IRF-3	interferon regulatory factor 3
ISDR	interferon sensitivity determining region
IVT	*in vitro* translation
JFH-1	japanese fulminant hepatitis 1
K	lysine
kDa	kilodalton
LDAO	*N,N*-dimethyldodecylamine *N*-oxide
Leu	leucine
LDL	low density lipoprotein
Mg	magnesium

MOPS	4-morpholinepropanesulfonic acid
N-	amino
N	asparagine
NF-kB	nuclear factor kB
NS2pro	NS2 protease domain
NANBH	non-A, non-B transfusion associated hepatitis
NS	non-structural
nt	nucleotide
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
PCR	polymerase chain reaction
peg-IFN	pegylated interferon
R	arginine
RdRp	RNA dependent RNA polymerase
RIG-I	retinoic acid-inducible gene
RNA	ribonucleic acid
RRL	rabbit reticulocyte lysate
S	serine
ss	single-stranded
SDS	sodium dodecyl sulphate
Ser	serine
SR-BI	scavenger receptor BI
SRP	signal recognition particle
STAT-C	specifically targeted antiviral therapy for hepatitis C
SVR	sustained virological response
TCEP	tris(2-carboxyethyl)phosphine
TLR-3	toll-like receptor 3
TM	transmembrane
TRIF	TIR-domain-containing adapter-inducing interferon-β
UTR	untranslated region
VBP-1	von Hippel-Lindau binding protein 1

VHL	von Hippel-Lindau
VLDL	very low density lipoprotein
Zn	zinc

OVERVIEW OF THE THESIS

Hepatitis C virus (HCV) is an important global health problem, with current estimates of 5 times more HCV infected individuals worldwide than those infected with HIV. There is no vaccine available to prevent infection and the current standard of care treatment is inadequate in treating all those infected. There is therefore the need for improved therapeutics with improved efficacy, specificity and side effect profiles. In order to be able to specifically target the virus in novel ways, it is of critical importance to fully understand the roles of viral proteins during infection and pathogenesis and the molecular mechanisms involved in the viral life cycle. The work presented in this thesis therefore focuses on the functional characterisation of two HCV proteins, the NS2/3 protease and the cleaved NS2 protein.

Chapter 1 presents an in depth literature review to introduce the hepatitis C virus. Its important clinical aspects are discussed, along with the cell systems used to study HCV and what is currently known about its molecular virology. The NS2/3 and NS2 proteins are then presented in more detail, with a focus on their potential roles and mechanisms of action. Finally, a brief overview of protein degradation, the ubiquitin-proteasome system and the relevance of these pathways for viral infection is given.

Chapter 2 consists of our work describing the importance of the active NS2/3 protease for HCV genome replication and the investigation of the possible reasons for this, including the effect of uncleaved NS2 on the catalytic activities and stability of the NS3 protein.

Chapter 3 presents a lysine mutagenesis study performed to further investigate the rapid degradation of the uncleaved NS2/3 protein and we also expanded these studies to the short-lived cleaved NS2 protein itself. The role and regions of NS2 involved in viral infectivity are also investigated by the mutational analysis presented in this chapter.

Finally, in Chapter 4, the major findings presented in Chapters 2 and 3 are further examined in light of more recently published data and their significance for the understanding of the HCV viral life cycle is discussed.

CHAPTER 1:

INTRODUCTION

Sections of this introduction were previously published in:

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1.1 HEPATITIS C VIRUS: CLINICAL ASPECTS

1.1.1 Epidemiology

Hepatitis C virus (HCV) infection is an important global health problem. The World Health Organisation estimates that over 170 million people worldwide have been infected with HCV, approximately 3% of the world's population [1]. However, prevalence of the infection varies according to region with North America and northern Europe showing low infection rates (<2.5%) while prevalence in other regions can be much higher, particularly in Egypt where approximately 15% of the population is infected (see Figure 1.1).

HCV is a blood-borne infection and therefore the major mode of transmission of the pathogen involves direct contact with contaminated blood or blood products, either through transfusion or the use of contaminated needles and syringes. While transmission through contaminated blood transfusions was a major method of infection in the past, the ability to screen the blood supply for HCV has almost completely eliminated this route of transmission in western countries [2], where now most new cases occur due to unsafe injection practises among injection drug users (IDUs) [3]. Indeed, the prevalence of HCV infection among IDU populations is believed to be between 50-90%, with co-infection with HIV being common [4]. Other sources of HCV transmission include infection through contaminated therapeutic injections (particularly in developing countries lacking appropriate sterilization/screening procedures/resources), accidental needlestick injuries among health care workers, and a low (4-7%) risk of infection from perinatal transmission. Sexual transmission of the virus has also been reported, but is not thought to be a major route of infection [3].

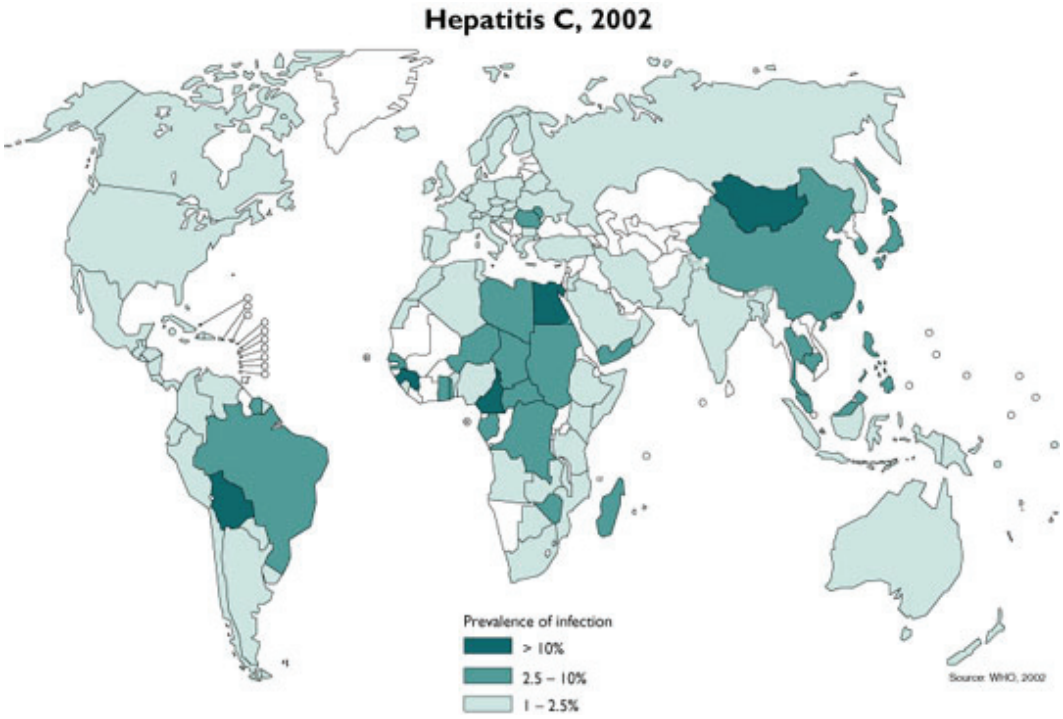

Figure 1.1: Global HCV prevalence distribution.

(from World Health Organization. International Travel and Health. 2008)

1.1.2 Clinical consequences of HCV infection

HCV leads to a chronic infection in up to 80% of individuals initially infected with the virus. However, this chronic infection is often asymptomatic and can lead to various hepatic consequences over a prolonged period of time [5-6]. The most common consequence of HCV infection is liver fibrosis, and approximately 10-15% of patients will then advance to liver cirrhosis over a period of several decades. The mechanism underlying the fibrosis development in HCV patients is thought to be mainly due not to the virus infection itself (although emerging roles of viral proteins in this regard are being reported), but to the immune response of the individual, which, while often inadequate in clearing the virus, persists and causes chronic inflammation of the liver, leading to fibrosis, which can eventually progress to cirrhosis and hepatocellular carcinoma (HCC) [7]. Indeed, HCV infection is the main cause of liver transplantations in developed countries and it has been reported that 27% of the cases of liver cirrhosis and 25% of the cases of hepatocellular carcinoma identified in patients are in HCV positive individuals [8].

While many HCV infected individuals will take decades before they develop symptoms of the disease, several clinical factors have recently been identified as promoting the rate of disease progression, such as age at time of infection, male gender, obesity, alcohol consumption, co-infection with HIV and genetic factors [9-14]. Moreover, in addition to the hepatic consequences, HCV infection has also been associated with several extrahepatic effects, such as lichen planus, cryoglobulinemia, porphyria cutanea tarda, kidney disease and in particular insulin resistance and diabetes [15]. Therefore, while recent studies have shown a general decrease in the incidence of new HCV cases since the 1990s, predictions show a likely considerable increase in the morbidity and mortality of the disease in the years to come [16].

1.1.3 HCV therapy

There is currently no vaccine available to prevent HCV infection. Unfortunately, the current "standard of care" therapy for HCV infection is relatively unspecific. It involves injections of pegylated-interferon (peg-IFN) combined with administration of the orally bioavailable general antiviral nucleoside analogue, ribavirin [17-19]. Interferon can cause serious side effects such as flu-like symptoms (fever, headache, chill, myalgia), neuropsychiatric disorders (irritability, apathy, fatigue, depression) and autoimmune syndromes [20]. In addition, haemolytic anemia has been particularly associated with ribavirin treatment [21]. As HCV treatment must be administered over a period of 24-48 weeks, these side effects are significant and cause frequent withdrawal (10-20% of patients) or modification (20-30%) of therapy [20]. In addition to these side effects and the high cost of treatment, a major issue is that peg-IFN-ribavirin therapy is only able to achieve an SVR (sustained virological response; undetectable viral RNA in serum 6 months after cessation of therapy) in between 40-80% of treated individuals [22]. This depends on the viral genotype the patient is infected with with genotype 2 and 3 patients achieving high rates of SVR while the widespread genotype 1 infection is more difficult to treat.

Therefore, new therapeutics against HCV are urgently needed and there has been significant progress achieved in recent years towards the development of a "Specifically Targeted Antiviral Therapy for hepatitis C" (STAT-C). Most efforts in the development of STAT-C agents have focused on the NS3 protease and NS5B polymerase. Several compounds targeting these enzymes are in clinical trials and are showing promising results in being able to reduce viral load when used in combination with peg-IFN and ribavirin [23-24]. However, resistance to individual compounds has been shown to occur both while investigating inhibitors in cell culture systems as well as in patient trials. It seems therefore likely that any future STAC-C therapy will comprise a combination of agents directed against several drug targets simultaneously to minimize the emergence of resistance.

1.2 HEPATITIS C VIRUS: THE VIRUS ITSELF

1.2.1 HCV discovery, identification and classification

Since the 1970s, after the development of testing for hepatitis A and hepatitis B, it has been known that there were additional agents causing liver disease in transfused patients [25]. These were referred to as cases of non-A, non-B transfusion associated hepatitis (NANBH) and were seen in 5-10% of transfused individuals in the United States. Much work was put into identifying the causative agent of NANBH and by 1985, enough properties of the causative agent had been described (see Tables 1.1 and 1.2) for it to be suggested to be a small enveloped RNA virus [26]. In was however in 1989 that a cDNA clone derived from the NANB viral hepatitis genome was isolated, with the resultant virus termed HCV [27]. The identified cDNA clone was found to be derived from an approximately 9600bp extrachromosomal RNA. This same year, these same researchers generated an assay to identify HCV antibodies in patients and showed HCV was indeed the major cause of NANBH throughout the world [28]. Once identified, the genetic organisation of this enveloped, positive-strand RNA virus was found to be similar to that of pestiviruses (such as bovine viral diarrhea virus (BVDV)) and flaviviruses (yellow fever virus, dengue virus) [29-30]. HCV is now classified as the sole member of the *Hepacivirus* genus in the Flaviviridae family [31].

1.2.2 HCV genotypes

Like most RNA viruses, there is a large amount of HCV sequence variability due to high mutation rate of the viral polymerase [32]. HCV sequences have been classified into 6 major genotypes, with additional subtypes within each group (eg 1b, 3a etc) (see Table 1.3) [33]. These genotypes have distinct geographical distributions, with genotypes 1a, 1b, 2a, 2b being widespread around

Table 1.1: Physiochemical properties of a tubule-forming, posttransfusion non-A, non-B hepatitis agent (virus)

(1) Infectivity is destroyed by:
   (a) formalin 1 : 1,000. 37°C. 96 h
   (b) heat 100°C. 5 min or 60°C. 10 h
   (c) treatment with 2O% v/v $CHCl_3$
(2) Agent contains essential lipid (enveloped)
(3) Diameter of infectious agent is <80 nm
(4) Can be pelleted from plasma
(5) Agent can be recovered from chronic-phase plasma by a multi-step procedure used for the purification of small, enveloped RNA viruses

Table 1.2: Pathogenetic properties of a tubule-forming posttransfusion non-A, non-B hepatitis agent (virus)

(1) Agent normally causes persistent infection and/or slowly-resolving disease
(2) Agent interferes with the replication of other hepatotrophic viruses (HAV, HBV)
(3) Specific ultrastructural changes associated with replication in chimpanzees are confined to hepatocyte cytoplasm
(4) Ultrastructural changes in chimpanzee hepatocytes are most similar to those induced by some enveloped mammalian RNA viruses
(5) Titer of virus in majority of inocula reported to be < 1 x IO^3 CID/ml
(6) Recrudescence of disease may spontaneously occur >3 yr after the initial infection

Adapted from reference [26]

Table 1.3: HCV genomic heterogeneity

Term	Definition	Nucleotide Similarity*
Genotype	Heterogeneity among different viruses	66% to 69%
Subtype	Closely related viruses within each genotype	77% to 80%
Quasispecies	Complex of genetic variants within individual viruses	91% to 99%

Data from [34]
* Full length genome sequence identity

Adapted from reference [35]

the world and together with genotype 3 comprise the strains most frequently found in North America. Genotype 4 is mainly found in Africa and the Middle East, genotype 5 in South Africa and genotype 6 in Southern China and Southeast Asia [35-36]. Interestingly, the different HCV sequences have different properties with respect to interferon treatment, clinical effects and their ability to grow in cell culture systems. For example, as mentioned previously, patients infected with genotype 2 or 3 respond much better to interferon therapy than genotype 1 patients and also only need to be treated for a shorter period of time (24 vs. 48 weeks) [22]. In addition, genotype 3 patients have been shown to be more likely to develop liver steatosis than patients infected with other genotypes [37], which could be linked to amino acid differences in the viral core protein [38]. And, for reasons still unknown, the only HCV sequence able to efficiently propagate in cell culture is based on a specific strain of genotype 2a, JFH-1 [39-41]. The variability between the different sequences may also be an issue when designing inhibitors of specific viral proteins as a compound designed against a genotype 1 enzyme [42] may not work as well against the slightly different enzyme of another genotype [43].

Even within a single patient, due to high replication rates of the virus and the viral polymerase lacking proofreading ability, HCV exists not as a single sequence but as millions of slightly different sequences termed the "quasispecies" [44]. This could also be an issue when designing new specific antiviral drugs as it is likely that resistance mutations to these compounds already "pre-exist" in a patient and could become the dominant sequence upon treatment with the inhibitor as they outgrow the "wildtype" sequence and cause viral rebound and treatment failure [45-47].

1.2.3 HCV genome organisation

The HCV genome is a positive strand RNA sequence of approximately 9.6kb. The RNA itself contains several highly conserved structural elements (see

Figure 1.2), in particular sequences in the 5' and 3' untranslated regions (UTRs) that are important for viral protein translation and virus replication and infectivity. An internal ribosomal entry site (IRES) in the 5'UTR is used to direct translation of a single polyprotein, approximately 3000 amino acids in length. This polyprotein is cleaved both co- and post-translationally by both cellular and viral factors. Whereas host signal peptidases are responsible for cleavages in the structural region, the viral NS2/3 protease cleaves between NS2 and NS3 and the NS3 serine protease cleaves the rest of the downstream non-structural polypeptide (see Figure 1.3).

1.2.3.1 HCV 5' and 3'UTRs

The HCV 5'UTR is highly conserved, 341nt in length and is separated into 4 structural domains (I to IV) that contain various stem loops (see Figure 1.2) [48-49]. The IRES from which polyprotein translation is initiated encompasses domains II-IV as well as continues on to nucleotide 354 in the core coding region [48, 50]. Translation initiation at the HCV IRES is independent of most canonical translational initiation factors required for cap-dependent translation (eg eIF4A, eiF4B, eiF4F) requiring only eIF3, eiF2-GTP-initiator tRNA for initiation complex formation [51]. Other factors such as the cellular protein La may also be involved in HCV translation [52] and the 3'UTR also likely plays a role in enhancing translational efficiency, possible through protein intermediates [53]. The HCV 3'UTR ranges from 200-235nt in length and is composed of a variable region, shown to not be essential for replication, a poly U/UC stretch, and a conserved 98nt X-region at the C-terminus [54-55]. Although this X-region is involved in RNA replication and has been shown to interact with sequences in both the 5'UTR [49] and in NS5B [56], the exact mechanisms involved in RNA synthesis remain to be determined.

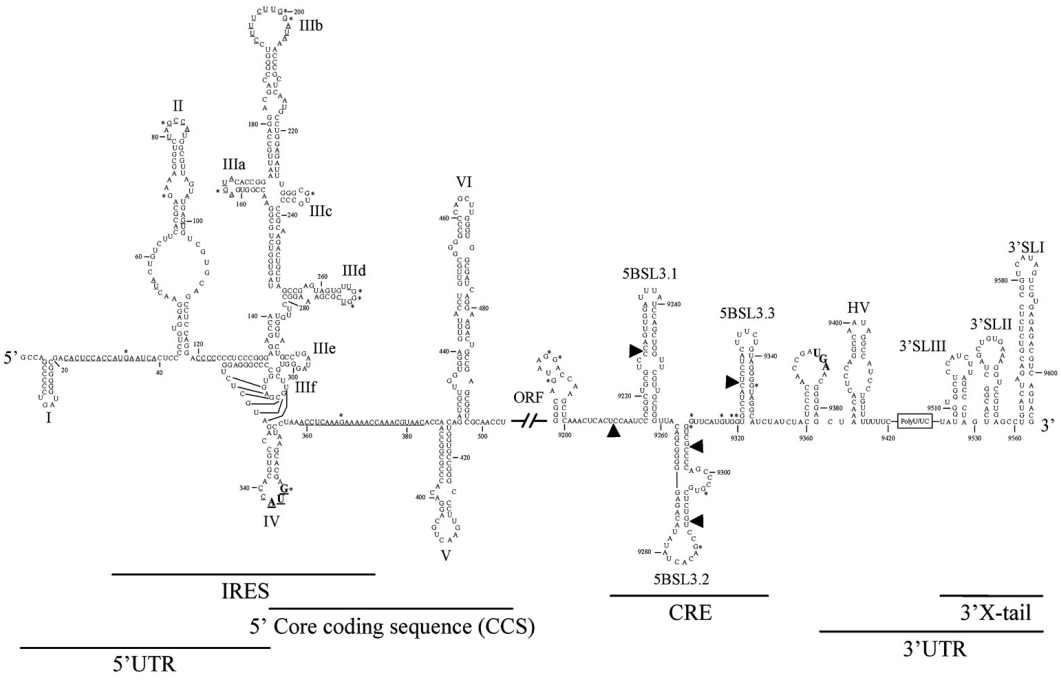

Figure 1.2: Sequence and secondary structure for the 5′ and the 3′ ends of the HCV genome.

The highly structured 5' and 3' unstranslated regions (UTRs) are indicated, as well as the 5' internal ribosome entry site (IRES) that overlaps with the core coding region and the *cis*-acting replication element (CRE) located in the NS5B coding region.

From reference [49]

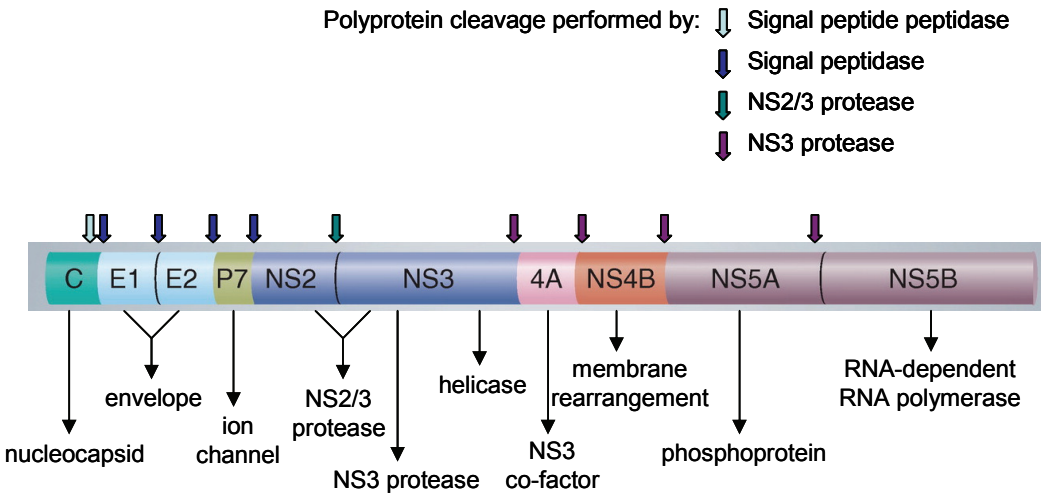

Figure 1.3: HCV polyprotein

The HCV polyprotein gets cleaved by host and viral proteases into 10 structural (core-p7) and non-structural (NS2-NS5B) proteins as indicated above. The major known functions of each HCV protein are indicated.

Adapted from reference [57]

1.2.3.2 HCV structural proteins

The amino-terminal portion of the viral polyprotein is made up of the structural proteins, core, E1, E2 and p7 that get cleaved into individual proteins by host signal peptidases in the ER membrane. These proteins are not required for genome replication and with the exception of p7, whose presence in the virion is still under debate, they make up the elements of the HCV viral particle itself.

Core

The HCV core protein is the nucleocapsid protein that binds the RNA genome for packaging. It is originally translated as a 23kD protein that is released from the polyprotein by ER signal peptidase cleavage at the core-E1 junction [58]. However, signal peptide peptidase then cleaves the E1 signal peptide located at the extreme C-term to yield the mature 21kD protein [59]. The N-terminus of core (domain I) is highly basic and responsible for binding RNA [58], whereas domain 2 is hydrophobic and important for interaction with membranes [60]. In particular, core was recently shown to induce the redistribution of, and localize to, lipid droplets proximal to the ER [61-62]. Core also co-localises with NS5A on lipid droplets and these are now thought to be sites important for viral assembly [63]. In addition to its role in viral packaging, the core protein has also been suggested to interact with many cellular proteins and influence various processes such as cell signalling, apoptosis and lipid metabolism and therefore be important for HCV persistence and pathogenesis [64].

ARFP

Various forms of the ARFP (alternative reading frame protein), F (frameshift) or core+1 protein have been described to be encoded in the core region of the genome and expressed via ribosomal frameshift or internal translation initiation [65-68]. While a protein encoded in this reading frame is not strictly required for viral replication [69], antibodies recognizing these sequences

are present in HCV patient serum [65-67], suggesting expression *in vivo*. Therefore, the roles and biological significance of this protein remain under investigation.

E1 and E2

E1 and E2 are the envelope proteins expressed on the surface of the HCV viral particle and are essential for viral entry [70]. They are both highly glycosolylated type 1 transmembrane proteins that together form non-covalent heterodimers [71] and are required for the binding of the HCV particle to the target cell, through interactions with receptors expressed on the hepatocyte. Although no direct interaction between envelope proteins and claudin 1 has been reported, E2 has been suggested to bind to the other three obligatory HCV entry factors, CD81, scavenger receptor B1 (SR-BI) and occludin [72-74]. In addition to their roles in binding the virus to the hepatocyte, although the mechanisms involved in this process are still under debate/investigation, it has also been suggested that sequences in E1 may play a role in viral fusion, after clathrin-dependent endocytosis of the viral particle [75]

p7

The HCV p7 protein is located in the viral polyprotein at the junction between the structural and non-structural proteins. While not required for genome replication, it has been shown to be essential for the generation of a productive infection in a chimpanzee [76]. p7 is a membrane protein consisting of 2 transmembrane helices separated by a cytosolic loop that oligomerizes into either heptamers or hexamers and possesses ion channel activity that can be blocked by several classes of compounds (eg. amantadine and long-alkyl-chain imino-sugar derivatives) [77-80]. Although recent work has shown the importance of p7 for HCV particle assembly and infectivity, its exact role in these processes remains to be investigated [81-82]. However, as these processes depend on the basic loop

residues required for ion channel activity, p7 is also under investigation as a target for future HCV antiviral drugs [83].

1.2.3.3 HCV non-structural proteins

NS2

NS2 is the first HCV non-structural protein translated in the polyprotein. The N-terminus of NS2 is cleaved from the adjacent p7 protein by host signal peptidases and together with the N-terminal third of NS3, this protein makes up the NS2/3 autoprotease, which is responsible for the cleavage at the NS2-NS3 junction. Although all NS proteins are proposed to play a role in viral replication, the exact functions of HCV NS2/3, as well as cleaved NS2 remain largely unexplored; however, some interesting potential functions have emerged in recent years, particularly with respect to roles in viral replication, assembly and modulation of host cell signalling. As the main focus of this thesis, both the NS2/3 and NS2 proteins will be introduced in detail in a later section of this introduction (see Section 1.4).

NS3/4A protease

The NS3/4A protease is a chymotrypsin-like serine protease responsible for the polyprotein cleavages at the NS3/NS4A (*cis* cleavage) and NS4A/NS4B, NS4B/NS5A, NS5A/NS5B (*trans* cleavage) junctions [84-87]. The NS3 protein contains in its N-terminal third the protease active site (His1083, Asp1107 and Ser1165) as well as a structural zinc binding site (Cys1123, Cys1125, Cys1171, His1175) required for proper folding of the protease[88-89]. The protease activity of NS3 has been shown to be enhanced by, and in some cases absolutely require, the presence of the middle stretch of amino acids of the NS4A protein [90-92]. NS4A (or peptides derived from it) acts as a co-factor to stimulate NS3 serine protease activity by binding the N-terminal domain of NS3. In the absence of NS4A, the crystal structure of the protease domain of NS3 shows an extreme N-

terminus that is largely unstructured [89]. However, upon NS4A binding, this co-factor leads to an organisation of the N-terminus of NS3, and indeed, contributes an integral strand to the NS3 N-terminal β-sheet [88, 93]. This also causes a conformational change in the NS3 active site that promotes efficient protease activity of the enzyme [88, 93]. NS4A also contains an N-terminal hydrophobic domain which is required to target NS3 to cellular membranes, the site of HCV replication[94].

In addition to its role in polyprotein processing, NS3/4A has been shown to play a role in virus escape from the host cell innate immune system. NS3/4A, through its protease function, specifically interferes with both TLR-3 and RIG-I pathways of IRF-3 (and NF-kB) activation and interferon production by cleaving the adaptor proteins TRIF and IPS-1 [95-98].

NS3 has long been one of the main HCV targets for drug development. Indeed, it was a peptidomimetic inhibitor of this enzyme that was used in the first proof-of-principle experiments that showed inhibition of viral replication by the inhibition of a viral enzyme, both in cell culture systems [99] and in patients [42]. Moreover, the most clinically advanced STAT-C agents, Boceprevir and Telaprevir (currently in phase 3 trials), are both NS3 inhibitors.

NS3 helicase

The C-terminal two thirds of the NS3 protein contain nucleic acid stimulated ATPase [100] as well as helicase activity [101]. Although the protease and helicase sections of NS3 fold into separate domains that can be independently functional when assayed on their own [102], there is also evidence of the NS3 protease and helicase domains modulating each others enzymatic activity in the context of the full length protein [103-104]. ATPase activity of NS3 was shown to be required for viral infection *in vivo* [105], however the exact role(s) of the NS3 helicase in the viral life cycle are still unclear. The most basic function of the NS3 helicase would involve unwinding of viral RNA secondary structure

and/or replication intermediates during genome replication. Indeed, RNA unwinding activity of the enzyme has been shown to be required for the replication of subgenomic replicons [106] . However, this helicase may have additional roles, as it is interesting to note that it is also a processive DNA helicase, a nucleic acid itself never present in the HCV genome replication cycle [107-108]. Perhaps even more interesting is the recent observation that the helicase domain of NS3 may also play an important role in HCV particle assembly [109].

NS4B

NS4B is a very hydrophobic 27 kDa integral membrane protein, whose role has not yet been extensively characterised [110]. It is proposed to contain four transmembrane domains in the central region of the protein, with both N-and C-termini located in the cytosol [111]. However, amphipathic helices at both ends of the protein likely still interact with the membrane and have roles in membrane targeting and formation of a functional replication complex [112-114]. Indeed, the most well described function of NS4B in HCV replication is its ability to induce membrane rearrangements into the ER-derived "membranous web" which is thought to be the site of HCV replication complex formation [115-116]. Furthermore, a nucleotide binding motif and GTPase activity have also been associated with NS4B, and although mutagenenic studies have shown it to be required for RNA replication, the function of this enzymatic activity in this process remains unclear [117]. Interestingly, a recent mutation analysis of the C-terminal domain of NS4B revealed an amino acid mutation that had no effect on membrane rearrangement or genome replication, but could modulate viral assembly and/or release, implicating a possible role for NS4B in these processes as well [118].

NS5A

NS5A is a phosphoprotein with many proposed functions in HCV replication, assembly and the modulation of host cell processes. NS5A consists of 3 domains (I, II and III) and includes an N-terminal amphipathetic helix for membrane association [119-120]. Domain I contains a structural zinc binding site and an RNA binding grove important of NS5A's function in genome replication [119, 121]. Domain II contains the "Interferon Sensitivity Determining Region"-ISDR), so named because polymorphisms in this region have correlated to response to interferon treatment in patients [122]. NS5A exists in two forms, basally phosphorylated (p56) and hyperphosphorylated (p58) [94]. It has been proposed that the modulation of the differentially phosphorylated forms of the protein serves to regulate various processes of the viral life cycle. For example, adaptive mutations in NS5A that decrease hyperphosphorylation have been shown to increase replication [123] but likely interfere with other steps required for viral infectivity [124]. Similarly, phosphorylation sites in domain III, which has been shown to be dispensable for genome replication [125-126], are critical for viral particle assembly [127] and have been suggested to be important for association with core at the surface of lipid droplets [128]. Therefore, NS5A and its phosphorylation level have been suggested to act as a switch between replication and assembly steps although much more work remains to be done [123, 129]. In addition to these roles, NS5A has been proposed to interact with a wide variety of cellular proteins and signalling pathways. These include modulating interferon response and the cellular innate immune system pathways, growth factor signalling, apoptosis, protein trafficking, and many others (reviewed in [130]).

NS5B

NS5B is the RNA-dependent RNA polymerase (RdRp) responsible for the replication of the viral genome. It has been shown to be capable of primer-independent *de novo* initiation [131] and replicates the genome by first using the positive strand RNA to synthesize a negative (replicative) strand RNA that is then used as a template for more production of the positive strand genome. The crystal structure of NS5B shows the typical polymerase "right hand" configuration with

palm, fingers and thumb domains and the active site located in the palm domain [132-133]. In addition, like most HCV proteins, it is anchored to membranes, via a C-terminal transmembrane helix that is not required for polymerase activity *in vitro* but is necessary for genome replication in cells [134]. NS5B has also been shown to interact with other viral (NS3, NS4B [135], NS5A [136]) and cellular (cyclophilin B [137]-in a genotype specific manner [138]) proteins which may modulate its activity during viral replication in cells. Together with the NS3 protein, NS5B has been a major target for HCV drug development. Several classes of inhibitors of this enzyme are under investigation: nucleoside analogues, that bind the active site and cause chain termination, and various classes of non-nucleoside inhibitors that bind to one of four different allosteric sites identified on the enzyme [23]. However, as with NS3 inhibitors, resistance against these compounds has been observed and therefore future therapy will likely require one or more NS5B inhibitors in combination with an NS3 inhibitor (and/or compounds targeted against yet another target) to minimize this problem.

1.2.4 HCV life cycle

The proposed HCV life cycle is illustrated in Figure 1.4. The virus first interacts with target hepatocytes through interaction between HCV envelope glycoproteins E1 and E2 and receptors expressed at the surface of the cell. HCV particles bind specifically to CD81 [72] and SR-B1 [73] receptors, although other molecules (eg LDL receptor [139], glycosaminoglycans [140], lectins [141]) may be involved in initial interactions or as co-receptors. It has then been suggested that the viral particle is then "transferred" to the other two HCV receptors, claudin-1 [142] and occludin [143] present in tight junctions at the basolateral surface of the cell. The particle is then internalized by clathrin-mediated endocytosis and fusion, uncoating and RNA genome release occur in an ill-defined, pH-dependent manner [144].

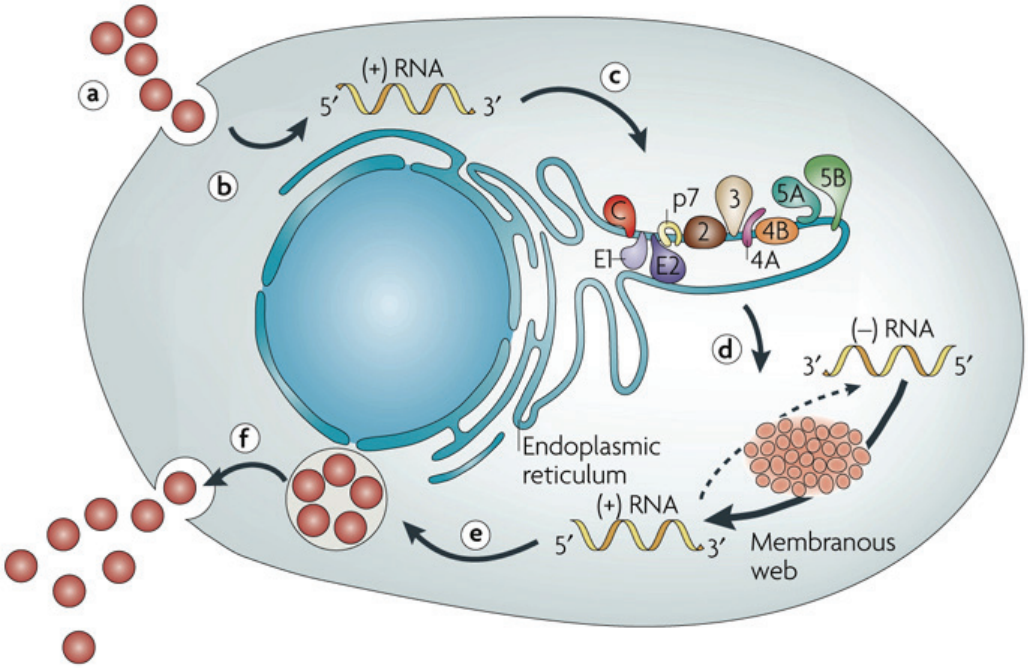

Figure 1.4: HCV life cycle

The major steps of the viral life cycle are illustrated: a) virus attachment and entry, b) uncoating, c) translation of HCV proteins on the ER membrane and polyprotein processing, d) formation of a "membranous web" and HCV genome replication, e) RNA genome encapsidation and HCV particle assembly, f) HCV particle release. See text for details.

Adapted from reference [145]

Translation of the positive-strand genome occurs at the ER, where the resultant polyprotein is cleaved by both cellular and viral factors into structural proteins and the non-structural proteins required for genome replication. Replication complexes form at the "membranous web" induced by NS4B [116] and replicate the RNA through a negative strand intermediate. The positive-strand RNA generated can then be used for further translation, replication or for packaging into new viral particles, however the mechanisms regulating the transition between each of these events remain largely undefined.

The process of viral particle assembly and release has been increasingly investigated in recent years with the availability of the JFH-1 cell culture system. It is clear that core (the nucleocapsid, RNA binding protein) has an important function in this process and it has been found to localize to the surface of lipid droplets, in proximity to the ER-derived replication complexes, likely the site of assembly [63]. Furthermore, p7 and NS2 have also been shown to be important for the early steps in viral assembly [81-82, 146]. Other non-structural proteins, such as NS3 [109], NS4B [118] and NS5A [127, 129] likely also play a role in assembly in addition to their functions in genome replication. Indeed, genetic evidence points to interactions between all of these proteins being important for viral particle assembly [147-149]. While the mechanisms involved in viral assembly, maturation and release require a lot more investigation to be fully defined, recent work has demonstrated that HCV virions may be secreted from the cells in a manner very similar to VLDL (very low density lipoprotein) [150-151]. Indeed, inhibition of this pathway also inhibits HCV secretion and HCV virions are found to interact very closely with VLDL itself [151-152].

1.3 STUDYING HCV

1.3.1 Animal models

1.3.1.1 The chimpanzee

The only other animal known to be susceptible to HCV infection is the chimpanzee. Despite several disadvantages, the chimpanzee has proved to be a very useful model. It has proved invaluable for the original isolation of the virus [27], the generation of infectious cDNA clones of the different genomes [153] and for the original functional analysis of the viral protein products and genetic sequences [105]. The major advantage of this model is that the infection in chimpanzee is very similar to human infection. The clinical course after infection involving the increasing viral titers, rise in ALT (alanine aminotransferase) levels, seroconversion and frequent viral persistence very well models the human infection, although the chimpanzee usually presents with milder disease than humans [154]. As the chimpanzees used in these studies are immunocompetent, much work has been elucidated (and correlated to human infection) with respect to the role of the immune system in the control and pathogenesis of the disease [155]. This also makes the chimpanzee a good model in which to measure the efficacy of potential preventative and therapeutic anti-HCV vaccines [156]. Despite these advantages, this model is extremely expensive and its endangered status also causes limited availability, thus limiting studies to 2-3 animals. Therefore several other models have emerged to complement the chimpanzee, particularly in the area of preclinical drug testing.

1.3.1.2 HCV mouse models

Although HCV is unable to infect and replicate in mouse cells, several groups have generated transgenic mice that express HCV proteins alone or in combination (for review see [157]). While these models may have some use in determining the roles of individual proteins in liver pathologenesis, the most useful HCV mouse model to date is the SCID-Alb/uPA chimeric mouse [158]. These are immunocompromised mice with a liver specific transgene that results in accelerated hepatocyte death. The damanged mouse liver can then be repopulated with human primary hepatocytes from a healthy donor. Upon infection with HCV, the chimeric mouse/human livers support HCV infection in a sustained and transmissible manner. While the immunocompromised status of these mice

prevent recapitulation of many aspects of the human HCV infection, this model can however be used for small molecule testing of antivirals as a preclinical step which encompasses not only pharmacodynamic and pharmacokinetic data, but also efficiency against the virus infection [159]. As these mice are significantly cheaper than the chimpanzee model and more widely available, statistical and dose-response studies can be performed with significantly larger numbers of animals. However, the mice are still fairly costly and considerable skill is required to maintain the fragile mouse colony and perform the hepatocyte transplantations (which is also dependent on the availability of fresh human hepatocytes).

1.3.2 Cell systems

1.3.2.1 The replicon system

For many years after the identification of HCV, it was impossible to study the HCV life cycle in cell systems. It was only in 1999 that a replicon system was developed that, which while not producing HCV particles, allowed the investigation of HCV genome replication [160]. The replicon system is illustrated in Figure 1.5 Bicistronic constructs are generated that contain the authentic 5' and 3' HCV UTRs and the non-structural proteins required for HCV genome replication. In the place of the structural genes (core-NS2, which are dispensable for genome replication), a selectable marker, such as a neomycin resistance gene, is added, whose translation depends on the HCV IRES in the 5'UTR. Following the selectable marker, a second IRES is inserted (e.g. EMCV, encephalomyocarditis virus, IRES) to direct translation of the NS3 to NS5B proteins. An RNA construct of the replicon is generated by *in vitro* transcription of the cDNA sequence and then transfected (commonly by electroporation) into an appropriate cell line (Huh-7, a liver hepatoma cell line). Upon treatment with antibiotic, only cells that contain autonomously replicating RNAs will be able to produce enough of the resistance protein to survive the selection. Therefore,

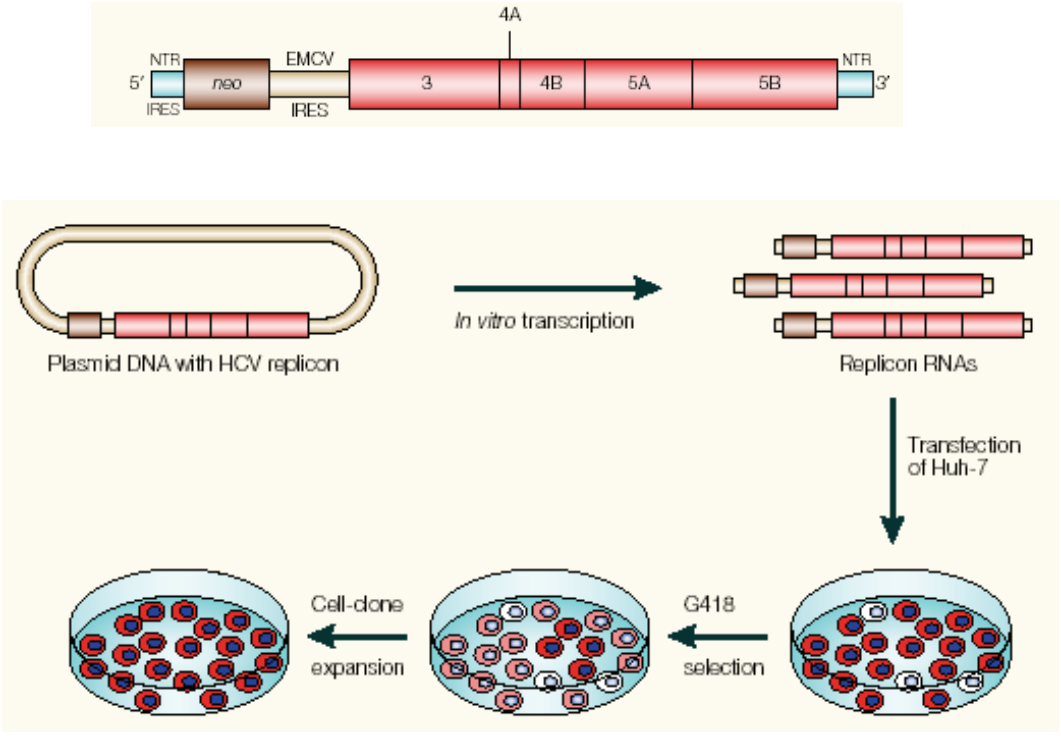

Figure 1.5: The HCV subgenomic replicon system.

An RNA transcript containing the HCV genes required for replication (NS3-NS5B) as well as a selectable marker (neomycin resistance) is transfected into Huh-7 cells. Cells that are not transfected (white), or which do not contain an actively replicating construct (pink) will eventually die off during G418 selection as they will not express enough of the resistance gene to survive. However, those that contain actively replicating RNA (red) will survive and form colonies. Therefore, efficiency of colony formation upon selection correlates with efficiency of replication of the replicon construct. See also text.

Adapted from [161]

efficiency of colony formation upon selection correlates with efficiency of replication of the replicon construct. In this way, the roles of viral proteins, their important residues/domains, as well as the importance of cellular factors and the effects of inhibitors on genome replication can be evaluated.

In addition to the selectable replicons used in stable colony-formation assays, transient replication assays have also been developed using replicons that contain a reporter gene, such as luciferase, in place of the selectable marker [162]. In this case, after transfection into cells, replication of the construct can be assessed 3-4 days later by measuring the levels/activity of the reporter. Although NS3 to NS5B proteins are required and sufficient for autonomous replication of the constructs, replicons can also be generated that include NS2 [160] or indeed all the structural proteins (genomic replicons) [163]. However, these genotype 1 based constructs do not produce infectious particles in this system.

The replication of these genotype 1a and 1b replicons in Huh-7 cells is still however a very inefficient process [160, 164]. It was found that replication efficiency can be increased in two ways. The first was the discovery of "adaptive" mutations that evolved in the replicon sequence of stably transfected cell lines. These mutations have been found mainly in NS3, NS4B and NS5A and when reinserted into wildtype replicons significantly increase their replication capacity [162, 164]. However, the effect of these same mutations on viral infectivity, at least *in vivo*, could in fact be deleterious [124]. The second way to increase replication efficiency was the development of highly permissive "cured" Huh-7 cell lines. These were generated from stable replicon-containing Huh-7 cells in which the replicon was then cleared using interferon (Huh-7.5 [165]) or a specific inhibitor (Huh-7 Lunet [166]), leaving a subpopulation of cells "pre-selected" to be permissive to HCV replication. The most widely used of these, Huh-7.5s, are highly permissive to HCV replication due to a mutation in the host cell innate immune response protein RIG-I [167].

However, as the replicon system only allows the investigation of genome replication and not other important aspects of the viral life cycle such as viral entry, assembly and release, other systems to study theses processes continued to be developed over the years.

1.3.2.2 The HCV pseudoparticle system (HCVpp)

The HCV pseudoparticle system (HCVpp) was developed in 2003 in order to allow the study of viral entry [168]. This system consists of transfecting a packaging cell line with plasmids encoding: 1) the HCV EI and E2 glycoproteins 2) lentiviral or retroviral packaging genes and 3) a defective lentiviral or retroviral genome containing a reporter gene, such as luciferase or GFP. This packaging cell will then produce lentiviral or retroviral particles that express the authentic HCV envelope proteins on their cell surface that can be used to study the requirements and mechanisms of viral particle attachment and cell entry [144, 169].

1.3.2.3 The JFH-1 cell culture infectious system (HCVcc)

In 2005, three groups finally published the description of a much awaited cell culture system that supports the full HCV life cycle [39-41]. This system is based on a specific strain of HCV genotype 2a, JFH-1 (Japanese Fulminant Hepatitis 1) that was isolated from a Japanese patient presenting with fulminant hepatitis [170]. Subgenomic replicons of this strain are able to replicate to high levels without the need for adaptive mutations [171] and the full length genome, when transfected into Huh7 based cells, replicates and for reasons still unknown, actually secretes viral particles, unlike constructs derived from other genotypes. These particles are infectious, both to naïve Huh-7 cells [39-41] and in an *in vivo* chimpanzee model [40, 172]. This system therefore allows for further investigation of what was known about HCV entry and genome replication, but now in the context of the full life cycle, as well as for the first time allowing the

investigation of viral particle assembly and release and much work in the past few years has focused on these processes.

The original JFH-1 system has also quickly been adapted. For example, by the insertion of reporter genes into the constructs to facilitate infectivity measurements [81, 173-174] and by the construction of functional chimeric constructs in which the JFH-1 non-structural proteins have been replaced with those from other, more clinically relevant, HCV strains [175-176].

1.4 THE NS2/3 PROTEASE AND NS2 PROTEIN

1.4.1 NS2/3 catalytic cleavage

1.4.1.1 General structural features of NS2/3

The NS2/3 protease is responsible for the intramolecular cleavage of NS2 from NS3 between aa 1026 and 1027 [177-178]. Figure 1.6 shows the main structural and functional domains of the protein. NS2 contains a highly hydrophobic N-terminal region suggested to contain multiple transmembrane segments. However, this region is not required for efficient cleavage at the NS2/3 site [178-180]. The minimal domain for activity of the enzyme *in vitro* has been mapped to aa 907-1206 [179]. This encompasses the C-terminal portion of NS2, immediately following the hydrophobic region, as well as the N-terminal protease domain of NS3. Although these sequences are required and sufficient for cleavage activity, processing is not dependent on the NS3 serine protease activity [177-178]. This differs from the NS2B protein of flaviviruses, in which the NS3 protease performs a *cis*-cleavage at the NS2B site and then uses NS2B as a co-factor for the processing of the downstream polypeptide [181-183].

1.4.1.2 NS2/3 processing requirements

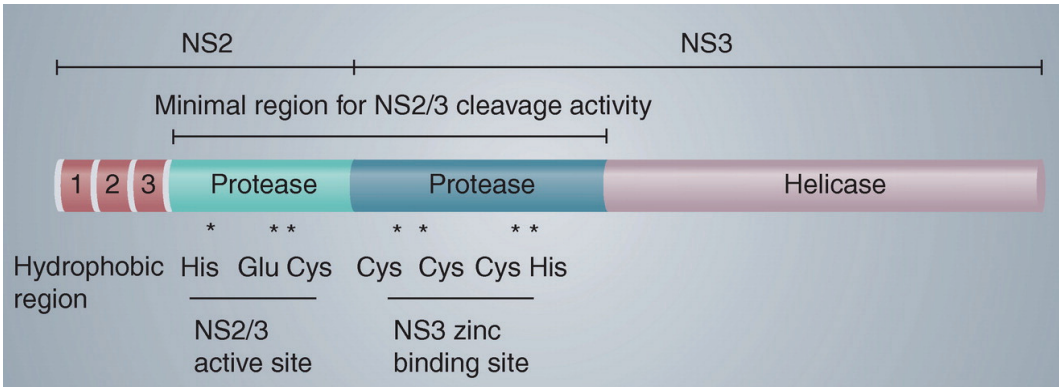

Figure 1.6: Functional domains of the NS2/3 protease.

The NS2 protein contains an N-terminal hydrophobic region containing a putative three transmembrane domains, as well as a protease domain containing the histidine, glutamic acid and cysteine residues of the NS2/3 protease active site. The NS3 protein contains serine protease activity in its N-terminus and helicase activity in its C-terminus. NS3 also contains a structural zinc binding site in its protease domain that is required for both NS2/3 and NS3 protease activities. The minimal region of NS2/3 required for cleavage at the NS2-NS3 junction is shown.

From reference [57]

The HCV NS2/3 protease shows no sequence motifs typical of known proteases; however, sequence alignments show similarity with the GB-virus NS2/3 protein as well as the bovine viral diarrhea virus (BVDV) NS2/3 protein [184]. Residues H952, E972 and C993 are conserved among all genotypes of HCV and mutation of any of these amino acids to alanine completely inhibits NS2/3 cleavage activity [177-178, 185]. Furthermore, although NS3 serine protease activity is not required for NS2/3 processing, the NS3 protease domain cannot be substituted for another NS protein [186]. In addition, mutation of cysteine residues 1123, 1127 and 1171 in NS3, which together with H1175 participate in the coordination of a zinc molecule [88-89], abolishes both NS3 and NS2/3 activities [178, 187], presumably by disrupting folding of the enzymes. Indeed, while for many years it was believed that the NS2/3 protease required the full NS3 protease domain in *cis* for cleavage, very recent evidence in cell expression systems has demonstrated that the NS2 protein itself may be a *bona fide* protease that requires only the structural zinc-binding domain of NS3 as a co-factor for efficient cleavage [188].

Proper folding of the NS2/3 protein and cleavage site plays an important role in the efficiency of NS2/3 processing. Residues surrounding the cleavage site, WRLL↓APIT, are highly conserved between HCV genotypes, but are remarkably resistant to mutations [189-190]. Only mutations severely affecting the conformation of the cleavage site (such as deletion or proline substitution of P1 or P1') severely inhibit cleavage. Furthermore, NS4A-derived peptides that upon binding cause a conformational rearrangement of the NS3 N-terminus are potent inhibitors of NS2/3 activity, likely by altering the positioning of the cleavage site [180, 191]. The presence of microsomal membranes or non-ionic detergents has been found to be required for *in vitro* processing at the NS2/3 site in certain genotypes [186, 192], while increasing the efficiency of cleavage of others [177, 186], suggesting the hydrophobic environment is necessary for proper folding of the enzyme and positioning of the cleavage site. Similarly, Waxman *et al*. have demonstrated the requirement for the ATP hydrolyzing ability of the molecular chaperone HSP90 for efficient cleavage in *in vitro* and

cell bases assays [193]. A similar phenomenon has been described for the BVDV NS2/3 protein where a cellular DnaJ chaperone protein, Jiv, has been found to associate with and modulate NS2/3 activity, possibly by causing a conformational change in the protein [194]. Further evidence of a role for cellular chaperones in inducing/maintaining the proper conformation of NS2/3 required for cleavage comes from the very recent observation that the peptidyl-prolyl-isomerase activity of cyclophilin A may be important for NS2/3 protease function in the replicon system [195].

1.4.1.3 Mechanism of action: a novel cysteine protease

Initial studies showing NS2/3 activity was inhibited by EDTA and stimulated by zinc led to the early suggestion that NS2/3 functions as a zinc-dependent metalloprotease [178]. However, with the discovery of the importance of zinc for the structural integrity of the NS3 protease domain, others then proposed NS2/3 may be a novel cysteine protease with a catalytic dyad composed of H952 and C993, with the possible involvement of E972 as the third residue of a catalytic triad. Inhibition studies both in *in vitro* translation systems and with purified proteins failed to yield a definite classification [179-180, 192]. Although inhibited by metal chelators such as phenanthroline and EDTA, this inhibition was relieved by the addition of $ZnCl_2$, $CdCl_2$ or $MgCl_2$. This could therefore point to a structural rather than catalytic role for the zinc molecule as Cd has not traditionally been able to functionally replace Zn in other metalloproteases [196-198]. However, although classical cysteine protease inhibitors iodoacetamide and N-ethylmaleimide showed strong inhibition of NS2/3 processing, no single cysteine was found to be more susceptible to these alkylating agents [179] and it was only with the recently elucidated crystal structure of its NS2 portion (NS2pro) that the enzyme was firmly established as a novel cysteine protease [199].

The crystal structure of the N-terminal truncated NS2 portion (aa 904-1026) of the NS2/3 enzyme is shown in Figure 1.7. This structure shows a

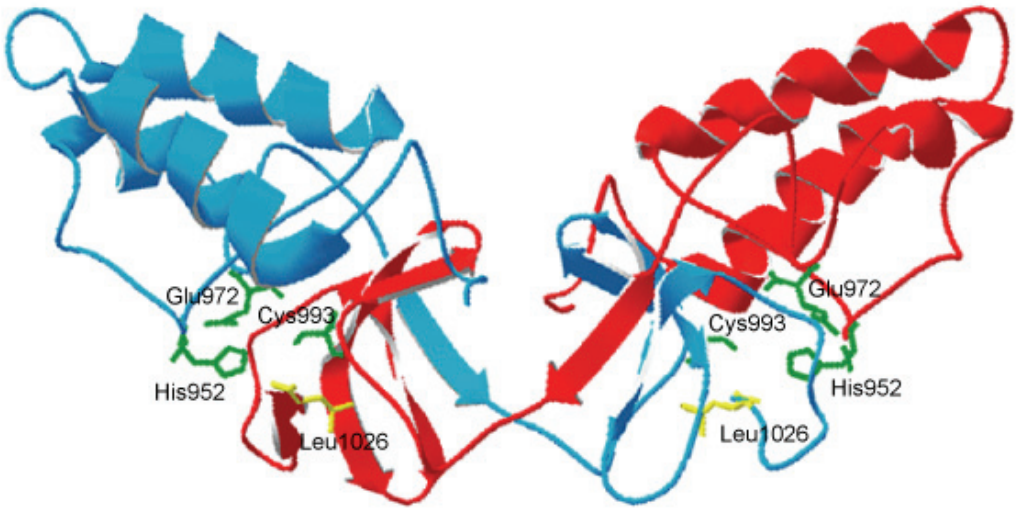

Figure 1.7: Crystal structure of the protease domain of NS2

Structure of the NS2 protease domain showing the composite active sites (green) with His952 (Con-1 polyprotein numbering), Glu972 provided by one monomer of the dimer and Cys993 provided by the other chain. The C-terminal residue of NS2 (Leu1026, yellow) is shown in close proximity to the active site. Figure was created using coordinates from [199] deposited in the protein database, ID number 2HD0.

Adapted from reference [57].

dimeric enzyme containing two composite active sites. Each active site shows the catalytic histidine and glutamic acid residues present on one monomer with the catalytic cysteine of the triad contributed by the monomer. As this structure essentially depicts the NS2 cleavage product of the NS2/3 enzyme, it is interesting to note that the C-terminal leucine of the protein remains coordinated in the active site, which was suggested to potentially function to inactivate the protease after cleavage into NS2 and NS3 has occurred [199].

1.4.1.4 NS2/3 bimolecular cleavage

Bimolecular cleavage of NS2/3 had been shown to occur, albeit inefficiently, in cell transfection experiments [177, 190]. In this system, NS2/3 proteins with mutations/deletions in either the NS2 or NS3 domains could support cleavage provided the missing functional region was co-expressed on a separate polypeptide. In addition, catalytically inactive NS2/3 mutants were also found to inhibit processing of a wildtype protein when expressed in *trans*. The observation that a recombinant NS2/3 protein forms dimers *in vitro* was consistent with these findings [179]. However, no *trans* cleavage has been observed using purified proteins [179-180]. Interestingly, NS2/3 activity *in vitro* was found to be concentration-dependent, supporting the notion that dimer formation is essential for the reaction [179]. Dimitrova *et al.* have also demonstrated the homo-association of the NS2 protein in various systems and suggest that the cleavage between NS2 and NS3 could potentially be performed by dimers of NS2/3 encoded on neighbouring polyprotein chains [200]. These observations were recently confirmed by the dimeric crystal structure of NS2 [199]. In addition, co-transfection in mammalian cells of mutant NS2/3 constructs in which either the catalytic cysteine or histidine residues are mutated to alanine, still results in some cleavage of the NS2/3 precursor due to the presense of one functional active site on the dimer (contributed by the cysteine residue on the histidine-mutant monomer and the histidine residue on the cysteine-mutant monomer) [199]. As NS2/3 cleavage was widely believed to be an intramolecular event, the

significance of these findings with respect to the polyprotein processing events of HCV infection will be of interest to determine.

1.4.2 Role of NS2/3 cleavage in viral replication

NS2/3 cleavage is required for viral replication *in vivo*, as demonstrated by an HCV clone devoid of NS2/3 activity that fails to cause a persistent infection in a chimpanzee [105]. However, NS3-5B subgenomic replicons not encoding the NS2 protein replicate efficiently in Huh-7 cells [160], suggesting that NS2 itself is not strictly required for genome replication. If cleavage at the NS2/3 site occurs solely for the release of the NS2 protein, what is the advantage for the virus of encoding two distinct proteases for polyprotein processing? Although several roles have been proposed for the cleaved NS2 protein, the NS2/3 protease itself appears unique in that its activity subsequently causes its inactivation.

1.4.2.1 NS2 as part of the replication complex?

HCV RNA replication has been proposed to occur via the formation of a membrane bound replication complex that comprises the association of the non-structural proteins required for genome replication (NS3-5B) [116]. However, as an infectious cell culture system to study the HCV life cycle has only recently been established, studies focusing on the replication complex have so far mainly used a subgenomic replicon system where NS2 is not expressed. Several studies have indicated that NS2 is an integral membrane protein that is targeted to the ER [186, 201]. Interestingly, NS2 has been found by one group to be inserted into the membrane only when expressed in the context of the NS2/3 protein, and only after cleavage from NS3 [186]. NS2 has also been found to interact with all the other HCV non-structural proteins in *in vitro* pull downs, as well as cell-based co-localisation and co-immunoprecipitation experiments [86, 200, 202]. Moreover, both NS2 and NS2/3 have been suggested to affect HCV translation and NS5B activity [203-204]. Therefore, although not absolutely required for RNA

replication, the possible presence of NS2 in this complex as an accessory protein is plausible.

1.4.3 Role of NS2 in viral particle assembly and release

The recent construction of intergenotypic infectious chimeras containing Con1 (genotype 1b), H77 (1a) or J6 (2a) structural proteins in the JFH-1 (2a) backbone suggests that the optimal junction point of the chimera is within NS2, immediately following the first putative transmembrane domain [175]. The most efficient chimeras contained the C-term of NS2 of the same strain as the replicase and the N-term of the same strain as the structural proteins. This was therefore the first suggestion that NS2 may play a dual role in the viral life cycle and, in addition to its role in NS2/3 cleavage, may also be involved in virus assembly and release, possibly through association of the N-terminus of NS2 with structural proteins and/or p7.

Although the exact role of NS2 in viral particle assembly and infectivity is still under intense investigation, several studies have been published in the last few years confirming its important role in these processes. Jones *et al.* and Jirasko *et al.* showed that seemingly the entire NS2 protein is required for viral infectivity, as deletion of either the whole protein itself, any of the putative transmembrane helices or the protease domain inhibited the secretion of infection viral particles in a cell culture system [81, 146]. Interestingly, both groups also showed, using bicistronic constructs in which NS2 is physically separated from NS3 using an IRES, that the protease active site contained in NS2 does not have an essential role in viral infectivity after NS2/3 cleavage is accomplished. Furthermore, the role of NS2 in viral assembly/infectivity is able to be *trans* complemented by expression of the functional wildtype protein [146, 205]. NS2 has been shown to act at a stage prior to virion release from the cell as the formation of infectious intracellular particles is inhibited by defects in NS2 [81]. Indeed, Yi *et al.* very recently proposed NS2 to act at a "maturation" stage of

virion morphogenesis [205]. However, the exact details, as well as the confirmation of the many protein-protein interactions (NS2 with core, E1, E2, p7, NS3, NS5A and possibly other unknown cellular factors [147-148, 175, 205-207]) genetically suggested to contribute to this process remain to be fully defined.

1.4.4 Other proposed roles of NS2

1.4.4.1 NS2 is an integral membrane protein

The NS2 protein derived from the cleavage of NS2/3 is inserted into the ER membrane through its N-terminal hydrophobic domain. However, the exact mechanisms of translocation as well as the membrane topology of the protein remain controversial. Membrane association has been found to be dependent on SRP(signal recognition particle)-SRP receptor targeting [186]. It was originally proposed that a signal sequence present in upstream p7 was required co-translationally, although NS2 translocation has subsequently been demonstrated by several groups to be p7 independent [186, 201]. Furthermore, although the cleavage at the p7-NS2 junction is performed in a membrane-dependent fashion by signal peptidase [208-209] and the presence of membranes is stimulatory (and for some strains required) for NS2/3 cleavage, one group has shown that the integration of NS2 into the membrane is performed post-translationally, and only after cleavage from NS3 [186]. However, Yamaga and Ou have since proposed that translocation could occur co-translationally and therefore the exact mechanisms of integration remain unclear [201]. The amino terminal region of NS2 is likely to span the membrane several times [179, 201]. While the exact number of transmembrane domains, and their orientation in the membrane, have not been conclusively determined, one current model suggests three transmembrane domains with a cytosolic protease domain [146] (see Figure 1.8). However, the possibility that only one helix is inserted into the membrane, or even that NS2 may adopt different conformations relating to its various functions, has not been excluded.

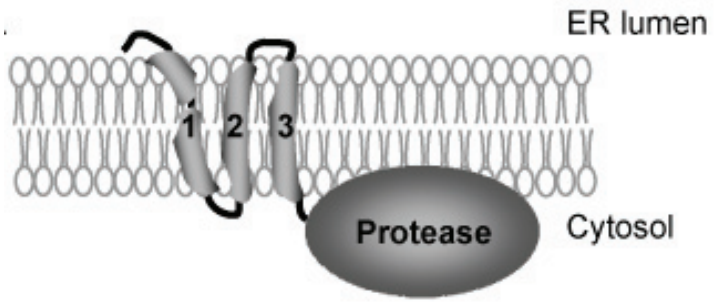

Figure 1.8: Schematic representation of the membrane association of the NS2 protein

Putative transmembrane domains (1, 2, 3) and the cytosolic protease domain are shown.

Adapted from reference [210]

1.4.4.2 NS2 and NS5A hyperphosphorylation

HCV NS5A has many roles in RNA replication, viral infectivity and the modulation of the host cell environment during infection and has been found to be present in two distinct phosphorylated forms: p56 and p58. Liu *et al.* have reported the importance of NS2 for the generation of hyperphosphorylated NS5A (p58) [211]. Using plasmids expressing various section of the HCV polyprotein in transient transfection experiments, they demonstrate the requirement of NS2 generated by the cleavage of NS2/3 for the formation of p58. However, while performing similar experiments, other groups have demonstrated the appearance of p58 without the presence of NS2 [212-213]. Neddermann *et al.* therefore suggested that NS2 itself is not required for the hyperphosphorylation process, but rather that is could be the authentic N-terminus of NS3, generated by NS2/3 cleavage that is of importance [213]. However, as these two proteins have been suggested to cooperate during HCV particle assembly [205], the importance of the various phosphorylated forms of NS5A for interaction with NS2 remains an interesting question for future studies.

1.4.4.3 NS2 inhibition of gene expression

NS2 may also play a role in modulating cellular gene expression in infected cells. One study by Dumoulin *et al.* found that NS2 exerted a general inhibitory effect on the expression of a reporter gene expressed from a variety of different promoters (human ferrochelatase promoter, NFkappaB binding sites, SV40 promoter/enhancer sequences, full length, as well as minimal TNF-alpha promoters and cytomegalovirus immediate-early promoter) in several different hepatic and non-hepatic cell types [214]. The amino-terminal (810-940) region of NS2 was sufficient to cause this effect, suggesting inhibition of gene expression is not dependent on the activity of the NS2/3 protease itself. It was therefore suggested that NS2 could potentially regulate host cell protein levels by interfering with a general aspect of transcription or translation. Indeed, Kaukinen *et al.* have also shown NS2 to inhibit a variety of cellular promoters involved in

cytokine gene expression, such as IFN-β, CCL5/RANTES and CXCL10/IP-10 promoters [215]. Several other HCV-encoded proteins, including core, NS3/4A, NS4B and NS5A, have also been demonstrated to alter cellular gene expression through a variety of mechanisms [215-219]. This aspect of NS2 function will require further confirmation and careful investigation as it indicates a potential role for NS2 in the modulation of the host cell environment which has important implications for both the establishment of persistent infection and the pathogenesis of chronic hepatitis C.

1.4.4.4 NS2 and apoptosis

In order to establish a persistent infection, many viruses have evolved mechanisms to interfere with cellular apoptosis. In this manner, the virus is able to replicate to sufficient levels without the elimination of the host cell. Several HCV proteins have been implicated in the modulation of cell signaling and apoptosis, including core, E2, NS5A and NS2 [220-223]. Machida *et al.* have reported that Fas-mediated apoptosis is inhibited in transgenic mice expressing HCV core, E1, E2 and NS2 proteins [222]. The expression of these proteins in the liver prevented cytochrome c release from the mitochondria as well as prevented the activation of caspase 9 and caspase 3/7, but did not affect caspase 8. Therefore, this implicates these HCV proteins in the mitochondrial intrinsic apoptotic pathway, which involves mitochondrial membrane permeabilization and the release of pro-apoptotic factors, resulting in cell death. Furthermore, Erdmann *et al.* showed that NS2 inhibits CIDE-B-induced apoptosis in co-expression experiments [224]. CIDE-B (cell death-inducing DFF45-like effector) is a mitochondrial pro-apoptotic protein whose overexpression has been shown to induce cell death [225]. CIDE-B-induced apoptosis requires mitochondrial localization and dimerization of the protein, both of which are mediated by a region in its C-terminal domain [226]. NS2 was found to interact specifically with the C-terminal region of CIDE-B and block cytochrome c release from the mitochondria as well as cell death [224]. NS2 could therefore potentially prevent the dimerization of CIDE-B required for activity. However, the mechanism of

inhibition remains unclear as NS2 is thought to be localized to the ER membrane. In this case, NS2 could potentially bind and sequester CIDE-B, preventing its localization to the mitochondria.

1.4.4.5 NS2 and cell cycle progression

Expression of the NS2 protein has been shown to inhibit cell proliferation in both transient (Huh-7) and stable (Vero, HeLa) cell assays [227]. Expression of NS2 was found to be associated with a 40-50% decrease in the growth rate of the cells, resulting from cell cycle arrest in the S-phase. This was also correlated with a decrease in the levels of cyclin A RNA and protein. Although the mechanisms involved in these observations are currently unknown, it is suggested that NS2 may modulate the cell cycle to help provide a cellular environment that is advantageous for viral replication [227].

These observations that NS2 could potentially modulate the host cell environment are of particular interest. However, most of these studies have been performed using individual expression of the NS2 protein from DNA vectors transfected into a variety of cell lines; now that it is possible, it would be interesting to have validation of these finding in the more physiological setting of the complete viral life cycle.

1.5 PROTEIN DEGRADATION

Controlling protein levels is an important way to regulate many cellular processes. Indeed the regulated degradation of many specific proteins is important for progression of the cell cycle [228], the transient action of transcription factors [229], oncogenes and tumour suppressors [230], as well as many other cell signaling pathways that are modulated via this process (for review see [231]).

1.5.1 The ubiquitin-proteasome pathway

The most common, and most well studied, mechanism of protein degradation involves the ubiquitin-proteasome system (reviewed in [232], Figure 1.9). This pathway involves the covalent attachment of ubiquitin (a small 76 amino acid protein) chains to lysine residues of the protein to be targeted for degradation. This attachment involves the action of at least three enzymes: E1, the ubiquitin-activating enzyme, E2, the ubiquitin-carrier protein, and E3, the ubiquitin ligase responsible for the final transfer of ubiquitin to the target protein. This last protein is as well often responsible for the specificity of this process (for example by recognizing special binding domains or phosphorylation sequences on the target protein). The ubiquitinated protein can then be recognized by the 26S proteasome and degraded in an ATP-dependent manner. Although it contains threonine protease active sites, several aldehyde cysteine and serine protease inhibitors can also inhibit the proteasome [233]. The most commonly encountered proteasome inhibitor, MG132 (*N*-Carboxybenzyl-LeuLeuLeu-Aldehyde), is indeed a peptide aldehyde and has been widely used to block proteasomal activity in cell culture systems in order to study protein degradation.

1.5.2 Protein degradation and viral infection

Protein degradation can have an important role in the regulation of viral infectivity. For example, the EMCV and HAV 3C proteases, as well as the Sindbis virus RNA polymerase (among many others) have been shown to be rapidly degraded, which is suggested to be a possible mechanism of regulating the different steps of the various life cycles [234-236]. Similar to this, the HIV-1 integrase was recently shown to be selectively degraded at a post-integration step of HIV-1 infection, a process that was found to be mediated by VBP-1 (von Hippel-Lindau (VHL) binding protein 1) and VHL E3 ligase activity [237]. In this case, the timing of the degradation was suggested to facilitate the transition between the genome integration and transcription steps of the viral life cycle. In

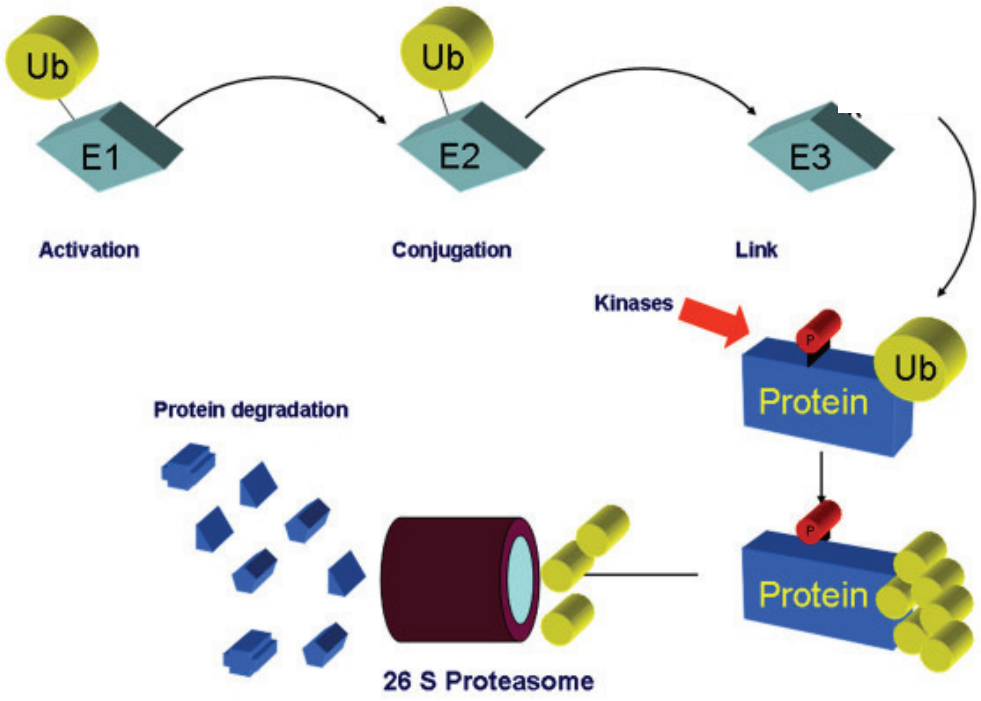

Figure 1.9: The ubiquitin-proteasome system

Ubiquitin conjugation involves activation of the ubiquitin molecule by an E1 enzyme, followed by transfer of the ubiquitin to an E2 enzyme and subsequent attachment of the ubiquitin to a lysine residue in the protein target by an E3 ubiquitin ligase. The E3 ligase specifically recognizes the protein target to be degraded, often by a phosphorylation signal. The ubiquitinated protein is then degraded by the 26S proteasome and the ubiquitin molecules recycled.

Adapted from reference [238]

addition to viral proteins themselves being targeted for degradation, there are several examples of viral proteins targeting other cellular factors for degradation in order to facilitate virus replication (eg. HIV proteins Vif and Vpu dependent degradation of restriction factors APOBEC3G and tetherin [239-240]).

Interestingly, the levels of several HCV proteins have also been suggested to be regulated in ways that could affect viral replication and infectivity. The nucleocapsid protein core has many potential roles in virus assembly and modification of the host cell environment and one group has shown it to interact with E6AP (E6-associated protein), an E3 ubiquitin ligase, and be ubiquitinated and degraded in a proteasome-dependent manner [241]. Indeed, increasing levels of E6AP resulted in a decrease in levels of core and this correlated with a decrease in infectivity titers in a JFH-1 infectious virus experiment. Correspondingly, siRNA knockdown of E6AP resulted in an increase in both core levels and supernatant infectivity titers. Similarly, the ubiquitin-like protein hPLIC1 has been reported to bind the viral NS5B polymerase and be capable of modulating its levels [242]. Overexpression of hPLC1 was able to decrease NS5B half-life significantly, which resulted in a decrease in NS5B levels and subsequent decrease in the replication of subgenomic replicons. Interestingly, HCV NS2 has also been reported to be rapidly degraded in a CKII-phosphorylation dependent manner [243]. However, these studies were mainly performed using overexpression of tagged NS2 encoded on DNA constructs and the mechanism and significance of NS2 degradation remains unclear. Whether levels of these, or any other HCV protein, are regulated in a temporal manner during viral infection remains an important and interesting question, as this could potentially involve them in regulation of the various steps of the HCV viral life cycle.

1.6 RATIONALE FOR THE THESIS WORK

The preceding introduction summarizes the current state of HCV knowledge (2009). However, at the time this thesis project was initiated in 2003

much less was known, particularly with respect to the functions of the NS2/3 and NS2 proteins, which were largely uncharacterised. While it had been shown in 2000 that NS2/3 cleavage was required for the generation of a productive infection in a chimpanzee [105], the cause for this had not been further investigated. This effect seen *in vivo* could have been due to a defect in viral genome replication and/or the requirement for the cleaved NS2 and/or NS3 protein in another aspect of the viral life cycle. We therefore initiated our work to further characterise NS2/3 by looking at the importance of NS2/3 cleavage for HCV genome replication itself using the replicon cell system.

CHAPTER 2:

HEPATITIS C VIRUS NS2/3 PROCESSING IS REQUIRED FOR NS3 STABILITY AND VIRAL RNA REPLICATION

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2.1 ABSTRACT

The hepatitis C virus NS2/3 protease is responsible for cleavage of the viral polyprotein between non-structural proteins NS2 and NS3. We show here that mutation of three highly conserved residues in NS2, H952, E972 and C993, abrogates NS2/3 protease activity and that introduction of any of these mutations into subgenomic NS2-5B replicons results in complete inactivation of NS2/3 processing and RNA replication in both stable and transient replication assays. The effect of uncleaved NS2 on the various activities of NS3 was therefore explored. Unprocessed NS2 had no significant effect on the *in vitro* ATPase and helicase activities of NS3, whereas immunoprecipitation experiments demonstrated a decreased affinity of NS4A for uncleaved NS2/3 as compared to NS3. This subsequently resulted in reduced kinetics in an *in vitro* NS3 protease assay by the unprocessed NS2/3 protein. Interestingly, NS3 was still capable of efficient processing of the polyprotein expressed from a subgenomic replicon in Huh-7 cells in the presence of uncleaved NS2. Importantly, we show that fusion with NS2 leads to the rapid degradation of NS3, whose activity is essential for RNA replication. Finally, we demonstrate that uncleaved NS2/3 degradation can be prevented by the addition of a proteasome inhibitor. We therefore propose that NS2/3 processing is a critical step in the viral life cycle and is required to permit the accumulation of sufficient NS3 for RNA replication to occur. The regulation of NS2/3 cleavage could constitute a novel mechanism of switching between viral RNA replication and other processes of the HCV life cycle.

2.2 INTRODUCTION

Hepatitis C virus is the primary causative agent of parenterally transmitted and community acquired non-A, non-B viral hepatitis and an important cause of chronic liver disease leading to cirrhosis and hepatocellular carcinoma in humans [27-28, 244]. It is estimated that nearly 200 million individuals worldwide are currently infected with HCV. Of particular concern is that the virus establishes a

chronic infection in approximately 85% of cases and there are no specific and broadly effective anti-HCV compounds to date [244].

HCV is a single stranded positive sense RNA virus of the *Flaviviridae* family [29-30]. It encodes a single polypeptide of approximately 3000 amino acids in length that is cleaved co and post-translationally into both structural (core, E1, E2 and p7) and non-structural (NS2, NS3, NS4A, NS4B, NS5A, NS5B) proteins (reviewed in ref. [245]). Host signal peptidases are responsible for cleaving the structural proteins, while two virally encoded proteases (NS2/3 and NS3) process the non-structural proteins required for viral replication. NS3 is a serine protease that mediates the *cis*-cleavage at the NS3/4A site, as well as the *trans*-cleavage of NS4B, NS5A and NS5B [84-87]. NS4A forms a stable complex with the N-terminus of NS3 and acts as an essential cofactor for its protease activity, while also anchoring NS3 to the ER membrane where a replication complex is thought to form [86, 94, 246]. The C-terminal segment of the NS3 protein also harbours nucleoside triphosphatase and RNA helicase activities [101, 247]. Although much work has focused on the elucidation of the function of both protease and helicase domains of NS3, the role of the NS2/3 protease remains to be extensively characterised.

NS2/3 is an autocatalytic protease that is responsible for the intramolecular cleavage of NS2 and NS3 at amino acids 1026-1027 [177-178]. Encoded by NS2 and the N-terminal third of NS3, NS2/3 extends from amino acids 810-1206 with a minimal region required for activity beginning at amino acid 907 [179]. Although NS2/3 cleavage does not require NS3 protease activity, the protease domain of NS3 cannot be substituted for another protein, suggesting it plays a structural role in the folding of the NS2/3 enzyme [177, 186]. No homology between NS2/3 and other proteases has been identified and the catalytic mechanism of action remains unclear. Although the observation that NS2/3 activity is stimulated by zinc and inhibited by EDTA has led some groups to suggest NS2/3 is a novel metalloprotease, others have proposed it may function as

a cysteine protease and studies performed with classical protease inhibitors have not yielded a definite classification [179-180, 192]. Mutagenesis studies have identified amino acids His 952 and Cys 993 within NS2 as being essential for NS2/3 protease activity [177-178] and in addition, mutations thought to perturb the local conformation of the cleavage site also inactivate the enzyme [190]. Furthermore, molecular chaperones have been proposed to be required for efficient cleavage at the NS2/3 site [193].

Recently, a few studies have focused on the possible roles of NS2 after its release from NS3 [186, 214, 224, 243], however, the exact role of NS2/3 processing in viral replication remains unclear. Although NS3-3' UTR replicons have been shown to replicate efficiently in Huh-7 cells in the absence of NS2 [160], NS2/3 activity has been shown to be essential for productive replication *in vivo*, as demonstrated by an HCV clone devoid of NS2/3 protease activity that fails to establish a productive infection in a chimpanzee [105]. In this study, we further investigated the role of NS2/3 cleavage in viral replication by using the replicon system. Our results demonstrate the critical importance of NS2/3 autoprocessing for RNA replication. The ability of uncleaved NS2/3 to perform the various NS3 catalytic functions was therefore examined in order to determine the mechanism by which unprocessed NS2/3 could interfere with viral RNA replication. We show that the protease, ATPase and helicase functions of NS3 are not significantly affected by uncleaved NS2 but that the presence of NS2 leads to a rapid degradation of the NS3 protein, possibly constituting for the virus a novel mechanism of regulating viral RNA replication.

2.3 EXPERIMENTAL PROCEDURES

2.3.1 Strains and constructs.

To generate an adapted NS2-3'UTR genotype 1b replicon (pNeo/2-5b), a PmeI-BsrGI fragment from I389neo/NS2-3'/wt [160] was inserted into adapted

replicon pFKNeo/3-3'/5.1 [162]. To construct NS2/3 mutants, DNA fragments representing aa 806-1106 were generated from this template by overlapping PCRs using synthetic oligonucleotide primers for the insertion of the mutations. The resulting DNA was digested with SnaBI and BsrGI and then ligated back into the corresponding site of pNeo/2-5B to create pNeo/2-5B WT, H952A, E972A and C993A. For *in vitro* translation constructs, a 2x FLAG tag was generated with overlapping oligonucleotide annealing, digested, and ligated to the HindIII-BamHI fragment of pcDNA3. DNA containing NS2/3 (aa810-1657) and NS3 (aa 1027-1657) was amplified from the replicon constructs and ligated to the EcoRI-XhoI fragment of pcDNA3 2xF to generate 2xF NS2/3 and 2xF NS3. A DNA fragment containing NS4A was amplified with PCR from pNeo/2-5B, digested, and ligated to the BsrGI-XhoI sites 2xF NS2/3 and 2xF NS3 to form 2xF NS2/3/4A and 2xF NS3/4A. NS2/3 protease mutants were generated by inserting a BsiWI-BsrGI fragment (aa 947-1100) from pNeo/2-5B replicon constructs containing the NS2/3 mutations, into the BsiWI-BsrGI site of the 2xF NS2/3/4A construct to form 2xF NS2/3/4A H952A, E972A and C993A. For bacterial expression constructs, the NS2/3(904-1206) and NS3(1027-1206) sequences were amplified from pNeo/2-5B. An N-terminal His6 tag was included in the synthetic oligonucleotide primer to facilitate downstream purification. The DNA was then inserted into a pET11d vector via XhoI-BamHI sites that were included in the oligonucleotide primers used in the amplification. Plasmid pFK repPI-luc/NS2-3'/ET (referred to in this report as PI-luc/NS2-3'/ET) was generated by insertion of a fragment encompassing part of the EMCV-IRES, NS2 and the amino terminus of NS3 from plasmid I389neo/NS2-3'/wt [160] into pFK rep PI-luc/ET [248], using Hind III and a Sfi I restriction sites. The replicon harbours adaptive mutations E1202G, T1280I and K1846T, referring to the amino acid position of the polyprotein from the Con1-isolate (EMBL-database accession number AJ242654). Plasmid pFK repPI-luc/NS2-3'/GND, which is replication deficient due to a aspartic acid to asparagine substitution in the active centre of the NS5B-polymerase at position 2737 of the polyprotein was generated by replacing the NS5B region in pFK repPI-luc/NS2-3'/ET with a corresponding fragment from

plasmid pFK I341 sp PI luc EI3420-9605/GND [249] using a Xho I restriction site within NS5A and a Spe I restriction site directly adjacent to the 3'NTR. Mutation H952A was introduced by site-directed mutagenesis in a PCR-reaction, the PCR-fragment containing the mutation was introduced into pFK repPI-luc/NS2-3'/ET using BsiWI and BsrGI restriction sites to obtain pFK repPI-luc/NS2-3'/H952A. E972A, C993A, S977A as well as double mutations were introduced by site-directed mutagenesis of an NS2/3 fragment in pNEB193 (QuickChange Site-Directed Mutagenesis kit, Stratagene) and then ligated into pFK repPI-luc/NS2-3'/ET using BsiWI and BsrGI restriction sites. In all cases, correct insertion of mutations and fidelity of DNA sequence was verified by sequencing.

2.3.2 *In vitro* transcription and purification of RNA

Circular DNA plasmids were linearized with XbaI for the 2xF expression constructs, Sca1 for the pNeo/2-5B replicon contructs and AseI/ScaI for PI-luc/NS2-3' replicon constructs and purified using phenol/chloroform extraction. DNA was transcribed with T7 RNA polymerase (Ambion Megascript kit, Ambion) following the manufacturers suggested protocol, template DNA was removed by digestion with DNase 1 and the RNA was purified by passing through a column (RNeasy mini kit, Qiagen) and dissolved in RNase-free water. The RNA concentration was determined by measuring the optical density at 260nm and RNA integrity was checked by denaturing agarose gel electrophoresis.

2.3.3 Cell culture

Huh-7 cells were grown in Dulbecco's modified Eagles medium (DMEM; Gibco-BRL, Invitrogen Life Technologies) supplemented with 10% fetal bovine serum, 100 U penicillin, 100 μg streptomycin as well as 100 μM non-essential amino acids. A Huh-7 clone cured with a selective inhibitor cells was used for transient replication assays [166].

2.3.4 Electroporation of replicons and G418 selection

Sub-confluent Huh-7 cells were electroporated with RNA (10 µg) and selected with G418 as described previously [250]. Four weeks after transfection, colonies were stained with Coomassie Blue stain.

2.3.5 Transient replication assays with luciferase replicons

Transient replication assays were performed as described previously [248] with several modifications. In brief, 1 µg RNA was mixed with 400 µl cured Huh-7 cells (10^7 cells/ml in Cytomix [251] containing 2 mM ATP and 5 mM glutathione), electroporated as described, immediately transferred to 12 ml complete DMEM and seeded into 6-well plates. Cells were harvested 4, 24, 48 and 72 h after electroporation. For luciferase activity assays, cells were scraped from the plate with 350 µl luciferase lysis buffer (1% Triton X-100, 25 mM glycylglycine pH 7.8, 15 mM $MgSO_4$, 4 mM EGTA, 1 mM DTT). Cleared lysate (100 µl) was mixed with 360 µl luciferase assay buffer (25 mM glycylglycine, 15 mM $MgSO_4$, 4 mM EGTA, 1 mM DTT, 2 mM ATP, 15 mM K_2P0_4; pH 7.8) and was measured for 20 s in a luminometer (Lumat LB9507, Berthold Technologies) after addition of 200 µl of a 200 µM luciferin solution. Values obtained with cells harvested after 4 h were used to correct for transfection efficiency.

2.3.6 Immunoblotting

For western blot analysis, 20 µg RNA was electroporated as described above for transient replication assays and cells were seeded into 10 cm plates. Cells were harvested by scraping the cells of the plate in phosphate buffered saline (PBS), centrifuged and lysis was performed by resuspension of the pellet in 5% SDS and multiple passages of the cells through the tip of a 21 gauge needle. Following quantitation (DC Protein Assay, Bio-Rad), total cell proteins were separated by 8% SDS-PAGE and transferred to a nitrocellulose membrane. Membrane blocking and antibody dilutions were performed in 5% milk in PBS

with 0.2% (vol/vol) Tween 20 for NS3 or luciferase (Chemicon International) detection and in 2% milk in PBS with 0.5% Tween 20 for NS5B analysis. For antibody detection, appropriate species-specific horseradish peroxidase (HRP)-conjugated secondary antibodies (anti-rabbit from Amersham Biosciences, anti-mouse from Jackson ImmunoResearch laboratories) and Western Lightning Chemiluminescense Reagents (Perkin Elmer) were used. To ensure equal loading of lanes, a non-specific band present on all membranes was used as an internal control.

2.3.7 Proteasome inhibitor treatment

Cells were electroporated as described above for western blot assays. 4 hours after plating, cells were treated with 10 μM MG132 (Sigma) or vehicle control (dimethyl sulphoxide) for an additional 4 or 20 hours. Cells were then processed for western blot analysis as described above.

2.3.8 Immunoprecipitation of *in vitro* translated NS2/3/4A mutants and NS3/4A

Purified RNA was translated for 90 minutes at 30ºC in the presence of [^{35}S] methionine and cysteine (Redivue Pro-mix, Amersham), using a rabbit reticulocyte lysate (RRL) system (Promega) following manufacturers suggested protocol. Lysates were then incubated with Ez view Red ANTI-FLAG M2 Affinity gel (Sigma) at 4ºC for 2 hrs in IP buffer containing 20 mM Hepes-KOH pH 7.7, 150 mM NaCl, 10% glycerol and 0.5% Triton X-100. Following centrifugation at 8200 x g for 30 seconds, beads were washed three times with 1ml cold IP buffer. Immunoprecipitated protein was eluted from the beads with 3X Flag peptide (Sigma, final concentration 150 ng/μl). Radiolabelled proteins were visualized by SDS-PAGE followed by autoradiography. Relative band intensity was measured using a FUJI X BAS 2000 phosphorimager.

2.3.9 ATPase and Helicase Assays

NS3 ATPase assays were performed based on the colourimetric method described by Chan *et al.* and Kyono *et al.* [252-253]. Equimolar concentrations of *in vitro* translated, immunoprecipitated FLAG-tagged proteins were mixed with 50 mM Hepes pH 7.5, 2.5 mM $MgCl_2$, 0.04 μg/μl poly(U) and the reaction initiated by the addition of 2 mM ATP. The reaction was allowed to proceed at room temperature for 30 min and the quantity of released phosphate was determined by the addition of 4 volumes of malachite green/molybdate/polyvinyl alcohol reagent prepared as described by Chan *et al*. [252]. The absorbance of the coloured complex was measured at 630nm on a SPECTROmax Plus 384 spectrophotometer (Molecular Devices) and the amount of phosphate present was determined by using a standard curve obtained using potassium dihydrogen phosphate solutions of known concentration. NS3 helicase activity was measured based on methods used by Gallinari *et al.* [247] and a DNA probe developed by Pang *et al*. [108]. Briefly, an 18 bp release strand oligonucleotide was labelled at the 5' end using T4 polynucleotide kinase and [γ-^{32}P] ATP (Perkin Elmer) and purified by passing through a G-25 sephadex column. Annealing to a partially complementary 36 bp strand was performed by heating the labelled and unlabelled strands in a 1:3 ratio to 95ºC for 2 min followed by slow equilibration to room temperature in 10 mM Tris pH 8, 1 mM EDTA and 100 mM NaCl. Unwinding was measured by preincubating immunoprecipitated proteins in 25 mM MOPS-NaOH pH 7, 3 mM $MgCl_2$, 2 mM DTT and 0.1 μg/μl BSA for 10min with 1.25 fmol/μl labelled probe. The reaction was initiated by the addition of 4 mM ATP and allowed to proceed for 30 min at 30ºC. The reaction was stopped by the addition of 2X stop buffer (50 mM EDTA pH 8, 0.8% SDS, 0.04% NP-40, 20% glycerol, 0.4 mg/ml bromophenol blue) and excess cold release strand. The reaction products were run on a 20% polyacrylamide gel, visualized by autoradiography and quantified by phosphorimaging.

2.3.10 Enzyme expression and purification

NS3(1027-1206) and NS2/3(904-1206) were expressed in *E. coli* BL21(DE3)pLysS cells. Cells were grown at 37ºC to an OD600 of 0.4-0.5 and following induction with 1 mM IPTG were grown at 37ºC for a further 3 hours. Cells were harvested at 6000x g and the pellet was stored at -80ºC. Protein was purified and folded as described in Thibeault *et al*. [180]. In brief, cells pellets were thawed at room temperature (21ºC) and sonicated at 4ºC in a lysis buffer containing 100 mM Tris, pH8.0, 1% Triton X-100, 5 mM EDTA, 20 mM $MgCl_2$, 5 mM DTT. Following centrifugation at 30,000 x g for 30 minutes at 4ºC, the insoluble pellet was homogenized in extraction buffer (100 mM Tris, pH 8.0, 6 M guanidine HCl, 0.5 M NaCl) using a glass tissue homogenizer. The supernatant was clarified at 30,000 x g for 1 hour at 4ºC. To purify the protein, the supernatant was mixed with Ni-NTA agarose (Qiagen) for 1 hour at 4ºC. Beads were recovered by centrifugation and washed twice with extraction buffer containing 20 mM imidazole. Protein was then eluted with 200 mM imidazole. Fractions containing the purified protein were pooled and quantified using the Bio-Rad Protein Assay (Bio-Rad). NS2/3 and NS3 proteins were folded on a superose 12 (10/300) column (Amersham Biosciences) equilibrated in 50 mM Tris, pH 8.0, 0.5 M arginine HCl, 1% LDAO, 5 mM TCEP as per Thibeault *et al*. [180].

2.3.11 NS3 protease kinetics

Enzymatic assays and kinetics were performed using a fluorogenic substrate and purified enzyme. Briefly, enzyme activity was determined by monitoring the fluorescence change associated with the cleavage of the fluorogenic substrate Ac-Asp-Glu-Asp(EDANS)-Glu-Glu-Abu-L-Lactoyl-Ser-Lys(DABCYL)- NH2 (Bachem Bioscience Inc. King of Prussia, PA) on a Perkin Elmer Victor 3 Fluorometer (λex = 355 nm, λem = 485 nm). Reactions were performed in black optiplate 96-well plates (Perkin Elmer), at room temperature for up to 1 hour in assay buffer (50 mM Tris, pH 7.4, 10% glycerol, 25 mM NaCl, 10 mMDTT, 0.1% n-dodecyl-β-D-maltoside, 1 mg/ml BSA) containing 2 nM enzyme and 10 μM cofactor peptide 4A (KKKGSVVIVGRIILSGR-NH2,

Anaspec, Inc. San Jose, CA). For kinetic studies, kinetic parameters were calculated from a nonlinear least-squares fit of initial rates as a function of substrate concentration (0.5-16 μM) using GraphPad Prism version 4.00 (GraphPad Software, San Diego, CA), assuming Michaelis-Menten kinetics.

2.4 RESULTS

2.4.1 NS2/3 cleavage mutants.

The catalytic activity of the NS2/3 protease has been previously demonstrated to require amino acids histidine 952 and cysteine 993 [177-178]. One study has also suggested the importance of glutamic acid 972 for NS2/3 autoprocessing from the observation that an E972Q point mutation reduces the catalytic activity of the enzyme [178]. Site directed mutagenesis was therefore used to generate point mutations in a NS2/3/4A expression construct to create H952A, E972A and C993A single mutants (Figure 2.1A). When processing of both the wild-type and mutant proteins were compared after *in vitro* translation in rabbit reticulocyte lysate, as expected, no cleavage is observed with the H952A and C993A mutants (Figure 2.2). In addition, the complete absence of NS2 and NS3 cleavage products clearly indicates that glutamic acid 972 is also a critical residue necessary for NS2/3 protease activity.

2.4.2 Cleavage at the NS2/3 site is required for replication of subgenomic replicons.

To further investigate the role of NS2/3 protease activity on the replicative capacity of HCV RNA, a series of NS2/3 mutants were generated in the context of an adapted NS2-3'UTR genotype 1b neomycin replicon (Figure 2.1B). *In vitro* synthesized RNA from replicons containing mutations affecting NS2/3 catalytic activity (H952A, E972A, C993A) were electroporated into Huh-7 cells and colonies selected using G418. Following selection, many colonies were visible

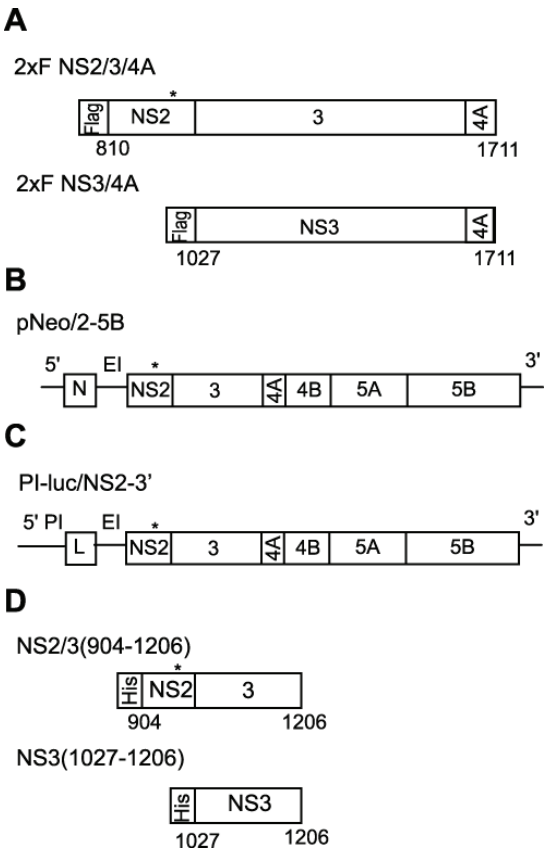

Figure 2.1: Schematic representation of the HCV 1b constructs used in this study.

A) *In vitro* translation expression constructs where a 2X tag (Flag) was added to the N-terminus of full length NS3/4A and NS2/3/4A. B) pNeo/2-5B replicon construct encompassing the 5' HCV NTR (5'), a neomycin resistance gene (N), non-structural proteins (NS2-5B) driven by an EMCV IRES (EI) and the 3' HCV NTR (3'). C) Luciferase replicon PI-luc/NS2-3' contains the HCV 5' NTR (5'), the gene encoding firefly-luciferase (L) under translational control of the Poliovirus-IRES (PI), the EMCV-IRES (EI), non-structural proteins NS2 to NS5B and the HCV 3'NTR (3'). D) Recombinant expression constructs where the protease domain of NS3 is preceded by a 6-histdine tag (His), as well as the same construct including a truncated form of NS2. Numbers under constructs refer to the amino acid residue of the beginning and end of each expressed protein while the asterisk (*) indicates either wild-type sequence or mutations at amino acids H952, E972, C993 or S977 in NS2.

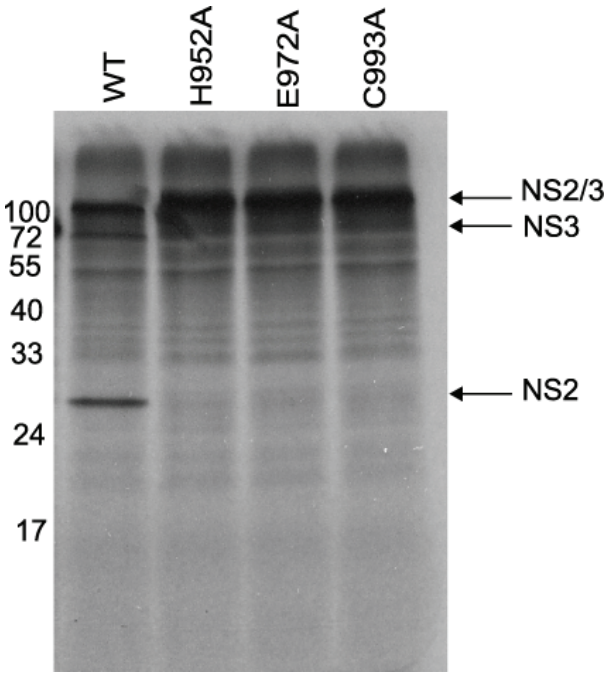

Figure 2.2: Effect of NS2/3 mutations on autoprocessing.

2xF NS2/3 WT (lane 1) and mutant (lanes 2, 3 and 4) proteins were *in vitro* translated in presence of [^{35}S] and the labelled bands separated by SDS-PAGE on a 15% gel. NS2/3, NS3 and NS2 proteins are indicated by arrows on the right.

with the wild-type replicon, while NS2/3 protease deficient mutants failed to produce any visible colonies (Figure 2.3A). In addition, these same mutations were introduced into a luciferase reporter replicon (Figure 2.1C) and also completely inhibited any replication in a transient transfection experiment (Figure 2.3B). Adapted wild-type (PI-luc/NS2-3'/ET) replicons showed a 10 fold increase of luciferase levels at 72 h compared to 4 h, while the H952A, E972A and C993A mutants showed a sharp decrease in luciferase activity over the course of the experiment. This decrease is similar to what we observed with the non-replicating GND replicon that contains an inactivating mutation in NS5B. This data suggests that efficient processing of NS2 and NS3 is a critical step in the viral RNA replication process. As replicons that do not contain NS2 are fully capable of replicating, these results indicate that the presence of NS2 still attached to NS3 could itself be interfering with the replication process. A likely mechanism would be that uncleaved NS2 is affecting the function of NS3. To test this hypothesis, the various catalytic activities of NS3 were investigated in the context of an uncleaved NS2/3 protein.

2.4.3 Presence of uncleaved NS2 has no effect on the ATPase and helicase activities of NS3.

The ATPase and helicase activities of NS3 have been shown to be required for viral replication [105] and therefore the effect of uncleaved NS2 on these processes was examined. Due to technical difficulties producing recombinant NS2/3(904-1597) encoding both protease and helicase domains of NS3 , FLAG-tagged NS3/4A and NS2/3/4A H952A, E972A or C993A constructs (Figure 2.1A) were used to generate RNA that was *in vitro* translated in rabbit reticulocyte lysate in presence of [^{35}S] methionine and cysteine. Upon anti-FLAG immunoprecipitation, followed by elution with FLAG peptide, the proteins were separated by SDS-PAGE and their relative concentrations determined. NS4A was included in the constructs since it has been proposed by several groups that in addition to functioning as a cofactor for NS3 protease activity, NS4A may have a role in modulating NS3 helicase activity, either positively [108, 254-255] or

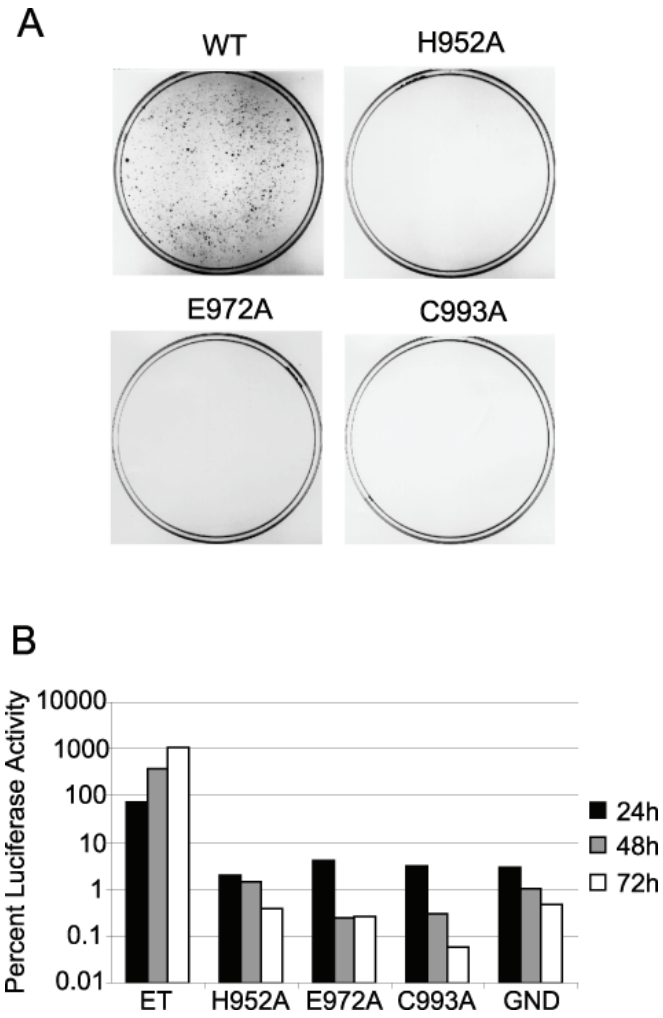

Figure 2.3: Effect of NS2/3 protease mutants on the replication of subgenomic NS2-5B replicons.

A) pNeo/2-5B replicon constructs expressing either wild-type or mutant NS2/3 were electroporated into Huh-7 cells. Following 4 weeks selection with G418, colonies were visualized by Coomassie Blue staining. B) Indicated PI-luc/NS2-3' luciferase replicon constructs were electroporated into cured Huh-7 cells, harvested at the indicated time points and luciferase activity measured. To correct for differences in transfection efficiency, values are reported as a percentage of the counts obtained 4 hours post-transfection (set at 100%).

negatively [247, 256]. Equi-molar quantities of wild-type NS3/4A and the catalytically inactive NS2/3/4A mutants were evaluated for NS3 helicase activity by measurement of their ability to unwind a radiolabelled dsDNA probe. Figure 2.4 shows that no significant difference in the helicase activity of NS3 could be measured in the presence or absence of NS2. Similarly, no significant change was observed in the ATPase activity of these enzymes (data not shown). To confirm that these results were due to the expressed proteins and not caused by a contaminating protein co-precipitated from the RRL, an NS3 protein containing a K1236N helicase inactivating mutation was also subjected to the above assays and showed activities similar to background (data not shown). NS3 is therefore a fully functional helicase in the presence of NS2 *in vitro*.

2.4.4 The ability of NS4A to associate with NS3 is decreased by the presence of uncleaved NS2.

It has been shown by x-ray crystallography that NS4A binds to the extreme N-terminus of NS3 [88, 93] and that this interaction is necessary for the efficient cleavage by NS3 of the downstream polyprotein [90-92]. We speculated therefore that the inability of NS2/3 to autoprocess NS2 and NS3 could leave NS4A unable to correctly associate with NS3. Indeed, Nedderman *et al.* demonstrated that the generation of an authentic N-terminus on NS3 is important for the formation of a stable complex between NS3 and NS4A [213]. To test this, N-terminal FLAG-tagged NS2/3/4A mutants and NS3/4A were *in vitro* translated and immunoprecipitated under conditions shown to allow NS3/4A complex formation [256]. The labelled proteins were then analyzed by SDS-PAGE and quantified by phosphorimager to determine the relative amount of NS4A pulled down with either the NS2/3 mutants or NS3. Figure 2.5 shows that NS3 is able to co-precipitate more NS4A than equal molar amounts of the uncleaved NS2/3 proteins (compare lane 2 with lanes 3, 4 and 5). A phosphorimager was used to quantify the relative intensities of the NS2/3, NS3 and NS4 bands and a 2 to 3 fold decrease in NS4A binding to NS2/3 was determined as compared with NS3. To confirm that this difference is in fact due to a decrease in NS3/4A complex

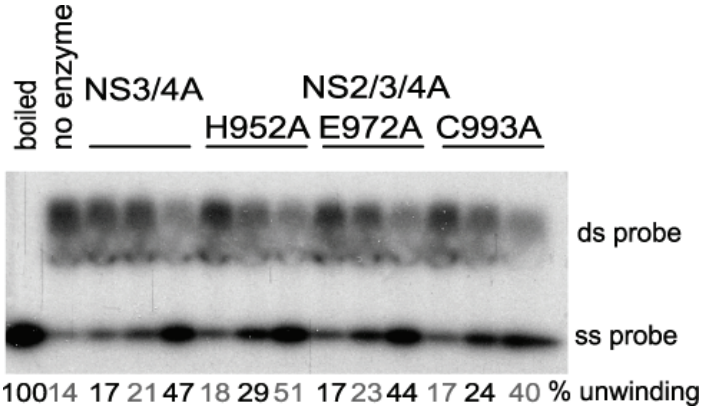

Figure 2.4: NS3 helicase activity in presence of uncleaved NS2.

Increasing amounts (1x, 2x and 3x concentrations) of equimolar, *in vitro* translated/ immunoprecipitated NS3/4A and NS2/3/4A mutants were incubated with a DNA probe and the reaction products visualized on a 20% non-denaturing acrylamide gel. The migration of the double-stranded (ds) probe and the released single-strand (ss) are indicated. Percent unwinding refers to the amount of single-stranded probe released by the enzyme as compared to the boiled control (lane 1).

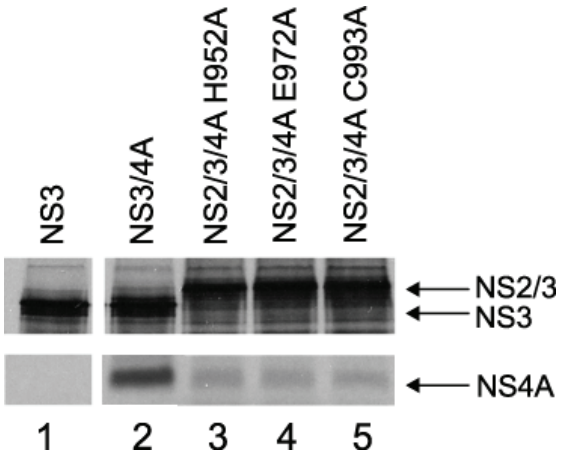

Figure 2.5: Effect of uncleaved NS2 on the ability of NS3 to associate with NS4A.

FLAG- tagged NS3/4A and NS2/3/4A mutants were *in vitro* translated in presence of [^{35}S] and immunoprecipitated using anti-Flag beads. The immunoprecipitated proteins were run on a 15% SDS-PAGE gel. Size of NS2/3, NS3 and NS4A proteins are indicated by arrows on the right. Lane 1 shows translation/IP of an NS3 construct not encoding NS4A as a control.

stability and not the effect of differential cleavage of 4A from the precursor proteins, it was determined that in our system, NS3/4A and NS2/3/4A show only a very slight difference in 4A cleavage (90% and 80% cleavage respectively) (data not shown). This therefore demonstrated that the effect seen after immunoprecipitation is in majority due to a decrease in NS3-NS4A complex stability as opposed to a cleavage effect. These results suggest that the addition of NS2 causes a conformational change at the NS3 amino terminus resulting in a decrease of its affinity for NS4A.

2.4.5 NS3 protease kinetics are affected by unprocessed NS2.

To understand the effect of NS2/3 catalytic mutants on NS3 protease activity, NS3(1027-1206) and inactive NS2/3(904-1206) proteins derived from the 1b genotype of HCV were expressed and purified from *E. coli*. These constructs are shown schematically in Figure 2.1D and encompass only the protease domain of NS3. Utilizing protocols for NS2/3 protease production [180], both the NS2/3 active site mutants and NS3 proteins were recovered from inclusion bodies. Following purification on a nickel column, the proteins were subsequently refolded on a gel filtration column. Although it was possible to produce soluble NS3, we chose to purify NS3 from the insoluble fraction, as was done with NS2/3, to control for any effects the folding buffers may have on NS3 protease activity. An *in vitro* study of NS3 protease kinetics was then performed. This involved measuring cleavage of a fluorescent substrate encoding the NS4A/NS4B cleavage site, a reaction which is dependent on the addition of an NS4A cofactor peptide. The results are summarized in Table 2.1. The fusion of NS2 to the NS3 protease domain does not cause a reduction in the ability of NS3 to bind its peptide substrate as indicated by the similar K_m values. However, a 4 to 5 fold decrease is seen in the catalytic constants of the enzyme in the presence of uncleaved NS2, which could be partially accounted for by the decreased stability of the NS3-NS4A complex as found in our immunoprecipitation experiments.

Table 2.1: Comparison of NS3 protease kinetics in presence or absence of NS2

Construct	K_{cat} **(s^{-1})**	K_m **(μM)**	K_{cat}/K_m **(M^{-1} s^{-1})**
NS3 (1027-1206)	0.55	11.9	46218.5
NS2/3 (904-1206) H952A	0.11	9.3	11828.0
NS2/3 (904-1206) E972A	0.09	10.9	8256.9
NS2/3 (904-1206) C993A	0.08	8.8	9090.9

2.4.6 NS3 dependent polyprotein processing occurs normally in NS2/3 mutant replicons.

To determine whether the decrease observed in the *in vitro* kinetics of NS3 in presence of uncleaved NS2 translates into an impairment of NS3 dependent polyprotein processing, the ability of mutant NS2/3 to cleave downstream proteins was investigated in the replicon context. pNeo/2-5B WT, H952A, E972A or C993A replicons were transfected into cured Huh-7 cells and the expression of NS2/3, NS3 as well as cleaved NS5B determined. The rabbit polyclonal antibody used in this study was raised against the protease domain of NS3 and has been found to recognize full-length uncleaved NS2/3 and NS3 to similar extents using recombinant proteins (data not shown). It has also been observed that in our system, mutant NS2/3 is visualized as multiple bands, the lower bands possibly representing proteolytic degradation fragments of the full length protein. Figure 2.6 shows that although expression of NS2/3 mutant replicon proteins is much lower compared to wild-type, discrete NS2/3 as well as NS5B can be seen, indicating the ability of uncleaved NS2/3 to correctly process the viral polypeptide despite the impaired kinetics of the NS3 protease activity seen *in vitro*. These results are in agreement with previously published data showing that a C993A single or H952A/C993A double mutant is still able to perform all NS3 mediated polyprotein cleavages when the HCV polyprotein is expressed in BHK-21 cells using a vaccinia system [105, 177].

2.4.7 Replicon encoded mutant NS2/3 is rapidly degraded in Huh-7 cells.

Recently, Franck *et al.*, have shown that NS2 is rapidly targeted for degradation in a cell line stably expressing a full-length replicon [243] and therefore, the possibility that NS3 could also have a decreased half-life due to the presence of uncleaved NS2 was investigated. Adapted wild-type (PI-luc/NS2-3'/ET), as well as a non-replicative NS5B mutant (PI-luc/NS2-3'/GND) and H952A NS2/3 luciferase replicons were transfected into cured Huh-7 cells and NS2 and NS3

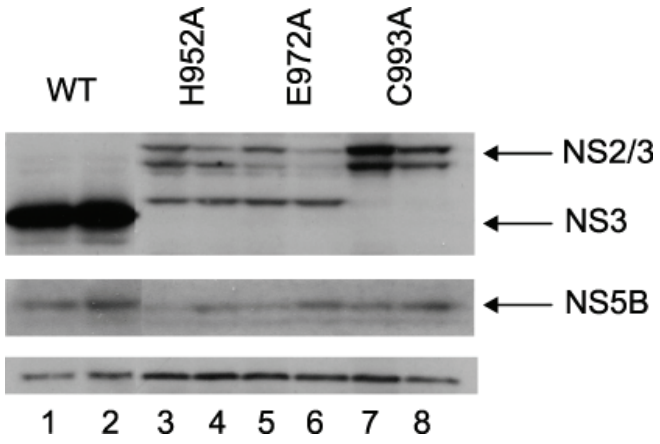

Figure 2.6: Effect of NS2/3 mutants on polyprotein processing.

Wild-type and NS2/3 mutant pNeo/2-5B replicons were electroporated into cured Huh-7 cells. 75 μg of total cell protein obtained after 4 (lanes 1,3,5,7) and 6 (lanes 2,4,6,8) hours were separated by SDS-PAGE and subjected to western blot analysis using NS3 and NS5B specific antibodies. The lower panel indicates a non-specific band used as a loading control.

levels were visualized at several time points post-electroporation. As seen in Figure 2.7A, the ET replicon shows NS3 levels that increase over time, whereas the non-replicating GND construct, in addition to much lower initial expression levels, shows stable levels of NS3 for the first 24 hours, followed by a decrease in levels after 48 hours. In contrast, the H952A construct shows very low levels of NS2/3 after 4 hours which decreases further after 8 hours and is not detectable after 24 hours. To ensure that the low levels of NS2/3 observed were not due to a decrease in the transfection efficiency or stability of the mutant RNA, luciferase levels were also visualized and are shown in similar amounts for both ET and H952A replicon constructs after 4 hours. However, it is interesting to note that the GND construct shows much lower luciferase levels compared to ET, as is consistent with the levels of NS3 protein expressed. As similar results to those observed with the H952A mutant were obtained with E972A and C993A mutants (data not shown), this suggests that the addition of NS2 has a destabilizing effect on the NS3 protein. This data strongly suggests that inactivation of the NS2/3 protease prevents accumulation of the NS3 protein which is required to drive HCV RNA replication.

As Franck *et al*. have also shown that NS2 can be phosphorylated at serine 977 and that mutation of this residue prevents degradation of NS2 by the proteasome [243], we investigated whether this amino acid could also be involved in NS2/3 degradation. A serine to alanine mutation of amino acid 977 was therefore introduced into both wild-type ET and NS2/3 proteolytically inactive luciferase replicons and NS2/3 levels were determined after electroporation into cured Huh-7 cells. As can be seen in Figure 2.7B, the S977A mutant behaves as does the original ET construct in that NS2/3 is completely cleaved into NS2 and NS3 and levels of NS3 increase over time. Interestingly, the H952A/S977A double mutant was found to be rapidly degraded after 4 hours as was observed with the H952A single mutant. Furthermore, to confirm the S977A mutation does not interfere with another aspect of polyprotein processing or RNA replication, luciferase based transient replication assays were performed and it was found that

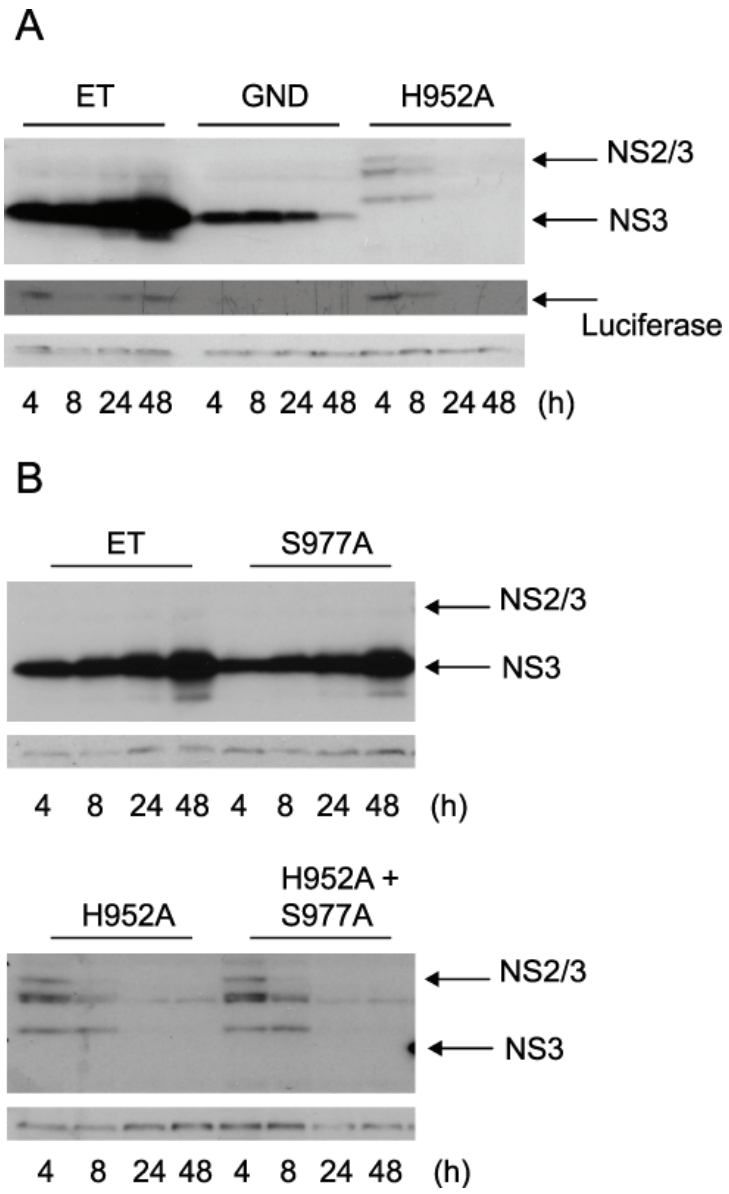

Figure 2.7: Degradation of NS2/3 mutant compared to cleaved NS3.

Wild-type ET, or mutant PI-luc/NS2-3' luciferase replicons were electroporated into cured Huh-7 cells and the amount of NS2/3, NS3 or luciferase visualized by western blot analysis using an NS3 or firefly luciferase specific antibody at the time points post-electroporation indicated. A) Comparison between ET, GND and H952A constructs. B) Effect of an S977A mutation on stability of NS2/3 and NS3 proteins. In each case, the lower panel indicates a non-specific band used as a loading control.

although the H952A/S977A double mutant failed to replicate, the S977A single mutant could replicate efficiently in Huh-7 cells (data not shown). Similar results were obtained for E972A/S977A and C993A/S977A double mutants (data not shown). These results therefore suggest that although an S977A mutation was previously found to be sufficient to prevent degradation of NS2, this is not the case for NS2/3 where additional factors might be involved.

2.4.8 Uncleaved NS2/3 levels are increased by a proteasome inhibitor.

As NS2 degradation has been shown to require the proteasomal degradation pathway [243], we investigated whether this is also the case for the uncleaved NS2/3 protein. Non-replicative GND and H952A replicon constructs were electroporated into cured Huh-7 cells. After attachment (time 0), cells were treated with a proteasome inhibitor (MG132) for an additional 4 and 20 hours. Figure 2.8 shows that the levels of NS2/3 seen with the H952A replicon were significantly increased upon the addition of MG132 after both 4 and 20 hours (lanes 8 and 10), as compared to untreated cells (lanes 7 and 9). However, treatment of the GND replicon cells with the proteasome inhibitor had no significant impact on the levels of cleaved NS3. These results indicate that the rapid degradation of uncleaved NS2/3 observed in our system is most likely also proteasome mediated.

2.5 DISCUSSION

Although one study has shown the importance of HCV NS2/3 protease cleavage for viral infectivity in the chimpanzee model [105], its exact role in viral RNA replication has remained elusive due to the observation that NS3-5B subgenomic replicons replicate efficiently in Huh-7 cells [160]. In this report, we explored the critical role of the HCV NS2/3 protease in viral RNA replication

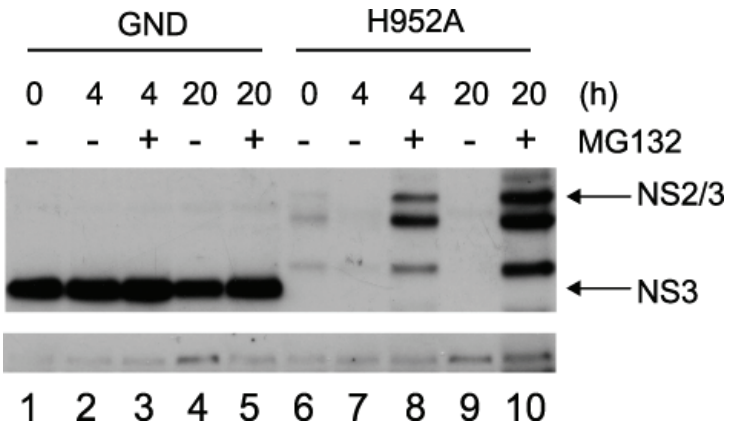

Figure 2.8: Effect of a proteasome inhibitor on uncleaved NS2/3 degradation. GND and H952A mutant PI-luc/NS2-3' replicons were electroporated into cured Huh-7 cells. After attachment (time 0), cells were treated with 10 μM MG132 or DMSO control and harvested at the time points indicated. NS3 and NS2/3 levels were visualized by western blot analysis using an NS3 specific antibody. The lower panel indicates a non-specific band used as a loading control.

using NS2/3 catalytically inactive mutants. Previous studies have suggested that E972 could be an important residue for NS2/3 protease activity [178] and we confirm here the importance of this residue for autoprocessing and viral replication in the context of the replicon system. This gives further evidence that E972 could be the third residue in a catalytic triad also involving H952 and C993. Indeed, it has recently been shown by Lackner *et al.* [184], that these three residues, in addition to being conserved in all HCV isolates, are also found to be conserved and of importance for the NS2/3 protease of the bovine viral diarrhea virus (BVDV), a pestivirus related to HCV and often used as a surrogate model for its study [257].

NS2/3 cleavage is an important and essential step in the replication of NS2-5B replicons as shown here by protease inactivating mutants that fail to support replication in Huh-7 cells. This confirms the importance of NS2/3 autoprocessing as was shown in the in vivo chimpanzee model [105], while suggesting that NS2/3 cleavage plays a role in viral RNA replication itself. However, this does not eliminate the possibility that NS2/3 cleavage could have additional functions in other aspects of the viral life cycle in addition to its requirement for genome replication. Nevertheless, the results reported here further substantiate inhibition of NS2/3 cleavage as a valid target for development of anti-HCV therapies.

It is the presence of NS2 fused to the N-terminus of NS3, and not the absence of the NS2 protein, that interferes with RNA replication, and we therefore explored the possible mechanisms by which uncleavable NS2/3 could be having this effect. Although NS3 ATPase and helicase activities are unaffected in the context of uncleaved NS2, this protein has two to three fold lower affinity for NS4A than cleaved NS3 does. This could be due to the manner in which NS4A has been shown to bind NS3, forming an integral part of its amino-terminus structure [88, 93]. A conformational change induced by the presence of uncleaved NS2 may leave NS4A unable to associate as tightly with the enzyme.

However, the fusion of NS2 to the amino terminus of NS3 does not completely abolish the interaction with NS4A. In fact, several groups have reported synthetic 4A peptides as potent inhibitors of NS2/3 autoprocessing [180, 191]. Nevertheless, we find a decrease in NS3 protease kinetics in an *in vitro* assay dependent on the addition of a 4A peptide using purified NS2/3(904-1206). In addition to the decreased stability of the NS2/3-4A complex, 4A binding may not be able to provoke the rearrangement of the catalytic triad of NS3 necessary to stimulate protease activity.

It has been demonstrated by several groups, both by *in vitro* translation [178] and in cell expression systems [105, 177] that uncleavable NS2/3 causes no defect in NS3-dependent processing. Our results here are in agreement with these studies and confirm that the same is true when NS3 is expressed in Huh-7 cells as part of the HCV polyprotein in the replicon context. However, despite its ability to correctly process the viral polyprotein, this does not exclude the possibility that the NS3 protease kinetics of mutant NS2/3 may be reduced sufficiently *in vivo* to cause an effect on RNA replication.

Uncleaved NS2/3 is rapidly degraded when expressed in Huh-7 cells as part of an HCV replicon. In the replicon system, polyprotein translation and processing are rapid events as demonstrated by the appearance of non-structural proteins soon after transfection (within 4h). However, it takes significantly longer for RNA replicaton to occur. Transfected RNA, once translated, is rapidly degraded by the cell, while new RNA is found to be synthesized only 24 hours post-transfection [162]. We therefore suggest that uncleaved NS2 may be preventing RNA replication by destabilizing NS3 and causing its rapid elimination from the cells. In this case, although NS3 functions may not be disturbed by uncleaved NS2, the NS2/3 protein is not present in sufficient quantities to support RNA replication. It is of importance to note that the rapid degradation of uncleaved NS2/3 has not been previously observed likely due to the differences inherent to the systems used. Uncleaved NS2/3 is easily detected

in a vaccinia-induced expression system in BHK-21 cells [105, 177]. However, in this system a large and continuous overexpression of the HCV proteins is achieved, which might saturate the degradation pathway. The replicon system used here allows the investigation of the NS2/3 protein at more physiologically relevant levels.

Cleaved NS2 has also recently been reported to be a short-lived protein in cells [243]. In that case, NS2 degradation was found to be regulated in a phosphorylation-dependent manner by protein kinase CKII. It would therefore be interesting to determine whether inhibition of the protein kinase CKII itself could have an effect on NS2/3 stability and possibly help rescue RNA replication. Unfortunately, these experiments have proved difficult due to the observed toxicity of the curcumin CKII inhibitor added soon after transfection (data not shown). However, a serine to alanine mutation of conserved residue 977 that has been demonstrated to be sufficient to prevent proteasome mediated degradation of NS2 was found not to be sufficient to restore levels of NS2/3 in our system. NS2/3 degradation was found here to be proteasome dependent, however, a replication rescue experiment could not be performed due to the toxicity of the proteasome inhibitor after a 24 hour incubation. It is likely that NS2/3 therefore behaves differently than cleaved NS2 and that there are either other residues or additional mechanisms involved in the regulation of its degradation. For example, association of NS3 with NS4A is essential for NS3 membrane localization and stability [94] and as NS4A is shown here to have a decreased affinity for NS2/3, this could also be a contributing factor to the short half life observed. Further study is required in order to determine the exact mechanisms involved in NS2/3 degradation and how this process could be regulated.

Bovine viral diarrhea virus NS2/3 cleavage plays a crucial role in the generation of different BVDV strains. In this pestivirus, uncleaved NS2/3 is present in non-cytopathic strains causing persistent infection, while discrete NS3 is present in cytopathic strains required to cause disease [258-259]. For this virus,

viral RNA levels have been shown to correlate with cleaved NS3 protein [184], however, uncleaved NS2/3 is required for viral infectivity [260]. It has recently been suggested that BVDV NS2/3 is an autoprotease whose temporal regulation is involved in modulating the different stages of RNA replication and virus morphogenesis [184]. It is possible that HCV NS2/3 could perform a similar regulatory role. By causing the degradation of NS3, uncleaved NS2/3 could potentially constitute a switch between synthesis of viral RNA and the later events of the viral life cycle, such as virion packaging and release.

It has previously been reported that NS5B levels are decreased by the presence of a cellular ubiquitin-like protein and that this may function to regulate viral RNA replication [261]. The controlled cleavage and degradation of NS2/3 may therefore constitute an additional level of regulation for the virus. Although the mechanism of NS2/3 degradation has yet to be elucidated, it is likely that cellular proteins are involved. Cellular factors have also been proposed to be required for efficient NS2/3 protease activity [192-193], and the availability of these factors may modulate the degree of NS2/3 cleavage over the course of infection. In addition, the NS2/3 cleavage products themselves could potentially be involved in the regulation of these processes through their actions on host cell proteins. The exact role of NS2 after cleavage has not yet been firmly established, although NS2 has been shown to inhibit cellular gene transcription [214]. NS3 is known to have several functions in modulating cell signaling events, as demonstrated by its cleavage of the Toll-like receptor 3 adaptor protein TRIF [96] and disruption of retinoic acid-inducible gene 1 (RIG-1) signaling [97, 262], preventing IRF-3 activation and the host cell interferon response [95]. Regulation of NS2/3 cleavage and degradation are therefore both possible mechanisms the virus could use to control the stages of its own life cycle and further study is required in order to fully understand these events.

2.6 ACKNOWLEDGEMENTS

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CONNECTING TEXT

In Chapter 2 we have shown that HCV NS2/3 protease activity is required for HCV genome replication and that while NS3 catalytic activities appear to be unaffected by the presence of uncleaved NS2, the resultant fusion protein is rapidly degraded in a proteasome-dependent manner. As this degradation could be an important way for the virus to regulate its own life cycle, in Chapter 3 we use a lysine mutagenesis study to further investigate the mechanism of degradation of the NS2/3 protein and determine whether a stabilized NS2/3 would be competent for genome replication. This lysine mutagenesis approach is then also used to characterise the cleaved NS2 protein itself, both with respect to its rapid degradation and its role in viral assembly.

CHAPTER 3:

INVESTIGATION OF A ROLE FOR LYSINE RESIDUES IN NON-STRUCTURAL PROTEINS 2 AND 2/3 OF THE HEPATITIS C VIRUS FOR THEIR DEGRADATION AND VIRUS ASSEMBLY

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3.1 ABSTRACT

It has been demonstrated that both uncleaved, enzymatically inactive NS2/3 and cleaved NS2 proteins are rapidly degraded upon expression cells, phenomena described to be blocked by the addition of proteasome inhibitors. As this degradation and its regulation potentially constitute an important strategy of the hepatitis C virus (HCV) to regulate the levels of its non-structural proteins, we further investigated the turnover of these proteins in relevant RNA replication systems. A lysine-mutagenesis approach was used in an effort to prevent protein degradation and determine any effect on various steps of the viral replication cycle. We show that while NS2-lysine mutagenesis of protease-inactive NS2/3 results in a partial stabilisation of this protein, the increased levels do not rescue the inability of NS2/3 protease inactive replicons to replicate, suggesting that uncleaved NS2/3 is unable to functionally replace NS3 in RNA replication. Furthermore, we show that the cleaved NS2 protein is rapidly degraded in several transient and stable RNA replicon systems and that NS2 from several different genotypes also has a short half-life, highlighting the potential importance of the regulation of NS2 levels for the viral life cycle. However, in contrast to uncleaved NS2/3, neither ubiquitin nor proteasomal degradation appear to be significantly involved in NS2 degradation. Finally, although NS2 lysine to arginine mutagenesis does not affect this protein's levels in a JFH-1 cell culture infection system, several of these residues are identified to be involved in virion assembly, further substantiating the importance of regions of this protein for production of infectious virus.

3.2 INTRODUCTION

Hepatitis C virus is a single-stranded positive sense RNA virus of the *Flaviviridae* family [29-30] and is an important cause of chronic liver disease. The RNA genome encodes a single polyprotein that comprises both structural as well as non-structural proteins. While host signal peptidases cleave the

polyprotein in the structural region, the non-structural proteins are cleaved by two virally encoded proteases: NS2/3 and NS3. NS3 is a serine protease that cleaves at the NS3/4A and downstream junctions [86] and also harbours C-terminal ATPase and helicase activities [101].

The HCV NS2/3 protease is an autocatalytic cysteine protease that is responsible for the cleavage between NS2 and NS3 and whose activity has been shown to be required for both viral infectivity and RNA genome replication [105, 177-178, 185]. With the recent introduction of the JFH-1 HCV cell culture infection system, the presence of the cleaved NS2 protein has been shown to be required for HCV virion assembly and viral infectivity, although its mechanism of action in this process remains unclear [81, 146].

Cellular protein levels are tightly regulated by the balance between transcription/translation and degradation. Interestingly, many virally-encoded proteins have also been demonstrated to be quickly degraded, suggesting control of their levels could regulate their role in the viral life cycle [235, 237]. Furthermore, levels of several HCV proteins such as core, E2 and NS5B have been suggested to be regulated at the level of protein stability [242, 263-264], which may in turn modulate viral replication. Indeed, core degradation by the ubiquitin-proteasome pathway has recently been shown to involve interaction with E6AP. When levels of this ubiquitin ligase were increased, a decrease in core levels was observed as a well as a decrease in viral titers in a cell culture system [241].

Most cellular proteins are degraded via the ubiquitin-proteasome pathway. In this system, poly-ubiquitin chains are attached to lysine residues of the target protein, which is then recognized for degradation by the 26S proteasome (for review see [232]).

Interestingly, both uncleaved mutant NS2/3 and processed NS2 have been described by us and others to be rapidly degraded in cells, phenomena which were inhibited by the addition of proteasome inhibitors [185, 243]. The degradation of these proteins could constitute an important way for the virus to regulate the levels of its own proteins and therefore its own replication or assembly. However, most of the work on NS2 degradation was performed using tagged NS2 expressed alone [243] which could differ from what occurs with the untagged protein in its viral RNA context. We decided to further investigate the degradation of these proteins in more relevant replication and infection systems. We used a lysine-mutagenesis approach in an effort to prevent protein degradation and determine any effect on various aspects of the viral life cycle.

We report here that while NS2-lysine mutagenesis of protease inactive NS2/3 results in a partial stabilisation of this protein, the increased levels are unable to rescue the inability of NS2/3 protease inactive replicons to replicate, suggesting uncleaved NS2/3 is unable to functionally replace NS3 in genome replication. Furthermore, using several different DNA and RNA replicon cell systems, we show that the cleaved NS2 protein from several relevant HCV genotypes is rapidly degraded, highlighting the potential importance of the regulation of NS2 levels for the viral life cycle. Although NS2 lysine mutagenesis is found not to affect this protein's levels, we identified several residues in NS2 to be required for infectious HCV virion assembly in a JFH-1 cell culture infection system, further substantiating the importance of regions of this protein for viral infectivity.

3.3 METHODS

3.3.1 Constructs

To generate pcDNA32xFNS2 DNA constructs and to insert NS2 mutations into pNeo/2-5B and pFK-repPI-luc/NS2-3' [185], subgenomic Con1

replicons containing NS2 to NS5B, pFK-I_{389}neo/Core-3'/5.1 [163] and pJFH-1 [40] standard subcloning techniques and Quick Change mutagenesis (Stratagene) were used.

To insert NS2 lysine mutations into pNeo/2-5B and pFK-repPI-luc/NS2-3' [185] subgenomic replicons containing NS2 to NS5B, Quick Change mutagenesis (Stratagene) of NS2 was performed in a pNEBNS2/3 vector in which a portion of the EMCV IRES, all of NS2 and part of the NS3 protease were present [185]. For insertion into pNeo/2-5B, a portion of this replicon (part of the neomycin resistance gene to part of the NS3 protease) was amplified by PCR and inserted into pBluescript II KS+ (pBS, Stratagene) using XbaI and XhoI sites included in the oligonucleotide primers. A HindIII-BsrGI fragment of this construct was replaced with the equivalent mutated fragment from pNEBNS2/3. The mutated segment was then inserted back into the original pNeo/2-5B replicon via RsrII and BsrGI sites. For insertion into PI-luc/NS2-3', a portion of this replicon was removed and inserted into the HindII-XhoI sites of pcDNA3. The appropriate mutated fragments (HindII-BsrGI) were then inserted from pNEBNS2/3 into this vector and then reinstated into the original PI-luc/NS2-3' replicon via HindIII-XhoI sites. To generate NS2 lysine mutants in the full length replicon, a fragment of pFK-I_{389}neo/Core-3'/5.1 [163] (referred to here as core-3'/5.1) encompassing part of E2 and most of NS2 was removed via SacII digestion and inserted into the same site of pBS. Quick change mutagenesis was then performed on this construct and then the mutated fragment reinstated back into core-3'/5.1. To generate lysine mutations in the JFH-1 infectious clone, a BsiWI-SpeI fragment from pJFH-1 [40] was inserted into a pNEB193 vector (New England Biolabs) into which these sites had been inserted by oligonucleotide annealing. Mutagenesis was then performed on this construct using the Quick Change method and then the mutated fragment was reinserted into pJFH-1 using the same sites. To generate pcDNA32xFNS2 constructs, PCR amplification of the full length NS2 from Con1 (pNeo/2-5B), H77c [265], HC-$J6_{CH}$ (pJ6CF [266]) and JFH-1 (pJFH-1) was performed and the protein inserted via EcoRI and XhoI sites

into pcDNA32xF[185]. To generate pcDNA32xFNS2/3(2a), PCR amplification of NS2/3 from pJFH-1 was performed and the product inserted into pCDNA32xF via BamHI and EcoRI restriction sites included in the oligonucleotide primers. Quick change mutagenesis was performed on pcDNA32xFNS2/3(2a) to generate the K1028R mutant. pTM NS2-3' ET and pTM NS2-3' H952A were generated by transfering fragments encompassing the entire NS2-NS5B region including the 3' NTR from PiLuc NS2-3' and the respective mutant to pTM1 [267] using NcoI-SpeI restriction sites. In all cases, correct insertion of mutations and fidelity of DNA sequence generated by PCR was verified by sequencing.

3.3.2 Cell Culture

Huh-7, Huh7.5 (generously provided by C. Rice) and Huh7-Lunet cells were grown in Dulbecco's modified Eagles medium supplemented with 10% fetal bovine serum, 100 U/ml penicillin, 100 μg/ml streptomycin and 100 μM non-essential amino acids. 500 μg/ml G418 was also added to stable replicon cell lines.

3.3.3 *In vitro* transcription

DNA plasmids were linearized with ScaI for the pNeo/2-5B and core-3'/5.1 replicon contructs, AseI/ScaI for PI-luc/NS2-3' replicon constructs and XbaI for pcDNA32xFNS2/3(2a) constructs and purified using phenol/chloroform extraction. pJFH-1 was linearized with XbaI and treated with mung bean nuclease prior to purification. Linearized DNA was transcribed with T7 RNA polymerase (Ambion MegaScript kit, Ambion) following the manufacturers suggested protocol, template DNA was removed by digestion with DNase 1 and the RNA was purified by either lithium chloride precipitation or by passing through a column (RNeasy mini kit, Qiagen) and dissolved in RNase-free water. The RNA concentration was determined by measuring the optical density at 260nm and RNA integrity was checked by denaturing agarose gel electrophoresis.

3.3.4 Generation of stable replicon cell lines

Sub-confluent Huh-7 (pNeo/2-5B WT), Huh-7.5 (pNeo/2-5B WT, pNeo/2-5B K-6-R, core-3'/5.1 WT, core-3'/5.1 K-6-R) or Huh7-Lunet (pNeo/2-5B WT) cells were electroporated with RNA (1 μg) and selected with 500 μg/ml G418 as described previously [250]. Individual clones were expanded except in the case of the full-length constructs where pools were generated.

3.3.5 Transient replication assays and production of infectious HCV

Transient replication assays were performed as described [185]. For production of infectious HCV, Huh7.5 cells were transfected with 5 μg of a given *in vitro* transcript and processed as described.

3.3.6 Transient transfection for pulse-chase analysis

For DNA constructs, Huh7.5 cells were transfected using lipofectamine 2000 (Invitrogen) whereas luciferase replicons (20 μg RNA) were electroporated into Huh7-Lunet cells as described above. Transfected cells were used for pulse-chase analysis 48 h or 72 h after transfection as described below.

3.3.7 Pulse-chase analysis

For analysis of protein half-life, the relevant cells were methionine-starved for 1h using methionine and cysteine-free DMEM supplemented with 10% serum, penicillin-streptomycin and L-glutamine. Cells were then pulsed in the same media containing 250 uCi/ml [^{35}S]methionine/cysteine (Redivue Pro-mix, GE Healthcare) for 3 h. After washing with PBS, complete media supplemented with 0.05% cold methionine was added for the relevant chase periods after which the cells were harvested and lysed using lysis buffer (50 mM Tris-HCl pH8.0, 150 mM NaCl, 1% NP-40) with addition of protease inhibitors. Cleared cell lysates

were immunoprecipitated by incubation with NS2 antibody for 2 h followed by addition of 50/50 protein A/G coupled agarose beads (Upstate Cell Signalling Solutions) for an additional 2 h. NS3 immunoprecipitation was then performed in the same way. For FLAG-tagged proteins, lysates were incubated for 3 h with EZview Red ANTI-FLAG M2 Affinity Gel (Sigma). The beads were then washed 4 times with wash buffer (50 mM Tris-HCl PH8.0, 900 mM NaCl, 1% NP-40) and once with lysis buffer. Bound proteins were eluted with SDS-sample buffer, run on SDS-PAGE, and visualised by autoradiography. Relative band intensity was measured using a FUJI X BAS 2000 phosphorimager.

3.3.8 Immunoblotting

For immunoblot analysis of protein levels after transient electroporation, 20 μg RNA (5 μg for JFH-1 and derivatives) was electroporated as described above. For proteasome inhibitor treatment, cells were seeded and 10 μM MG132 (Sigma) or vehicle control (Me_2SO) were added 4h after plating. Cells were harvested at the indicated time points by scraping in PBS, lysed in lysis buffer and cleared by centrifugation. Following quantitation using Bio-Rad protein assay (Bio-Rad), total cellular proteins were separated by SDS-PAGE, transferred to nitrocellulose membrane and immunoblotted as described [185]. Cell extracts from stable cell lines were processed similarly.

3.3.9 Antibodies

The antibodies used in this study were: a rabbit polyclonal anti-NS3 antibody generated against the NS3 Con1 protease domain [185], a rabbit polyclonal anti-NS2 antibody generated against NS2 Con1 residues 904-1026, anti-luciferase (Chemicon International, Inc), anti-Flag M2 (Sigma) and anti-actin (AC40, Sigma). For the immunoblot in Fig. 6 a rabbit polyclonal antibody raised against the helicase domain of NS3 Con1, a rabbit polyclonal antibody raised

against two NS2-derived peptides spanning residues 148-163 and 202-217 of JFH-1 NS2 and the core-specific polyclonal antibody C-830 [268] were used.

3.3.10 Core ELISA

Quantitative detection of intracellular and extracellular HCV core protein was done using the Trak-C Core ELISA assay (Ortho Clinical Diagnostics, Germany) as recommended by the manufacturer and as described [146].

3.3.11 Infectivity assays

Cell-associated infectivity was determined as described elsewhere [269]. In brief, cells were extensively washed with PBS, scraped off the plate and centrifuged for 5 minutes at 400 x g. Cell pellets were resuspended in 1 ml DMEM complete and subjected to three cycles of freezing and thawing using liquid nitrogen and a thermo block set to 37°C. Samples were then centrifuged at 10,000 x g for 10 minutes at 4°C to remove cell debris. Infectivity titres of these lysates, and cell culture supernatants, were determined by using limiting dilution assay on Huh7.5 cells as described recently [270]. Briefly, target cells (1.1×10^4 cells per well of a 96-well plate in a total volume of 200 µl complete DMEM) were inoculated with serial dilutions of virus containing supernatant or cell lysate by using 8 wells per dilution. Two to three days later, cells were fixed, and stained by using an NS5A-specific monoclonal antibody (9E10; kindly provided by C.M. Rice and T. Tellinghuisen, New York) at a dilution of 1:2,000 (hybridoma supernatant). Bound antibody was detected by incubation with peroxidase – conjugated anti mouse antibody (Sigma – Aldrich, Germany) diluted 1:200 in PBS – saponin. Peroxidase activity was detected by using the Vector NovaRED substrate kit (Linaris Biologische Produkte GmbH, Germany). Virus titers [50% tissue culture infective dose ($TCID_{50}$/ml)] were calculated as described elsewhere [270].

3.3.12 *In vitro* translation

Purified RNA was translated for 2 hours at 30 °C in the presence of [^{35}S]methionine (NEN EasyTag™ L-[35S]-Methionine, Perkin Elmer) using a rabbit reticulocyte lysate system, following the manufacturers' suggested protocols. Radiolabeled proteins were visualized by SDS-PAGE, followed by autoradiography.

3.3.13 Metabolic labelling and immunoprecipitation of pTM constructs.

For metabolic labeling, proteins were expressed in Lunet-T7 cells after transfection of pTM vectors essentially as described recently [149]. In brief, total of 3 × 10^5 Huh7-Lunet/T7 cells were seeded in each well of a six-well cell culture, transfected with pTM NS2-3' ET, pTM NS2-3' H952A or empty vector using Lipofectamine 2000 (Invitrogen) according to the instructions of the manufacturer. After 7 h of transfection, cells were starved for 1 h in methionine/cysteine-free medium and further incubated for 14 h in 1 ml methionine/cysteine-free medium supplemented with 2 mM glutamine, 10 mM HEPES, and 100 μCi of Express protein labeling mix (Perkin-Elmer, Boston, MA). Cell lysates were prepared by using NPB (50 mM Tris-Cl [pH 7.5], 150 mM NaCl, 1% Nonidet P-40, 1% sodium deoxycholate, 0.1% sodium dodecyl sulfate) containing protease inhibitors (Complete, EDTA free protease inhibitor cocktail (Roche)) and cleared by centrifugation at 4°C. The cleared lysates were subjected to immunoprecipitation with polyclonal antisera directed against NS2, NS3, NS4B, NS5A or NS5B, respectively [212] and protein G sepharose (Sigma). Immunocomplexes were separated by 11% SDS- PAGE, and analyzed by autoradiography.

3.4 RESULTS

3.4.1 Lysine mutagenesis of NS2/3H952A replicons increases NS2/3 stability but fails to rescue genome replication.

We have previously shown that an NS2/3 protease active site mutant abolishes HCV RNA genome replication in the replicon system and that this uncleaved NS2/3 is rapidly degraded after transient electroporation into Huh7 cells [185]. However, the question remained as to whether the inability of the mutant NS2/3 replicons to replicate was due to insufficient NS3 levels (as a component of the rapidly degraded NS2/3) or because the NS2/3 protein can simply not functionally replace the role of NS3 in the replication complex. The initial goal was therefore to sufficiently increase the levels of uncleaved NS2/3 protein to determine any rescue in replication of the NS2/3 protease inactive replicons. As NS2 itself was previously suggested to have a short half-life compared to NS3 [243] and levels of both NS2 and mutant uncleaved NS2/3 could be increased by treatment with the proteasome inhibitor MG132, the role of the ubiquitin/proteasome pathway in the degradation of NS2/3 was investigated. Because of the inability to use the MG132 inhibitor in replication assays due to toxicity at the time scales involved, and as ubiquitin chains are generally attached to lysine residues, a mutation analysis of the lysine residues in NS2 was performed. Genotype 1b NS2 (Con1 isolate) contains 6 lysine residues, 3 of them conserved amongst all genotypes (see Figure 3.1). Single lysine to arginine mutations were introduced into PI-luc/NS2-3'/H952A luciferase reporter replicons and electroporated into Huh7-Lunet cells, a highly permissive subclone of the Huh-7 cell line [271]. After attachment (4 h post-electroporation), 10 μM MG132 was added for an additional 5 hours. Figure 3.2A shows that while levels vary, none of the single mutants is able to prevent NS2/3 degradation, with all constructs showing very low levels prior to MG132 treatment, similar to what is observed with the H952A mutation alone. As seen previously [185], mutant NS2/3 was visualized as multiple bands, all three of which reacted with both anti-

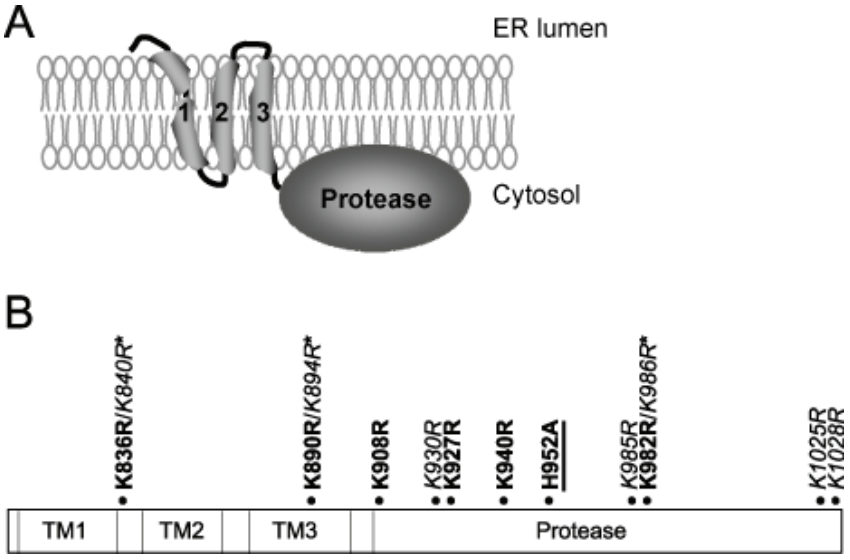

Figure 3.1. NS2 mutations used in this study.

A) Schematic representation of the NS2 protein indicating putative transmembrane domains (1, 2, 3) and the protease domain. B) Schematic representation of the NS2 mutations used in this study. **Bold** numbering refers to genotype 1b (Con1) residues while genotype 2a (JFH-1) mutations are indicated in *italics*. Asterisks (*) refer to lysines conserved amongst all HCV genotypes.

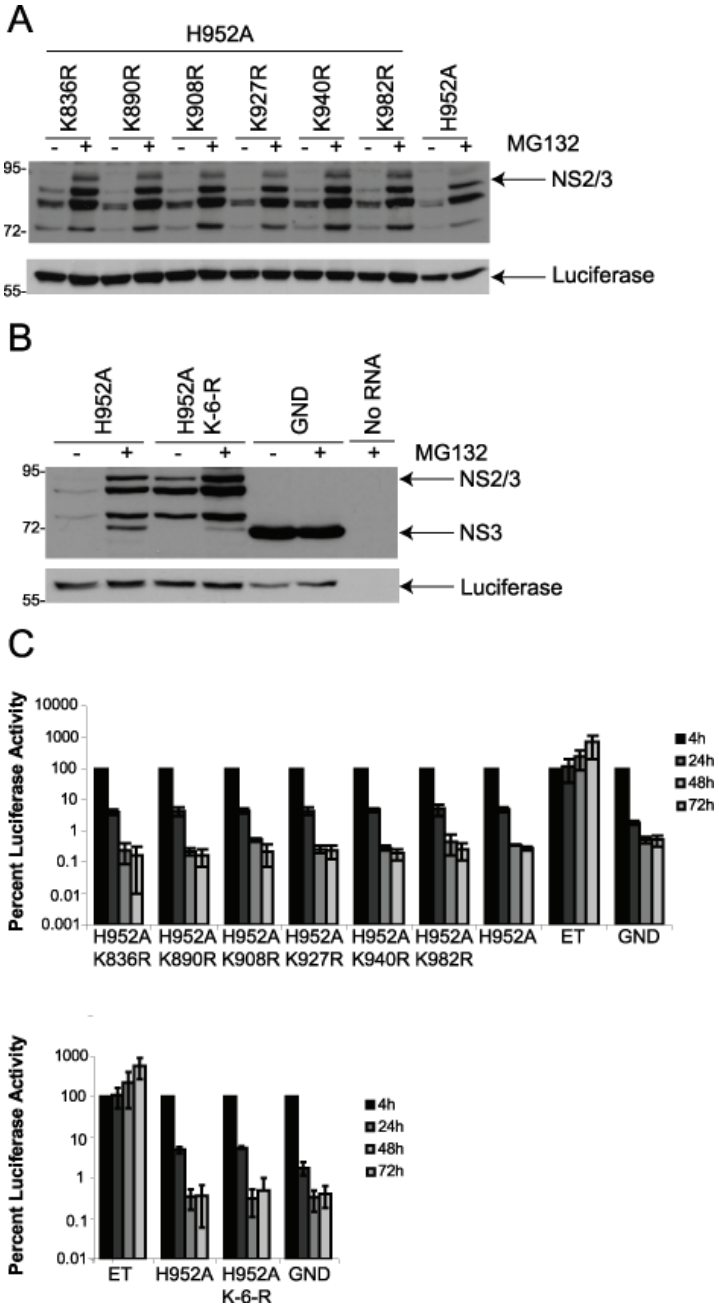

Figure 3.2. Effect of NS2 lysine mutations on uncleaved NS2/3 levels and replication of subgenomic NS2/3 protease inactive replicons.

Single (A) and multiple (B) NS2 lysine-to-arginine mutations were introduced into pFK-repPI-luc/NS2-3'/H952A replicons and electroporated into Huh7-Lunet cells. After attachment, cells were treated with 10μM MG132 or Me_2SO control for 5 h. NS2/3 and NS3 levels were visualized by immunoblot analysis using an

anti-NS3 specific antibody. Luciferase detection was also included as a control for RNA levels. C) Indicated pFK-repPI-luc/NS2-3' luciferase constructs were electroporated into Huh-Lunet cells, harvested at the indicated time points, and luciferase activity measured. Values are reported as a percentage of the counts obtained 4 h post-transfection. ET: adapted wildtype replicon, GND: polymerase inactive negative control. Error bars indicate standard deviation of a minimum of 2 independent experiments.

NS3 and anti-NS2 antibodies (data not shown). As it has been shown with other proteins that mutation of a single lysine is often not sufficient to prevent ubiquitination and targeting for degradation [272], a construct was also generated in which all six NS2 lysines were converted to arginines. As seen in Figure 3.2B, this NS2-lysine-free NS2/3 (K-6-R) is partially stabilized and is readily detectable without MG132 treatment at levels comparable to the treated H952A mutant alone. Luciferase levels were also found to be similar, indicating the stabilization seen is in fact due to the different mutations, not varying RNA levels.

Transient luciferase replication assays were performed to determine if the additional stabilization observed was sufficient to rescue replication. Figure 3.2C reveals that the single-lysine mutants as well as the NS2-lysine-free H952A constructs fail to replicate, showing a similar effect as the H952A mutation alone or a GND polymerase-inactive negative control. Furthermore, when the same mutations were introduced into pNeo/2-5B neomycin-selectable replicons, no rescue of replication was seen by colony-formation assay (data not shown). The stabilization of NS2/3 is therefore not sufficient to rescue viral replication suggesting that the uncleaved NS2/3 fusion cannot replace NS3 in the replication complex.

3.4.2 NS2 is rapidly degraded in the replicon system.

Figure 3.2 indicates that an NS2-lysine-free NS2/3 H952A mutant is stabilized compared to the H952A protein alone. It was therefore interesting to investigate whether the same effect is seen with the mature NS2 protein. NS2 itself was previously shown to be rapidly degraded in a proteasome-dependent manner [243]. However, as these studies were mostly performed using tagged DNA constructs in HepG2 cells, we wanted to investigate this degradation in the more relevant context of RNA replicons in Huh-7 based cells. A stable cell line containing pNeo/2-5B was established to investigate degradation of NS2 in the context of the replicating viral genome. A pulse-chase analysis was performed in

which these cells were pulsed with [^{35}S] methionine/cysteine and then chased for the indicated time periods. Labelled proteins were immunoprecipitated with anti-NS2 antibody followed by anti-NS3 immunoprecipitation. Figure 3.3A shows rapid decrease in the levels of NS2 as compared to the slower decrease in NS3 levels. Quantitation of the amounts relative to the initial levels at time 0 is shown in Figure 3.3B. Over several experiments the half-life of NS2 is found to be between 3-6 hours, slightly longer than what had been reported using DNA constructs, but much quicker than what is seen with NS3 and what has been reported for other non-structural proteins [273]. Therefore these experiments confirm the short half-life of NS2 in the context of a replicating viral RNA genome.

3.4.3 Lysine mutagenesis of NS2 does not increase its stability in replicon systems.

Since we observed stabilisation of uncleaved NS2/3 by replacement of NS2 lysines with arginine, we wanted to determine if any of these mutations would increase levels of the cleaved protein in a subgenomic replicon system. Lysine to arginine changes were therefore generated in pFK-PI-luc/NS2-3' luciferase replicons. These RNA constructs were electroporated into Huh7-Lunet cells and after attachment, 10 μM MG132 was added. The NS2 levels after 8 hours are shown in Figure 3.4A. No significant increase in levels as compared to wildtype NS2 is seen with any of the lysine single mutants with the effect of MG132 also being consistent in most cases: a very slight but reproducible increase. NS3 levels are also shown to be similar, indicating similar RNA levels in all cases. Interestingly, unlike the NS2/3H952A protein which was stabilized by the addition of NS2 lysine mutations, when all three conserved NS2 lysines (K836, K890, K982) were replaced by arginine (K-3-R) or when all six lysines in NS2 were replaced together (K-6-R), no effect was seen on levels of cleaved NS2 as compared to wildtype (Figure 3.4B). Furthermore, a S977A mutation, suggested to stabilize genotype 1a NS2 in DNA-transfected cells by preventing

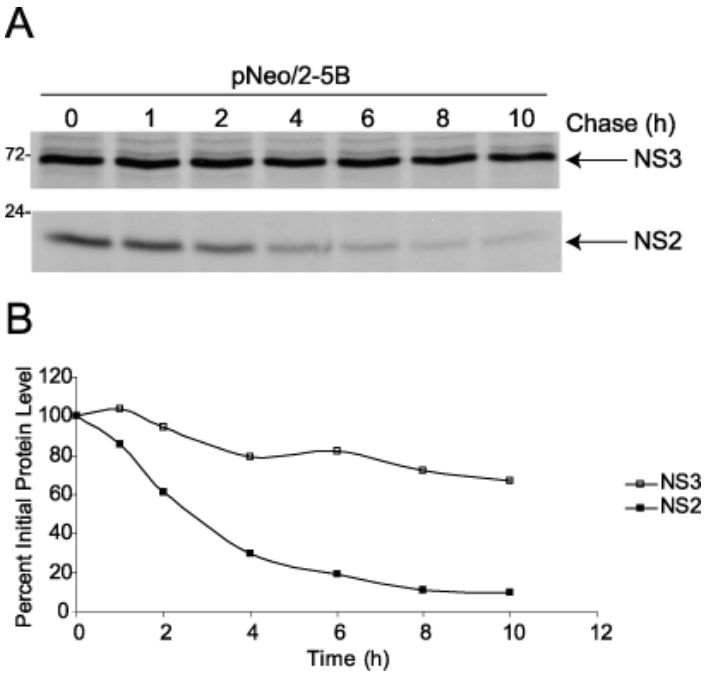

Figure 3.3. Degradation of NS2 in replicon stable cell lines.

A) Pulse-chase labelling of pNeo/2-5B replicon cell lines was performed for the time points indicated followed by anti-NS2 and anti-NS3 immunoprecipitation. Labelled protein levels were visualized by autoradiography and quantified (B) by phosphorimaging. Initial protein level at time 0 was set at 100%.

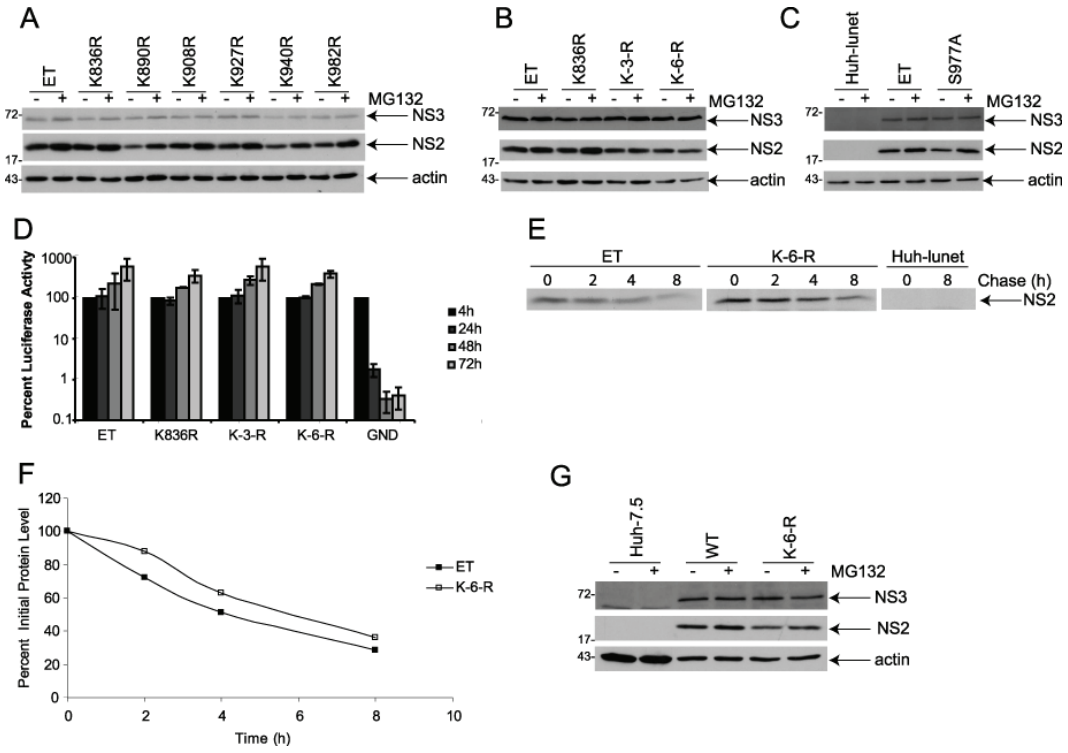

Figure 3.4. Effect of lysine mutations on NS2 stability.

A, B and C) The indicated pFK-repPI-luc/NS2-3' luciferase replicons were electroporated into Huh7-Lunet cells. After attachment, cells were treated with 10 μM MG132 or Me_2SO control for 8h. NS2, NS3 and actin levels were then visualized by immunoblot analysis using specific antibodies. D) The indicated pFK-repPI-luc/NS2-3' luciferase replicons were electroporated into Huh7-Lunet cells and luciferase activity measured after 4, 24, 48 and 72 h. Values are reported as a percentage of the counts obtained after 4 h. Error bars indicate standard deviation of a minimum of 2 independent experiments. E) Pulse-chase analysis of NS2 half-life was determined 72 h after electroporation of wildtype (ET) and NS2-lysine-free (K-6-R) luciferase replicons into Huh7-Lunet cells. Labelled proteins were isolated by anti-NS2 immunoprecipitation at the chase times indicated and visualized by autoradiography. F) Quantification of NS2 levels in E) as determined by phosphorimaging and expressed as a percentage of NS2 present at time 0. G) Full-length core-3'/5.1 WT and NS2-lysine-free core-3'/5.1 K-6-R cell lines were treated with 10 μM MG132 for 8 h and the levels of NS2, NS3 and actin visualized using specific antibodies.

phosphorylation of this site by CKII [243], also had no effect in this system (Figure 3.4C). Interestingly, a recent report showed that use of a specific CKII inhibitor also did not increase levels of a genotype 2a NS2 protein [127], suggesting that perhaps the stability of NS2 proteins from different genotypes could be regulated in different ways. In addition, the similar levels seen here of both NS2 and NS3 for all constructs indicate that the mutations are not interfering with NS2/3 cleavage. As this cleavage is required for genome replication, this was also confirmed by a transient luciferase replication assay where the NS2-lysine-free replicon was found to replicate to very similar levels as the wildtype control (Figure 3.4D).

In order to more precisely determine any effect of the lysine mutations on NS2 stability, a pulse-chase analysis of NS2 half-life was performed after transient transfection of both the wildtype (ET) and NS2-lysine-free (K-6-R) luciferase replicons. NS2 levels shown in Figure 3.4E and quantified in Figure 3.4F, show that indeed the lysine mutations are not sufficient to stabilize NS2 in this system. Furthermore, stable cell lines were generated containing wild type and NS2-lysine-free pNeo/2-5B neomycin-selectable replicons in Huh7.5 cells. Pulse-chase analysis of NS2 half-life in these subgenomic cell lines also shows little difference in the degradation of the wildtype or lysine-free NS2 proteins (data not shown), therefore confirming in the context of stably replicating viral genomes the results obtained above.

NS2 lysine mutations were also introduced in combination into pFK-core-3'/5.1 full-length genotype 1b neomycin replicons and stable cell lines generated from Huh-7.5 cells. This allowed us to determine any additional effect the presence of structural proteins could have on NS2 levels. As recent genetic evidence suggests that HCV structural proteins may interact with NS2 [148, 175] it is also conceivable that their presence could affect its stability. Wildtype and NS2-lysine-free full-length replicon cells were therefore treated with MG132 or DMSO for 8 hours and NS2 levels visualized (Figure 3.4G). As compared to

actin (loading control) and NS3 (RNA replication level control), the levels of NS2 do not increase in the lysine-free mutant, and as is seen with subgenomic cell lines, proteasome inhibition has very little effect. These results are in contrast to what was reported by Franck *et al*, who showed very low amounts of NS2 in genotype 1a full-length replicon cells, levels which were greatly increased by MG132 treatment. One possibility to explain this discrepancy is the difference in genotypes used as mentioned above.

3.4.4 Effect of MG132 on NS2 levels.

Despite the fact MG132 increases levels of uncleaved mutant NS2/3, the effect of this proteasome inhibitor on cleaved NS2 levels is less obvious in the systems described above. This was further confirmed with cell lines containing pNeo/2-5B in either Huh-7, Huh-7.5 or Huh7-Lunet cells which were treated with MG132 for 8 hours and the effect on NS2 levels visualized (Figure 3.5A). Again, in contrast to what is shown with NS2/3 and what has been observed with cleaved NS2 using transient DNA systems ([243] and data not shown), proteasome inhibition shows only a very slight increase in NS2 levels in these cell lines. Interestingly, when MG132-dependent induction of a non-related protein (Hifα) is measured as a control for proteasome inhibition, the parental Huh-7 cell line (with and without replicon) shows a much larger increase than what is observed with cured cell lines (data not shown), suggesting there could be differences in these Huh-7-based cells with respect to MG132-induced proteasome inhibition. However, when MG132 is added to pulse-chase analysis of pNeo/2-5B-containing Huh-7 cell lines, no significant stabilization of NS2 is observed (Figure 3.5B and C) suggesting NS2 degradation could at least in part be proteasome-independent.

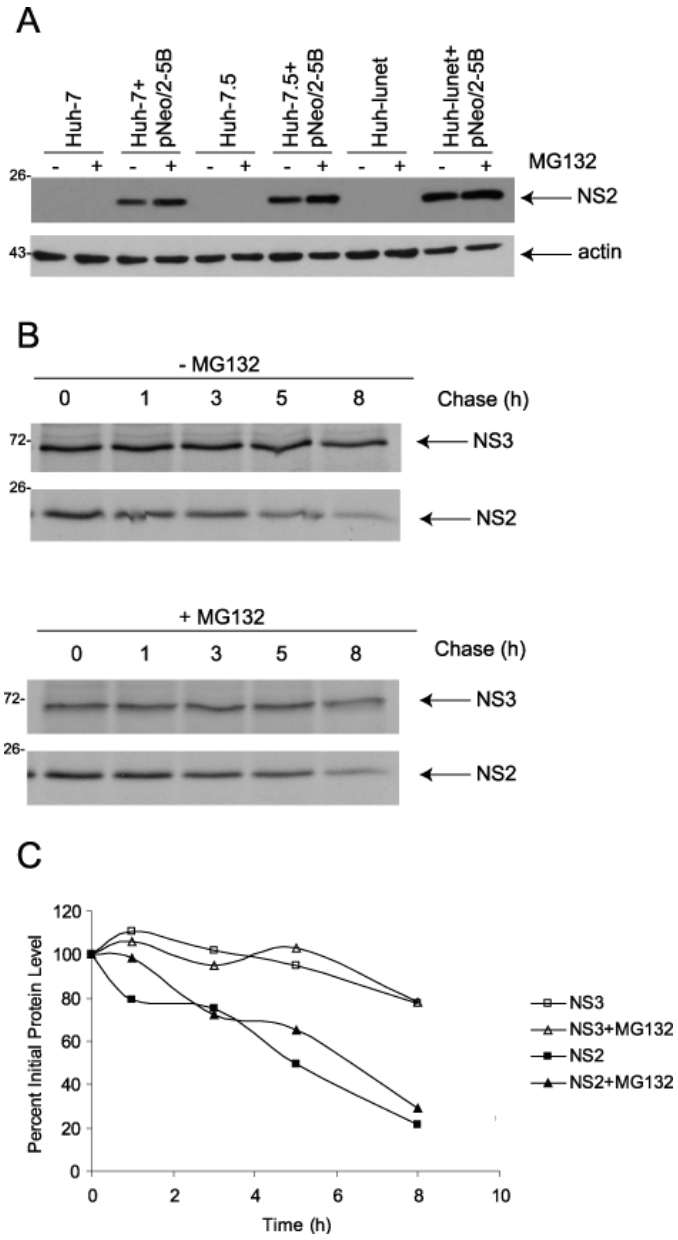

Figure 3.5. Effect of proteasome inhibitor MG132 on NS2 half-life.

A) Stable cell lines containing pNeo/2-5B in Huh-7 based cells were treated with MG132 or Me_2SO control for 8 h and NS2 and actin levels visualized by immunoblotting. B) Pulse-chase analysis was performed in Huh-7+ pNeo/2-5B cells in absence and presence of MG132. After the indicated chase periods, NS2 and NS3 were isolated by anti-NS2 and anti-NS3 immunoprecipitation and levels of radiolabelled proteins visualized by autoradiography and quantified (C) relative to time 0.

3.4.5 NS2 proteins from several different genotypes are also rapidly degraded.

As the characteristics of NS2 stability reported here with genotype 1b replicons differ from those previously published using genotype 1a DNA constructs, it is conceivable that NS2 stability could therefore differ between genotypes and in different systems. To examine the effect of genotype and determine whether the short half-life of NS2 is specific for genotype 1 or conserved amongst other genotypes, NS2 stability measurements were performed using NS2 proteins derived from Con1 (genotype 1b), H77c (1a), J6 (2a) and JFH-1 (2a) sequences. In order to measure the effect of the different genotype sequences using a uniform system, while also controlling for antibody immunoprecipitation efficiency, the NS2 proteins were all inserted into pcDNA3 vectors allowing the expression of NS2 proteins containing an amino-terminus FLAG tag. The constructs were transiently transfected into Huh7.5 cells and showed sufficient levels after 48 hours (Supplementary Figure 3.S1A) for pulse-chase analysis. Supplementary Figures 3.S1B and 3.S1C clearly show that the different NS2 proteins are all rapidly degraded; suggesting NS2 degradation could likely be a phenomenon common to several genotypes and important for some aspect of the viral life cycle.

3.4.6 Effect of lysine mutations in the genotype 2a JFH-1 infection system.

Stability analysis of NS2 derived from other strains (H77c, Con1, J6, JFH-1) was performed in DNA expression systems and they were all found to be rapidly degraded (Supplementary Figure 3.S1), suggesting the short half-life of NS2 is not unique to genotype 1. As JFH-1 NS2 was rapidly degraded in this DNA-based system, NS2 levels were also investigated in the genotype 2a cell culture infection system. Single NS2 lysine mutations, as well as a seven mutant lysine-free NS2 sequence, were introduced into pJFH-1. After transfection, very

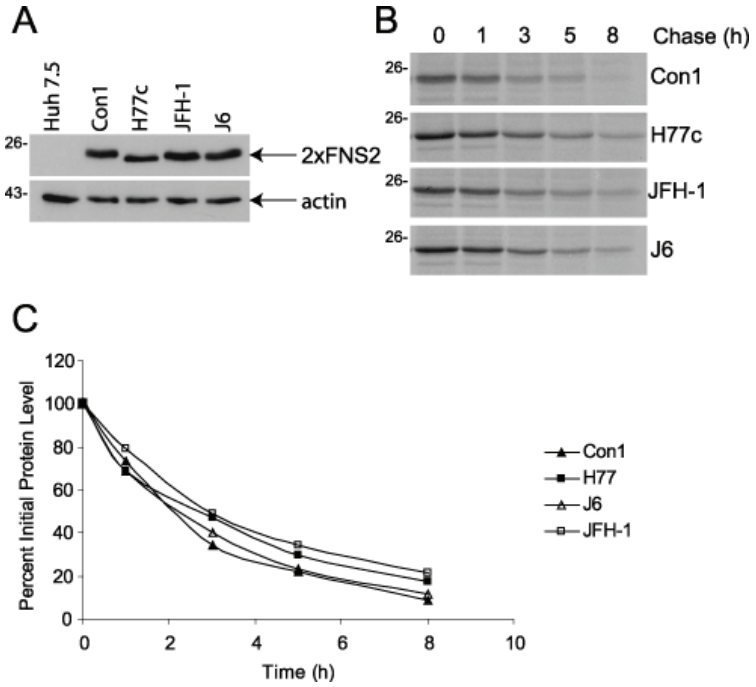

Figure 3.S1. Effect of genotype on NS2 half-life.

A)pcDNA32xFNS2 constructs from the indicated genotypes were transfected into Huh7.5 cells and after 48h the levels visualized by immunoblotting with anti-FLAG antibody. B) Pulse-chase analysis was performed after transient transfection of the DNA constructs. At the indicated chase periods, labelled NS2 levels were determined by immunoprecipitation using anti-FLAG beads followed by autoradiography and quantitation (C).

similar levels of core and NS3 proteins were achieved with all constructs, indicating similar replication capability of all mutants compared to wildtype (Figure 3.6A). Furthermore, NS2 is easily detected at similar levels, indicating no difference in production or stability of the protein in this system, consistent with what is described above with genotype 1b replicons. Consistent with the comparable replication efficiency of the mutants, accumulation of intracellular core protein was comparable between them and the wildtype (Figure 3.6B). However, in the case of K894R and K-7-R mutants, core release was reduced arguing that these mutants were impaired either in virus assembly or release. In support of this, release of infectious virus particles was reduced up to 100-fold with these two mutants (Figure 3.6C). This reduction was however much stronger than the effect on core release arguing that primarily infectivity of the virus particles rather than their release is affected. In support of this assumption, intracellular infectivity titers were also reduced by almost 2 logs for K894R (Figure 3.6D) thus showing that this residue plays a critical role in assembly of infectious HCV particles. Interestingly, the K1028R substitution resulted in an adaptive phenotype with release of infectious HCV enhanced more than 10-fold at 24h post transfection (Figure 3.6C) even though the overall infectivity titers achieved at later time points were not higher than wildtype. A kinetic analysis at even earlier time points showed that while neither wildtype nor K1028R show significant infectious virus release before 20 h, core release from the mutant genome appears at least 4 hours before that of the wildtype and infectivity levels continue to be higher with the mutant until about 48 h (Supplementay Figure 3.S2). All other lysine mutants were comparable to the wildtype arguing that they do not play an important role in viral replication and assembly.

3.4.7 NS2 K1028R shows no detectable change in NS2/3 cleavage efficiency

As lysine 1028 is located close to the NS2-NS3 junction (Figure 3.1), it is conceivable that the K1028R mutation could be modulating the kinetics of cleavage at the NS2/3 site. To investigate this possibility, WT and K1028R JFH-

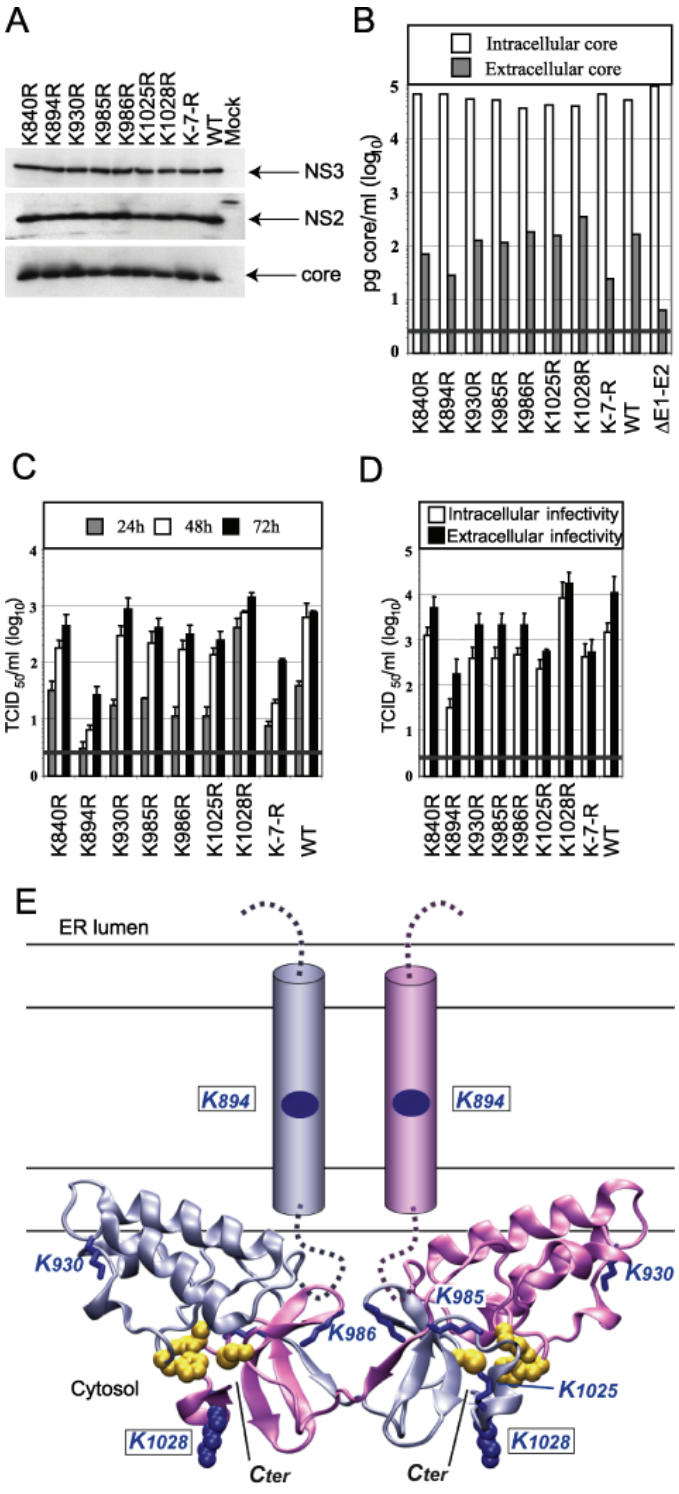

Figure 3.6. Effect of NS2 lysine mutations in the JFH-1 infectious system.

A) Immunoblot analysis of Huh7.5 cells harvested 48 h after transfection with the constructs specified. Blots were probed with primary antibodies on the right. B)

Determination of intra- and extracellular amounts of core proteins detected 48h after transfection of Huh7.5 cells with constructs specified. A representative result of two independent experiments with error ranges is shown. C) Kinetics of release of infectious HCV particles from transfected Huh7.5 cells as determined by $TCID_{50}$ assay. D) Intracellular and extracellular infectivity 48h post transfection as determined by $TCID_{50}$ assay. A representative result with values measured in triplicate is shown in panels (C) and (D) with error ranges. The experiments were independently repeated 3 times. E) Model structure of JFH-1 derived NS2 ectodomain based on the X-ray structure of a genotype 1a variant (PDB entry 2HD0, [199]). Ribbon diagrams of the dimer subunits are coloured in ice blue and light magenta. The sidechain atoms of active-site residues His 956 (143), Glu 976 (163) and Cys 997 (184) are represented as yellow spheres. Lysine residues are represented as blue sticks, except K1028 for which the side chain atoms are represented as blue spheres. NS2 ectodomain is positioned relatively to the membrane according to [199]. According to this model, the putative transmembrane segment 3 (shown here as cylinders and connected to the ectodomain chains by dotted lines) would extend into the membrane. K894 is cpredicted to belong to TM3 and to be located within the hydrophobic core of the membrane. The putative N-terminal transmembrane segments 1 and 2 are not represented. This 3D model was constructed with Swiss-PdbViewer program [274]. The Figure was generated using Visual Molecular Dynamics (http://www.ks.uiuc.edu/Research/vmd) and rendered with POV-Ray (http://www.povray.org)

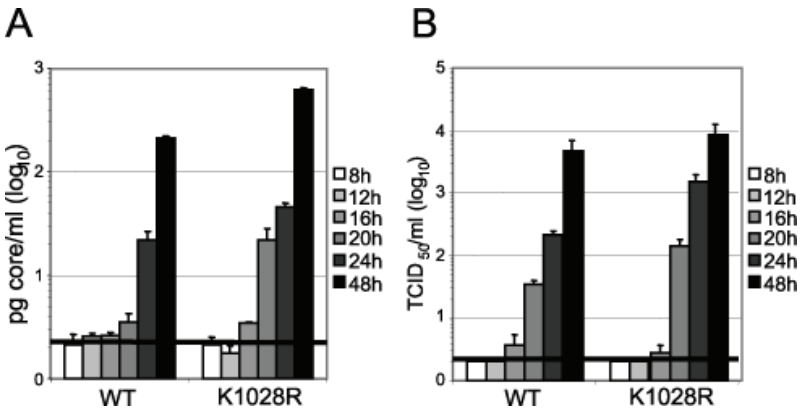

Figure 3.S2. Effect of K1028R on infectivity at early time points.

A) Kinetics of core release after transfection of 20 μg of RNA into Huh7.5 cells as determined by core ELISA. B) Kinetics of extracellular infectivity as determined by $TCID_{50}$ assay. The horizontal black bar is set at background level, as determined by a ΔE1/E2 mutant.

1-derived NS2/3 sequences were inserted into a pcDNA3 vector with an N-terminal FLAG tag. 48 hours after transfection of these constructs into Huh-7.5 cells, the cells were pulsed with ^{35}S-methionine and at the indicated chase times, anti-FLAG beads were used for immunoprecipitation of 2xFNS2 and 2xFNS2/3. Supplementary Figure 3.S3A shows that although similar amounts of NS2 are seen with both constructs, no precursor NS2/3 is detected in either case. This result is not unexpected in the case of the WT as the autocatalytic cleavage of NS2/3 is thought to occur very rapidly, without detection of the uncleaved precursor. That no precursor is seen with the mutant suggests that K1028R does not cause a significant decrease in cleavage efficiency, however it is not possible using this system to measure an increase in the already rapid cleavage kinetics of this protein. To investigate this, an *in vitro* translation system was used where cleavage kinetics are sufficiently slowed down to detect uncleaved NS2/3 as well as the cleaved NS2 and NS3 products. *In vitro* transcribed RNA generated from pcDNA32xFNS2/3(2a) WT and K1028R were used as templates for *in vitro* translation in rabbit reticulocyte lysates with and without canine microsomal membranes. Supplementary Figure 3.S3B shows that while in both cases membranes increase cleavage efficiency, there is no significant difference in levels of cleavage between WT and K1028R when levels of cleaved NS2 and NS3 are compared to the uncleaved NS2/3. These results therefore suggest that the cleavage capability of the K1028R mutant does not differ significantly from the wildtype protein.

Overall, while NS2 is easily detectable in the JFH-1 infectious system and lysine mutations have no effect on its levels, some of these mutations affect steps in viral assembly and infectivity, an interesting observation that will require further study.

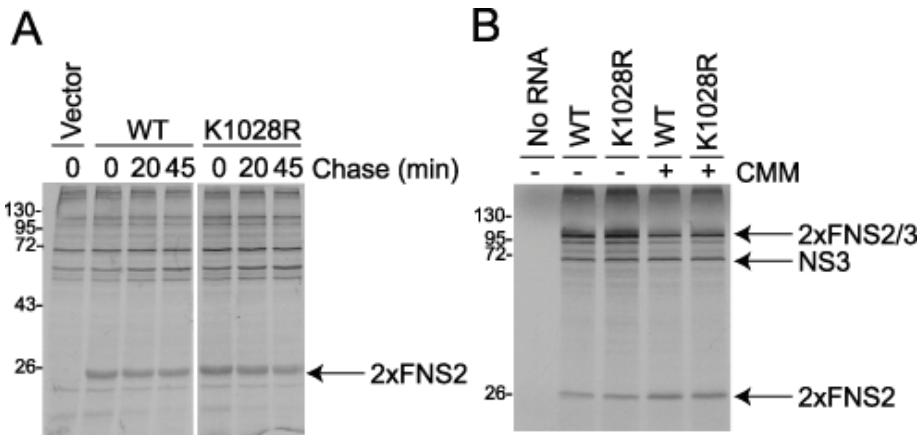

Figure 3.S3. Effect of K1028R on NS2/3 cleavage.

A) pcDNA32xFNS2/3(2a) constructs were transfected into Huh7.5 cells and after 48h cells were metabolically labelled for 90 min. At the indicated chase periods, immunoprecipitation using anti-Flag beads was performed, followed by detection of labelled proteins by autoradiography. B) RNA generated from pcDNA32xFNS2/3(2a) constructs was *in vitro* translated in presence or absence of canine microsomal membranes (CMM). The translated products were separated by SDS-PAGE and visualized by autoradiography.

3.5 DISCUSSION

One goal of this study was to further investigate the mechanism by which HCV NS2/3 mutant replicons are rendered inactive for RNA replication. Our previous work [185] looked at the NS3 catalytic activities of mutant uncleaved NS2/3, and while a small decrease in NS3 protease activity was seen *in vitro*, no significant effect on polyprotein processing was seen in cells (see also Supplementary Figure 3.S4) and therefore for this study we decided to focus on the observation that the resulting NS2/3 protein was rapidly degraded. We therefore performed a lysine-mutagenesis study to determine if a stabilized NS2/3 protein would be functional for RNA replication. While an NS2-lysine-free protein was found to be substantially stabilized, we show here that the longer-lived mutant H952A NS2/3-containing replicon is still unable to replicate suggesting uncleaved NS2/3 may not be able to functionally replace NS3 for genome replication.

This is in line with recent evidence from bovine viral diarrhea virus (BVDV) suggesting that even at high levels, uncleaved NS2/3 may simply not be able to functionally replace NS3 in viral genome replication. For this pestivirus, the uncleaved NS2/3 protein is stable and required for viral infectivity [275] while viral RNA levels have been shown to correlate with levels of cleaved NS3 [184]. For BVDV therefore, it would appear that uncleaved NS2/3, while present, simply cannot functionally replace cleaved NS3 in genome replication and this could also be the case for HCV NS2/3. It is important however to point out several differences between the NS2/3 proteins of these two viruses. Any potential role for uncleaved NS2/3 has yet to be established in HCV infection as cleavage at the 2/3 junction appears to be very efficient in cell systems, without the identification of an uncleaved NS2/3 precursor. Indeed, recent evidence argues against a role for such a precursor. It was reported that, using bicistronic constructs to physically separate NS2 from NS3 in a viral system, no effect was seen on

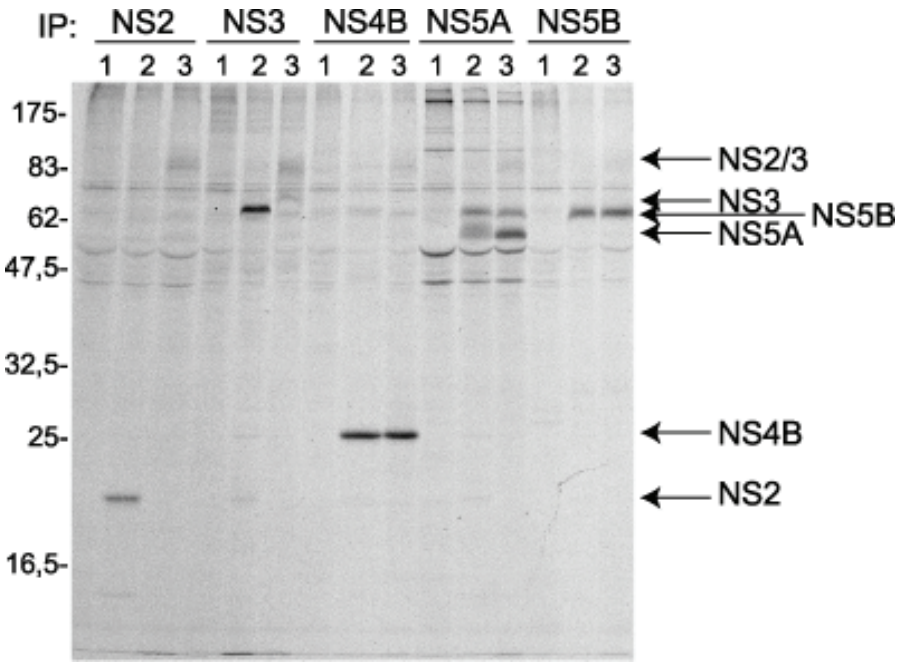

Figure 3.S4. Polyprotein processing in presence of mutant NS2/3.

Lunet-T7 cells were transfected with pTM NS2-5B Con1 WT (lanes 2) or H952A mutant (lanes 3) constructs or mock treated (lanes 1) followed by metabolic labelling and immunoprecipitation using the antibodies specified at the top of the figure as described in supplementary methods.

Mutant uncleaved NS2/3 is still capable of efficient polyprotein processing

As our previous work showed a slight decrease in the kinetics of NS3 cleavage *in vitro* in presence of uncleaved NS2/3, we re-examined the ability of H952A NS2/3 protease inactive mutant to process the viral polyprotein. pTM vectors containing NS2-3' (either wildtype or H952A) were transfected into Lunet-T7 cells and metabolic labelling and immunoprecipitations with anti-NS2, NS3, NS4B, NS5A and NS5B antibodies were performed. Supplementary Fig. S4 shows efficient cleavage at all sites and the similar intensities of NS4B and NS5B indicate no decrease in the generation of these proteins by the mutant NS2/3 enzyme, confirming results reported previously [105, 177]. The difference in NS5A bands confirms the inability of NS5A to be hyperphosphorylated in absence of cleaved NS2/3 as shown previously [211].

infectious virus production [81, 146], unlike with BVDV where uncleaved NS2/3 is required for infectivity [275].

Rapid degradation of several viral proteins has been reported and could constitute an important way for either the host cell or the virus itself to regulate the various events of the viral life cycle. While a previous study focussed on NS2 degradation in DNA-transfected cells using tagged constructs, we show here that NS2 is also a short-lived protein in various RNA-based replication systems. This was important to investigate as expressing untagged NS2 in the context of the other viral proteins could have important differences in terms of its function, localisation or degradation. Moreover, our results differ from what was previously reported by Franck *et al*, in that in all systems used here, NS2 was readily detectable without proteasome inhibition, likely due to differences with respect to the genotypes used. The relevance of this degradation for the virus remains elusive, as does the mechanism of degradation.

Lysine mutagenesis, which would abolish potential ubiquitination sites, had no effect on NS2 levels. Several proteins have been reported to be degraded by both ubiquitin dependent and independent pathways. Some were suggested to interact directly with the proteasome for degradation, such as IκBα [276]. Ornithine decarboxylase is degraded in a similar fashion via recognition by the proteasome after binding to an antizyme co-factor [277]. However, our observation that MG132 has only a very slight effect on NS2 levels suggests that other, non-proteasomal pathways could be involved in the degradation of this protein. While further study is required, it is possible that NS2 is being targeted for degradation via lysosomal pathways or that targeted proteolytic cleavage may be involved. Indeed, although its significance remains unknown, Jirasko *et al.* (2008) have recently shown the generation of a truncated form of NS2 in infectious cell systems.

Interestingly, while no change in NS2 levels is seen upon introduction of lysine mutations into the JFH-1 infectious construct, several of the mutants themselves affect viral infectivity, despite showing no defect in NS2/3 processing or RNA replication. Recently, it was reported that the NS2 protein is required for HCV infectivity by functioning at a stage prior to virion assembly [81, 146] although its exact role has yet to be fully defined. This is consistent with the K894R mutation observed here to block the formation of intracellular infectious viral particles. Indeed, this mutation likely disturbs some intra- and/or intermolecular interactions within or between NS2 or its biological partners. Recent genetic evidence has suggested NS2 may function through interaction with other structural and non-structural HCV proteins. The generation of intergenotypic chimeras showed an ideal junction site to be within NS2, suggesting one part of this protein could interact with structural proteins and/or p7, while its C-terminus required compatibility with the other non-structural proteins [175]. This is further substantiated by the appearance of compensatory mutations in these proteins upon culture of an H77c/JFH-1 chimera [147]. These mutations significantly increased viral titers, potentially by correcting incompatibilities between interactions sites of the proteins from different genotypes. Interestingly, K890R was identified in that study, and we show here that this residue (K894 in JFH-1, see below) is indeed affecting viral assembly. Similarly, compensatory mutations in NS2 and p7 were also identified to increase viral assembly of defective constructs containing core mutations, suggesting many interactions between the structural proteins, p7 and NS2 could be required for virion assembly [148]. Alternatively, it is also possible that NS2 interacts with other, yet to be identified, cellular proteins during the assembly process and the mutation identified here could also be affecting this process.

In order to investigate the relationship between the location of the mutated lysines and their effect on viral infectivity, a model structure of the JFH-1 NS2 ectodomain (Figure 3.6E) was constructed by homology modeling using the three-dimensional X-ray structure of the genotype 1a NS2 variant [199]. As

shown in Figure 3.6E, four of the lysines belonging to the NS2 ectodomain are located at the protein surface (K930, K985, K986, K1025). This could explain why their mutation to arginine has no detectable effect, as they would be unlikely to alter NS2 structure. Interestingly, K1028R resulted in a stimulation of viral infectivity titers at early time points. This residue is located close to the NS2-NS3 cleavage site and while we detected no significant effect on NS2/3 cleavage, it could also likely be acting by modulating intra- and/or intermolecular interactions. In contrast, K840 and K894 reside in the membrane domain of NS2. While K840 belongs to the putative cytosolic loop connecting TM1 and TM2, K894 is located within predicted TM3 (Figure 3.6E). The large decrease of infectious virus particle release induced by the mild mutation of K894 to arginine is therefore consistent with the critical role of the NS2 membrane domain in the processes of viral assembly, as reported previously [81, 146]

Overall, although much work is still required to understand NS2's role in the viral life cycle, we show in this study that NS2 levels could play an important part in this process and confirm that NS2 is a critical component of the viral assembly mechanisms by identifying residues in NS2 required for virus assembly.

3.6 ACKNOWLEDGEMENTS

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CHAPTER 4:

DISCUSSION

During the time span of this thesis work (2003-2009), many important advances were made in the field towards better understanding the virology of HCV. These breakthroughs included the description of the first cell culture infectious systems in 2005 [39-41], the elucidation of several viral protein structures [80, 121, 199], the discovery of tight junction receptors important for virus entry [142-143] and the importance of lipid droplets [63] and the VLDL pathway [150] for viral assembly and secretion, to mention a few. In particular, the number of studies focussing on NS2, the subject of this thesis, has risen exponentially in recent years thanks to the JFH-1 cell culture infectious system and the discovery of this protein's essential role in viral assembly.

This thesis project started out simply investigating the role of NS2/3 cleavage in genome replication. We showed that mutation of the catalytic active site of this enzyme completely abolished the ability of NS2-5B replicons to replicate. While the catalytic activities of NS3 did not seem to be affected by the presence of NS2, the uncleaved NS2/3 protein appears to be rapidly degraded in a proteasome-dependent manner. Interestingly, further experiments showed that stabilization of uncleaved NS2/3 by lysine mutagenesis failed to rescue genome replication of the NS2/3 protease inactive replicons, suggesting NS2/3 cannot functionally replace NS3 for RNA replication. We then expanded these degradation studies to the NS2 protein itself, which we found to be degraded by what appears to be a ubiquitin and proteasome independent mechanism. Interestingly, during the course of these studies, we were also able to identify residues in NS2 that, when mutated, modulate HCV infectivity, further confirming the emerging role of this protein in the viral particle assembly process. While these major findings were analysed in the results sections of this thesis, in this chapter their significance will be discussed in more detail and in light of recently published information not available when these studies were initiated.

4.1 NS2/3 PROCESSING IS REQUIRED FOR VIRAL GENOME REPLICATION: BUT IS IT A REALISTIC DRUG TARGET?

In Chapter 2, we demonstrated that NS2/3 cleavage activity is absolutely required for genome replication as mutation of the active site residues of this protein completely abolished the ability of NS2-5B replicons to replicate (Figure 2.3). This result therefore further validates targeting the NS2/3 protease activity as a novel HCV drug target. However, despite the development of several high throughput screens to identify compounds [278-281] and now the availability of the NS2 crystal structure for rational drug design [199], no specific inhibitor of NS2/3 activity has yet been shown in the literature to be able to decrease HCV RNA levels in a cell system.

Therefore, NS2/3 is proving to be very challenging as a drug target. Many inhibitors were identified *in vitro* (see Table 4.1) during the initial characterization of the enzyme, however, in most cases, this was before the availability of the replicon and JFH-1 systems, and a proof of principle experiment that an NS2/3 active site inhibitor can inhibit HCV replication is still lacking. So why are there still no NS2/3 cleavage inhibitors in development? One possibility is that it may be much more difficult to inhibit NS2/3 during the events of the full viral life cycle than it is to target a single protein in an *in vitro* assay. NS2/3 cleavage during polyprotein processing is thought to be a very efficient process that could be occurring extremely quickly after co-translational folding of the enzyme, making it potentially very difficult to target with an active site agent. In addition, unlike NS3 that is required to perform many different *trans* cleavages during the viral life cycle, targeting NS2/3 may well involve targeting a single autoproteolytic event. Indeed, several lines of evidence argue against the cleaved NS2 protein having additional proleotytic substrates. First of all, while the crystal structure of NS2 shows an apparently complete active site [199], and recent evidence suggests it may indeed be a *bona fide* protease able to cleave in *cis* with the addition in *trans* of the NS3 zinc binding domain as a co-factor [188], this

Table 4.1: HCV NS2/3 inhibitors

Inhibitor Class	Examples	Potency	Assay	Ref.
Cysteine protease inhibitors	Iodoacetamide	>80% inh. at 0.5mM	IVT	[192]
		25% inh. at 0.5mM	Purified	[179]
		100% inh. at 1mM	Purified	[180]
	N-ethylmaleimide	>80% inh. at 4mM	IVT	[192]
		70% inh. at 5mM	Purified	[179]
		100% inh. at 0.1mM	Purified	[180]
Metalloproteinase Inhibitors	EDTA	>60% inh. at 2mM	IVT, purified	[178-180, 192]
	1,10-phenanthroline	>70% inh. at 2mM	IVT	[192]
		80% inh. at 1mM	Purified	[180]
HSP90 Inhibitors	Geldanamycin	EC50 ~1.8μM	IVT	[193]
		IC50 12-40nM	Cell-based	[193, 282]
	Radicicol	EC50 ~0.22μM	IVT	[193]
		IC50 13-22nM	Cell-based	[193, 282]
NS4A-derived peptides	KGSVVIVGRIILSGRK	IC50 ~ 5.7nM	IVT	[191]
	KKGSVVIVGRIILSGRK	IC50 ~ 0.6μM	Purified	[180]
NS2/3 cleavage-site derived peptides	EQGWRLL*APITAYS	No sig. inhibition	IVT, purified	[179, 191]
	SFEGQGWRLL*APITAYQQT	IC50 270μM	Purified	[180]
	SFEGQGWRLL	IC50 90μM	Purified	[180]

IVT: *in vitro* translation NS2/3 cleavage assay, Purified: *in vitro* purified NS2/3 cleavage assay, Cell- based: cell-based reporter assay for NS2/3 cleavage

Adapted from reference [57]

same structure also shows the extreme C-terminal leucine residue located in close proximity to the active site (see Figure 1.7). This was suggested to cause autoinactivation of the enzyme and prevent further cleavages from occurring after NS2-NS3 processing [199]. Indeed, no *trans* cleavage of peptides using NS2 has been reported *in vitro*, and no additional cellular targets of this protease have been identified. In addition, recent work has shown that beyond its role in NS2/3 processing, the protease active site located in NS2 is not required for the HCV viral life cycle. Indeed, mutation of the active site cysteine in NS2, in the context of a bicistronic construct is which NS2 is already physically separated from NS3 by an IRES, has no effect on HCV replication or infectivity [81, 146]. However, it cannot be excluded that NS2 undergoes conformational rearrangements to allow for the cleavage of cellular factors not strictly required for the viral life cycle, but that could be involved in for example the host response to infection or viral pathogenesis.

While targeting NS2/3 through an active site inhibitor remains a challenging goal, there has been some very recent success at targeting NS2/3 function through inhibition of a cellular host factor. While HSP90 was suggested several years ago to be required for NS2/3 processing [193], Ciesek *et al.* recently implicated another chaperone protein, cyclophilin A, as being important for this process [195]. They showed that an inhibitor of this protein, cyclosporin A, already known to act on HCV through cyclophilins and the NS5B protein, was much more effective at decreasing HCV replication levels with constructs that depended on NS2/3 cleavage than in those that did not. While no direct effect on NS2/3 processing was observed, it was interesting to note that the peptidly-prolyl-isomerase activity of the enzyme was required for susceptibility to cyclosporine A. It is therefore possible that cyclophilin A is required for proper folding of the NS2/3 protein as the NS2 structure does indeed contain an unusual *cis* proline which could contribute to the correct conformation of the active site [199].

In summary, our data showing the essential role of NS2/3 cleavage in genome replication does indeed justify the continued investigation of ways to inhibit this process. However, for reasons discussed above, this is not a straightforward endeavour and while additional structural information on the uncleaved NS2/3 protein, not just the NS2 reaction "product", might help in the rational design of novel inhibitors, it is likely that targeting the active site of this enzyme directly will continue to be extremely challenging. However, targeting this enzyme through its essential interactions with cellular factors currently appears to be a more immediately successful route.

4.2 HOW DOES UNCLEAVED NS2 INTERFERE WITH NS3 FUNCTION?

The NS2 protein itself is not required for HCV genome replication [160]. However, we showed that if this protein is present, its cleavage from NS3 is essential for replication and we therefore further investigated the reasons for this by determining whether the uncleaved precursor NS2/3 could perform known NS3 enzymatic functions. While the ATPase and helicase activities of NS3 were unchanged *in vitro* (Figure 2.4, data not shown), there was a slight decrease in NS3 protease kinetics observed in presence of uncleaved NS2 when using recombinant proteins (Table 2.1). However, as has been shown previously [105, 177], this did not correlate to an inability to process the downstream non-structural polypeptide in cells (Figure 2.6 and 3.S4). However, whether or not the kinetics of this polyprotein processing could be affected has not been determined.

Recently, it has been suggested that the NS3 protease domain could exist in two distinct conformations, depending of whether it is acting as a co-factor for NS2/3 cleavage or performing its own role as a serine protease [188]. Evidence for this also includes the different susceptibilities of the NS2/3 and NS3 enzymes to zinc chelators that imply structural differences between the conformation of the NS3 zinc binding site in the two enzymes [187], as well as our *in vitro* evidence presented here showing a reduced affinity of the NS4A NS3 co-factor to the

uncleaved NS2/3 precursor (Figure 2.5). This decreased affinity for NS4A suggests that with an uncleavable NS2/3, the NS3 portion of the protein is still partly in an "NS2/3 cleavage" state. Decreased NS4A binding could therefore make it more difficult for this co-factor to cause the conformational rearrangement to the NS3 protease form required for serine protease activity, which could explain the slight decrease in NS3 protease kinetics seen *in vitro*. The dependence on NS4A of the inhibition observed could be further tested *in vitro* by determining whether the slight decrease seen in our experiments performed with a substrate derived from the NS4A/NS4B cleavage site would be significantly magnified if a cleavage sequence absolutely depending on NS4A binding (such as NS4B/NS5A) were to be used. However, the fact that NS4A has been shown to inhibit NS2/3 activity *in vitro* [191] and that the ability of NS4A peptides to inhibit NS2/3 cleavage activity correlates with their ability to promote NS3-dependent cleavage, suggests NS4A can still to some extent bind to the uncleaved precursor and "force" NS3 into the serine protease form, away from the conformation of NS2/3 required for cleavage at the NS2/NS3 junction. The extent to which NS3 is functional is clearly sufficient for what appears to be normal polyprotein processing in cells (Figures 2.6 and 3.S4 and [105, 177]). Whether or not the kinetics of processing are affected *in vivo* would be technically challenging to determine. While most polyprotein processing studies have involved transfection of vaccinia virus driven expression constructs, metabolic labelling and immunoprecipitation in order to have sufficient levels of the proteins for analysis, performing a detailed kinetic analysis in this way may be technically difficult (for example Figure 3.S4 was metabolically labelled for 14 h, and others have labelled for at least 4 h [283], timescales which could mask any small kinetic changes). Until this experiment is performed, we cannot completely exclude the possibility that a defect in NS3 protease kinetics in the context of uncleaved NS2/3 could contribute to an effect on replication, however, it is unlikely that this alone would have the dramatic affect seen in our experiments (a complete loss of replication ability).

Our results showed that uncleaved mutant NS2/3 is very rapidly degraded, in a proteasome-dependent manner, soon after transfection into cells (Figure 2.7 and 2.8). We therefore initially proposed in Chapter 2 that this could be a reason for the inability of NS2/3 protease-inactive replicons to replicate. While the NS3 portion of NS2/3 may be catalytically active for replication, it may just not be present in sufficient quantities to perform that role. We therefore used a lysine-mutagenesis approach in Chapter 3 in an effort to stabilize this protein and determine any rescue in replication. While we found that mutation of all NS2 lysines in NS2/3 to arginine significantly increased levels of this protein, this stabilization was not sufficient to confer replication capability (Figure 3.2), suggesting NS2/3 is unable to functionally replace NS3 for genome replication. It is however possible that the levels of the uncleaved protein were not sufficiently increased. Treatment of the NS2 lysine-free replicon cells with MG132 resulted in an even further increase in levels (Figure 3.2B) suggesting additional ubiquitin-independent but proteasome- dependent pathways, or indeed the NS3 portion of the protein (in which the lysine residues were not mutated) could be contributing to the degradation phenotype.

We also cannot exclude the possibility that the NS2/3 catalytic active site mutations could be modifying the uncleaved NS2/3 protein structure. A very recent study has shown that in particular the H952A mutant, in addition to its affect on catalytic activity, may be affecting the overall fold of the protein [284]. This effect is less pronounced with the C993A mutation and while our degradation studies were performed with all three active site mutant constructs (Figures 2.6, 2.7 and data not shown), the lysine stabilization experiment was only performed with the H952A mutant protein (Figure 3.2). However, as all these mutants had similar NS3 catalytic activities to cleaved NS3 (Chapter 2), and another group has shown similar NS3 protease activity *in vitro* for the NS2/3 WT and mutant proteins [187], it is likely that a "WT" uncleaved NS2/3 would also not be functional for replication. This is indeed the case for BVDV, where a WT uncleaved NS2/3 exists but cleaved NS3 is required for replication [184].

So why can HCV NS2/3 not perform the role of NS3 in replication? Perhaps having NS2 still attached changes NS3 localization. NS4A usually targets NS3 to membranes [94] (and presumably the replication complex), however, the membrane bound NS2 could interfere with this targeting. Immunofluorescence studies could be used to compare the subcellular localizations of NS2, NS3 and uncleaved NS2/3. Indeed, for BVDV, NS2/3 and NS3 proteins have in fact been shown to associate with different intracellular membranes [285]. It is also possible that uncleaved NS2/3 is preventing incorporation of NS3 into the replication complex itself. In addition to the above-mentioned immunofluorescence experiment, replication complexes could themselves be isolated [286] and probed for NS3 and NS2/3 as well as for NS2, which while never shown to be part of the replication complex, may however co-purify in the replication complex membrane fraction.

4.3 ROLE FOR UNCLEAVED NS2/3 IN THE HCV VIRAL LIFE CYCLE?

As mentioned above, for BVDV, the cleaved NS3 protein is required for viral genome replication. For this virus, there is cellular co-factor dependent temporal control of NS2/3 cleavage to modulate replication levels. In fact, cytopathic strains of this virus are highly replicative and have high levels of the cleaved NS3 protein (generated by various insertions/duplications of the genome) [258-259, 287-289]. However, cytopathic strains are found together with non-cytopathic strains which express the uncleaved NS2/3 protein [258-259] and Agapov *et al*. have shown that uncleaved NS2/3 is required for viral particle formation/morphogenesis [275]. It is therefore tempting to speculate that HCV uncleaved NS2/3 could also have a role in modulating the various steps of the viral life cycle. However, there is no evidence to date to support such a proposition. HCV NS2/3 cleavage appears to be extremely efficient in the polyprotein context in cell systems and no uncleaved precursor has been detected. Moreover, as mentioned in Section 1.4.3, work with IRES separated NS2-NS3

HCV constructs has demonstrated no requirement for the uncleaved NS2/3 precursor in the viral life cycle. Although BVDV NS2/3 is required for virion assembly, HCV NS2 has also been shown to be important for this process, and could even be interacting with NS3 post-cleavage [202] for a role in assembly similar to the BVDV uncleaved NS2/3. Whether NS2/3 could have an accessory role in the viral cycle has however not been formally ruled out. It is possible that both NS2/3 cleavage and NS2/3 degradation are regulated in a specific manner and the specific conditions in which NS2/3 may be present have simply just not yet been determined. While it is difficult to work with the WT NS2/3 protein that undergoes autocleavage, co-transfection of a stabilized NS2/3 uncleavable mutant, such as the one generated in our study, might be possible to determine if presence of this protein in *trans* in any way affects viral replication or assembly. As mentioned above, it is also possible that NS2/3 may also play an additional role in HCV persistence and/or pathogenesis which cannot be measured in a simple cell culture based infection assay.

4.4 NS2 DEGRADATION: MECHANISM AND SIGNIFICANCE?

In Chapter 3, we showed that cleaved genotype 1b Con1 NS2 protein is rapidly degraded in RNA replication systems. However, unlike uncleaved NS2/3, mutation of all the lysine residues in this protein failed to stabilize it (Figure 3.4), suggesting a ubiquitin-independent mechanism of degradation. While several examples of ubiquitin-independent degradation exist in the literature (some proteins have been found to bind directly to the proteasome [276], or to do so via interaction with another cellular co-factor [277]), what was most surprising was that the MG132 proteasome inhibitor also did not have a significant effect on the stability of this protein (Figure 3.5). Other possible mechanisms for its degradation could include specific protease dependent cleavages or lysosomal degradation. N- and C-terminal truncated forms of NS2 have been reported for the JFH-1 genotype 2a and H77 genotype 1a proteins, respectively, which could indicate NS2 is a target for cytosolic or membrane bound proteases [146, 205].

Lysosomal degradation of NS2 is another possibility. To test this, levels of NS2 could be measured with and without treatment of the cells with lysosomal inhibitors such as chloroquine or ammonium chloride.

When our study on the stability of genotype 1b Con1 NS2 was initiated, there was a report showing rapid degradation of the H77 genotype 1a NS2 protein [243]. While the studies in that paper were largely performed using DNA expression vectors and *in vitro* assays, they showed that NS2 was degraded after phosphorylation of serine 168 (NS2 amino acid numbering, equivalent to serine 977 in the Con1 polyprotein) by casein kinase II (CKII) and they demonstrated that mutation of that specific serine to alanine, or treatment with curcumin, a CKII inhibitor, prevented NS2 degradation. However, we found that this mutation had no effect on the stability of NS2 expressed from our genotype 1b Con1 constructs (Figure 3.4C), and other groups have also shown no effect of either a CKII inhibitor [127] or this serine mutation [146, 207] on genotype 2a JFH-1 NS2 levels. Further evidence confirming the apparently genotype-specific mechanisms of degradation of this protein is also presented in a recent paper comparing the differing role of the proteasome and S168 on the levels of JFH-1 and H77 NS2 [205]. Interestingly, S168 appears to be important not only for genotype 1a NS2 stability, but also for the assembly of infectious particles even in genotypes whose NS2 levels are not affected by this amino acid [146, 205, 207].

So is there any significance to the degradation of NS2? Is NS2 degradation regulated? Are levels of NS2 important to modulate the different events of the viral life cycle? As NS2 is dispensable for genome replication but required for assembly of infectious viral particles, it is tempting to speculate that NS2 (and its levels) could be involved in which of these processes will be preferentially occurring at a given time. For example, levels of NS2 could be regulated until sufficient replication has occurred and an increase in NS2 levels could facilitate a switch over to the assembly process. While many *trans* complementation studies have been reported showing the addition of wildtype

NS2 in *trans* being able to rescue infectivity of deficient genomes [207, 290-291], it would be interesting to determine what effect increasing or decreasing NS2 levels in a WT infectious background could have. Would the kinetics of viral release change? One study has reported a slight decrease in levels of infectious virus produced at 48 h by an infectious genome containing WT NS2 after introduction of a helper virus also containing WT NS2 (as well as other HCV proteins) [146]. However, no kinetic analysis was performed and as this helper virus was somewhat cytotoxic and as replication of the HCV-based helper virus itself could compete with replication of the full length virus construct, this result is hard to interpret. Further work on elucidating the mechanism of NS2 degradation and its potential regulation may however provide the means to be able to control NS2 levels during the viral life cycle and further investigate the consequences of its modulation.

The control of NS2 levels could also play a role in the regulation of other NS2 functions not strictly involved in viral replication, assembly and infectivity. For example, NS2 has been suggested to affect several cellular processes such as gene transcription, cell cycle regulation and apoptosis (see Section 1.4.4). Regulation of NS2 levels could be important for the balance between disrupting these pathways (which could help provide the ideal environment for virus replication), while also limiting toxicity to the cell.

And what is the signal for genotype 1b NS2 degradation, if unlike genotype 1a, it does not require CKII phosphorylation of S168? Phosphorylation at a different site is a possibility and a few other putative serine or threonine phosphorylation concensus sites have been suggested by bioinformatics searches. Surprisingly, no study has yet been performed to determine the phosphorylation status of NS2 (derived from any genotype) in cell replication systems. Labelling such cells with ^{32}P orthophosphate, followed by anti-NS2 immunoprecipitation, could give some indication as to whether or not this protein gets phosphorylated during the viral life cycle. In addition to potentially being a contributing factor

involved in its degradation, phosphorylation of NS2 could also be a way of modulating the roles of this protein and therefore this experiment would be of interest to perform as it could help further characterise NS2 and its functions. Furthermore, while we have performed large scale immunoprecipitation experiments, we have been unable to identify NS2 binding partners by MudPIT analysis that could also help to characterise this protein (data not shown).

4.5 ROLE OF NS2 RESIDUES IN VIRAL ASSEMBLY/INFECTIVITY

During the course of our lysine mutagenesis study in Chapter 3, we identified several residues in JFH-1 NS2 that were able to modulate viral infectivity without affecting NS2 levels, NS2/3 cleavage or genome replication (Figure 3.6). Mutation of K894 had the most significant effect, severely impairing virus infectivity. K894 is located within the proposed 3rd transmembrane region of NS2 and this region of the protein could be required for transmembrane-based interaction either with other parts of NS2 itself or with other HCV or cellular proteins. Interestingly, further characterization of this NS2 residue was published by Phan *et al.* in an NS2 mutagenesis study six months after the publication of Chapter 3 [207]. Their paper describes the effect on viral infectivity of 36 conserved residues of NS2. These included additional mutations of several of the amino acids also investigated in our study (K840A, K894A, K894E, K986A), all of which showed severe defects in viral infectivity in their experiments. It is interesting to note that while even a highly conserved mutation of K984R had a strong effect in our study, arginine substitution of K840 (located in the cytosolic loop between TM1 and TM2) and K986 (located on the surface of the protease domain) had very little effect compared to the several log decrease observed with alanine substitution by Phan *et al.*, suggesting these amino acids/regions may be slightly more tolerant to conserved mutation (for example may simply require the positive charge) as compared to the extreme sensitivity of the 894 residue which may strictly require a lysine. In addition, Phan *et al.* also selected for compensatory mutations to both K894A and K894E. In both cases

reversions to WT were observed, although for K894A an E1 mutation was also selected which was then able to suppress the assembly defect, suggesting an interaction between E1-E2 and this region of NS2 is important for some aspect of viral assembly.

It is difficult at this time to explain the mechanism behind the defects caused by NS2 mutations as not enough is known about NS2 function in assembly and most of the information available concerning protein-protein interactions is of a genetic nature. Several mutagenesis studies have been performed with NS2 in the past year or so, several of which also included the selection of compensatory mutations [146, 205-207]. There is therefore a very long list of mutations in NS2 that either do not affect infectivity, inhibit it, or even increase it. Furthermore, NS2 has been shown to genetically interact with most other HCV proteins [147-148, 175, 205, 207] but biochemical significance and evidence of these interactions during viral assembly is largely lacking. While specific co-immunoprecipitation experiments involving these proteins are difficult due to membrane association and the likely formation of large complexes, recent evidence for co-localization of NS2 with E2 and NS5A (but not with core) was reported [205], which is a first step in providing the tools necessary to determine whether any of the identified mutations affect localisation of NS2 and/or its co-localisation with any of these proteins.

NS2 was proposed to act at a maturation step after initial particle formation but prior to formation of infectious intracellular virus [205], however, until we know more about the precise functions of NS2 in assembly it is difficult to assign which regions of the protein are important for them. Importantly, our mutational results confirm the accumulating evidence for an essential role for NS2 in viral infectivity. Figuring out the steps and protein-protein interactions involved in these processes will be important not only in order to understand the molecular mechanisms of the viral life cycle, but knowledge of the regions of NS2 involved in these various protein-protein interactions could provide

additional targets for the identification and design of novel therapeutics against the virus.

4.6 CONCLUSION

The work presented in this thesis contributes important findings to the understanding of the virology of HCV by further characterising the NS2/3 and NS2 proteins and their important roles in the viral life cycle. We have demonstrated the critical importance of NS2/3 protease activity for genome replication and investigated the possible reasons for this. We have also investigated the rapid NS2 degradation we observed in RNA replication systems and elucidated the importance of several amino acids of this protein for viral infectivity. However, determination of the full implications of our findings will require even further research and understanding of the virus. Work on the NS2 protein, in particular its role in viral assembly and infectivity, has recently become a major focus in the field of HCV research. It will therefore be exciting in the future to uncover just how the results presented here, in particular those involving NS2 degradation and the residues of this protein involved in infectivity, fit into, and may help characterise, the rapidly emerging molecular functions of NS2.

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ORIGINAL CONTRIBUTIONS TO KNOWLEDGE

1) Demonstrated NS2/3 protease activity is required for HCV genome replication.
2) Demonstrated that NS3 helicase and ATPase activities are not affected by the presence of NS2 *in vitro*.
3) Showed uncleaved NS2/3 binds NS4A less efficiently than NS3 *in vitro*.
4) Showed inactivated uncleaved NS2/3 to be rapidly degraded in cell systems in a proteasome dependent manner.
5) Showed that lysine mutation of NS2 can stablilize mutant uncleaved NS2/3 but that increased levels of this protein are unable to rescue replication of NS2/3 protease inactive replicons, demonstrating that NS2/3 cannot functionally perform cleved NS3's role in genome replication.
6) Demonstrated that genotype 1b con1 NS2 has a short half life when expressed in RNA replicon systems, which suggests its levels could be important for the viral life cycle.
7) Demonstrated that genotype 1b NS2 is degraded by what appears to be a ubiquitin and proteasome independent mechanism.
8) Showed that using DNA constructs, NS2 degradation is conserved amongst several genotypes. However, the regulation and mechanisms of this degradation may be genotype specific in replication systems. Unlike genotype 1a previously shown to require S168 for degradation, we showed that degradation of genotype 1b NS2 is not dependent on this amino acid.
9) Identified JFH-1 NS2 residues K894 and K1028 that impair or increase the kinetics of HCV viral assembly, respectively, in a cell culture infection system.