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Hepatitis C virus intracellular host interactions

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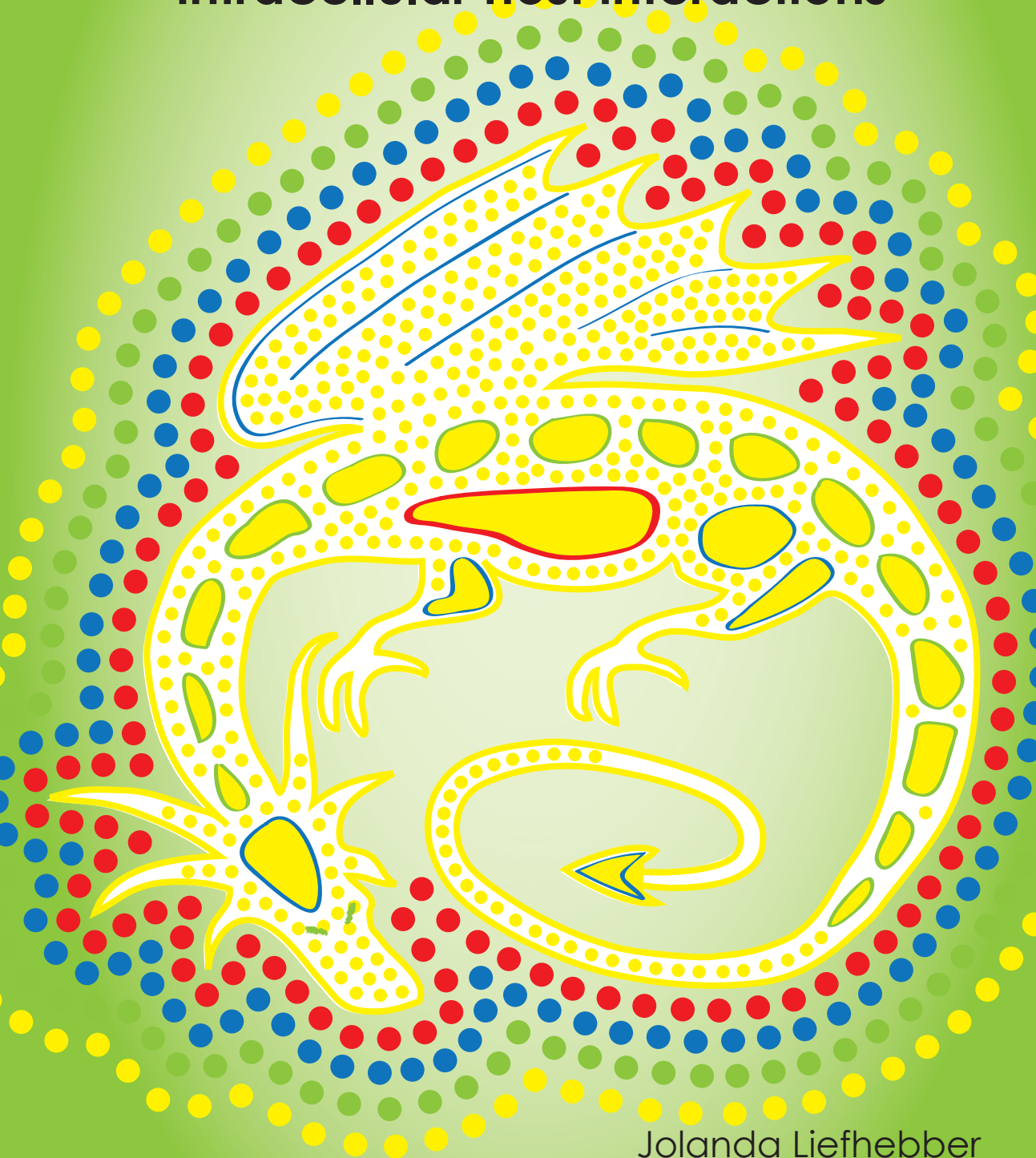
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Hepatitis C virus intracellular host interactions



Jolanda Liefhebber

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Hepatitis C virus intracellular host interactions

Proefschrift

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Voor mijn ouders

*“Wanneer je verstandig bent, vermeng je het ene ding met het andere:
Hoop niet zonder twijfel en twijfel niet zonder hoop.”*

- Lucius Annaeus Seneca

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General introduction



An inflammation of the liver is called hepatitis, which can be the effect of certain chemicals, medication, alcohol abuse or a viral infection. The main cause of viral hepatitis was identified in 1989 as Hepatitis C virus (HCV). Hepatitis C was originally known as non-A non-B hepatitis ¹. This thesis will concentrate on HCV. In the two decades following discovery, researchers have acquired quite some knowledge on the virus. How HCV is transmitted, the sequence of the viral genome and what it encodes, and the virus life cycle is starting to be unravelled. This resulted in the identification of several new drug targets. Still, much remains to be elucidated about the virus life cycle, including intracellular interactions of HCV-proteins with the host cell.

Transmission, clinical course of infection and current treatment

Transmission of HCV is through exposure to infected blood. Healthcare related procedures are therefore a risk factor. To decrease healthcare-related infections, blood and blood-products have been screened for HCV since 1991. Presently the main risk factor in developed countries is intravenous drug usage, due to sharing of injecting equipment ^{2,3}. Additional risk factors include mother-to-child transmission, high-risk sexual behaviour and perhaps tattooing and body piercing ².

When infected with HCV, three stages are described in the clinical course of infection. First, there is the acute phase. In about 20-50% of the cases the infection is cleared, depending on virus genotype and genetic background of an infected individual ⁴. For example, a genetic variation in the gene IL28b is strongly associated with spontaneous clearance of HCV ⁵. After 6 months, the infection is called a chronic infection and fibrosis will continue. Fibrosis is a process of regeneration of damaged liver tissue, through proliferation of connective tissue and rearrangement of extra cellular matrix components, which can result in progressive loss of liver function. Hepatic stellate cells play a major role in fibrinogenesis. As a result of inflammation and cell damage these cells are activated, ultimately leading to more production of extracellular matrix components ⁶. In this way, the chronic infection can slowly progress (20-30 years) toward end stage liver disease, the third stage. Of the chronically infected people, 10-20% develop liver cirrhosis (scar tissue development) and of those 75% are diagnosed with severe cirrhosis and hepatocellular carcinoma (HCC). In

patients with HCV-related cirrhosis the annual incidence rate of HCC is 1-4%⁷. These percentages do not indicate a major healthcare problem. Yet, the World Health Organisation estimates that world-wide around 170 million people are infected with HCV, of whom 130 million are chronic HCV carriers. Each year around 2.3 to 4.7 million people become newly infected⁸. As a result HCV infection is currently the leading indication for liver transplantation⁹.

The present standard treatment of chronic HCV infection is a combination therapy of PEGylated interferon alpha and ribavirin. However viral eradication is only reached in 40-80% of the cases and side effects of the treatment are grounds for 10-15% of patients to discontinue the treatment⁷. New medication is therefore being developed, their targets being the viral enzymes and host-virus interactions¹⁰. In general, clinical trial phases are difficult to reach. The anti-viral drug development against HCV faces another challenge; that is the rapid evolution of the virus, resulting in quick emergence of resistance to the newly developed drugs. This rapid evolution of the virus is a consequence of high genetic diversity of the virus, an error prone polymerase (high mutation rate) and a high magnitude of replication¹¹⁻¹⁴. Hence, a better understanding of the viral proteins and the HCV lifecycle is needed.

Viral phylogenetics

HCV is a single-stranded positive-sense RNA virus. It belongs to the family of *Flaviviridae*, which includes the Flavi-, Pesti- and Hepacivirus genera¹⁵. Well-known members of the Flavivirus genus are Yellow Fever, Dengue and West Nile virus. The genus pestivirus contains viruses infecting non-human mammals and includes bovine viral diarrhoea virus that is the closest relative of Hepaciviruses^{15,16}. HCV is a member of the Hepacivirus genus, which is classified into 6 major genotypes (1-6) and related subtypes (a, b, c, ...) ^{17,18}. The three most prevalent subtypes among Dutch blood-donors, detected during screening, are 1a, 1b and 3a. Of these, type 1b is associated with blood transfusions in the past and 1a and 3a are associated to intravenous drug usage¹⁹.

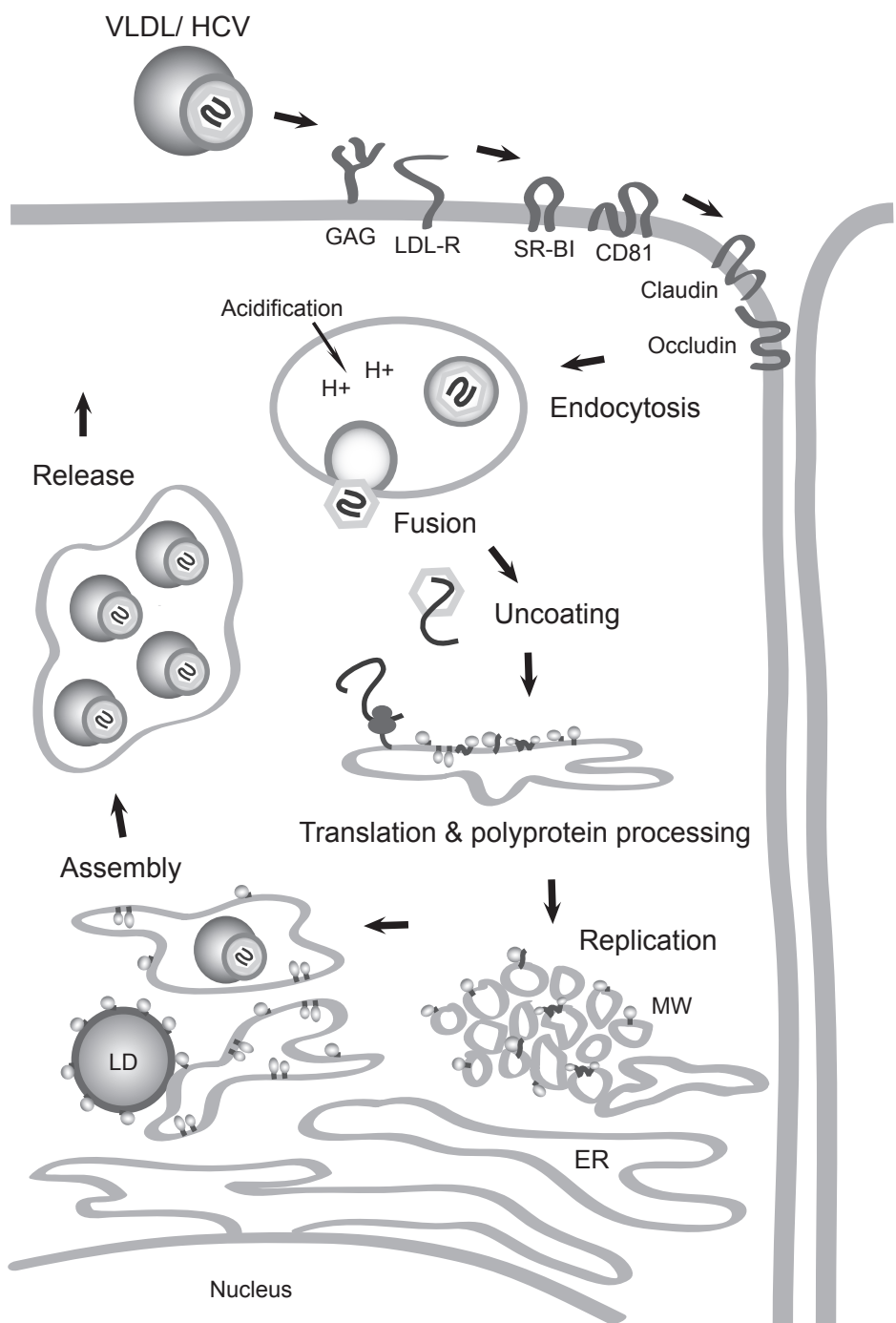


Figure 1 - The Hepatitis C virus life cycle

This figure illustrates the life cycle of hepatitis C virus. Abbreviations: very low density lipoprotein (VLDL), hepatitis C virus (HCV), glycosaminoglycans (GAG), low density lipoprotein receptor (LDL-R), scavenger receptor class B type I (SR-BI), membranous web (MW), endoplasmic reticulum (ER), lipid droplet (LD). For more details, see main text.

The viral life cycle

The host range of HCV are humans and closely related primates. HCV mainly infects hepatocytes^{20, 21}. The viral life cycle includes several steps, which are depicted in Figure 1.

Firstly, the virus life cycle starts with entry of the virus particle into the host cell. After that, the viral genome, of about 9.6 kb, is released from the virion via membrane fusion and uncoating (Fig.1). This is followed by translation of the viral genome using the host cell machinery, producing one large polyprotein of about 3000 amino acids at the endoplasmic reticulum (ER) membrane. The polyprotein is cleaved by viral and host proteases into at least 10 proteins. The genomic order of the proteins being: Core, E1, E2, p7, NS2, NS3, NS4a, NS4b, NS5a and NS5b (Fig.2). The next step is viral genome synthesis, called replication, which occurs on ER derived membranes (Fig.1). The ready produced genome is packaged into a new virion, a process associated with lipid droplets (Fig.1). Subsequent to virus particle assembly, the virion is released from the cell and can infect new hepatocytes (Fig.1).

These steps will be discussed in more detail in the next paragraphs regarding virus-host interactions including protein-protein interactions, membrane-protein interactions and modifications of viral proteins by the host-machinery.

Entry

Virus entry is the process that comprises all the steps from particle binding to the cell surface up to the delivery of the viral genome to the site of translation within the cell (Fig.1). Virus-host interactions herein will be described in this section.

Glycoproteins E1 and E2

The HCV particle has a size of about 50-60 nm²² and comprises the viral genome, a capsid containing Core proteins and a host cell-derived membrane envelope²³. In the envelope the heterodimer complex of the two glycoproteins E1 and E2 is present, which is essential for virus entry (Reviewed in^{24, 25}). The glycoproteins are anchored

in the membrane envelope by transmembrane domains at the carboxy-terminal end of E1 and E2 ²⁶⁻²⁹. Via binding to host-cell receptors, this complex mediates virus particle internalisation (Reviewed in ²⁴).

Binding of the virus particle to the cell surface

Initial virus attachment involves low affinity binding to receptors. For HCV, the receptors low density lipoprotein receptor (LDL-R), C-type lectins and glycosaminoglycans (GAGs) were shown to mediate this initial binding ³⁰⁻³³ (Fig.1). Proteoglycans, supramolecular complexes of 'core' proteins with covalently attached GAG chains, are ubiquitously expressed on the cell surface and for that reason may be the primary docking site for many viruses, including several of the *Flaviviridae* members, Dengue virus ³⁴, classical swine fever virus ³⁵ and tick-borne encephalitis virus ³⁶. Heparin, a highly sulphated linear polysaccharide, is a GAG that was shown to associate with E1 and E2 of HCV ³¹. The negatively charged GAG is proposed to bind to positive-charged residues in the glycoproteins ^{31, 37-39}

The envelope glycoproteins of HCV are highly glycosylated, up to 6 potential glycosylation sites are present in E1 and up to 11 sites in E2 ⁴⁰. Since C-type lectins contain a calcium-dependent carbohydrate recognition domain, these receptors could bind to virus carbohydrates (Reviewed in ⁴¹). DC-SIGN and L-SIGN, two C-type lectins, both interact with soluble E2 and with E1/E2 heterodimers on HCV pseudoparticles ^{32, 33}. The high mannose-type oligosaccharides on E1 and E2 are necessary for this binding ^{32, 33}. Expression of these C-type lectins on non-permissive cells, such as dendritic cells, lymphocytes and liver sinusoidal endothelial cells, suggests that they may act as capture receptors to deliver the virus particles to permissive cells ^{32, 42, 43}. In addition, a liver-expressed C-type lectin, asialoglycoprotein receptor, was identified as a potential attachment factor ⁴⁴, although its relevance in entry needs to be demonstrated.

Another receptor that can initiate virus particle binding to the host cell surface is the LDL-R, which is a receptor for the uptake of high, low and very low density lipoproteins (HDL, LDL and VLDL) ³⁰. It may seem surprising that this receptor takes part in the entry process, however, infectious in the serum circulating HCV is mainly associated with beta-lipoproteins ^{30, 45} (Fig.1). Moreover, highly infectious Lipo-Viro-Particles (LVPs) isolated from serum contain, in addition to the viral capsid and the envelope glycoproteins, triglyceride-rich lipoproteins including ApoB

and ApoE^{46, 47}. No direct interaction between LDL-R and HCV glycoproteins has been reported; instead the binding of the LDL-R to ApoE seems to mediate entry⁴⁸. As HCV particles are associated with lipoproteins, the LDL-R was proposed to be an initial binding factor^{30, 48-50}.

Essential entry co-factors

A different lipoprotein receptor is scavenger receptor class B type I (SR-BI), which was also suggested to be involved in HCV uptake (Fig.1). SR-BI is able to interact with E2⁵¹⁻⁵³. However, another study shows that HCV from sera of infected individuals attaches to SR-BI-expressing cells in an ApoB-dependent and E2-independent manner⁵⁴. Taken together, binding of the virus particle to SR-BI can potentially occur via direct or indirect interactions. In any case, blocking this scavenger receptor with antibodies reduces HCV entry into hepatoma cells, defining SR-BI as an essential entry co-factor⁵⁴⁻⁵⁶. In fact, SR-BI expression levels might be rate limiting for HCV uptake^{52, 57, 58}, because overexpression of SR-BI enhances internalisation of HCV^{52, 58}. It is noteworthy that alpha-interferon, in addition to other effects, decreases expression of SR-BI and LDL-R at the cell surface⁵⁹.

During HCV entry, SR-BI functions cooperatively with CD81^{56, 60}, a tetraspanin protein shown to bind directly to E2⁶¹⁻⁶³ (Fig.1). Moreover, SR-BI and CD81 can be indirectly linked to each other via soluble E2 or HCV pseudo particles containing E1/E2 heterodimers⁶⁴, pointing out dual binding to E2. CD81 is suggested to be a post-attachment co-receptor^{65, 66}, because HCV particles can attach to CHO cells expressing SR-BI, but not to CD81 expressing cells⁶⁶. Additionally, antibodies against CD81 inhibit virus entry at a post-binding step⁶⁵. After association with CD81, actin rearrangements are induced and the virus-receptor complex moves to the tight junctions, which are closely associated areas between cells forming a barrier to fluids in the extracellular space⁶⁷.

Expression of both SR-BI and CD81 is required, but is not sufficient for HCV entry. This observation initiated a quest for other co-receptors⁵¹. A lentivirus based repackaging screening was set up to identify genes that render HCV-resistant cells susceptible to HCV pseudoparticle infection, using a complementary DNA library derived from HCV permissive cells. With this approach two new co-receptors were identified that localise to the tight junctions: Claudin-1 and Occludin^{57, 66} (Fig.1).

Claudins and Occludins play a role in the gate function of tight junctions, establishing a barrier to the apical-basolateral movement of ions and solutes through the intracellular space^{68, 69}. Co-immunoprecipitation experiments indicate Occludin interacts with E2^{70, 71}. Interestingly, CD81 and Occludin are both able to bind directly to E2 and were shown to determine HCV species-specific tropism between human and mouse cells^{57, 72}. Claudins comprise four transmembrane helices and have two extra-cellular loops that are involved in paracellular tightening and pore formation⁶⁹. The first and larger extra cellular loop of Claudin-1 was shown to be essential for HCV internalisation⁶⁶. In fact, the necessary motif in the extracellular loop was suggested to play a role in cell-cell contact⁷³, which might indicate a functional requirement of Claudin-1. No direct interaction with E2 could be observed, though Claudin-1 interacts with CD81, implying co-receptor complex formation^{66, 74, 75}. Together these four receptors seem to be the crucial entry co-factors for virus particle entry at the cell surface.

Endocytosis, fusion and uncoating

The next step in entry is endocytosis of the virus particle, which occurs in a clathrin-dependent manner⁷⁶ and requires an intact microtubule network^{77, 78}. The internalised virus particle is transported to the early endosome⁷⁷. At present, there is no data on whether one of the entry co-receptors is present in the endosome together with the virion. In the maturing endosome the pH will drop, which initiates fusion of the virus particle membrane with the early endosomal membrane^{79, 80} (Fig.1). The exact mechanism of fusion is unknown, but it entails close contact of the two membranes to merge into one membrane.

The envelope glycoproteins play a crucial role in fusion because they are presumed to be able to associate with both membranes directly. E1 and E2 are anchored into the membrane envelope^{29, 81}. Additionally, a still unidentified surface exposed hydrophobic peptide in the glycoproteins, the fusion peptide, probably makes contact with the early endosomal membrane. This fusion peptide destabilizes the membrane by insertion into the lipid bilayer and might prime fusion (Reviewed in⁸²). Several studies indicate hydrophobic peptides in E1 and E2 that could be the fusion peptide, though no consensus has been established⁸³⁻⁸⁶.

The low pH in the endosome seems to induce conformational changes in the glycoproteins because acidification results in exposure of new E2 epitopes²⁹. These

could be the fusion peptides, or otherwise the altered fold could bring the two membranes close together. Besides acidification, another trigger is likely needed to prime the envelope proteins for fusion as HCV infectivity is not affected when cell surface bound particles are temporarily exposed to low pH^{87, 88}. Furthermore, lipid composition of the virion and of the target membrane seems to play a critical role in fusion because cholesterol in the target membrane enhances the process^{56, 80}. Additionally, internalisation, but not membrane attachment, of the virion is affected when cholesterol and sphingomyelin composition is changed in the virus particle^{89, 90}.

After membrane fusion, the nucleocapsid containing the HCV genome is released into the cytosol of the host-cell and the viral genome has to be delivered to the ER where, subsequent to uncoating, translation is initiated (Reviewed in^{25, 91}) (Fig.1). A recent report demonstrates that treadmilling microtubules are involved in a step after nucleocapsid release into the cytoplasm⁷⁸. Additionally, the HCV Core protein was shown to interact with the alpha- and beta-chains of tubulin⁷⁸. Taken together this implies that the HCV genome is transported to the ER via the interaction of Core with treadmilling microtubules.

Uncoating of the viral genome requires the disassembly of the nucleocapsid. Core is incorporated into the capsid after being proteolytically cleaved by signal peptide peptidases⁹². This mature form of Core forms less stable viral capsids, which does not affect virus assembly, though might prepare the capsid to disintegrate in the newly infected cells⁹³.

Translation of the polyprotein

After uncoating, the viral genome is exposed in the cytosol, where it can be captured by the translation machinery of the host cell and serve as a template for translation (Fig.1).

Viral translation initiation

The open reading frame in the viral genome is flanked by highly conserved 5'- and 3'-non-translated region (NTR), which contain signals for translation and replication⁹⁴ (Fig.2). In the 5'NTR an internal ribosome entry site (IRES) is found, which is a highly structured RNA domain of multiple stem-loops and a pseudoknot that initiates cap-

independent translation⁹⁵⁻⁹⁸. This IRES can be directly bound by the 40S ribosomal subunit, devoid of other translation initiation factors⁹⁹⁻¹⁰¹. The 40S ribosomal subunit is positioned onto the AUG at the 'P' site in such a way that translation can start directly, without scanning or unwinding of the RNA¹⁰². Subsequently, the 43S pre-initiation complex is formed by recruitment of the eukaryotic initiation factor 3 (eIF3) and eIF2-GTP-initiator tRNA complex^{100, 103}. Finally, the 60S subunit can join this complex after GTP hydrolysis and eIF2 release, to form the 80S ribosome that initiates translation¹⁰⁴.

In addition to the 5'NTR, the 3'NTR is also important for translation. The three regions described in the 3'NTR are the variable region, the poly (U/UC) tract and the 98 nucleotide X-region^{105, 106}. The 3'NTR seems to enhance translation of the viral genome^{107, 108}. 3'NTR RNA-binding proteins, such as Insulin-like growth factor-II mRNA-binding protein 1 (IGF2BP1) and polypyrimidine tract-binding protein (PTB) could be involved in increasing translation efficiency. PTB binds to itself and can associate with the X-region in the 3'NTR and three sites in the 5'NTR¹⁰⁹⁻¹¹¹. In this way circulation of the genome can be mediated, which could enhance translation similar to other flaviviruses¹¹². A comparable mechanism is proposed for IGF2BP1¹¹³. Additionally, IGF2BP1 is co-immunoprecipitated with HCV RNA, 40S subunit and eIF3, implicating recruitment of eIF3 to the 5'NTR¹¹³. Many proteins have been shown to associate with the 5'NTR. For example La autoantigen that binds close to the AUG start sequence, possibly promoting binding of the 40S ribosomal subunit to the IRES¹¹⁴.

Polyprotein cleavage

During translation, one large polyprotein is synthesised that is processed co- and post-translationally into at least 10 proteins (Reviewed in¹¹⁵) (Fig.2). Host endoplasmic reticulum signal peptidase(s) release the structural proteins, core, E1 and E2, and the peptide p7 from the polyprotein (Reviewed in¹¹⁶) (Fig.2). In the C-terminus of core protein a signal sequence is present, therefore additional processing of core is mediated by a signal peptide peptidase⁹² (Fig.2). The non-structural proteins are separated by the viral proteases NS2-3 and NS3-4a (Reviewed in¹¹⁶) (Fig.2). The cysteine proteinase NS2-3 forms a dimer with two composite active sites, which autocleaves at the NS2/ NS3 junction¹¹⁷⁻¹¹⁹. Folding of this protease could be facilitated by the heat shock protein 90 that associates with NS2-3¹²⁰.

After NS2/ NS3 processing, the other non-structural proteins NS3 to NS5b are cleaved off by the NS3-4a serine protease, where NS4a functions as a co-factor to the NS3 protease¹²¹⁻¹²⁵. Binding of NS4a to NS3 stabilizes the protease¹²⁶. Processing of NS3 to NS5b occurs in the order of NS3/ NS4a, NS5a/ NS5b, NS4a/ NS4b and NS4b/ NS5a^{121, 127}. Cleavage at the NS4b/ NS5a might be facilitated by the interaction of NS4a with the NS4b/ NS5a polypeptide¹²⁸.

Membrane association of the HCV proteins

Since the HCV proteins originate from one polypeptide, their membrane topologies are related to each other. Additionally, each HCV protein contains intrinsic properties to determine their association with the lipid bilayer, mainly the membranes of the ER (Fig.2). This is discussed in the section below.

Translation starts with the sequence of core. The capsid protein is observed at the ER and later in infection also on the surface of lipid droplets^{92, 129, 130}. Core is suggested to initially localise at one point of the lipid droplet, possibly an ER-lipid droplet contact site, and is subsequently loaded onto the lipid droplet¹³¹. This re-localisation might be regulated by the cleavage of the signal sequence at the C-terminal end of core^{92, 132}. A domain essential for ER membrane association and targeting to lipid droplets, termed D2, was identified in the C-terminal one-third of core. This domain seems to fold upon interaction with the phospholipids into two amphipathic helices that are in plane with the membrane¹³³⁻¹³⁵ (Fig.2). Just behind the D2 domain a palmitoylation site was identified. This seems to be involved in the localisation of core to ER membranes that are closely associated with lipid droplets¹³⁶. A second function of the signal sequence at the end of core is directing the growing polypeptide to the rough ER²³. Via recognition of the signal sequence in core and binding to the ribosome by the signal recognition particle (SRP), the ribosomal complex is recruited to an SRP-receptor in the ER membrane. This is followed by docking of the ribosome with the nascent chain onto the translocon (Reviewed in¹³⁷). Now E1 can be translocated into the ER.

E1 and E2 contain a C-terminal transmembrane domain, which anchor and retain the proteins in the ER membrane^{26, 27} (Fig.2). Both transmembrane domains contain two hydrophobic stretches separated by charged residues in a short hydrophilic segment¹³⁸. Work from Cocquerel et al. shows that, before signal peptide cleavage, the

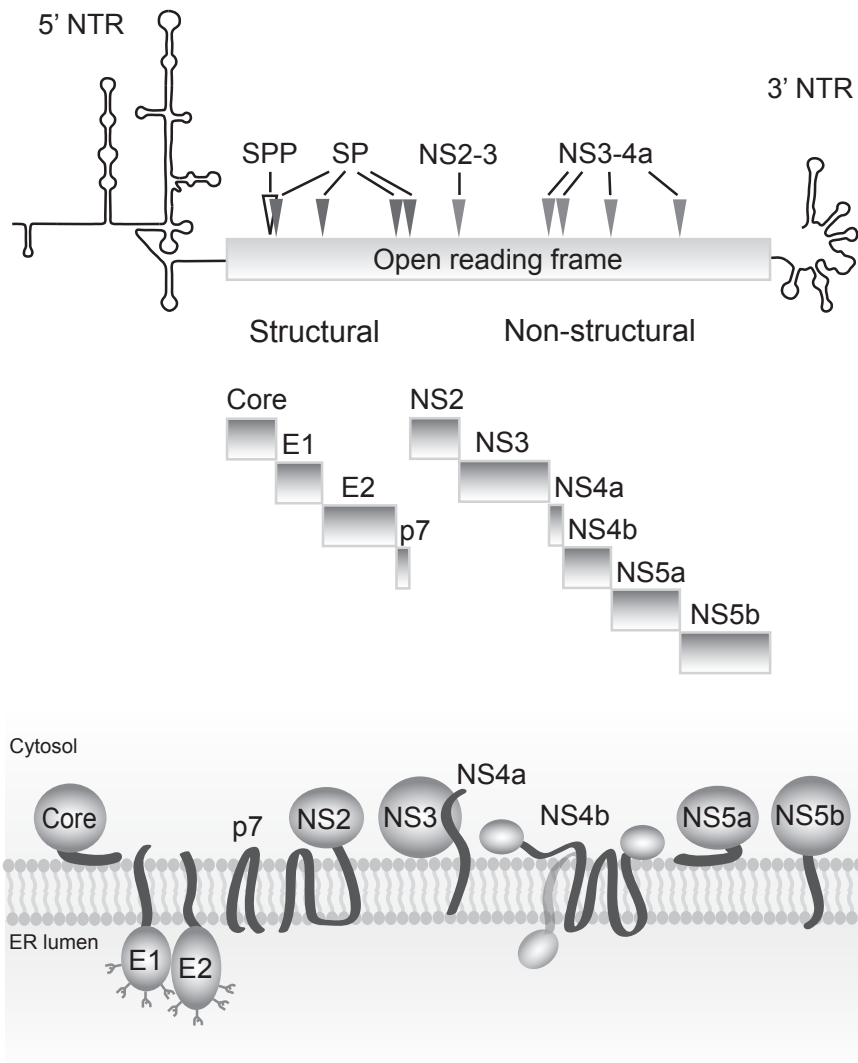


Figure 2 - Hepatitis C virus genome organisation and membrane association of the proteins

The hepatitis C virus open reading frame is flanked by the 5' and 3' non-translated regions (NTR). Translation results in one large polyprotein that is cleaved by signal peptide peptidase (SPP), signal peptidase (SP), non-structural protein 2-3 protease (NS2-3) and NS3-4a protease (NS3-4a) into 10 proteins. These can be roughly divided into structural proteins Core, envelope protein 1 and 2 (E1 and E2), peptide p7 and non-structural proteins (NS) 2, 3, 4a, 4b, 5a and 5b. In the bottom panel the HCV proteins are schematically drawn, to illustrate their interactions with the endoplasmic reticulum (ER) membrane.

transmembrane domains form a hairpin like structure with their C-terminus facing the ER lumen. Upon cleavage the transmembrane domains form a single membrane-spanning segment, with the C-termini on the cytosolic side ¹³⁹. Correct folding of the E1E2 heterodimer depends on the transmembrane domains ^{28, 140, 141}. E1 and E2 are modified by glycosylation and possess up to 6 and 11 potential N-glycosylation sites respectively ^{40, 142}. Several of these glycosylations contribute to the folding of the glycoprotein complex and receptor binding ^{40, 143, 144}. Furthermore, ER chaperone proteins facilitate the assembly of the E1-E2 complex. Calnexin is such a protein and has been shown to interact with the HCV envelope proteins ¹⁴⁵⁻¹⁴⁷.

P7 is a polypeptide of two transmembrane domains connected by a short cytoplasmic loop and is mainly localised at the ER and partially at the plasma membrane ¹⁴⁸. The N- and C-termini are towards the ER lumen ¹⁴⁸ (Fig.2). Six p7 proteins fold into an ion-channel structure that resembles a flower, which is implicated in virus assembly and release ¹⁴⁹⁻¹⁵².

The first non-structural protein to be translated is NS2, which also localises at the ER ¹⁵³. Since the C-terminus of p7 is at the ER luminal side of the membrane, where cleavage by the signal peptidase takes place, the N-terminus of NS2 seems to be in the lumen of the ER ¹⁵⁴ (Fig.2). The NS2/ NS3 junction is processed in the cytosol, where the NS3-4a protease resides, which would leave the C-terminus of NS2 at the cytosolic side of the ER membrane ^{117, 118} (Fig.2). However, in the N-terminal 96 residues of NS2, three to four transmembrane segments have been predicted ¹⁵⁵. Unpublished, data from Jirasko et al. suggest this region contains three transmembrane segments and an alpha-helix associating with the membrane leaflet at the ER luminal side ¹⁵⁶ (Fig.2). Instead, if this hydrophobic segment would span the membrane four times, post-cleavage reorientation is expected.

NS3 binds to the central domain of NS4a, which stabilizes the protease and through this interaction NS3 is associated to membranes ^{126, 157}. In the N-terminus of NS4a, a hydrophobic domain is present that connects the complex to membranes ¹⁵⁸ (Fig.2). This segment is inserted into the lipid bilayer, after cleavage of the NS3/ NS4a junction, with the N-terminus of NS4a towards the ER-lumen ¹⁵⁹. Via the interaction with NS4a, NS3 co-localises with the ER and partially with mitochondria ^{158, 160}. Another determinant for membrane association was identified in the N-terminus of

NS3, an amphipathic helix that was suggested to properly position the protease onto the lipid bilayer¹⁵⁹.

NS4b is a multi membrane-spanning protein and it traverses the membrane 4 to 5 times^{161, 162} (Fig.2). The N- and C-termini are, as a consequence of polyprotein processing, at the cytosolic side of the ER membrane¹⁶¹. Topology studies indicate four transmembrane domains in the central part of the protein (aa 70-190)^{161, 162}. In the N-terminal fraction of NS4b there is an amphipathic helix (aa 42-69) that also has the potential to translocate from the cytosolic side of the ER membrane into the lipid bilayer^{162, 163} (Fig.2). Only a fraction of the NS4b pool adopts this transmembrane orientation with their amphipathic helix, which seems to be influenced by the expression of NS5a^{163, 164}. Another amphipathic helix is found in the N-terminus (aa 6-29) that could mediate membrane association¹⁶⁵. Disruption of this helix alters the localisation of the other NS proteins¹⁶⁵. Furthermore, in the C-terminal part of NS4b (aa 191-261) two palmitoylation sites are suggested, which might be involved in protein-protein interactions or binding to membranes¹⁶⁶.

An amphipathic helix at the N-terminus of NS5a associates the protein to ER membranes and to lipid droplets^{167, 168} (Fig.2). The helix is embedded flat into the cytosolic leaflet of the membrane, with the hydrophobic residues facing the lipids and the charged residues towards the cytosol¹⁶⁹. This charged side might be involved in specific protein-protein interactions¹⁶⁹.

NS5b, an RNA-dependent RNA polymerase, resides at the cytosolic side of the ER and is anchored to the lipid bilayer through the last 21 amino acids of the protein^{170, 171} (Fig.2). These C-terminal residues of NS5b form a hydrophobic helix that is inserted into the lipid bilayer by a post-translational mechanism¹⁷⁰⁻¹⁷².

Replication

After translation of the polyprotein, replication can start, using the produced HCV proteins to synthesise new HCV RNA genomes (Fig.1).

From translation to viral replication

The positive-strand viral RNA genome is used as a template for both translation and replication. Moreover, translation occurs in the 5' to 3' direction, whereas RNA

negative-strand synthesis goes from the 3' to 5' end. Therefore, there should be a switch between the two processes. It is possible that proteins binding to the RNA molecule regulate this functional change. A viral candidate could be core, which inhibits translation upon association to the IRES¹⁷³⁻¹⁷⁵. Also host-cell proteins, involved in the regulation of replication and translation, could mediate the switch^{109, 113, 114, 176-181}. Alternatively, there might be physical separation of the two processes, since replication seems to be shielded by membranes, while translation is not¹⁸².

Membranous web

Replication of most, if not all, positive-strand RNA viruses occurs on intracellular membranes (Reviewed in¹⁸³). Immuno-electron microscopy experiments to determine the cellular localisation of HCV RNA revealed that the viral genome is located at specific membrane structures in the cytoplasm, which are also termed the membranous web¹⁸⁴ (Fig.1). These HCV-induced membrane alterations appear as small irregular vesicles that are closely packed together^{184, 185}. Furthermore, immunofluorescence analysis of newly synthesised RNA by bromouridine triphosphate labelling, indicated that replication takes place at cytoplasmic dot-like structures^{184, 186}. Both structures observed in electron and immunofluorescence microscopy contain HCV non-structural proteins that are involved in replication¹⁸⁴⁻¹⁸⁶. Together this points towards the membranous web being the site of replication.

NS4b presumably is the HCV protein that induces these membrane alterations, because expression of this protein alone resulted in similar structures¹⁸⁵. In addition to some connections with the rough ER observed in electron microscopy, the localisation of NS4b to this organelle led to the supposition that the membranous web is derived from ER membranes^{161, 185}. Not only ER proteins are observed at the site of replication, also early endosomal proteins such as rab5 and rab7 are found, indicating recruitment of specific proteins towards the site of replication^{187, 188}. Immunofluorescence experiments examining the diffusion of NS4b indicate two pools: ER associated and fast moving NS4b and slower moving NS4b, which is clustered in cytoplasmic foci¹⁸⁹. The latter could be the replicase, because they resemble the dot-like structures observed for HCV RNA^{184, 189}. The cytoplasmically localised HCV-related foci seem to be connected to the cytoskeleton of cells, which might contribute to the transport of these structures¹⁹⁰.

Replication complex

The exact components required for HCV replication are not known, but will entail the viral genome, non-structural proteins and cellular host-factors. The viral non-structural proteins 3 to 5b are required for replication and form part of the replication complex^{191, 192}. They possess specific enzymatic activities, interact with each other and associate with host-cell proteins to facilitate the synthesis of new copies of the HCV genome^{193, 194}. A quantitative analysis of the replication complex indicates that of the total non-structural protein made, about 5% is actively participating in replication¹⁸². Additionally, it was calculated that a replication complex consists of several hundreds of NS proteins, one negative-strand RNA and two to ten positive-strand RNAs¹⁸². The functions of the NS proteins and their host protein interactions in relation to the replication complex will be discussed here.

Besides protease activity, NS3 has RNA unwinding activity via its NTPase-RNA helicase domain¹⁹⁴. This DExH/D-box RNA helicase, at the C-terminus of NS3, is capable of unwinding dsRNA in the 3' to 5' direction^{195, 196}. While NS3 translocates along its substrate, ATP is utilised^{197, 198}. The NS4a protein acts as a cofactor, to promote binding of NS3 to the RNA substrate^{199, 200}. Also NS5b can interact with and modulate the helicase activity of NS3, depending on the stoichiometry of both proteins^{201, 202}. Additionally, there is a genetic interaction between NS3 and NS4b that is important for replication²⁰³. The host-cell protein, creatine kinase B (CKB), forms a complex with NS3-4a, via direct interaction with NS4a¹⁷⁷. CKB, an ATP-generating enzyme, is suggested to support replication by enhancing NS3-4a helicase activity, through energy production¹⁷⁷. Another possibility to regulate enzymes is via protein modifications. The activity of NS3 helicase might to be controlled by methylation²⁰⁴.

Quinkert et al. showed that active replication complexes of HCV are protected from proteinases and RNases by membranes¹⁸². As mentioned above, the membranous web is induced by NS4b¹⁸⁵, indicating a role for NS4b in shielding the replicase. Additionally, NS4b is suggested to be an important protein-protein interaction platform during replication, because NS4b is able to bind to the other non-structural proteins^{193, 205}. Moreover, recent data suggest that when NS4b loses the ability to form foci, NS3 and NS5a also display an altered localisation^{163, 206, 207}. The host cell early endosomal protein Rab5 can be co-immunoprecipitated together with NS4b. In addition, siRNA knockdown experiments point to a function in replication^{187, 188}.

The exact function of NS5a in replication is not known, but this zinc-binding phosphoprotein is indispensable for replication¹⁹². NS5a has been shown to interact with RNA, other HCV proteins and multiple host-cell proteins^{193, 208, 209}. Several of these NS5a host cell-interacting proteins are involved in membrane transport, such as TBC1D20, a Rab1 GTPase activating protein^{179, 210}. Depletion of TBC1D20 or Rab1 resulted in decreased viral replication^{179, 211}. Since Rab1 is important for ER to Golgi trafficking of COPII vesicles^{212, 213}, the interaction might affect the membrane-associated replicase. A second example is tubulin to which both NS5a and NS3 associate. This interaction may possibly influence replication complex localisation¹⁹⁰. Thirdly, NS5a has been shown to bind to the lipid kinase PI4KIII α ¹⁷⁶. SiRNA knockdown of PI4KIII α inhibits replication and seems to reduce HCV induced membrane alterations²¹⁴⁻²¹⁷. Taken together NS5a could, via host-cell interactions, assist in the formation of the replication complex.

The role of NS5b in replication is obvious, because it is the RNA-dependent RNA polymerase (RdRp), a key enzyme responsible for RNA synthesis²¹⁸⁻²²⁰. Crystal structures show that NS5b folds into the classical right-hand tertiary-structure with fingers, palm and thumb domains²²¹⁻²²³. RNA can bind in a groove in the centre of the protein, in between the fingers, palm and thumb domains²²¹⁻²²³. The RdRp can initiate RNA synthesis *de novo*, meaning the enzyme can start without the need for a primer^{224, 225}. The activity of NS5b seems to be regulated by viral and host-cell proteins. NS3, NS4b and NS5b interact with one another^{201, 205}. *In vitro* experiments indicate that NS3 could stimulate NS5b template recognition, whereas NS4b seems to inhibit the NS3-NS5b complex, suggesting a balanced interaction to control replication²⁰⁵. Both cyclophilin A and B are able to bind to NS5b and facilitate viral replication^{180, 181}. For cyclophilin B a possible stimulatory mechanism has been suggested, which would be increasing the binding of NS5b to RNA^{180, 226}. The isomerase or chaperone activity of cyclophilin A might assist in proper incorporation of NS5b into the replication complex^{227, 228}. A different kind of host interaction with NS5b is that of protein kinase C related kinase 2 (PRK2), which is proposed to increase replication via NS5b phosphorylation^{178, 229}.

Virus particle assembly and release

After copying of the HCV genome, the newly synthesised RNA has to be packaged into the virions, which is followed by secretion of the particles (Fig.1).

From replication to assembly

In the switch from viral replication to virus particle production, NS5a has been suggested to play a major role^{230, 231}. NS5a has a basal- and a hyperphosphorylation level that seem to correlate with replication and assembly respectively because blockage of hyperphosphorylation stimulates replication²³²⁻²³⁴, but hampers virus production^{235, 236}. Therefore, the change from RNA synthesis to assembly is apparently regulated by the phosphorylation status of this non-structural protein. A consequence of NS5a phosphorylation was investigated by Evans *et al.*²³³. NS5a and also NS5b interact with VAP-A and -B, two proteins that function in membrane trafficking, lipid transport and microtubule stabilization²³⁷⁻²³⁹. Moreover these proteins are required for viral replication^{237, 240, 241}. Upon phosphorylation of NS5a the interaction with VAP-A seems to be impaired, which results in decreased replication activity²³³. Therefore, the regulatory role of NS5a in the virus life cycle might be through specific interactions that are modulated by phosphorylation. Hyperphosphorylation of NS5a is influenced by the other HCV proteins NS3, NS4a and also NS4b^{242, 243}.

Lipid droplets

Virus particle assembly is closely linked to lipid droplets in the cytosol of the cells²⁴⁴ (Fig.1). Two viral proteins, Core and NS5a are connected with lipid droplets, via amphipathic helices in the C- and N-terminal domains, respectively^{244, 245}. Additionally, their association with lipid droplets is required for virus particle production^{131, 244, 246}. Core induces lipid droplet aggregation close to the nucleus, which might increase ER-lipid droplet interactions and enhance virus production²⁴⁷. Redistribution of lipid droplets is dependent on an intact microtubule network and dynein, a motor protein²⁴⁷. Furthermore, plus and minus strand HCV RNA was shown to closely associate with lipid droplets, with around 5% of HCV RNA co-purifying with lipid droplets²⁴⁴. Together, these data suggest that assembly of virions is linked to lipid droplets.

Lipid droplets are involved in VLDL production, an important function of hepatocytes. Apolipoprotein E, DGAT-1 (diacylglycerol acyltransferase), MTP (microsomal transfer protein) contribute to VLDL synthesis (Reviewed in²⁴⁸). When these proteins are reduced or inhibited in HCV infected cells, virus production is hampered²⁴⁹⁻²⁵¹. Given that virus particles are indicated to associate with apolipoproteins^{30, 45}, it suggests that assembly and release proceeds co-operatively with VLDL production.

Functions of HCV proteins in assembly and release

All HCV proteins, except NS5b, seem to play a role in the assembly and/ or release of virus particles. The structural proteins core, E1 and E2 are part of the virions. The other HCV proteins seem to be accessory proteins, because mutations in p7, NS2, NS3, NS4b and NS5a influence infectious virus production^{150, 230, 236, 252-256}. Firstly, p7 exhibits ion-channel activity that is essential for assembly and release^{149, 150, 152}. The NS2 protease domain, but not its ability to cleave, is critical^{150, 252}. A mutation in the NS3 helicase domain, which does not affect its activity, reveals a function for NS3 in the assembly of virus particles²⁵⁴. Interestingly, these data imply that both the NS2 protease and the NS3 helicase domains carry out a second function. Another mutational study suggests a regulatory function of NS4b in virus production²⁵³. Lastly, NS5a together with Core seem to play a crucial part in initiating genome packaging and nucleocapsid assembly. Serines in the last 20 amino acids of NS5a are required for an interaction with Core²⁵⁷. Moreover, mutagenesis of these serines is deleterious for virus particle release, indicating their importance of the interplay between the two proteins²⁵⁷. This protein-protein interaction might facilitate transfer of the viral RNA from the site of replication (where NS5a participates in replication) to the lipid droplets (where core is involved in lipid droplets redistribution)^{244, 247}. Moreover, in the presence of Core the viral RNA and other non-structural proteins are observed on lipid droplets, though in the absence this localisation fails²⁴⁴. Again the phosphorylation of NS5a might determine the association with Core²⁵⁷. One more interaction partner of NS5a is apolipoprotein E, indicating an additional connection between synthesis and virus particle assembly^{258, 259}.

Outline of thesis

In order for a virus to generate progeny, it needs components of the host cell. Therefore, interactions between the virus and host are essential. This relation between the virus and the host cell is clearly illustrated in the introduction, Chapter 1. The objective of the work described in this thesis was to study virus-host interactions, at the level of protein-membrane association, protein-protein interactions and viral-protein modifications.

To study protein-membrane association, the NS4b protein was investigated. In the HCV proteome, this protein is required for replication and is essential for reorganisation of the host membranes, inducing the membranous web. NS4b interacts with the lipid bilayer in several ways. Chapter 2 addresses the membrane association of the carboxy-terminal domain of NS4b.

Most viral proteins have more than one task in the virus life cycle and need to be tightly regulated. NS3 is a multifunctional protein as outlined in the Introduction and little is known about the regulation of its functions. We therefore set up experiments to examine the protein modifications of NS3. These studies are presented in Chapter 3.

The third subject we focused on, are protein-protein interactions between the virus protein NS3 and the host cell. Chapter 4 describes experiments to purify NS3 and interacting proteins in the context of replication. Several methods were explored and we identified two new NS3 interacting proteins. One of the interacting proteins is GLT25D1 and little was known about its cellular localisation. Since this is required to be able to examine the NS3-GLT25D1 interaction, we investigated the protein characteristics of GLT25D1, which is shown in Chapter 5. Subsequent studies on the interaction between NS3 and GLT25D1 are reported in Chapter 6.

All these interactions of the virus with the host cell are further reviewed in Chapter 7. The topics that will be discussed are the interactions of NS4b with the membrane, NS3 protein modifications, NS3 interactome studies and the implications of the newly discovered NS3 interacting proteins. Ultimately, this thesis indicates that the virus life cycle is regulated at the level of protein-membrane association, protein modifications, sub-cellular localisation, and protein-protein interactions.

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**Hepatitis C virus NS4b carboxy-
terminal domain is
a membrane-binding domain**

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Abstract

Hepatitis C virus (HCV) induces membrane rearrangements during replication. All HCV proteins are associated to membranes, pointing out the importance of membranes for HCV. Non-structural protein 4b (NS4b) has been reported to induce cellular membrane alterations like the membranous web. Four transmembrane segments in the middle of the protein anchor NS4b to membranes. An amphipathic helix at the amino-terminus attaches to membranes as well. The carboxy-terminal domain (CTD) of NS4b is highly conserved in Hepaciviruses, though its function remains unknown. A cytosolic localisation is predicted for the NS4b-CTD. However, using membrane-floatation assays and immunofluorescence, we now show targeting of the NS4b-CTD to membranes. Furthermore, a profile-profile search, with an HCV NS4b-CTD multiple sequence alignment, indicates sequence similarity to the membrane-binding domain of prokaryotic D-lactate dehydrogenase (d-LDH). The crystal structure of *E. coli* d-LDH suggests that the region similar to NS4b-CTD is located in the membrane-binding domain (MBD) of d-LDH, implying analogy in membrane association. Targeting of d-LDH to membranes occurs via electrostatic interactions of positive residues on the outside of the protein with negative headgroups of lipids. To verify that anchorage of d-LDH MBD and NS4b-CTD is analogous, NS4b-CTD mutants were designed to disrupt these electrostatic interactions. Membrane association was confirmed by swapping the membrane-contacting helix of d-LDH with the corresponding domain of the NS4b-CTD. Furthermore, the functionality of these residues was tested in the HCV replicon system. Together these data show that NS4b-CTD is associated to membranes, similar to the prokaryotic d-LDH MBD, and is important for replication.

Introduction

Hepatitis C virus (HCV) preferentially infects hepatocytes¹. Although this does not have a direct cytopathic effect, infection often becomes persistent, slowly progressing into chronic liver diseases like cirrhosis and hepatocellular carcinoma^{2,3}. Phylogeny of HCV places this positive-sense RNA virus, within the genus *Hepaciviruses* of the family *Flaviviridae*⁴. The single-stranded RNA genome contains one open reading frame flanked by two non-translational regions (NTRs) at the 5' and 3'-end. An internal ribosomal entry site in the 5'-NTR facilitates the translation of the polyprotein⁵. Cellular and viral-encoded proteases process the polyprotein into three structural proteins (core and two glycoproteins, E1 and E2), a hydrophobic peptide p7 and six non-structural (NS) proteins^{6,7}.

During infection, the conformation of cellular host membranes changes in a number of ways. One of these membrane alterations is the membranous web (MW), composed of small vesicles embedded in a membrane matrix⁸. Ultra structural analysis of HCV replicon cells in combination with labelling of viral RNA revealed that this membranous web is the site of RNA synthesis⁸.

The non-structural (NS) proteins NS3 to NS5b are required for viral replication⁹. They localise to the cytosolic leaflet of membranes derived from the endoplasmic reticulum (ER)¹⁰. NS3 possesses RNA helicase as well as protease activity. Membrane anchoring of NS3 is mediated through an amphipathic helix at the N-terminus of NS3 and a transmembrane segment in NS4a, which is also a co-factor for NS3 protease^{11,12}. New HCV RNA strands are synthesised by NS5b, the RNA-dependent RNA polymerase. NS5b is targeted post-translationally to membranes via a carboxy-terminal hydrophobic domain^{13,14}. NS5a, a peripheral membrane-binding protein, associates with lipids via an amphipathic helix at its amino-terminus¹⁵. Importance for both replication and virus production has been suggested for NS5a^{16,17}. A central role for the integral membrane protein, NS4b, in the formation of the membranous web was suggested when Egger et al. showed that very similar structures could be induced by the NS4b protein in the absence of any other HCV proteins¹⁸. These NS4b induced structures were defined as swollen, partially vesiculated membranes and clustered aggregated membranes¹⁹.

NS4b is a hydrophobic protein with a molecular weight of approximately 27 kDa and has a modular domain organisation with the amino- (N) and carboxy- (C) terminal ends being cytoplasmic and a central region, which is inserted in the ER membrane. A topology study of NS4b indicated that the central domain has four transmembrane segments^{20, 21}. The N-terminal part, approximately 70 to 90 amino acids long, has several reported functional properties. The extreme N-terminal segment of NS4b revealed the presence of a putative amphipathic helix (AH, aa 6 – 29), which mediates membrane association through its hydrophobic side²². Disruption of this helix alters its ability to rearrange intracellular membranes and the localisation of HCV replication proteins^{21, 22}. The region next to this amphipathic helix is predicted to form a large amphipathic helix (aa 22 – 49), with the characteristics of a basic leucine zipper motif (bZIP)²³. The first 72 amino acids from the N-terminus of NS4b have been suggested to be involved in multimerisation²⁴, which may involve intramolecular leucine zipper interactions. A post-translational relocation of the N-terminus to the ER lumen was proposed for a fraction of the NS4b pool, giving the protein a dual transmembrane topology with either four or an extra fifth transmembrane domain (TMx)^{20, 21}. The C-terminal domain (CTD) of NS4b is oriented towards the cytosol and seems well conserved throughout hepaciviruses. Despite this sequence conservation not much is known about the CTD, though lately several studies describe possible characteristics of the domain²⁴⁻²⁷. A genetic interaction between NS3 with the extreme C-terminus of NS4b has been postulated²⁷. Besides protein-protein interactions^{24, 27}, a protein-RNA interaction has also been suggested²⁵. Furthermore the CTD of NS4b is involved in RNA synthesis and virus production²⁶.

The most widely suggested function for NS4b is the creation of a platform in the cell that concentrates the virus template, replication and host cell proteins, thereby increasing the efficiency of replication^{18, 28}. Alternatively, distortion of cellular membranes can reduce the transport of cell surface proteins in infected cells in order to escape from the host immune response¹⁹. Other functions attributed to NS4b are inhibition of host as well as viral protein translation^{29, 30} and modulation of NS5a hyper-phosphorylation³¹. Clearly, NS4b is involved in a wide range of activities, which seem to point to a role in modulating the host cell environment either for evasion of the host response or optimising the setting for viral replication.

In this study, we investigate the most conserved, though least characterised, domain of NS4b, the CTD. Expression of this domain in Huh7 cells, a human hepatoma cell

line, revealed membrane targeting of the NS4b-CTD, in contrast to its predicted cytosolic localisation. Based on similarity with D-lactate dehydrogenase (d-LDH) membrane-binding domain and mutational studies, we suggest that the NS4b-CTD is a membrane-binding domain. The importance of this membrane targeting during replication was analysed in replicon studies. Taken together our results show that in addition to the N-terminus and the transmembrane domains, NS4b can associate with intracellular membranes via its CTD. Furthermore, mutational studies suggest that, for membrane targeting, positive residues in the NS4b-CTD interact with the negatively charged headgroups of lipids.

Table 1 - Primers used to generate expression constructs

NS4b FL
Forward primer, GTGGGTACCATGTCACACCTCCCTTACATCGAACAG Reverse primer, TAGTCTAGAGAGCCGGAGCATGGCGTGGAGCAGTC
NS4b-CTD
Forward primer, GTGGGTACCATGGCGATACTGCGTCGGCACGTGGGC Reverse primer, as NS4b FL
NS4b-deltaCTD
Forward primer, as NS4b FL Reverse primer, TAGTCTAGAGACCGACGCAGTATCGCTGCGCACACGAC
NS4b-CTD sub-Cys
Forward primer, as for NS4b-CTD Reverse primer, AGATCTAGAGAGCCGGAGGATGGCGTGGAGGAGTCCTCGTTGATC-CACTG
d-LDH MBD
Forward primer, GTGGGTACCATGAAATACGGCAAAGACACCTTCC Reverse primer, TACTCTAGAGAATGCTCGTATTATCGC

Methods

Plasmid Construction

To construct Myc-epitope-tagged expression plasmids, the sequence was amplified by PCR from pFK5.1Neo³² or *E.coli* DNA using specific primers, see Table 1. The PCR products were digested with *KpnI* and *XbaI* and ligated into pCDNA3.1mychisB (Invitrogen) similarly digested with *KpnI* and *XbaI*. This resulted in the construction of expression vectors containing a 10-residue Myc-epitope-tag at its C-terminus. In

order to construct HA-epitope-tagged NS4b expression constructs, the Myc-epitope sequence was *Xba*I – *Pme*I cut and replaced with an *Xba*I – *Pme*I fragment coding for the HA-epitope.

Cell culture and transfection

Human hepatoma cell line Huh7 was grown in Dulbecco's Modified Eagle's Medium supplemented with non-essential amino acids, L-glutamate, Penicillin and Streptavadin. Cells were subcultured using trypsin and transfected using Eugene6 (Roche) at a DNA/reagent ratio of 1/3, according to manufacturers' instructions.

In vitro transcription, electroporation and selection of selectable replicon cells

In vitro transcription, electroporation and selection of G418-resistant cell lines was done as described previously ³³.

Antibodies

The following antibodies were used anti-PDI (Stressgen), anti-Myc (mouse) (Invitrogen), anti-Myc (rabbit) (Roche), anti-GAPDH (SantaCruz), anti-Transferrin receptor, clone H68.4 (Zymed Laboratories Inc), anti-COX-IV (Abcam), anti-Calnexin (BD) and anti-HA (Abcam).

Immunofluorescence microscopy

24 h post transfection cells were fixed with 3% paraformaldehyde (PFA) in PBS (154 mM NaCl, 1.4 mM phosphate, pH 7.5). PFA was quenched using 50 mM NH₄Cl in blockbuffer, which contained 5% fetal calf serum (FCS) in PBS. The cells were permeabilised with 0.1% TritonX-100 in blockbuffer and stained with primary antibodies diluted in blockbuffer for 1h. Next, the coverslips were washed with glycinebuffer, 10 mM glycine in PBS, and incubated with secondary antibody diluted in blockbuffer for 1h. After washing with glycinebuffer, PBS and water, the coverslips were mounted with Prolong (Invitrogen) mounting medium. Fluorescence images were captured using a Zeiss Axioskop 2 fluorescence microscope equipped with the appropriate filter sets, a digital Axiocam HRc camera and Zeiss Axiovision 4.4 software. Images were optimised with Adobe Photoshop CS2.

Membrane-floatation gradient

Transfected Huh7 cells were lysed after 24h in buffer that contained 20 mM

Tris pH 7, 1 mM MgCl₂, 15 mM NaCl and 240 mM sucrose using a ball bearing homogeniser (Isobiotec, Heidelberg Germany). Whole cells and cell debris was spun down at 500×g for 5 min and supernatant was collected. Cell extracts were mixed with sucrose to 80% w/v and overlaid with a linear sucrose gradient (80%- 10% w/v sucrose, 50 mM Tris pH 7, 1 mM MgCl₂, 15 mM NaCl). After centrifugation in a SW41 tube for 15h at 100,000×g (Beckmann ultracentrifuge), 500 µl fractions were collected from the top. The odd fractions were analysed by western blotting, either directly or subsequent to concentration. 200 µl of each fraction was concentrated using 9 volumes of ethanol and incubated overnight at -20 °C, followed by centrifugation at max in an Eppendorf 5417R for 1h. The protein pellets were dissolved in Laemmli-buffer.

SDS-Page and western blotting

After separation on SDS-PAGE gels, proteins were transferred to PVDF membranes (HybondP, GE-Healthcare) using a Semi-Dry blot apparatus (Biorad). Membrane blocking and antibody incubations were performed using 0.5% Tween-20, 5% non-fat, dry milk (Campina) in PBS. Since all secondary antibodies were conjugated to horseradish peroxidase, the proteins were visualised using enzyme-catalysed chemoluminescence (ECL+, GE-Healthcare) and Fuji Super RX medical X-ray film.

Profile searches of sequence databases

COMPASS (<http://prodata/swmed.edu/compass/>), database pfam21.0, 0 PSI-blast iterations, E-value threshold was set at 10. Profile comparer (PRC; <http://supfam.org/PRC>), database pfam22.0, E-value threshold was set at 10. HHpred (<http://toolkit.tuebingen.mpg.de/hhpred>), selected database pfamA_22.0, 0 PSI-blast iterations.

Results

NS4b carboxy-terminal domain localises to internal membranes

A well-conserved part of the HCV NS4b protein is the carboxy-terminal domain (NS4b-CTD), which is also conserved within the hepacivirus genus ²³. Along with the expected cytosolic localisation, it proposes a separate function of the NS4b-CTD. To study the localisation of NS4b-CTD various constructs were made. To each construct, a Myc-epitope-tag was fused at the C-terminus as a detection epitope.

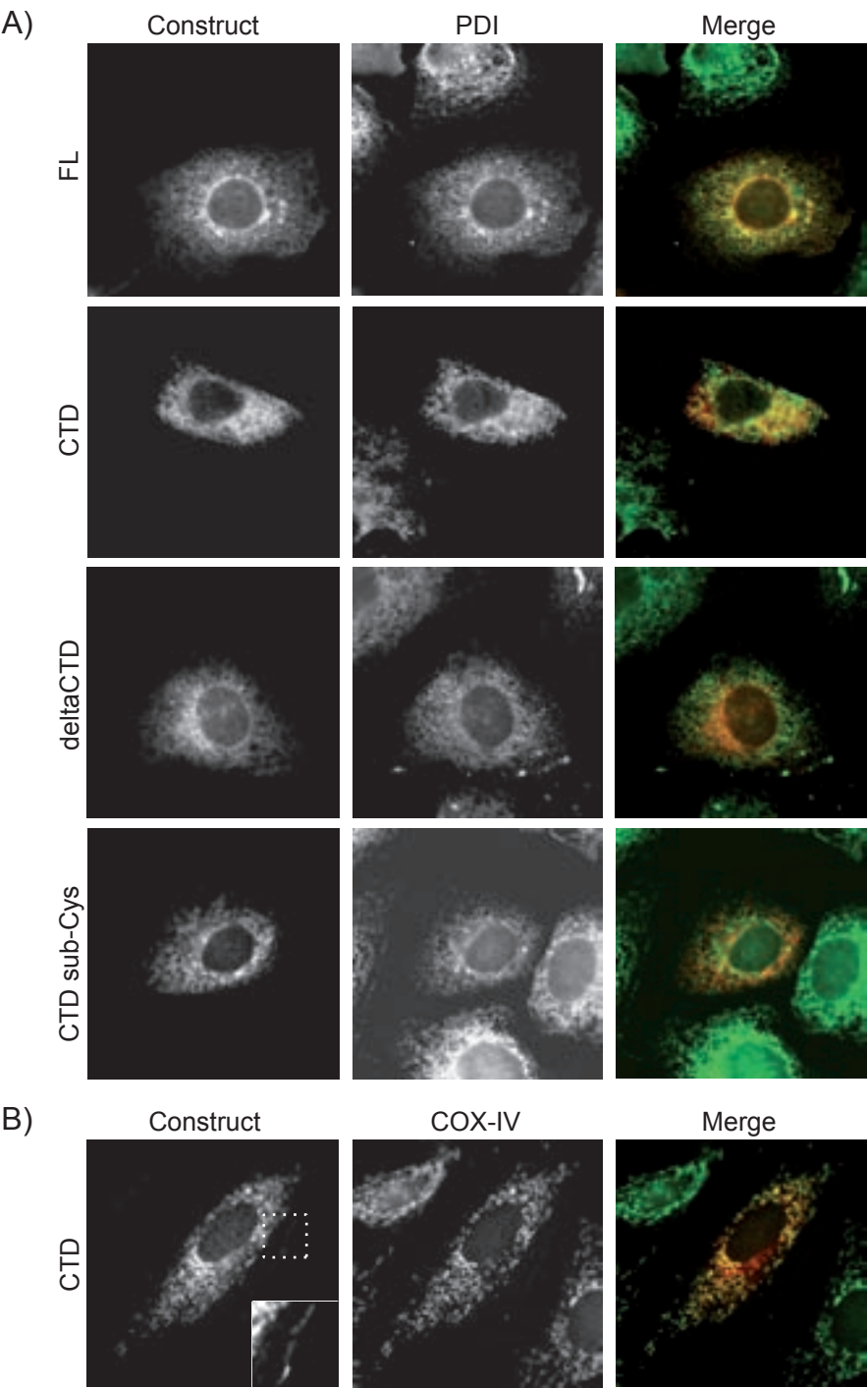


Figure 1 - Expression of different NS4b proteins in Huh7 cells

Huh7 cells were transfected with NS4b full-length (FL, aa 1-261), deltaCTD (aa 1-192), CTD (aa 188-261) or CTD substitution-Cysteines (CTD sub-Cys) and 24 h later processed for indirect immunofluorescence. Cells were double labeled with antibodies reacting against Myc-epitope-tag at the C-terminal end of each protein (in red) and **A.** protein disulphide isomerase (PDI) or **B.** Cytochrome C oxidase subunit IV (COX-IV) (in green), in first and second panels respectively. Third panels show merged images.

These constructs were transfected into Huh7 human hepatoma cells and analysed using immunofluorescence. Localisation of the constructs was first compared to the endoplasmic reticulum (ER), using Protein Disulphide Isomerase (PDI) as a marker. In Figure 1A, top left panel, Huh7 cells expressing full-length NS4b (NS4b-FL, aa 1-261) are shown. NS4b-FL has a perinuclear and reticular staining, typical for ER. Additionally, the pattern of NS4b-FL largely overlaps with PDI. This ER-like staining confirms the previously described localisation of native FL NS4b^{20, 34}. To our surprise, expression of the NS4b-CTD alone (aa 188-261) does not show a cytosolic staining, but displays small punctate or dot like structures throughout the cells (Fig.1A, CTD left panel). In the overlay of NS4b-CTD and PDI, some co-localisation is seen between the two (Fig.1A, CTD right panel). Together with the small punctate staining, this suggests that the NS4b-CTD might be associated to membranes.

Since the NS4b-CTD shows a dot like pattern, it might have an effect on the attachment to membranes or even localisation of NS4b-FL. Therefore, an NS4b lacking the CTD (NS4b-deltaCTD, aa 1-192) was constructed and examined in immunofluorescence. As shown in Figure 1A, NS4b-deltaCTD has a perinuclear and reticular staining, like NS4b-FL and PDI, indicating an ER-like localisation (Fig.1A). In addition, co-transfections of NS4b-FL and NS4b-deltaCTD show similar localisation (data not shown). Together this implies that the absence of CTD does not seem to alter the localisation of NS4b.

Two potential lipid modification sites for palmitoylation on cysteines, suggested by Yu and colleagues²⁴, might render the NS4b-CTD to membranes. We therefore investigated this possibility and mutated the two cysteines (cysteines 256 and 260) of the NS4b-CTD into serines (NS4b-CTD sub-Cys) (Fig.3A) and expressed this mutant in Huh7 cells. Localisation of the NS4b-CTD sub-Cys mutant was very similar to

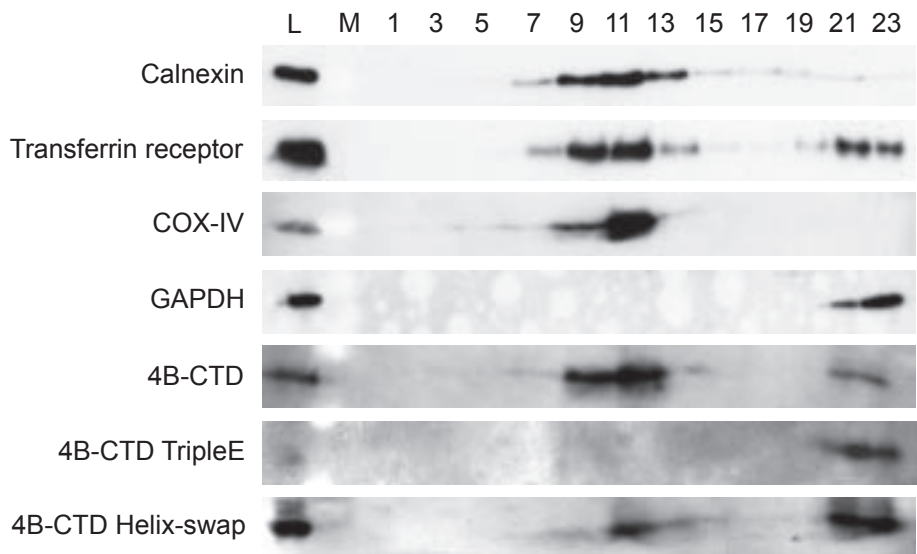


Figure 2 - Membrane association of NS4b carboxy-terminal domain

Huh7 cells transfected with NS4b-CTD-Myc, NS4b-CTD tripleE-Myc or NS4b-CTD Helix-swap-Myc were subjected to sucrose density gradient centrifugation. Cell lysates were loaded under a sucrose gradient from 10-80% w/v and part of the lysate was used as a loading control (L). Fractions were taken from top (fraction 1) to bottom (fraction 23) and separated by SDS-PAGE. This was followed by immunoblot analysis for Calnexin, Transferrin Receptor (TfR), Cytochrome C oxidase subunit IV (COX-IV) and Glyceraldehyde 3-phosphate dehydrogenase (GAPDH). NS4b-CTD and NS4b-CTD tripleE were assayed using an antibody against Myc-epitope. M indicates where molecular weight marker was loaded.

NS4b-CTD, exhibiting small punctate structures in the cells (Fig.1A). It shows that the dot-like membrane localisation of NS4b-CTD is caused by characteristics in the domain other than the cysteines at positions 256 and 260.

Membrane association of the carboxy-terminal domain of NS4b

Membrane association of proteins can be investigated in a membrane-floatation assay. In such an assay, a continuous-density gradient is loaded on top of a cell extract and subjected to centrifugation. Membranes and associated proteins float into the gradient, while cytosolic proteins stay in the loaded bottom fraction. To examine the suggested membrane-association characteristics of the NS4b-CTD a membrane-floatation assay was performed. Figure 2 shows the results of that

assay, in which a cell lysate of Huh7 cells transfected with NS4b-CTD was used. Fractions were collected from the top (10%) to the bottom (80%) of the gradient and the odd fractions were analysed by western blotting. As a control for cytosolic proteins, glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used. As expected, GAPDH was retained in the bottom fractions 21 and 23 of the density gradient, where the cell extract was loaded (Fig.2). Calnexin, Transferrin receptor (TfR) and Cytochrome C oxidase subunit IV (COX-IV) are transmembrane proteins and float into the gradient, they are mainly observed in fractions 9 and 11 (Fig.2). Since calnexin, TfR and COX-IV reside on different membranes in the cell (ER, the endocytic pathway and mitochondria), their distribution differs slightly (Fig.2). The NS4b-CTD is detected in fractions 7 to 13 and 21 and 23 with its highest signal in fraction 11 (Fig.2). In conclusion, similar to membrane proteins the NS4b-CTD floats into the gradient, implying membrane association of the NS4b-CTD. Together, the punctate structures in immunofluorescence and the floatation into the membrane-floatation gradient, suggest association of the CTD of NS4b to membranes.

Cellular localisation of the NS4b carboxy-terminal domain

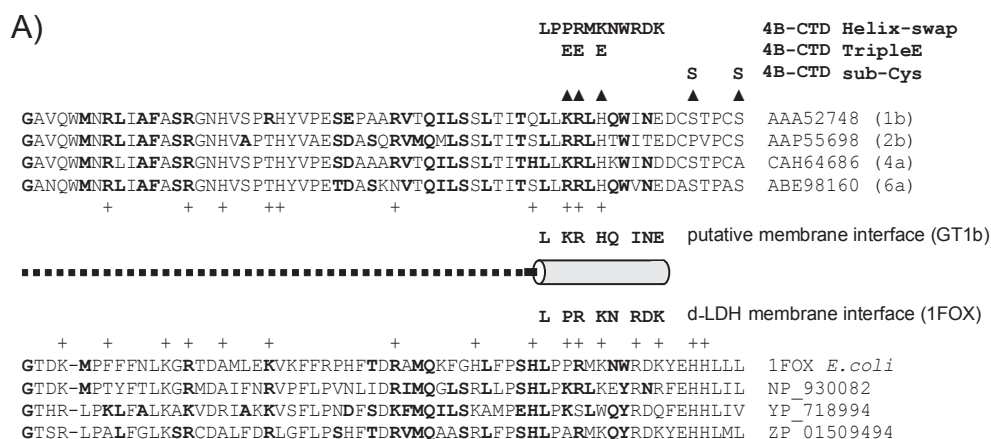
Since the CTD of NS4b only partially overlaps with the ER-marker, PDI (Fig.1A), we were interested in knowing on which other membranes the NS4b-CTD resides. Therefore, co-localisation studies with different organelle markers in Huh7 cells transfected with NS4b-CTD were performed. From the exocytic pathway, we examined the Golgi (Giantin) and the ER-Golgi intermediate compartment (ERGIC) and found no substantial co-localisation (data not shown). Similar results were obtained from co-localisation studies with markers from the endocytic pathway, such as Rab5 from early endosomes, mannose-6-phosphate receptor and LAMP1, proteins that resides in late endosomes and lysosomes (data not shown). Recently, lipid droplets were demonstrated to play an important role in the HCV life cycle ³⁵. However, no co-localisation of lipid droplets and the NS4b-CTD was observed (data not shown). HCV proteins, Core, NS3 and NS4a are suggested to localise to or close to mitochondria ^{36, 37}. For that reason, co-localisation of mitochondria and NS4b-CTD was investigated. We could observe considerable similarity in patterns between COX-IV, a mitochondrial protein marker and the NS4b-CTD (Fig.1B). However, the overlap is not complete. Even though we did not specifically preserve the plasma membrane during immunofluorescence, we could occasionally see a fraction of

NS4b-CTD at the plasma membrane (Fig.1B. Inset). Taken together the CTD of NS4b seems to be mainly targeted to mitochondria, ER membranes and the plasma membrane.

Profile searches with an HCV NS4b carboxy-terminal domain alignment suggest similarity to Lact-deh-memb

The importance of the NS4b-CTD might be reflected by the sequence conservation within hepaciviruses. Its sequence conservation may also provide a clue to its function. Identification of potentially remote protein homologues can help to predict protein properties, like folding, structure and most importantly function. Similarity between distantly related proteins can be effectively established using profile-based searches of databases of proteins families. In order to elucidate a possible function of the CTD of NS4b, we generated a multiple sequence alignment (profile) of the NS4b-CTD including all genotypes of HCV, Hepatitis GB virus A, B and C (Supplemental figure 1), which we manually refined. Programs for profile-profile comparisons have been developed and are available as web-based tools. We used three different tools for profile-profile comparison with our HCV NS4b-CTD query profile, namely PRC ³⁸, HHpred ³⁹ and COMPASS ⁴⁰ because each is sensitive to a different set of algorithms and the combination of the three tools reinforces independently detected relationships. This allows us to construct a consensus result with hits found by all tools. Using similar search parameters (see Materials and Methods) twelve, eight and five hits were found by PRC, HHpred and COMPASS respectively. Interestingly, only one protein family Lact-deh-memb (PF09330) was found by all three methods. This was the highest scoring profile for the three methods, next to the NS4b profile (E-values 0.057, 0.086 and 0.35 for the respective searches). According to HHpred the probability (which also includes the contribution from the secondary structure score) that Lact-deh-memb is significant similar to NS4-CTD is 44.8%. Members of this Lact-deh-memb family are predominantly found in prokaryotic D-lactate dehydrogenase (d-LDH), which is a peripheral-membrane respiratory enzyme located on the cytosolic site of the inner membrane ⁴¹. Comparison of the sequence similarity between HCV NS4b-CTD and d-LDH from *E. coli*, of which the crystal structure has been resolved ⁴¹, revealed that the common region lies in the membrane-binding domain (MBD) of d-LDH (Fig.3A and Supplemental figure 1) ⁴². Thus besides apparent sequence similarity, both domains seem to perform similar

functions, that is, they allow for membrane association. The MBD of d-LDH was suggested to bind non-specifically to the membrane through (the positively charged) basic residues (Lys, Arg), interacting with the negatively charged phospholipids of the membrane, rather than penetrating the lipid bilayer⁴¹⁻⁴³. Part of the d-LDH MBD



B)

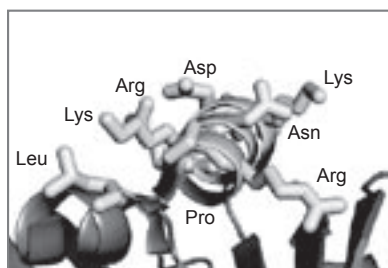


Figure 3 - Sequence similarity between NS4b carboxy-terminal domain and the membrane-binding domain of D-lactate dehydrogenase

A. Multiple sequence alignment of the carboxy-terminal domain of four genotypes of HCV NS4b proteins and the membrane-binding domain of four d-LDH family members (referenced by their accession numbers). Bold residues highlight amino acids present in both families. Basic residues (R, K, H) making up the potential electropositive surface are indicated (+). Dotted line indicates disordered region in the d-LDH crystal structure. Arrowheads point to mutations made in the CTD of NS4b.

B. Ribbon representation of the membrane-anchored side of d-LDH (PDB code 1FOX). Stick residues indicate the surface exposed amino acids of the ordered membrane-binding helix.

corresponding to the CTD of NS4b is disordered in the crystal structure and is thought to form a defined structure upon binding to the membrane⁴¹. The central alpha-helix of the d-LDH MBD (Fig.3B) corresponds to the extreme carboxy-terminal end of NS4b-CTD. The amino acids on the membrane interface of this alpha-helix and the corresponding residues of NS4b-CTD are indicated in Figure 3A.

Membrane targeting of NS4b carboxy-terminal domain and d-LDH is comparable

Profile-profile comparison E-values in the range of 0.1-0.001 can indicate a true relationship, but require additional evidence to conclude that there is a functional parallel between NS4b-CTD and d-LDH-MBD in membrane binding⁴⁴. Mutational studies could reveal functional similarity and were accordingly performed. D-LDH is a general membrane-binding protein in *E. coli* located on the cytosolic side of the inner membrane. The position of such a protein in eukaryotic cells is unknown. Therefore, we first investigated localisation of d-LDH MBD in Huh7 cells. As shown in Figure 4A d-LDH MBD (aa 319 to 390) mainly overlaps with COX-IV illustrating that when the d-LDH MBD is expressed separate from the enzyme part of the d-LDH protein, functionality of membrane binding is maintained. Moreover, co-transfection of NS4b-CTD and d-LDH MBD showed nearly complete overlap of the two patterns (Fig.4B). Furthermore these immunofluorescence assays indicate that d-LDH MBD, a general membrane-binding domain, has a preference for mitochondrial membranes in eukaryotic cells, which is comparable to the localisation of NS4b-CTD (Fig.4A), implying analogous membrane targeting of the two domains.

To test the hypothesis that the CTD of NS4b associates with the membranes in a way similar to the d-LDH MBD, we introduced mutations designed to disrupt the positive residues postulated to interact with the negative headgroups of lipids^{41, 42} (Fig.3A). The side chains of the d-LDH MBD pointing away from the protein, facing the membrane surface are indicated in Figure 3B. Three positively charged amino acids (Lys 247, Arg 248 and His 250) in NS4b corresponding to the structured alpha-helix in d-LDH were simultaneously replaced with a negatively charged glutamic acid (K247E/ R248E/ H250E; NS4b-CTD tripleE), which should not be able to bind to phospholipid heads. The NS4b-CTD tripleE mutant was expressed in Huh7 cells and membrane association was investigated using immunofluorescence and a membrane-floatation assay. Mutation of all three positively charged residues results

in a dramatic change of localisation of the NS4b-CTD, from punctate structures in the perinuclear region to a diffuse distribution throughout the cell, possibly cytosolic (Fig.4A, compare NS4b-CTD to NS4b-CTD tripleE). Loss of membrane association was also shown in a continuous-density gradient, in which NS4b-CTD tripleE was detected in the same fractions as the cytosolic marker GAPDH (Fig.2).

A functional parallel can also be examined by swapping part of the membrane-binding domains of two proteins. A mutant was constructed, in which we exchanged the putative membrane-contacting helix of NS4b-CTD for the corresponding membrane-contacting helix of the d-LDH MBD (NS4b-CTD helix-swap) (Fig.4A). Huh7 cells expressing NS4b-CTD helix-swap display punctate structures in immunofluorescence, though the staining has a slightly more diffuse localisation compared to NS4b-CTD (Fig.4A). Similarity in patterns with COX-IV also indicated that the NS4b-CTD helix-swap is targeted to membranes while the NS4b-CTD tripleE mutant has lost membrane binding. Furthermore, a membrane-floatation assay showed that NS4b-CTD helix-swap is membrane associated (Fig.2), although compared to NS4b-CTD more was observed in the non-floating fractions. Altogether, these results illustrate that the CTD of NS4b can interact with membranes via the positively charged residues, comparable to d-LDH MBD.

Positively charged residues of NS4b carboxy-terminal domain are essential for replication

To examine the importance of the NS4b-CTD positively charged residues for RNA replication, we exchanged these amino acids involved in membrane association for negatively charged glutamic acids in selectable subgenomic replicons ⁹. Huh7 cells transfected with replicon RNA that carry the three negatively charged residues (tripleE) did not yield any viable colonies (Fig.5). Moreover, the single mutations K247E and R248E were replication defective and gave no colonies (Fig.5). Thus the positive residues are clearly indispensable for viral RNA replication in cell culture, suggesting that loss in membrane association leads to a replication defect. These results, together with the possible functional parallel between d-LDH MBD and the NS4b-CTD, prompted us to swap the membrane-binding helix from d-LDH MBD (PPRMKNWRDK) into replicons (helix-swap, Fig.3A) and determine colony formation. These replicons, in which eight amino acids are exchanged, indeed formed several viable colonies (40 colony-forming units per µg (CFU) of transfected replicon RNA) (Fig.5). Clearly, far less colonies were

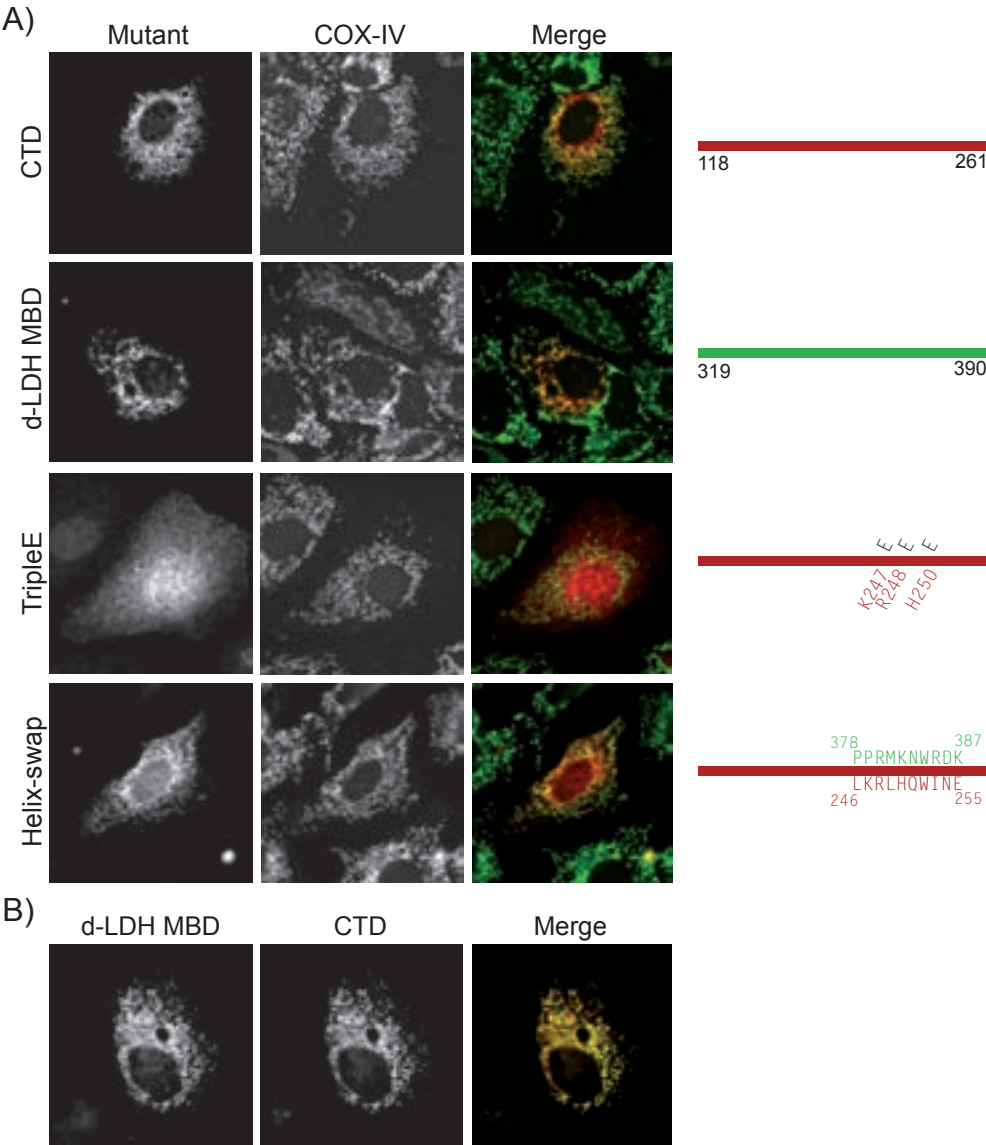


Figure 4 - Cellular distribution of NS4b carboxy-terminal domain mutants and D-lactate dehydrogenase membrane-binding domain in Huh7 cells

A. The panels on the right show Huh7 cells expressing different NS4b-CTD mutants or d-LDH membrane-binding domain (d-LDH MBD) after 24 h. Expression constructs are shown in red. Using COX-IV as a marker protein, mitochondria are shown in middle panels and in merged picture in green. On the right a schematic view of the different NS4b-CTD mutants is drawn; in red the sequence of NS4b-CTD-wt, in black the mutations made and in green the exchanged amino acids

from the membrane-contacting helix of the d-LDH-MBD.

B. Huh7 cells were co-transfected with NS4b-CTD-HA and d-LDH MBD-Myc and analysed by immunofluorescence after 24 h of expression. The first panel shows d-LDH MBD, which is presented as red in the merged picture. NS4b-CTD is displayed in the second panel and is shown in the merged picture as green.

formed relative to wild type (~10.000 CFU), but the replication defect from the helix swap is less than the negative charged mutations, where no colonies were formed. Two separate replicon colonies derived from the NS4b-CTD helix-swap were expanded, RNA isolated and sequenced to analyse whether they still contained the original mutations. Interestingly, the complete introduced helix was retained, confirming the importance of this membrane-contacting helix.

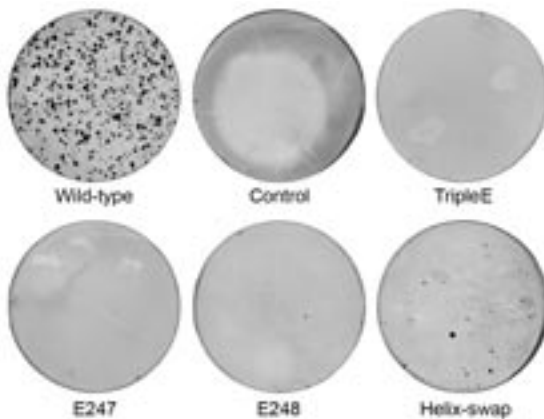


Figure 5 - Effect of NS4b carboxy-terminal mutations on colony formation using selectable replicons

Colony-formation assay in which Huh7 cells are transfected with *in vitro* transcribed replicon RNA that contain NS4b-CTD mutations. Colonies were stained using Coomassie blue. Wild-type is pFK5.1. Mock transfected cells as the control. The NS4b-CTD mutations TripleE, E247, E248 and helix-swap in pFK5.1 are explained in Figure 4.

Discussion

Compared to other HCV proteins NS4b is the least characterised. Besides involvement in replication and induction of membrane rearrangements little is known about the function(s) of the protein. A well-conserved part of NS4b is the carboxy-terminal domain (CTD) (Supplemental figure 1), which is predicted to contain two alpha-helices and expected to localise cytosolically^{20, 23}. Surprisingly, we found using different approaches that the NS4b-CTD is membrane associated. Immunofluorescence analysis of Huh7 cells expressing NS4b-CTD shows punctated

structures (Fig.1). Furthermore, in a membrane-floatation gradient, we could demonstrate that fractions containing floating membranes also have NS4b-CTD (Fig.2). Using profile-profile searches, we found similarity between the CTD of NS4b and the membrane-binding domain (MBD) of D-lactate dehydrogenase (d-LDH) (Fig.3A). D-LDH is a prokaryotic respiratory enzyme that is located on the cytosolic side of the inner membrane⁴¹. When we expressed the MBD of d-LDH from *E.coli* in mammalian Huh7 cells and performed immunofluorescence, we observe a pattern similar to NS4b-CTD (Fig.4A). Nearly complete overlap of both signals was shown in a co-transfection experiment of NS4b-CTD and the MBD of d-LDH (Fig.4B), indicating a functional parallel of both domains in membrane association. D-LDH is suggested to anchor to the membrane via interactions of positively charged amino acids with the negative heads of membrane phospholipids⁴¹⁻⁴³. Substitution of three positive residues in the NS4b-CTD resulted in complete loss of membrane association (Fig.4A). Together these experiments strongly suggest association of the NS4b-CTD to membranes.

The localisation of NS4b-CTD to mitochondria is the most prominent (Fig.1B and 4A). However, there is no complete co-localisation as a fraction is targeted to the ER (Fig.1A) and the plasma membrane (Fig.1B, Inset). In addition, the d-LDH MBD, a general membrane-binding domain that normally targets the enzyme towards the cytosolic side of the inner membrane of *E. coli* through electrostatic interactions, is largely located on mitochondrial membranes when expressed in human Huh-7 cells (Fig.4A)⁴¹. The apparent preference for mitochondria might be caused by the slow turnover rate of mitochondrial membranes compared to the rapid turnover of ER and Golgi membranes⁴⁵. A more general membrane-association characteristic of the NS4b-CTD is implied by these results.

Given the similarity between the CTD of NS4b and the d-LDH membrane-binding domain, the mode of general association to the membrane, through electrostatic interactions⁴¹⁻⁴³, might be comparable as well. When we substituted three positive residues in the NS4b-CTD region corresponding to the MBD of d-LDH into negative residues, to create repulsion towards the negative headgroups of lipids, membrane association is lost (Fig.2 and Fig.4A, NS4b-CTD tripleE), as well as the ability to form subgenomic replicon colonies (Fig.5). Single substitution of each positive residue in the NS4b-CTD resulted in a mild loss of membrane targeting (Data not shown), though a complete loss of replicon colony formation (Fig.5). It was previously shown that the

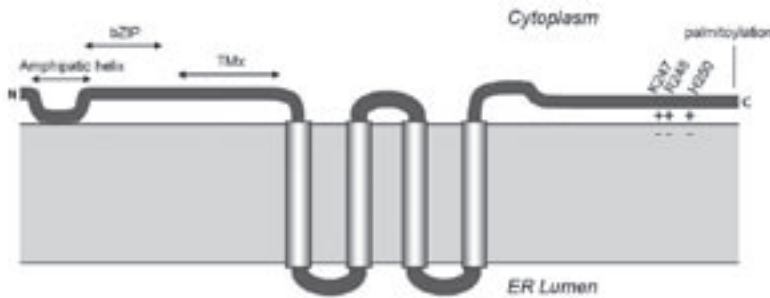


Figure 6 - Model of NS4b membrane association

Schematic of the proposed topology of NS4b relative to the ER membrane and reported functional properties (see introduction). Model for NS4b-CTD membrane association is discussed in this paper. Here we propose that positive residues (amino acids are indicated) are important for membrane targeting through the interaction with the negative headgroups of phospholipids.

Abbreviations: N, Amino terminus; C, Carboxy-terminus; bZIP, basic leucine zipper motif; TMx, transmembrane segment X.

integrity of NS4b is important for HCV replication; changes of only one amino acid can already influence replication^{46, 47}. Mutants in which we exchanged the complete membrane-contacting helix of NS4b-CTD with the d-LDH membrane-interface helix, retained membrane targeting (Fig.2 and Fig.4A, NS4b-CTD helix-swap) and selectable replicon colonies were obtained (Fig.5). Nonetheless, in this NS4b-CTD helix-swap fewer replicon colonies were formed compared to wild type. Moreover, the introduced sequence was unchanged in these colonies. In the NS4b-CTD helix-swap, mutant eight amino acids are substituted and it gains in total one positively charged residue compared to NS4b-CTD, though this charge is distributed differently along the helix. Both the immunofluorescence assay and the colony-formation assay illustrate that these mutations are allowed and indicate similar function of the two domains, NS4b-CTD and d-LDH MBD. Our experiments give an indication that the CTD of NS4b targets to membranes via electrostatic interactions of the positive residues in the NS4b-CTD with the negative phosphates of the phospholipids (Model in Fig.6), moreover that this protein-membrane interaction is important for HCV RNA replication.

The NS4b protein is associated with membranes in various ways. Four to five transmembrane domains in the central region^{20, 23} and an amphipathic helix at the N-terminus of the protein²² were described previously. In addition, we now show that the CTD of NS4b is a membrane-binding domain. This stresses the importance

of protein-membrane interaction throughout the protein. For the NS4b-CTD, we can envisage several possible functions. One possibility might be to position this domain of NS4b in a correct orientation. Recently, a membrane-binding amphipathic helix in NS3, together with the transmembrane domain of NS4a, were suggested to properly position the NS3-4a protease on the membrane¹¹. Positive residues in a MBD can also stabilize the orientation on the membrane surface⁴⁸. In analogy, membrane contacts of NS4b-CTD might position the domain on the membrane surface or facing towards the cytosol.

NS4b is involved in the formation of membranous web structures¹⁸. Therefore, a function of the NS4b protein might be the induction of membrane curvature. The N-terminal amphipathic helix could act as a wedge inserted into one leaflet of the lipid bilayer leading to membrane curvature^{49, 50}. Also transmembrane domains can influence membrane curvature, depending on their conical shape⁴⁹. The CTD of NS4b might induce or stabilize curvature by bracing the membrane like a scaffold^{49, 50}.

An interesting question for both the N-terminal amphipathic helix and the CTD membrane-binding domain of NS4b is whether these bind to the same membrane (cis) as the central transmembrane helices or that these can bind to other cellular membranes in close proximity (trans). In the latter situation it is conceivable that such a membrane-protein-membrane interaction would bring different membrane surfaces into close proximity, resulting in convoluted membranes¹⁹.

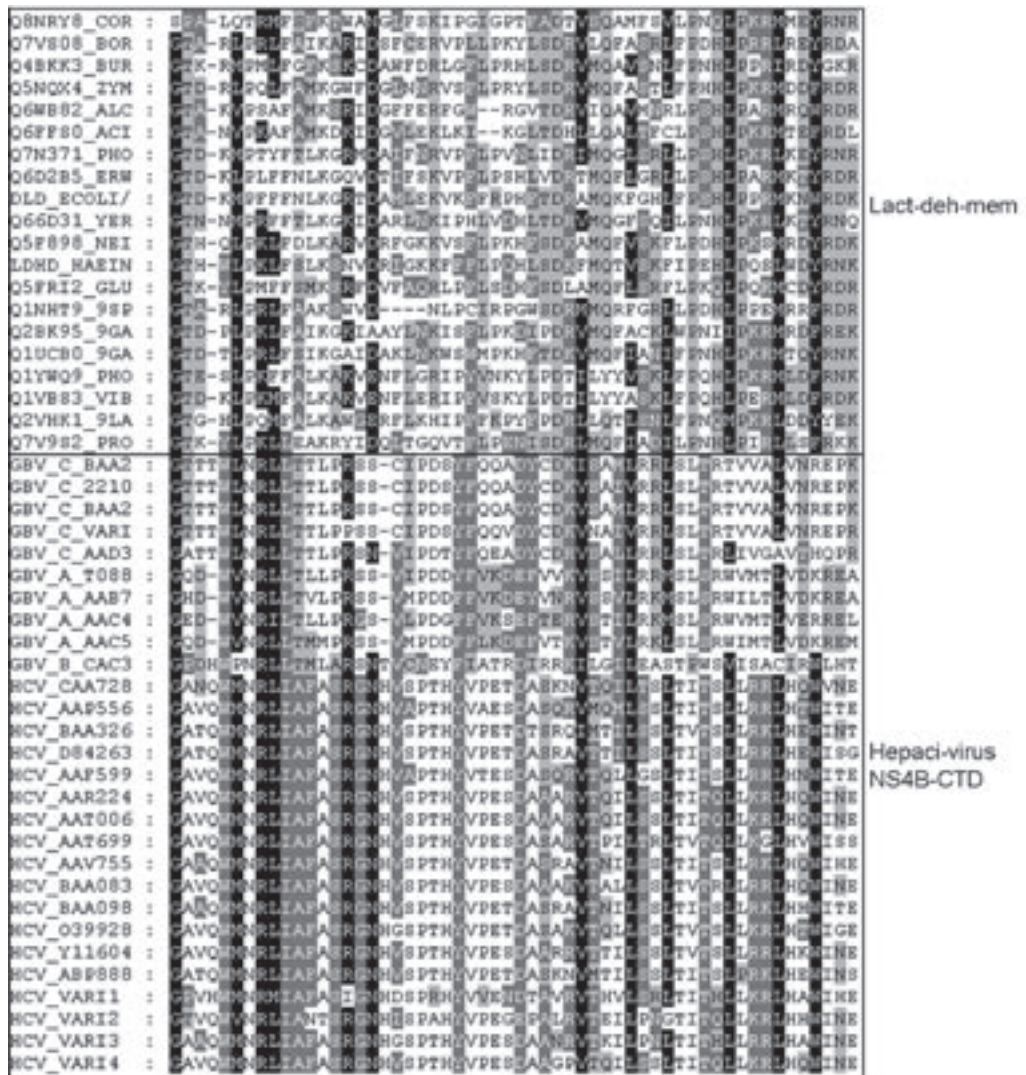
Recently, the positively charged amino acids that we propose to interact with the lipid headgroups, were also indicated in RNA-binding with an apparent preference for minus strand 3'NTR²⁵. When we co-transfected NS4b-CTD together with an excess of minus strand 3'NTR RNA, no change in localisation of the NS4b-CTD could be observed (data not shown). This indicates that membrane association is not affected by the suggested RNA-binding characteristics of the domain in the presence of RNA.

Clearly, the HCV life cycle is achieved by the interchange between membranes, protein membrane anchors and proteins. The membranous web formation for replication, possibly lipid droplet associated membranes are involved in virus particle assembly^{16, 35}. The switch between active replication and assembly of infectious

virus particles requires further levels of interactions between the membranous web and other associated membranes both in time and space^{35, 51, 52}. The modular domain architecture and association to membranes of NS4b suggests various functions throughout these processes.

Acknowledgements

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Supplemental figure 1 - Multiple sequence alignment of NS4b carboxy-terminal domain and Lact-deh-memb

Top panel contains a multiple sequence alignment of Lact-deh-memb (PF09330). A multiple sequence alignment of the NS4b-CTD, which includes all genotypes of HCV, Hepatitis GB virus A, B and C is shown in the bottom panel.

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**Characterisation of Hepatitis C
virus NS3 modifications in
the context of replication**

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Abstract

Post-translational modifications of viral proteins regulate various stages of infection and can even control cellular processes. With only 10 proteins Hepatitis C virus (HCV) can orchestrate its complete viral life cycle. The HCV non-structural protein 3 (NS3) has many functions. It has protease and helicase activity, interacts with several host cell proteins and plays a role in translation, replication and virus particle formation. Organisation of all these functions seems necessary and could be regulated by post-translational modifications. We therefore searched for modifications of the NS3 protein in the subgenomic HCV replicon. When performing a tag-capture approach coupled with two-dimensional gel electrophoresis analyses, we observed that isolated His6-NS3 yielded multiple spots. Individual protein spots were in-gel digested with trypsin and analysed by mass spectrometry. Differences observed between the individual peptide mass fingerprints suggested the presence of modified peptides and allowed us to identify N-terminal acetylation and an adaptive mutation of NS3 (Q1067R). Further analysis of other NS3 variants revealed phosphorylation of NS3.

Introduction

Post-translational modifications (PTMs) like phosphorylation, prenylation, methylation and acetylation play an important role in protein activity, localisation and protein-protein interactions ^{1, 2}. Not only eukaryotes use this mechanism to modulate the function(s) of their proteins. Especially viruses require PTMs because their genome is small and events like translation, replication, virus assembly and release all need to be well orchestrated ^{3, 4}. The small 9,6 kb genome of Hepatitis C virus (HCV), a positive-stranded RNA virus, yields a single polypeptide, which after processing results in three structural proteins Core, E1 and E2, a peptide p7 and six non-structural proteins NS2, NS3, NS4a, NS4b, NS5a and NS5b. Together these 10 proteins coordinate the complete life cycle of HCV ⁵. For some of these proteins it is known that they have a dual function involved in more than one process of the virus life cycle, requiring precise regulation ⁶⁻⁹.

An apparent example of a multifunctional protein within the HCV genome is NS3, which contains protease and helicase activity ^{10, 11}. The serine protease domain of NS3 is in the N-terminal one third of the protein. Together with NS4a it forms a stable complex, which cleaves the non-structural proteins, downstream of NS3 ¹²⁻¹⁴. Besides these cleavages, the NS3-4a protease is known to cleave MAVS/IPS-1/VISA/Cardif and TRIF, which affect signalling of the RIG-I and Toll-like receptor 3 pathways respectively, thereby abrogating the interferon response ¹⁵. Upon binding to NS4a the localisation of NS3 changes from cytosolic to endoplasmic reticulum (ER) membrane-associated, indicating that protein-protein interactions modulate its localisation ¹⁶.

In addition to the protease activity of NS3 in the N-terminus, the C-terminus of NS3 can unwind RNA through ATP-ase and helicase activity ^{11, 17}. RNA-unwinding activity seems to be modulated by other viral proteins like NS5b, the RNA-dependent RNA polymerase ^{18, 19} and NS4a ²⁰. A fine balance between the concentration of NS5b, NS3 and RNA has been indicated to influence activity ¹⁸. The helicase domain of NS3 can be post-translationally methylated at Arg1493 (polyprotein) by Protein Arginine Methyltransferase 1 (PMRT1) ²¹. Non-methylated NS3 helicase can unwind dsDNA, however when the helicase domain of NS3 is methylated unwinding of dsDNA is inhibited ²². This shows that methylation of the NS3 helicase domain can change its activity. The mechanism resulting in inhibition and the conditions for methylation

are still unclear, though proteins in the alpha interferon-induced signalling pathway have been implicated ²².

Besides the interaction of NS3 with PMRT1, several other host-protein interactions with NS3 have been described, including PKA, PKC, TBK1 and p53, which are all proteins involved in cell signalling ²³⁻²⁶. Recently a role in virus particle production has been suggested for NS3, by the identification of adaptation and compensatory mutations in NS3 using the infectious virus system ^{27,28}.

Taken together, the NS3 protein has various interaction partners and many functions which need to be regulated. Post-translational modifications could direct these processes. A first step towards understanding regulation is the identification of NS3 modifications. Besides methylation, which seems to regulate helicase activity and has been identified using over-expression of NS3, no other modifications are known. We therefore set up an assay to search for other NS3 modifications in the context of functional replication. In order to purify NS3 from the replicon, we introduced a His6-tag at the N-terminus of NS3 in the replicon RNA genome. After two-dimensional poly-acrylamide gel electrophoresis separation of purified NS3, at least 6 major protein spots corresponding to NS3 were observed. These spots point to several post-translational modifications of NS3. To identify post-translational modifications, we analysed spots by MALDI-ToF-ToF and LC-iontrap MS/MS and could reveal an N-terminal acetylation. In addition, an amino acid change in NS3, reported to enhance replication, was discovered. We furthermore show, by phosphate specific staining and phosphatase treatment, phosphorylation of NS3.

Methods

Plasmid Construction

To construct His6-tagged NS3-replicons, the sequence was amplified by PCR from pFK5.1Neo ²⁹ using the specific primers GTTCGGACCGTCTAGACATCATCACCATCACCATGCGCCTATTACGGCCTACTCC and GAAGTCGACTGTCTGGGTGACAATG. The PCR product was digested with *RsrII* and *BsrGI* and ligated into pFK5.1Neo similarly digested with *RsrII* and *BsrGI*. Start codon and plasmid were restored by introducing PCR product derived from specific primers GCTAAGCTTCGTAATACGACTC and ATTCTAGACATGGTATTATCGT-

GTTTTTC, digested with *RsrII* - *XbaI* back into the construct. This resulted in the construction of a replicon containing a His6-tag at the N-terminus of NS3.

Cell culture

Human hepatoma cell line Huh7 containing HCV replicons was grown in Dulbecco's Modified Eagle's Medium supplemented with Non-essential amino acids, L-glutamate, Penicillin, Streptavidin and Geneticin. Cells were subcultured using Trypsin.

Purification of His6-NS3

Huh7 cells containing His6-tagged NS3 replicons were sub-cultured at least two days before cell lysis with Ureum buffer (8 M ureum, 300 mM NaCl, 1% NP40, 50 mM Tris pH 7.0 and 10 mM imidazole pH 7.0). Total lysate volume of each 15 cm culture dish was 2 ml. To get rid of cell debris, mainly genomic DNA, total cell lysates were subjected to centrifugation at 14,000 $\times g$ for 15 min at 4°C and supernatant was collected. Eighty μl of 50% Cobalt²⁺-beads (Clontech) in Ureum buffer was added to the supernatant of one dish and incubated for 2 h at 4°C under continuous rotation. Subsequently the beads were washed three times with Ureum buffer. His6-NS3 was eluted from the beads using Ureum buffer containing 190 mM imidazole. Proteins in the eluate were precipitated overnight at -20°C by adding 9 volumes of ethanol, followed by centrifugation at 14,000 $\times g$ for 1 h at 4°C. The protein pellet was either dissolved in Laemmli buffer or 2D-PAGE buffer (8 M ureum, 4% CHAPS, 15 mM Tris pH 8.5, 1% DTT, Brome phenol Blue (BPB) and 0.5% IPG-buffer (pH 3-10, GE-Healthcare).

Two-dimensional poly-acrylamide gel electrophoresis (2D-PAGE)

Immobiline DryStrips pH 3-10, 13 cm (GE-Healthcare) were incubated for at least 6 h with 0.5% IPG-buffer in De-streak rehydration solution (GE-Healthcare), before starting isoelectric focusing of the sample. To apply the His6-NS3 samples (in 2D-PAGE buffer) onto the strip we used cup-loading at the basic end of the strip. Isoelectric focusing was performed on an Ettan IPGphorTM 3 Isoelectric Focusing System running the following program: 3 h at 150 V, 3 h at 300 V, gradually to 1000 V in 6 h, gradually to 8000 V in 1 h and 8000 V for 2 h. After first dimension separation the strip was equilibrated to SDS-PAGE conditions with two consecutive 15 min incubation steps with Equilibration buffer (6 M ureum, 50 mM Tris (pH 8.8), 30% glycerol and 4% SDS), which contained in the first step 2% DTT and in the

second 2.5% iodoacetamide and BPB. Subsequently the strips were applied to SDS-PAGE gels and covered with agarose. Following second dimension separation, gels were subjected to western blotting, silver staining, or ProQ diamond staining.

Western blotting

After separation on SDS-PAGE gels, proteins were transferred to PVDF membranes (Hybond P, GE-Healthcare) using a Semi-Dry blot apparatus (Biorad). Membrane blocking and antibody incubations were performed using 0.5% Tween-20, 5% non-fat, dry milk (Campina) in PBS. Monoclonal anti-NS3 was obtained from Novo Castra. Goat-anti-mouse (DAKO) conjugated to horseradish peroxidase together with enzyme-catalysed chemoluminescence (ECL+, GE-Healthcare) and Fuji Super RX medical X-ray film or Typhoon imager (GE-Healthcare) were used to visualise the signal.

Silver staining

SDS-PAGE gels were incubated in 50% ethanol, 12% acetic acid and washed with 50% ethanol. Sensitisation was performed using sensitise solution (1.5 mM sodiumthiosulfate) for 30 min, which was followed by washing with H₂O. Subsequently Silver solution (12 mM silvernitrate, 0.075% formaldehyde) was added for 30 min. After rinsing the SDS-PAGE gel in H₂O, it was stained with Develop solution (2.5% sensitise solution, 0.57 M sodiumcarbonate, 0.05% formaldehyde) until desired staining was reached. The staining reaction was stopped with 50% methanol, 12% acetic acid.

Phosphatase treatment

Purified His6-NS3 eluted from the Cobalt²⁺-beads was diluted to 1 M Urea using PBS (154 mM NaCl, 1.4 mM Phosphate, pH 7.5). Subsequently the phosphatase treatment was performed in the presence of 5 mM Tris pH 8.0 and 1 mM MgCl₂ with 1 unit Shrimp Alkaline Phosphatase (SAP) (USB) per 64 µl. Both the phosphatase-treated and the control sample, to which no SAP was added, were incubated at 37°C for 1h. After that, the samples were concentrated using ethanol precipitation as described above and the protein pellets were dissolved in 2D-PAGE buffer.

ProQ diamond staining

SDS-PAGE gels were fixed in 50% methanol, 10% acetic acid, washed with H₂O and stained for 1.5 h with Pro-Q Diamond phosphoprotein gel stain (Invitrogen). Then destaining was performed using 20% acetonitrile, 50 mM sodium acetate, pH

4.0 for a total time of 1.5 h. Until imaging, the gel was stored in H₂O. To visualise the ProQ diamond signal we used the Typhoon imager (excitation 532nm; emission filter 560nm; GE Healthcare).

In-gel tryptic digestion

Protein spots were excised, cut into small pieces and washed with 25 mM NH₄HCO₃ followed by dehydration with 100% acetonitrile (ACN) for 10 min. For reduction and alkylation, dried gel particles were first incubated with 10 mM dithiothreitol for 30 min at 56 °C. Following dehydration with ACN, gel plugs were subsequently incubated in 55 mM iodoacetamide for 20 min at room temperature. After washing with 25 mM NH₄HCO₃ and dehydration with 100% ACN, the gel particles were completely dried in a centrifugal vacuum concentrator (Eppendorf, Hamburg, Germany). Dried gel particles were re-swollen for 15 min on ice by addition of 10-15 µl of a trypsin solution (12.5 ng/µl in 25 mM NH₄HCO₃, sequencing grade modified trypsin, Promega, Madison, WI). Following this, 25-35 µl of 25 mM NH₄HCO₃ was added and samples were kept on ice for an additional 30 min. Tryptic digestion was subsequently performed overnight at 37 °C. Following tryptic digestion, the overlaying digestion-solution was collected (extract 1). Two additional rounds of extraction with 20 µl 0.1% TFA were used to extract peptides from the gel plugs and these were pooled with extract 1.

MALDI-ToF MS

For MALDI-ToF, tryptic digestions were desalted using C₁₈ ZipTips (Millipore) according to the manufacturer's instructions. Peptides were eluted using approx. 1 µl of 0.33 mg/ml alpha-cyano-4-hydroxycinnamic in acetone:ethanol 1:3 and spotted directly on a 600 µm AnchorChip MALDI target plate (Bruker Daltonics, Bremen, Germany) and allowed to dry. MALDI-ToF(-ToF) analyses were performed on an Ultraflex II time-of-flight mass spectrometer (Bruker Daltonics, Bremen, Germany) controlled by the Flexcontrol 2.0 software package. Spectra were recorded in the positive ion reflectron mode at a laser frequency of 50 Hz. For MS/MS analysis, precursors were accelerated and selected in a time ion gate after which laser-decomposed fragments were further accelerated in the LIFT cell and their *m/z* were analysed after passing the ion reflectron.

Nano-LC-iontrap MS

Tryptic digests were separated on a C₁₈ pepmap100 column (75 µm I.D., 15 cm

long; Dionex, Amsterdam, The Netherlands) coupled to an electrospray ionisation (ESI)-iontrap mass spectrometer (HCTultra, Bruker Daltonics, Bremen, Germany). The gradient profile started with 100% solvent A (20% ACN/0.1% formic acid) for 10 min. This was followed by a 20 min linear gradient from 20% solvent B (95% ACN/0.1% formic acid) up to 60% solvent B. The system remained at 60% solvent B for 25 min followed by a second linear gradient up to 90% solvent B in 10 min. After 30 min at 90%, solvent B the gradient was changed to 0% solvent B. The flow rate was maintained at 200 nL/min during the entire run. Eluting peptides were analysed in data dependent MS/MS mode over a 400–1600 m/z range. The five most abundant ions in each MS spectrum were selected for MS/MS analysis by collision-induced dissociation, using helium as collision gas. Mass spectra were evaluated using the DataAnalysis 3.1 software package (Bruker Daltonics, Bremen, Germany).

Results

Purification of NS3 from HCV selectable replicons

Besides methylation of the helicase domain of NS3 no other post-translational modifications (PTMs) of NS3 have been reported. Our objective was to identify novel NS3 PTMs in the context of actively replicating HCV RNA. For this purpose, we introduced a His6-tag to NS3 in the HCV replicon (Con1 genotype 1b containing NS3 to NS5b, see Methods), providing an unlimited source of HCV proteins actively replicating HCV RNA (Supplemental figure 1). Addition of the His6-tag did not influence replicon colony formation efficiency (Supplemental figure 1). RT-PCR sequencing of the replicon cell-line furthermore confirmed that the six histidines remained unchanged at passage 30 at which stage our experiments were conducted (Supplemental figure 1). In addition, lysates of the His6-NS3 replicon cell line show that the extra tag resulted in a slightly higher molecular weight compared to the control cell line (Fig.1A, lanes 1 and 2). Based on western blot, using NS3 antibody (NCL-HCV-NS3, Novagen) similar amounts of NS3 are expressed by both cell lines (Fig.1A).

An additional advantage of a His6-tag is that it allows for affinity purification under denaturing conditions. Hence, to maximise yields of purified NS3, an ureum/NP40-based buffer was used to lyse the cells and, following centrifugation, the His6-NS3 was purified from the supernatant using Cobalt²⁺ beads (See Methods). Figure 1 shows

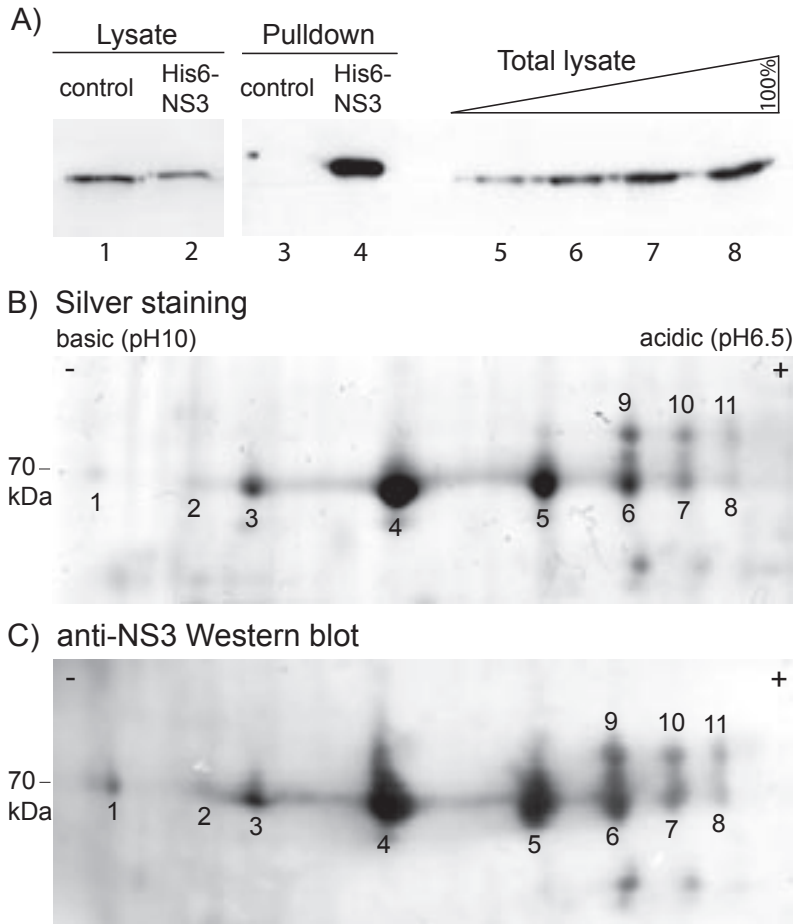
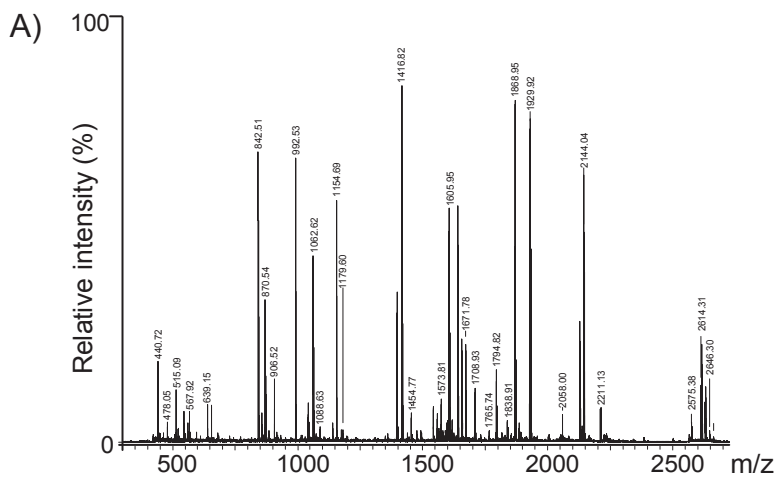


Figure 1 - Affinity purification and 2D-PAGE analysis of NS3 isoforms from Huh7 replicon cell line

A. Lysates of Huh7 replicon cells containing non-tagged NS3 (Control, lane 1) or His6-tagged NS3 (His6-NS3, lane 2), standardised to protein concentration using Bradford reagent (Biorad), were separated by SDS-PAGE, followed by western blot analysis with anti-NS3. These lysates were subjected to affinity purification under denaturing conditions, using Cobalt²⁺-beads (See Methods for further details). Lanes 3 and 4 represent (His6-)NS3 bound to the beads from control or His6-NS3 cell lines, respectively. Lanes 5 to 8 contain increasing volumes of total cell lysate from His6-NS3 cells, with 100% corresponding to the total amount of His6-NS3 used for pulldown.

Isolated His6-NS3 was separated on 2D-PAGE, followed by either silver staining (**B**) or western blotting using NS3-antibody (**C**). Each His6-NS3 spot is labelled with a number. Basic and acidic indicate where the negative and positive pole are positioned.



B)

1	MSRHHHHHHA	PITAYSQQTR	GLLGCIITSL	TGRDRNQVEG	EVQVVSTATQ
51	SFLATCVNGV	CWTVYHGAGS	KTLAGPKGPI	TQMYTNVDQD	LVGWQAPPGA
101	RSLTPCTCGS	SDLYLVTRHA	DVIPVRRRGD	SRGSLSPRP	VSYLKGSSTG
151	PLLCPSGHAV	GIFRAAVCTR	GVAKAVDFVP	VESMGTTMRS	PVFDTNNSPP
201	AVPQTFQVAH	LHAPTGSQKS	TKVPAAYAAQ	GKVLVLNPS	VAATLGFGAY
251	MSKAHGIDPN	IRIGVRTITT	GAPITYSTYG	KFLADGGCSG	GAYDIIICDE
301	CHSTDSTTIL	GIGTVLDQAE	TAGARLVVLA	TATPPGSVTV	PHPNIEEVAL
351	SSTGEIPFYG	KAIPETIKG	GRHLIFCHSK	KKCELAAKL	SGLGLNAVAY
401	YRGLDVSVIP	TSGDVIVVAT	DALMTGFTGD	FDSVIDCNTC	VTQTVDFSLD
451	PTFTIETTTV	PQDAVSRQR	RGRTGRGRMG	IYRFVTPGER	PSGMFDSSVL
501	CECYDAGCAW	YELTPAETSV	RLRAYLNTPG	LPVCQDHLEF	WESVFTGLTH
551	IDAFLSQTK	QAGDNFPYLV	AYQATVCARA	QAPPPSWDQM	WKCLIRLKPT
601	LHGPTPELLYR	LGAVQNEVTT	THPITKYIMA	CMSADLEVVT	

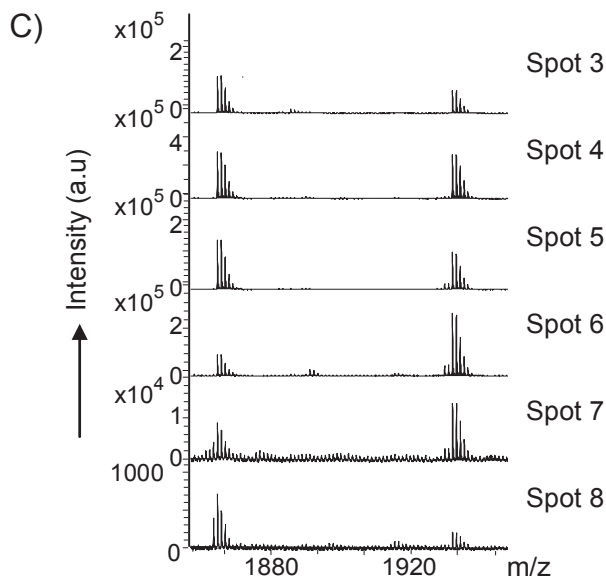


Figure 2 - MALDI-ToF analysis of affinity purified and 2D-PAGE separated NS3-isoforms

His6-tagged NS3 was affinity purified and separated using 2D-PAGE. Six individual proteins spots (3-8, Fig.1B) were digested with trypsin and analysed by MALDI-ToF MS. **A.** MALDI-ToF spectrum showing individual tryptic peptides from NS3. **B.** Primary sequence of His6-tagged NS3 with the sequence coverage obtained with MALDI-ToF MS in bold. **C.** Comparison of MALDI-ToF spectra showing two of the most abundant NS3 tryptic peptides (see A) in all the six protein spots.

specific pulldown of NS3, only when it is His6-tagged (compare lane 3 (control) and lane 4 (His6-NS3)). When relating the amount of purified His6-NS3 (lane 4) to the total amount of His6-NS3 in the lysate (lane 8), it is apparent that almost all the NS3 is pulled down under these purification conditions. This high yield makes it likely that all NS3 isoforms, if present, are isolated using this method.

2D-PAGE separation of NS3 isoforms

Protein isoforms may arise from PTMs, which introduce a variation in the molecular mass and/or charge of a protein. These isoforms can be efficiently separated on the basis of isoelectric point and molecular weight in 2D-PAGE (two-dimensional polyacrylamide gel electrophoresis). To examine possible isoforms of NS3, we analysed purified His6-NS3 using this approach. His6-NS3 was visualised by means of silver staining (Fig.1B) or western blot using NS3 specific antibodies (Fig.1C). With both techniques, at least 11 NS3 protein spots were resolved, with a pI ranging from approximately 7 to 9 and with an apparent molecular mass around 70 kDa. Lower molecular weight products were considered to be NS3 breakdown products. The split of NS3 into multiple pI variants indicate several NS3 protein modifications in the context of replication.

Tryptic digestion and MALDI-ToF analysis of individual NS3 spots

To unravel possible PTMs, responsible for the appearance of multiple spots of His6-NS3 after 2D-PAGE separation, tryptic digestion followed by mass spectrometric analysis was performed on spots 3 to 8. As an example, the MALDI-ToF spectrum of spot 5 is shown in Figure 2A. Additionally, samples were measured in a higher mass range (between m/z 3000-6000, data not shown). In total, peptides assigned to HCV NS3 add up to 60% coverage (Fig.2B, bold sequence). Although the sequence coverage was much lower in the digests of spots 7 and 8, NS3 specific peptides were also observed in these spectra (Fig.2C), confirming the results from the western blot

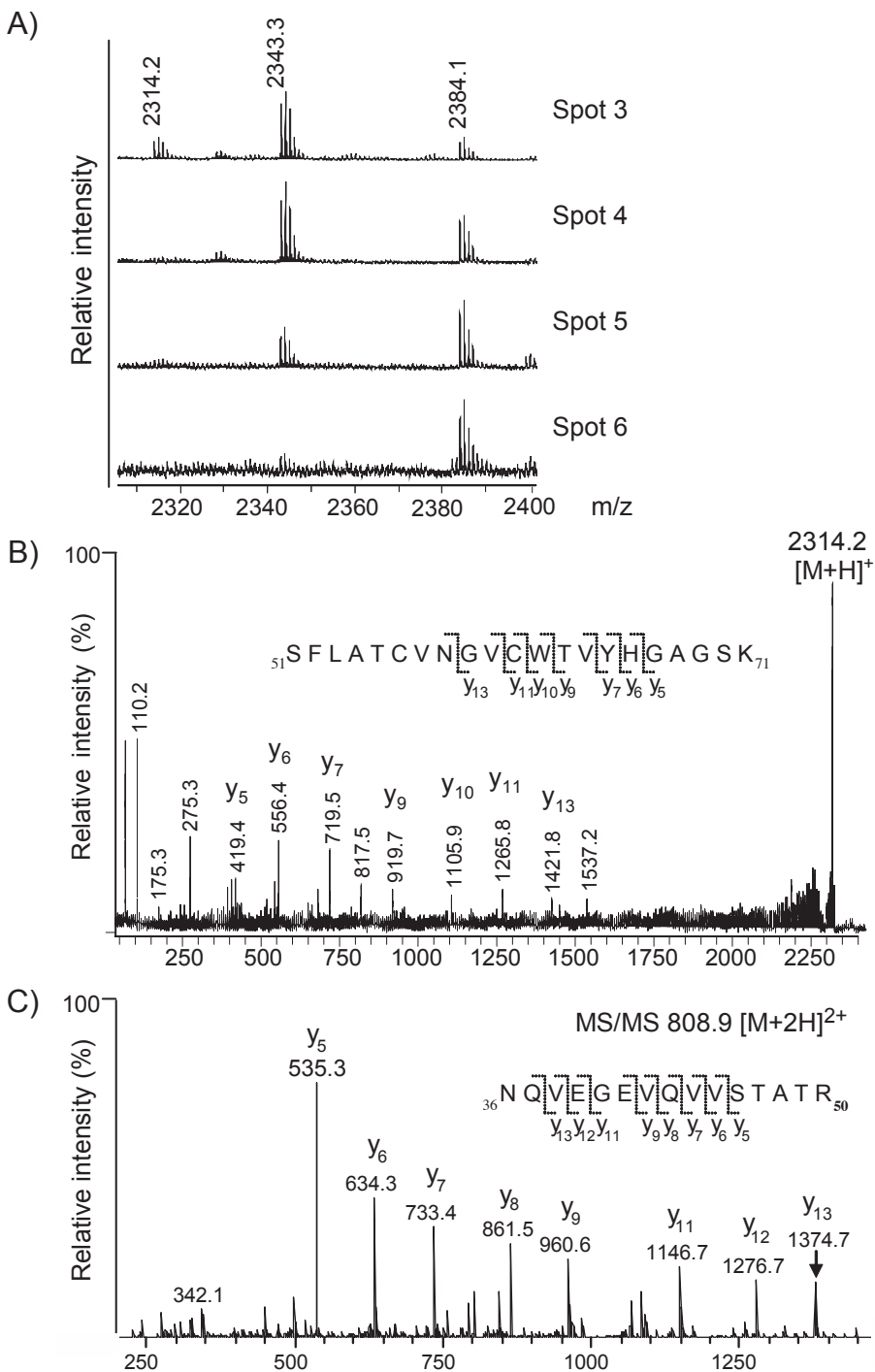


Figure 3 - Mass spectrometric characterization of NS3 mutation and post-translational modification

A. Comparison of MALDI-ToF spectra from NS3 isoforms in spots 3-6 (Fig.1B) The unique presence of a peptide at m/z 2314.2 in the tryptic digest of spot 3 and absence of the tryptic peptide at m/z 2343.3 in spot 6 are shown. MALDI-ToF-ToF revealed that the peptide at m/z 2343.3 corresponds to the acetylated N-terminal tryptic peptide of NS3, Ac-SRHHHHHHAPITAYSQQTR (data not shown)

B. MALDI-ToF-ToF analysis of the peptide at m/z 2314.2 showing that it matches with the peptide SFLATCVNGVCWTVYHGAGSK, corresponding to amino acid 51-71 in NS3 (Fig.2B). **C.** Identification of a peptide in the LC-MS run from the digest of protein spot 3, confirming Gln to Arg substitution at position 50 within NS3.

(Fig.1C). Since we observed similar intensities and comparable sequence coverages for the spots 3-6 (Fig.2C), these spectra were used for side-by-side comparison.

Identification of a Gln to Arg mutation in NS3

In the MALDI-ToF MS spectrum of spot 3, a peptide at m/z 2314.2 was observed, which was absent in the other spectra (Fig.3A). This mass could not be matched to an *in silico* tryptic peptide of NS3. MALDI-ToF-ToF analysis revealed that this ion corresponds to the peptide SFLATCVNGVCWTVYHGAGSK (aa 51-71, Fig.3B). This was unexpected because at position 50 within NS3 there is no tryptic cleavage site (Arg or Lys). We therefore speculated that NS3 corresponding to this spot was mutated at position 50. Indeed, using LC-MS, a peptide was observed with an Arg at position 50 (NQVEGVQVVSTATR (Fig.3C). Altogether, this revealed a Gln to Arg mutation in NS3 at position 50, corresponding to position 1067 of the polyprotein (HCV type 1b genome Con-1, AJ238799), which is likely to alter the isoelectric point of NS3.

Molecular mass based identification of HCV NS3 N-terminal acetylation.

During further analysis of the spots by MALDI-ToF a unique peptide at m/z 2343.3 was observed in spots 3, 4 and 5, which was absent in spot 6 (Fig.3A). This mass could not be assigned to any *in silico* tryptic peptide belonging to NS3. MALDI-ToF-ToF and manual inspection of the spectrum revealed a series of His residues in this peptide (data not shown). This indicates that the peptide corresponds to the N-terminus of NS3 and suggests that an N-terminal modification can explain these results. Indeed N-terminal acetylation (including removal of the Met) would

generate a m/z of 2343.3 (Acetyl-SRHHHHHHAPITAYSQQTR) corresponding to the observed mass and also would explain the MALDI-ToF-ToF fragmentation pattern (data not shown). This modification, which occurs on approximately 85% of all eukaryotic proteins ³⁰, could therefore at least explain some of the different spots we observed. It should be noted that normally the N-terminal end of NS3 is generated by (auto-) proteolytic cleavage of the HCV polyprotein and that the initiator methionine is added for construction in the HCV replicon ³¹.

Additional comparison of the MALDI-ToF spectra derived from the separate spots yielded no qualitative differences that could be explained by modifications. In conclusion, using this mass spectrometry based approach, we elucidated one modification and a mutation, but still lack an explanation for the other different isoforms observed.

HCV NS3 is phosphorylated

One of the most common post-translational modifications of proteins is phosphorylation (estimated one third of all cellular proteins ³²), which leads to a change of the net charge of proteins and thus the migration behaviour during 2D-PAGE. Consequently, comparison of 2D-gel spot patterns before and after treatment of the sample with a

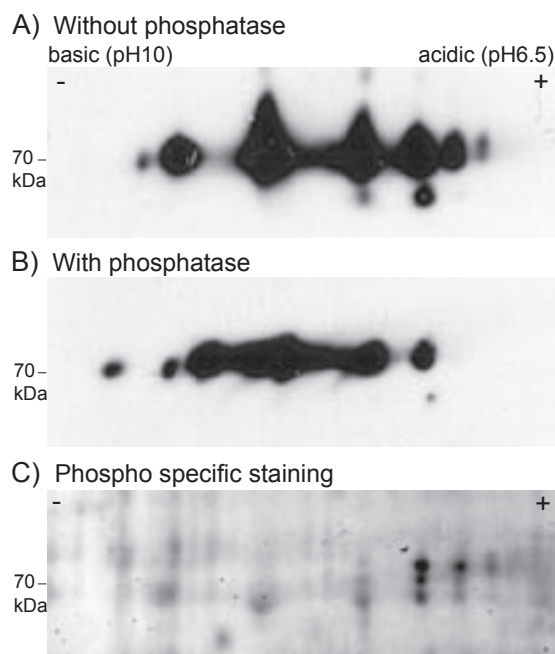


Figure 4 - NS3 phosphorylation

Purified His6-NS3 from Huh7 replicons was incubated without (**A**, **C**) or with alkaline phosphatase (**B**) at 37°C for 1 h. The samples were then concentrated, followed by separation on isoelectric point and molecular weight. Subsequently western blot detection was performed using anti-NS3 (**A**, **B**). Alternatively, the gel was directly stained with a phospho-specific stain (See Methods) (**C**). The negative and positive pole position are indicated with basic and acidic, respectively.

phosphatase can be utilised to assess the phosphorylation state of a protein ³³.

Therefore, phosphatase treated and untreated His6-NS3 samples were analysed by 2D-PAGE and stained with NS3 antiserum. In the untreated sample, we detected several spots in a similar pattern as shown before (Fig.4A). Upon treatment with phosphatase, the two most acidic spots disappeared and one extra spot appeared at the alkaline (basic) side of the gel (compare Fig.4A and B). This shift of spots towards the basic pole, after removing negatively charged phosphate group, clearly indicates phosphorylation of His6-NS3. Distinct spots resembling spots 9, 10 and 11 in Figure 1C were not detected in these experiments. This might be due to the long phosphatase procedure before separation in the two-dimensional gel electrophoresis (see Methods).

To further corroborate these results, we stained the 2D-PAGE gels directly with proQ diamond, a dye that selectively stains phosphorylated proteins in polyacrylamide gels (Fig.4C). The phospho-specific stain mainly coloured the acidic spots, which are the low abundant isoforms of NS3. Conversely, highly abundant NS3 isoforms were hardly stained. This is in accordance with the phosphatase treatment experiment, as the most acidic spots are lost after addition of phosphatase.

Taken together, the phosphospecific staining and the de-phosphorylation assay strongly suggest phosphorylation of NS3.

Discussion

Identification of NS3 modifications in the context of self-replicating HCV RNA is complicated, given that only low amounts of protein are available and a combination of processes can influence the modification states of NS3. For that reason we designed an isolation method to purify NS3 from replicon cells. A His6-tag was added to the N-terminus of NS3 in this system that comprises HCV proteins NS3 to NS5b. With this approach, high amounts of NS3 can be isolated (Fig.1A) and is thus likely to include all NS3 isoforms. Since modifications can induce changes in the isoelectric point (pI) and molecular weight of proteins, NS3 variants were visualised by two-dimensional gel electrophoresis (2D-PAGE). Purified NS3 was found to show multiple pI variants (Fig.1B and C), indicating several NS3 protein modifications in the context of replication. We uncovered protein modifications for some NS3 isoforms using various approaches.

Using mass spectrometry, we analysed four NS3 spots (spots 3-6) for modifications. Comparison of MALDI-ToF spectra from spots 3, 4 and 5 with spot 6 resulted in the identification of N-terminal acetylation of His6-NS3 (Fig.3A, m/z 2343.3). N-terminal acetylation is the most common covalent modification of eukaryotic proteins³⁰ and can affect protein-protein interactions, activity or stability of proteins³⁴⁻³⁶. Viral proteins can also be acetylated at their N-terminus, such as mature Core protein from HCV and Gag protein from L-A double-stranded RNA virus^{37, 38}. In the latter virus acetylation affects viral assembly³⁸. Since we use the HCV replicon system acetylation might be the result of the introduced starting methionine used in this construct. However, we cannot exclude N-terminal acetylation on proteolytically processed NS3 derived from the polyprotein.

Another change we observed was a Gln to Arg mutation at position 50 (corresponding to 1067 in the polyprotein), which would add a significant charge difference to the NS3 protein (pI 7.5 versus 7.8). This mutated arginine residue introduced an extra trypsin cleavage site resulting in two peptides (NQVEGVQVVSTATR at m/z 808.9 $[M+2H]^{2+}$ and SFLATCVNGVCWTVYHGAGSK at m/z 2314.2 $[M+H]^+$, respectively) in spot 3 of NS3 (Fig.3). This Q1067R is located close to the active site of the NS3 protease³⁹. An identical mutation at this position was described for a HCV genotype 1a replicon⁴⁰. Interestingly, this mutation was shown to compensate for a negative effect located in the proximal NS3 protease region of the HCV genotype 1a and found to enhance the replication capacity of the viral RNA⁴⁰. Surprisingly, we now observe this compensating mutation at the protein level in the Con1 HCV genotype 1b, which is normally not down modulated and does not accumulate mutations at this position.

Protein phosphorylation is an important regulator of diverse intracellular processes. HCV NS5a is a phosphoprotein, involved in replication and virus particle formation. In both processes the phosphorylation status of NS5a was shown to be critical^{8, 41-43}. The multifunctional protein NS3 might be regulated in a similar way. Since we observed a train of spots, which might indicate phosphorylation, we investigated this possibility for NS3. Both treatment with phosphatase and specific staining of phosphate groups by ProQ diamond illustrate phosphorylation of NS3 (Fig.4). Multiple phosphorylation sites are suggested within NS3 using the phosphorylation prediction program NetPhos 2.0, i.e. 17 serine-, 13 threonine- and 4 tyrosine-

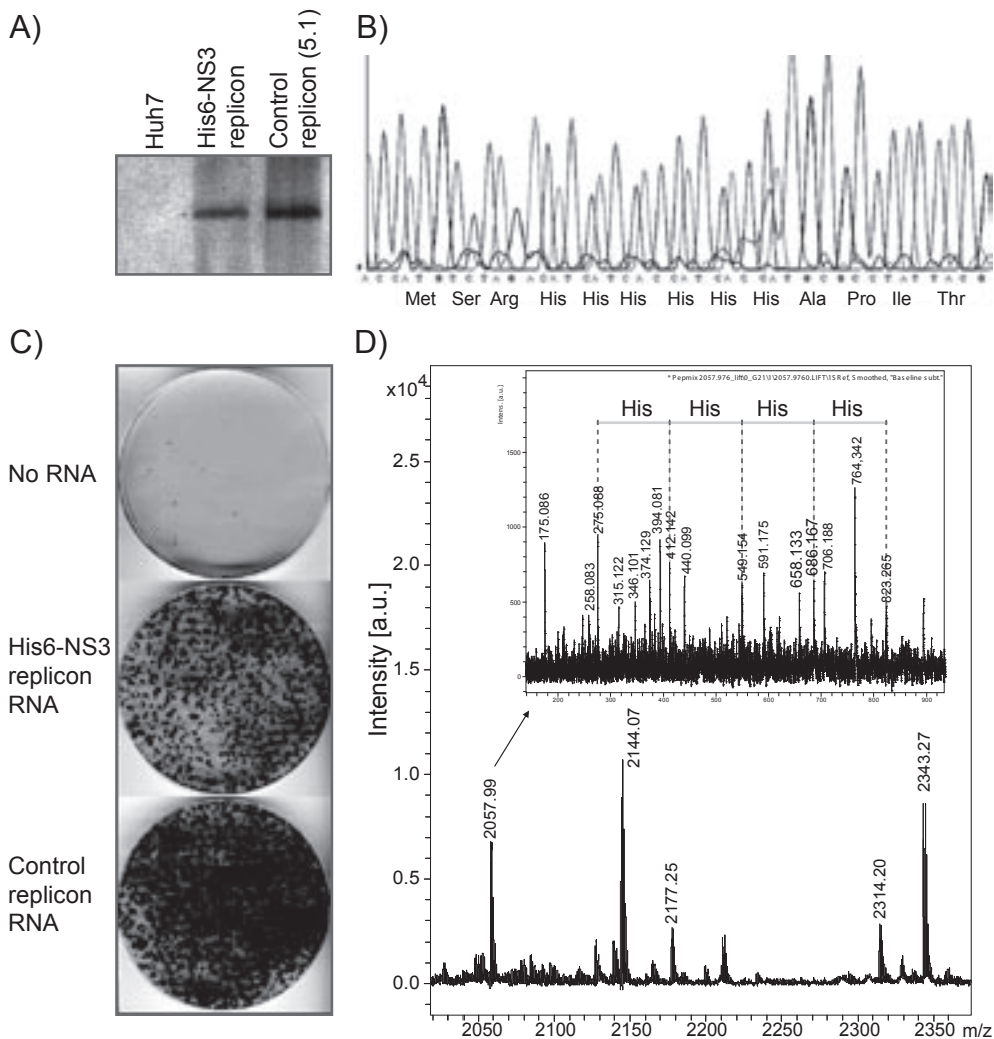
phosphorylation sites. As illustrated in Figure 4 phosphorylated NS3 protein is low abundant, therefore concentration of phosphorylated peptides was necessary to try to identify the phosphorylated amino acid in NS3. Phosphorylated peptides, generated from a pool of NS3 isoforms, were enriched using TiO_2 -beads prior to analysis by mass spectrometry. However, using this approach no phosphorylated peptides could be identified. As illustrated in Figure 3 phosphorylated NS3 protein is low abundant. Additionally lower signal intensities are often observed for phosphopeptides, possibly due to low ionisation efficiency ⁴⁴. Moreover, the phosphorylation site might be located within a tryptic peptide that is not well suited for our standard mass spectrometric analysis (Fig.2B). Usually tryptic digestions are measured in the mass range of 500 to 3500 Da. When only peptides between 500 and 3500 Da from the in-silico digest of His6-NS3 were taken into account, we observed coverage around 92%. Altogether, this might explain the difficulty to identify the site of phosphorylation.

pI heterogeneity of a protein can be generated by PTMs, chemical reactions like deamination ⁴⁵, or it could be due to conformational isomers of a protein ⁴⁶. All result in multiple isoforms observed in 2D-PAGE. In contrast a PTM that does not affect the pI of proteins is methylation ⁴⁷. Methylation was described to take place on amino acid 1493 and/or 1490 of NS3 (Fig.2B, aa 473 and 475) ²¹. In our experiment, we were not able to detect specific peaks in this region, since digestion with trypsin in the area of NS3 methylation generates very small peptides (5 amino acids), which are difficult to assign specifically to NS3.

Taken together we identified modifications of His6-NS3 in the context of self-replicating HCV RNA, such as N-terminal acetylation, a Q1067R point mutation and phosphorylation. Resolving the precise sites of phosphorylation and determining their function will be the next challenging step. Generation of replicons with mutations in the predicted phosphorylation sites, in total around 34 sites, followed by 2D-PAGE analysis could result in identification of specific phosphorylation sites, which might help our understanding of the orchestration of the HCV life cycle.

Acknowledgements

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Supplemental figure 1 - Analysis of the His6-NS3 replicon cell line.

A. [^3H] uridine labeling of total RNA isolated from 1×10^6 cells incubated for 12 hours in the presence of $50 \mu\text{Ci}$ of [^3H] uridine and 5mg/ml Actinomycin D.

B. Isolated RNA from the His6-NS3 replicon was subjected to sequence analysis after reverse transcriptase and PCR amplification of the 5' region of NS3.

C. Coomassie stained culture dish 3-weeks after electroporation of Huh-7 cells with no (top) or 10 ng of in vitro transcribed His6-NS3 tagged replicon RNA (middle) or untagged control replicon RNA in the presence of $500 \mu\text{g/ml}$ G418 (bottom).

D. MALDI-ToF and MALDI-ToF-ToF of $2058 [\text{M}+\text{H}]^+$ corresponding to N-terminal tryptic peptide of NS3 showing the presence of the consecutive histidines.

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**Isolation of Hepatitis C virus
NS3 interacting proteins in the
context of replication**



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Abstract

The life cycle of a virus greatly depends on interactions with the host cell. This is not different for the Hepatitis C virus, which is a positive single-stranded RNA virus and a major cause of chronic liver disease. An important Hepatitis C viral protein is non-structural protein 3 (NS3), because it participates in viral translation, replication and virus assembly. Moreover, it can modulate several cellular pathways by association with host cell proteins. Therefore, an assay was set-up to investigate virus-host protein interactions of this multifunctional protein. A His6-tag was introduced in the subgenomic replicon genome at the amino-terminus of NS3. This enabled us to isolate NS3 from Huh7 cells containing this subgenomic replicon, accordingly in the context of active replication. Several methods were explored to purify NS3 and interacting proteins, including pulldown under native conditions, NS3 isolation after *in vivo* crosslinking and purification with mild detergent Nonidet P-40 (NP40). In this chapter we describe, that *in vivo* crosslinking with paraformaldehyde resulted in co-purification of NS5a with NS3. Furthermore, we demonstrate that NS3 can be isolated using only 0.05% NP40. Additionally, we show by density gradient studies that, at this detergent concentration, endoplasmic reticulum membranes are disrupted. By means of mass spectrometry protein identification in combination with one-dimensional as well as two-dimensional polyacrylamide gel electrophoresis, two NS3 interacting proteins were identified i.e. Glycosyl transferase 25 containing domain 1 and lysyl hydroxylase 3. Both proteins are involved in lysine glycosylation of collagenous proteins.

Introduction

Hepatitis C virus (HCV) is a major healthcare problem, about 2.2–3% of the world's population is chronically infected ¹. The virus is transmitted by infected blood and blood products, therefore the widespread increase of iatrogenic procedures and intravenous drug use coincided with the global spread of HCV genotypes 1b and 1a in the 1940s and 1960s respectively ². Nowadays blood and blood products are screened for HCV, still yearly around 2.3–4.7 million people become newly infected ¹. The current treatment, a combination of ribavirin and pegylated interferon alpha, is effective in only 55% of the patients ³. Most drugs under development target the viral enzymes, though other strategies could be directed at affecting virus-host interactions. To design these new drugs more knowledge of the virus life cycle is required and especially of the virus-host interactions.

The genome of the positive single-stranded RNA virus encodes one large polyprotein, which is cleaved into 10 viral proteins ^{4,5}. The two main viral enzymes produced are the RNA-dependent RNA polymerase, NS5b and the multifunctional protein NS3 ⁶. NS3 contains ATPase, helicase and protease activities, which point out why it is essential during viral replication and translation ⁷⁻⁹. In addition, mutational studies show that virus assembly also requires NS3 ^{10,11}. Furthermore, NS3 is suggested to modulate the innate immune response (reviewed in ¹²), to adjust intracellular transport ^{13,14} and to alter various signal transduction pathways ¹⁵. These pathways are often affected by NS3 as a result of association with host proteins. For example, the TBK1-NS3 interaction abolishes downstream signalling of the interferon-alpha pathway by prohibiting TBK1 to bind to IRF-3 ¹⁶. TRIF and Cardif (also known as IPS-1, MAVS or VISA) are protease substrates of NS3 and are inactivated by cleavage; consequently, the interferon-alpha signalling is abrogated ¹⁷⁻¹⁹. Additionally, NS3 might have oncogenic properties as well, due to association with p53, protein arginine methyltransferase 5 (PMRT5) and Ataxia telangiectasia mutated (ATM) ²⁰⁻²³.

The interaction between NS3 and some of the host proteins have been identified using yeast-two-hybrid screens (Y2H) ^{13,20,23-25}. However there are certain drawbacks of this technique, since only binary interactions and not complexes can be investigated. Additionally, classical (or conventional) Y2H systems suffer from false negative and false positive protein-protein interactions. These are attributable to improper fold-

ing of the fusion protein, failure in nuclear localisation, dependence of interactions on protein modification or non-relevant interaction imposed by overexpression (Reviewed in ²⁶). Similar disadvantages have to be considered in immunoprecipitation of overexpressed epitope-tagged proteins (Reviewed in ^{27, 28}). Therefore, an alternative method to identify NS3 associating proteins was chosen here.

To investigate host cell interactions of NS3, we took the approach of protein purification by tag-affinity in the context of active replication. Therefore, a small His6-tag was introduced at the N-terminus of NS3 in the HCV subgenomic replicon genome. This tag was used to affinity purify NS3 from the human hepatoma cell line, Huh7, harbouring this replicon expressing NS3 to NS5b and actively replicating the HCV subgenome. Using this system, we could explore several possible methods to identify NS3 interacting proteins. First, native conditions were tried for NS3 purification. Secondly, *in vivo* protein crosslinking experiments were carried out, to stabilize NS3 interactions with host-proteins in their native environment, followed by purification of NS3 under denaturing conditions. Thirdly, low concentrations of detergent were used to isolate NS3 and interacting proteins. With this approach we could identify two new interacting proteins, both involved in lysyl-glycosylation of collagenous proteins.

Methods

Culturing Huh7 replicon cells

Human hepatoma cell lines, Huh7, harboring the HCV replicons were grown in Dulbecco's Modified Eagle's Medium without NaPyruvate, supplemented with non-essential amino acids, L-glutamate, Penicillin, Streptavadin and Geneticin. Cells were subcultured using trypsin.

Lysis of cells under native conditions and addition of 0.05% NP40

Huh7 replicon containing cells were washed three times with PBS (154 mM NaCl, 1.4 mM Phosphate, pH 7.5), after which the cells were scraped in an isotonic buffer, containing 50mM Tris pH 7.0, 15 mM NaCl, 1 mM MgCl₂, 240 mM sucrose and 20 mM imidazole. Subsequently, the cells were lysed with a ball-bearing homogeniser (Isobiotec, Heidelberg Germany), which has a clearance of 18 micron. Total lysate

volume of a 15 cm dish ($\sim 1.5 \times 10^7$ cells) was 2 ml. Whole cells and cell debris was spun down at $800 \times g$ for 10 min and supernatant was collected. Supernatants of the two cell lines with and without His6-tag at NS3 were standardised to protein concentration using Bradford reagent (Biorad). When NS3 isolation was performed in the presence of 0,05% NP40, detergent was added prior to beads incubation and left on ice for 30 min.

Lysis of cells with Triple detergent buffer

Huh7 cells harbouring the HCV replicon were lysed directly after washing with PBS in Triple detergent buffer, which is the isotonic buffer supplemented with 0.1% SDS, 0.5% DOC and 1% NP40. Cell lysates were subjected to centrifugation at $800 \times g$ for 10 min and the supernatants were used for NS3 isolation.

Crosslinking and lysis of the cells

After washing with PBS, Huh7 replicon cells (15 cm dish) were incubated with 3ml 0.5% paraformaldehyde (PFA) in PBS for 10 min. The crosslinking reaction was stopped with 5ml 1 M glycine, 1 M Tris buffer (pH 7.0) for at least 30 min. Subsequently the cells were washed with PBS and lysed with denaturing buffer (8 M Urea, 50 mM Tris pH 7.0, 300 mM NaCl, 1% NP40, 10 mM imidazole). Lysates were subjected to NS3 isolation after centrifugation at $14,000 \times g$ for 15 min.

NS3 isolation and elution from the beads

Cell lysate from one 15 cm dish ($\sim 1.5 \times 10^7$ cells) was incubated with 80 μ l of 50% Cobalt²⁺ bound-beads (Clontech) for 2 h under continuous rotation. The beads were washed three times with the appropriate lysis buffer. After washing, Laemmli was added to the beads and heated to 95°C for 10 min. Except for the PFA crosslinked samples, these were either heated to 65°C for 10 min or to 95°C for 20 min.

To elute associated proteins from the beads, the beads were incubated in lysis buffer containing 190 mM imidazole, under continuous rotation for 10 min. The supernatant was taken off the beads and pooled with other eluates.

Protein concentration using Trichloroacetic acid or ethanol

Eluate was adjusted to 25% trichloroacetic acid (-20°C) and incubated overnight at -20°C. After centrifugation at $14,000 \times g$ for 30 min, 0.05 N HCl Acetone was added to the pellet and subjected to identical centrifugation. This was followed by addition of Acetone to the pellet and another round of centrifugation. The pellet was taken up

in Laemmli-buffer and if required pH was corrected.

Another method applied to concentrate proteins is by the addition of at least 6 volumes of -20°C ethanol to the eluate. After overnight incubation the proteins were spun down at $14,000 \times g$ for 1 h. Pellets were taken up in 2D-PAGE buffer (see 2D-PAGE for composition of the buffer).

Membrane floatation assay

In total 15×10^7 Huh7 cells containing HCV replicon were scraped in isotonic buffer and spun down at $500 \times g$ for 5 min. The pellet was solved in 1 ml isotonic buffer, which was followed by mechanical lyses using the ball-bearing homogeniser. After centrifugation at $800 \times g$ for 10 min, the supernatant was split in two. To one part 0.05% NP40 and sucrose with a final density around 1.26 g/ml was added, the other part was only mixed with sucrose. These samples were loaded underneath a sucrose gradient ranging from densities 1.09 to 1.23 g/ml (20%–70% w/v sucrose, 50 mM Tris pH 7, 1 mM MgCl_2 , 15 mM NaCl) and subjected to centrifugation in a SW41 tube at $100,000 \times g$ for 14 h (Beckmann ultracentrifuge). Subsequently 500 μl samples were collected from the top to the bottom and the even fractions were analyzed by SDS-PAGE and western blotting.

SDS-PAGE and western blotting

After heating in Laemmli-buffer, proteins were separate on SDS-PAGE gels (sodiumdodecylsulfate polyacrylamide gel electrophoreses). Subsequently, proteins were transferred to PVDF-membranes (Hybond-P, GE-Healthcare) using a Semi-Dry blot apparatus (Biorad). Blocking of the membranes and the following antibody incubations were performed in PBS containing 0.5% Tween-20, 5% non-fat, dry milk (Campina). Since the secondary antibodies are conjugated to horseradish peroxidase (HRP), enzyme-catalyzed chemoluminescence (ECL+, GE-Healthcare) was used for detection. The signal was captured on Fuji Super RX medical X-ray film or visualised using a Typhoon Imager 9410 (GE-Healthcare).

Antibodies

Primary antibodies used in this study are anti-His6 (mouse, Abcam), anti-NS3 (mouse, Nova castra), anti-NS5a (mouse, Virostat), anti-NS5b (mouse, Virostat), anti-calnexin (mouse, BD Biosciences), anti-GAPDH (goat, Santa Cruz), anti-PDI (rabbit, Stressgen), anti-LH3 (Mouse, Abnova). The primary antibodies against GLT25D1 and NS4b were raised against a specific protein peptide in rabbits, CKT-

DWDRAKSQKMRE (Biogenes) and CRGNHVSPTHYVPES (Innovagen), respectively. The serum from GLT25D1 peptide immunised rabbits was peptide purified to increase specificity. Secondary antibodies goat-anti-mouse-HRP, rabbit-anti-goat-HRP and swine-anti-rabbit-HRP were all purchased from Dako.

Two-dimensional poly-acrylamide gel electrophoresis (2D-PAGE)

Protein pellets were solved in 2D-PAGE buffer, which contains 8 M ureum, 4% CHAPS (3-[(3-Cholamidopropyl)dimethylammonio]-2-hydroxy-1-propanesulfonate), 15 mM Tris pH 8.5, 1% dithiothreitol, 0.5% immobilised pH gradient (IPG) buffer pH 3-10 (GE Healthcare). Before starting isoelectric focusing of the sample, Immobiline DryStrips pH 3-10, 13 cm (GE-Healthcare) were incubated for at least 6 h with 0.5% IPG-buffer in De-streak rehydration solution (GE-Healthcare). Samples were applied to the strips at the basic end via cup-loading. Isoelectric focusing was performed on an Ettan IPGphor™ 3 Isoelectric Focusing System using the following program: 3 h at 150 V, 3 h at 300 V, gradually to 1000 V in 6 h, gradually to 8000 V in 1 h and 8000 V for 2 h. Thereafter the strips were equilibrated in two consecutive 15 min incubation steps with Equilibration buffer (6 M ureum, 50 mM Tris (pH 8.8), 30% Glycerol and 4% SDS), containing in the first step 2% DTT and in the second 2.5% iodoacetamide and brome phenol blue (BPB). Before starting second dimension separation, the strips, on top of the SDS-PAGE gel, were covered with BPB coloured agarose. Following SDS-PAGE, the gels were subjected to silverstaining.

Silverstaining

SDS-PAGE gels were fixed in 50% ethanol, 12% acetic acid, washed with 50% ethanol and sensitised for 30 min using sensitise solution (1.5 mM sodiumthiosulfate). After washing with H₂O, Silver solution (12 mM silvernitrate, 0.075% formaldehyde) was added for 30 min. Subsequently, the SDS-PAGE gels were rinsed with H₂O and stained with Develop solution (2.5% sensitise solution, 0.57 M sodium-carbonate, 0.05% formaldehyde) until desired staining was reached. 50% methanol, 12% acetic acid was used to stop the staining reaction.

In-gel digestion of proteins

The desired gel bands, isolated from gel, were digested with the Proteineer DP digestion robot (Bruker, Bremen, Germany), following the protocol supplied by the manufacturer, which is based on the protocol by Shevchenko et al ²⁹. The plate holder was modified in house to accommodate a Millipore solvinert hydrophilic filter plate,

type MSRLN04. After overnight digestion the peptide fragments were extracted with water/acetonitril/formic acid 1/1/1 v/v/v.

Mass spectrometry and database searching

The extracts were freeze dried and dissolved in 95/3/0.1 v/v/v/ water/acetonitril/formic acid and subsequently sequenced by tandem mass spectrometry. Peptides were analysed by nanoflow liquid chromatography using an Agilent 1100 HPLC system (Agilent Technologies) coupled on line to a 7-tesla LTQ-FT Ultra mass spectrometer (Thermo Electron, Bremen, Germany).

The chromatographic system consisted of the following components. ReproSil-Pur C18-AQ, 3 μm (Dr. Maisch GmbH, Ammerbuch, Germany) was used as a resin for the analytical nano column and AQUA-C18 5 μm was used as a resin for the trapping column. Peptides were trapped at 5 $\mu\text{l}/\text{min}$ on a 1 cm column (100 μm internal diameter, packed in house) and eluted to a 15 cm column (50 μm internal diameter, packed in house) at 150 nl/min in a 60 min. gradient from 0 to 50% acetonitrile in 0.1% formic acid.

The eluent was sprayed via emitter tips (made in house) butt-connected to the analytical column. The mass spectrometer was operated in data-dependent mode, automatically switching between MS and MS/MS acquisition. Full scan MS spectra were acquired in FT-ICR with a resolution of 25,000 at a target value of 5,000,000. The two most intense ions were then isolated for accurate mass measurements by a selected ion-monitoring scan in FT-ICR with a resolution of 50,000 at a target accumulation value of 50,000. These ions were then fragmented in the linear ion trap using collision-induced dissociation at a target value of 10,000. In a post analysis process, raw data were converted to peak lists using Bioworks Browser software, Version 3.1. For protein identification, MS/MS data were submitted to the SwissProt database using Mascot Version 2.1 (Matrix Science) with the following settings: 2 ppm and 0.8 Da deviation for precursor and fragment masses, respectively; trypsin was specified as the enzyme. All reported hits were assessed manually, and peptides with MASCOT scores lower than 40 were usually discarded.

RNA isolation and RT-PCR

RNA was isolated using High Pure Viral RNA Kit (Roche). In brief, 35 μl eluate was mixed with 400 μl binding buffer containing carrier RNA (10 $\mu\text{l}/\text{ml}$) and applied to the filter. After centrifugation at 8000 $\times g$, the filter was rinsed with 500 μl Inhibitor

Removal Buffer and washed two times with 450 µl Wash Buffer. Subsequently the RNA was eluted from the filter with 14 µl Elution Buffer and used for cDNA synthesis. 5x buffer, reverse transcriptase (Thermoscript), dNTP's, RNaseOUT and the primer GGCCTGGAGTGKTTAGCTCCCCRTTCA (HCV genome 9372-9398) were added to the isolated RNA and incubated for 1.5 h at 50°C. The cDNA synthesis reaction was terminated for 5 min at 85°C and RNA was degraded by RNaseH. PCR was performed using the RT-primer and CACAACATCCCTCAGCGCAAG (HCV genome 7717-7735), in the presence of dNTP's, DMSO, buffer 3, Expand enzyme Taq/T90 + proofreading and 2 or 4 µl of cDNA. After 40 cycles of PCR (15 sec 94°C, 30 sec 57.3 °C and 3 min 68 °C), the PCR products were analysed by electrophoresis on a 1% agarose gel.

Results and Discussion

Introduction of His6-tag into HCV replicon cell line

In order to identify NS3 interaction partners in the context of active replication, we introduced a His6-tag at the N-terminus of NS3 in the replicon RNA genome (genotype 1b expressing NS3 to NS5b) (pFK5.1Neo³⁰). This tag can be used for purification of NS3 from lysates of the human hepatoma 7 (Huh7) cell line containing this subgenomic HCV replicon. In addition, a tagged replicon cell line provides an unlimited source of functional (His6-tagged) NS3 protein. Our group previously showed that replicon colony formation efficiency of this N-terminally His6-tagged NS3 replicon cell line was not influenced by the addition of the His6-tag, compared to the control cell line³¹. In addition to that, we examined whether both cell lines express similar amounts of NS3 protein, which has a molecular weight of about 70 kDa (Fig.1). Cell lysates from the N-terminally His6-tagged NS3 (+) and the control Huh7 replicon cell lines (–, without His6-tag) were separated on SDS-PAGE. Subsequently western blot analysis was performed with an antibody against NS3. Figure 1 shows expression of NS3 at comparable protein levels in both replicon cell lines (compare lanes 1 and 2). Furthermore, demonstrated by RT-PCR sequencing of the replicon RNA, the His6-tag remained unchanged in the tagged NS3 cell line for at least 30 passages³¹. Before or at this stage our experiments were conducted. The presence of the His6-tag was also confirmed at protein level using SDS-PAGE and western blot analysis with an antibody against His6 (Fig.1). This antibody recognises

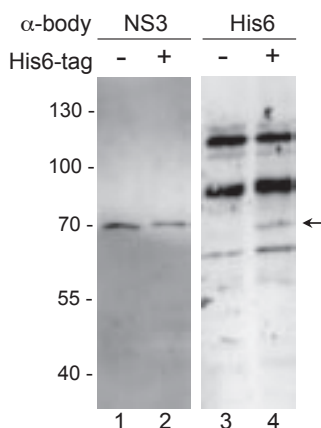


Figure 1 - Presence of His6-tag at amino-terminus of NS3 within replicon containing cells

Lysates of Huh7 cells, containing subgenomic replicons with (+) (lanes 2 and 4) or without (-) (lanes 1 and 3) a His6-tag at the amino-terminus of NS3, were loaded on SDS-PAGE gels. This was followed by western blotting using either anti-NS3 (left panel) or anti-His6 antibodies (right panel). Arrow indicates the NS3 protein.

a specific band in the NS3 His6-tagged replicon cell line (+) at 70 kDa (Fig.1; lane 4), corresponding to NS3 (Fig.1; lane 2). Given that the His6-tag is the only difference between the two replicon cell lines, these results clearly show that an N-terminally His6-tagged NS3 protein is present in the replicon cell line.

Purification of His6-NS3 from the HCV replicon cell line under native conditions

A first step towards identification of NS3 interacting proteins is the isolation of His6-NS3 from this replicon cell line, showing that the His-tag is available for purification. A pulldown experiment was performed using a buffer containing a mixture of three detergents, sodium dodecyl sulfate (SDS), sodium deoxycholate (DOC) and Nonidet P-40 (NP40) (Fig.2). Huh7 replicon containing cells with (+) or without (-) an N-terminal His6-tag at NS3 were lysed in this Triple detergent buffer. After centrifugation, removing nuclei and large cell debris at 800 ×g, Cobalt-beads were added to the supernatant (Lysates) for affinity purification of His6-NS3. The lysates and the proteins associated to beads (Bound) were separated on SDS-PAGE, followed by western blotting using an antibody against NS3. As shown in Figure 2, similar amounts of NS3 were present in the lysates of the His6-NS3 cell line (+) and the control cell line without the His6-tag (-) (Fig.2; top panel, lanes 1 and 2). In the lanes loaded with proteins associating to the beads, we observe that NS3 only binds to the Cobalt-beads when tagged with His6 (+) (Fig.2; bottom panel, lanes 1 versus 2). This clearly demonstrates that NS3 can be specifically purified from the replicon cell line, which is in line with the results from our previous report ³¹.

To preserve protein-protein interactions as much as possible, native conditions during isolation are essential. We therefore used an isotonic buffer, to harvest the cells in, in combination with a ball-bearing homogeniser for mechanical lysis of the cells (Iso), which keeps intracellular membranes intact. Compared to the Triple detergent lysed cells, less NS3 was observed in the 800g supernatant from cells lysed with the ball bearing homogeniser (Fig.2; top panel, lanes 3 and 4). Most NS3 is in the 800 g pellet, which is likely due to membrane structures, to which NS3 associates, ending up in this pellet fraction (data not shown). When focused on NS3 purification, we observe no to minimal binding of His6-NS3 to the beads (Fig.2; bottom panel, lanes 3 and 4), indicating that isolation of high amounts of NS3 under completely native conditions is not achievable. According to these results the His6-tag at the N-terminus of NS3 does not seem accessible during a native pulldown, whilst is available when detergents are present. Detergents dissolve cellular membranes and can solubilise proteins and protein-complexes, possibly resulting in the accessibility of the His6-tag for isolation.

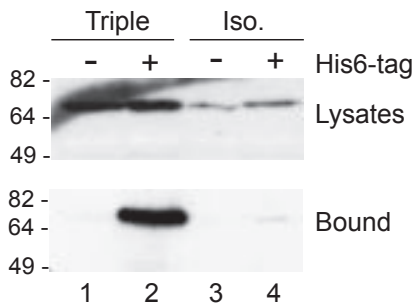


Figure 2 - Isolation of His6-tagged NS3 from replicon containing cells

Huh7 cells, harbouring replicons with (+) or without (-) a His6-tag at NS3, were lysed in a triple detergent buffer (Triple), containing sodium dodecyl sulfate, sodium deoxycholate and Nonidet P-40. Otherwise, the cells were lysed with a ball-bearing homogeniser in isotonic buffer (Iso). Following an 800 xg spin, the supernatant (Lysate) was subjected to NS3 purification using Cobalt-bound beads (for more details see Methods). The 800g supernatant (Lysate, top panel) and the proteins associated to the beads (Bound, bottom panel) were analysed by SDS-PAGE and western blotting with an antibody against NS3.

Purification of His6-NS3 and complexes from the HCV replicon cell line after *in vivo* crosslinking with paraformaldehyde

Apparently, more stringent conditions are required to make the His-tag available. Therefore chemical cross-linking followed by purification under denaturing conditions was performed. This is an alternative method to study protein-protein interactions *in vivo*, where crosslinks preserve protein associations that occur in replicon cells.

Paraformaldehyde (PFA) is a cross-linker that can be used for such purposes^{32, 33} because it can quickly permeate the cell membranes, it has a short distance crosslink arm of 2.3 to 2.7 Å and the formed crosslinks can be disrupted by heating, which makes subsequent analysis possible. Furthermore, the crosslinks stay intact at non-physiological conditions and for that reason can be subjected to stringent purification buffers for the removal of contaminants and non-specifically bound proteins³⁴. The latter is also compatible with the His6-tag, which can be used for isolation under denaturing conditions³¹.

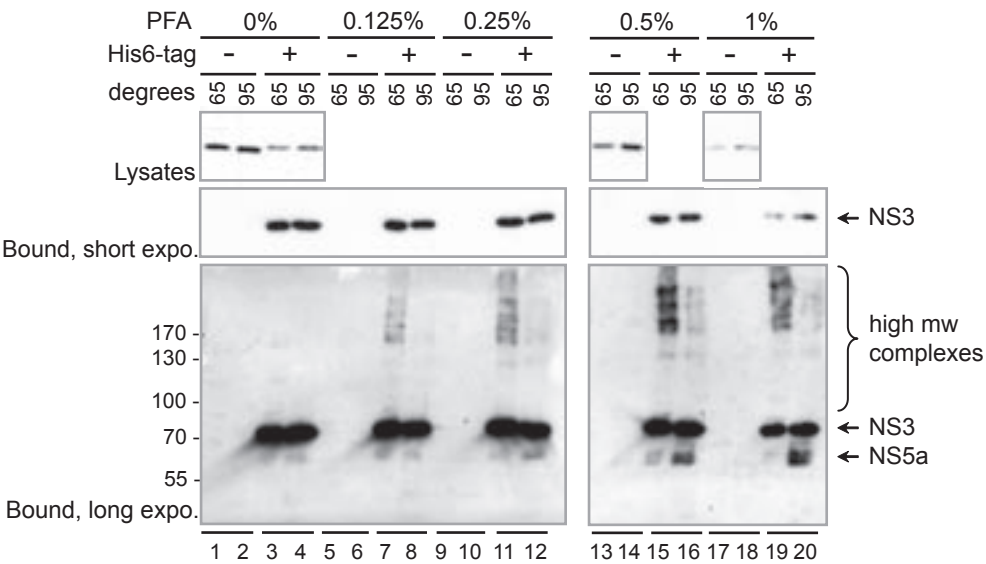


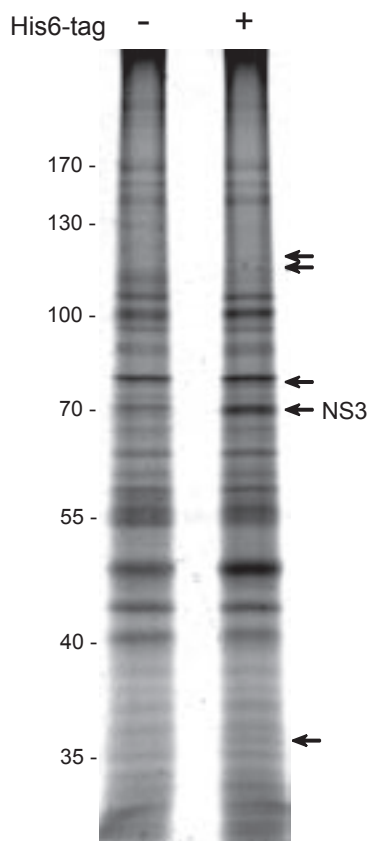
Figure 3 - Purification of NS3 and co-purification of NS5a from replicon containing cells after *in vivo* crosslinking

With increasing concentrations of PFA (0% - 1%), *in vivo* crosslinking was performed on replicon containing Huh7 cells with (+) and without (-) a His6-tag at NS3. The cells were lysed in an ureum-based buffer, which was followed by centrifugation at 14,000 xg (for more details see Methods). The supernatant (lysate) was used for metal-affinity purification of NS3. Subsequently, the lysates (top panel) and the proteins associated to the beads (Bound, middle and bottom panel) were examined by western blotting. Before separation on SDS-PAGE gels, the samples were heated to 65°C (odd lanes) or 95°C (even lanes), to preserve or break the crosslinks, respectively. In the top and middle panel, NS3 was detected using an antibody against NS3. The bottom panel was incubated with anti-NS3 and anti-NS5a antibodies. The middle panel is a short exposure of the long exposure bottom panel. The blocks in the top panel are from the same film.

Hence, we set up an assay to crosslink NS3 to interacting proteins and to isolate these complexes (Fig.3). Replicon containing cells with (+) and without (–) a His6-tag at NS3 were incubated with increasing concentrations of PFA during crosslinking. In a denaturing buffer, the cells were harvested and after centrifugation of the cell lysates at $14.000 \times g$, the supernatants (Lysates) were incubated with Cobalt-bound beads (See Methods for more details). Prior to separation on SDS-PAGE, proteins associated to the beads (Bound) were heated in Laemmli-buffer to 65°C or 95°C . Heating to 95°C will disrupt the crosslinks, while they remain intact at 65°C . After protein transfer, the western blot membranes were incubated with anti-NS3. Figure 3 shows that NS3 can be isolated after crosslinking, because NS3 signal is observed in the panel representing beads associated proteins (Fig.3; middle panel). However, binding of His6-NS3 to the beads decreased at a concentration of 1% PFA (Fig.3; middle panel, lanes 19 and 20). It seems that large complexes, formed by (extensive) crosslinking, are lost in the pellet during preparation of the cell lysates (Fig.3; top panel, compare lanes 1 and 2 with 17 and 18). Furthermore, crosslinking of NS3 might interfere with the availability of the His6-tag to bind to the beads. Since NS3 can be isolated in the presence of PFA, this method might allow isolation of interacting proteins crosslinked to NS3. Long exposure of the western blot membrane with proteins bound to the beads reveals that, after incubation with PFA, crosslinks are formed and purified (high molecular weight complexes) (Fig.3; bottom panel), indicating NS3 forms complexes. Comparison of samples heated at 65°C or 95°C confirms that heating disrupts crosslinks, causing high molecular weight complexes to dissociate (Fig.3; bottom panel even versus odd lanes). We furthermore tested whether NS5a, another HCV non-structural protein reported to bind to NS3^{35, 36}, can be co-purified with His6-NS3 in this crosslinking experiment. The western blot membranes were incubated with NS5a antibodies, in addition to the NS3 antibodies, to observe crosslinking as well as co-purification of NS5a (Fig.3; bottom panel). The apparent molecular weight of NS5a is 56 kDa and 58 kDa³⁷. If NS5a is crosslinked and co-purified with His6-NS3, we expect an increased signal at this molecular weight after disruption of the crosslinks. When comparing the lanes 65°C to 95°C from cells incubated with 0.5% or 1% PFA, a band corresponding to a molecular weight around 58 kDa is observed and intensified at 95°C (Fig.3; bottom panel, lanes 15 and 20). It should be mentioned that this 58 kDa band was also observed when western blot analysis was performed in the presence of only anti-NS5a antibody, showing co-purification of NS5a and NS3.

Together these results substantiate that this assay could be useful for the capture of NS3 interacting proteins.

With the aim of identifying the crosslinked proteins, we carried out an experiment to discover these unknown proteins by using mass spectrometry (MS). Replicon cells (– or + His6-tag) were incubated with 0.5% PFA and lysates were subjected to metal-affinity purification. After heating to 95°C, proteins associated to the beads were separated on SDS-PAGE. This was followed by silverstaining to visualise these proteins. NS3 and interacting proteins will appear as variation in the protein band patterns from the control (–) and the His6-tagged NS3 replicon (+) cell lines. Figure 4 shows the results, arrows indicate differences and the expected band of NS3 is specified. To identify these proteins (including NS3), the



bands were excised and in-gel digested with trypsin. The extracted peptides were separated on a nano-HPLC system and introduced into a LTQ-FT ultra mass spectrometer equipped with an electrospray ionisation interface (ESI MS). MS/MS analysis was performed for peptide identification (See for more detail Methods). Unexpectedly, despite assaying ample amounts of proteins after silverstaining, no proteins were identified, because peptide sequences could not be recovered from the MS. Given that we can identify NS3 by western blotting, there seems to be interference with MS analysis using this

Figure 4 - Isolated NS3 and associated proteins after *in vivo* crosslinking of replicon containing cells

Huh7 cells, harbouring a replicon with (+) and without (–) a His6-tag at NS3, were *in vivo* crosslinked using 0.5% PFA. NS3 was purified from the 14,000 xg supernatant and the proteins associated to the Cobalt-bound beads were subjected to SDS-PAGE. Subsequently, the proteins in the SDS-polyacrylamide gel were visualised by silverstaining. Arrows point to differences between the two lanes and NS3 is indicated specifically.

procedure. A possible explanation could be hindrance of PFA crosslinking with trypsin digestion, because formaldehyde is highly reactive with lysines, which are cleaved by trypsin used to generate peptides for MS³⁴. Therefore, chymotrypsin, which cleaves after aromatic amino acids tyrosine, tryptophan and phenylalanine, was applied to the putative NS3 band avoiding lysines. Again, no NS3 peptides were recovered. Another possibility could be that peptide modifications by PFA hamper subsequent MS analysis. Since PFA crosslinking is not protein-specific, PFA can crosslink proteins to DNA, RNA and other macromolecules. NS3, an RNA binding protein³⁸, could be crosslinked to RNA. Furthermore, two-dimensional PAGE (2D-PAGE) analysis already failed at the stage of isoelectric focusing of the crosslinked proteins, which suggests insolubility of the proteins or a change of protein charge. Together this could point to protein modifications that hinder further analysis. Although we thoroughly showed that PFA crosslinking intervenes with subsequent MS analysis, work by Tagwerker et al. showed identification of ubiquitinated proteins using a similar approach³².

His6-NS3 purification from HCV replicon cell line using NP40

An alternative strategy to isolate NS3 and interacting proteins is by the usage of detergents. Depending on the chemical properties and the concentration of detergents, proteins and protein complexes can be solubilised and purified. The triple detergent buffer, containing SDS, DOC and NP40, was shown to be useful for NS3 pulldown (Fig. 2). To investigate whether one of the three detergents alone is effective in NS3 isolation, three buffers were prepared with a single detergent (Fig.5A). His6-tagged NS3 replicon cells (+) were lysed with a ball bearing homogeniser and after an 800 ×g-centrifugation step detergents were added to the supernatant. Similar amounts of NS3 were present in the lysates (Fig.5A; top panel). Subsequent to affinity purification, NS3 bound to the beads was analysed by western blotting, using an anti-NS3 antibody. NS3 could be purified with buffers containing all detergents (triple), 1% DOC or 0.5% NP40, but not in the presence of 0.1% SDS or without detergents (Iso.) (Fig.5A; bottom panel). The concentration of SDS in the lysis buffer was below the critical micelle concentration (0.23% w/v) of the detergent, which might explain the failure to purify NS3. Since NP40 is effective in NS3 isolation, we decided to continue with this detergent. Especially for NP40 is a non-ionic detergent and less denaturing to proteins compared to the ionic detergents SDS and DOC. Additionally, non-ionic detergents do not interfere with isoelectric focusing of proteins used in two

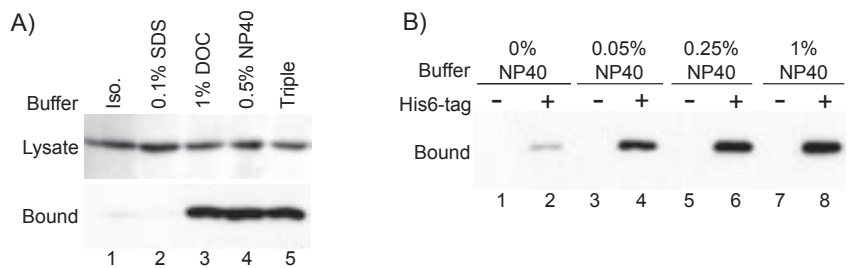


Figure 5 - Purification of NS3 using detergents

A. Huh7 cells, containing HCV subgenomic replicon with a His6-tag at NS3, were lysed in isotonic buffer using a ball-bearing homogeniser. To the lysate of an 800 xg centrifugation step none (Iso.), sodium dodecyl sulfate (SDS), sodium desoxycholate (DOC), Nonidet P-40 (NP40) or all these three detergents (Triple) were added. NS3 was affinity purified from each condition and detected with anti-NS3 during western blot analysis (Bound, bottom panel). Also the lysate was subjected to western blotting (lysate, top panel).

B. A similar experiment as in A was carried out. This time NS3 isolation was performed in the presence of increasing concentrations of NP40 (0% - 1%) (bound, bottom panel). NS3 was purified from 800g supernatant of Huh7 cells containing replicons with (+) and without (–) a His6-tag at the N-terminus of NS3 (lysate, top panel).

dimensional poly acrylamide gel electrophoresis (2D-PAGE), a technique later used for analysis of complexes.

Besides chemical properties, the concentration of detergents during purification of proteins and protein complexes can be critical. On one hand a too low detergent concentration will not solubilise proteins and therefore not succeed in isolation of proteins. On the other hand a too high concentration of detergents might disrupt protein-protein interactions, consequently loosing associated proteins during purification. We therefore determined the lowest effective concentration of NP40 for NS3 isolation (Fig.5B). NS3 was purified from replicon cells with (+) and without (–) a His6-tag at NS3 in the presence of different concentrations of NP40, ranging from no detergents to 1% NP40. Pulldown of NS3 was examined by western blot analysis (Fig.5B). There was no isolation of NS3 from the control cell line (–, no His6-tag). Some NS3 was purified without any detergents (0%), but a large increase in the amounts of NS3 was observed in the presence of NP40. The minimal concentration of NP40 required for NS3 isolation was determined at 0.05% NP40 (Fig.5B) and was used in the following experiments to purify NS3 and interacting proteins.

Characterisation of His6-NS3 purified material by fractionation

All HCV proteins are associated to membranes, including NS3⁶. NS3 is associated indirectly to the membranes via the small HCV non-structural protein 4a. Through its central region, NS4a is non-covalently bound to the N-terminal part of NS3³⁹⁻⁴¹.

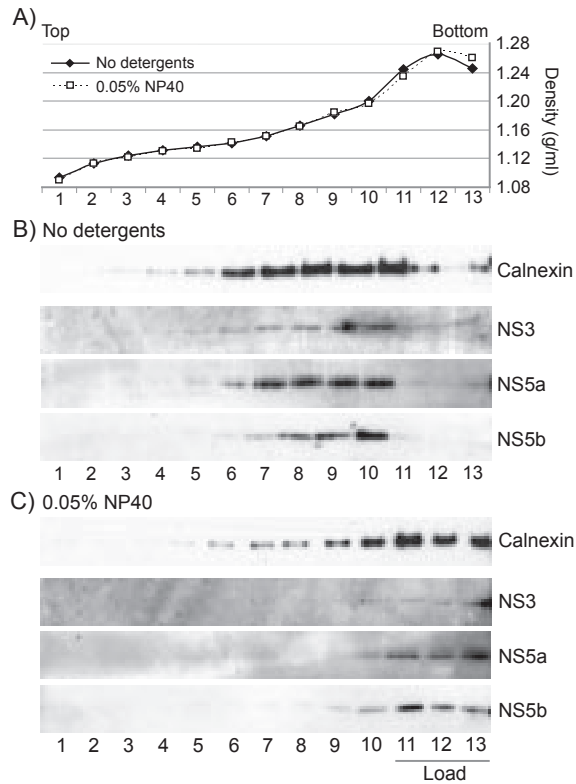


Figure 6 - Membrane floatation gradient of replicon containing Huh7 cell lysates

A. Plot showing densities of sucrose gradient fractions from replicon containing Huh7 cell lysates without detergent (black triangles) or in the presence of 0.05% NP40 (open squares).

B. Huh7 cells harbouring HCV replicon were lysed in isotonic buffer using a ball-bearing homogeniser. Lysates were subjected to 800 xg centrifugation and the supernatant was adjusted to a sucrose density concentration of ~1.26 g/ml and loaded underneath a sucrose gradient with densities ranging from 1.23 to 1.09 g/ml. After overnight centrifugation, samples were taken from the top. The fractions were analysed by SDS-PAGE and western blotting using antibodies against calnexin, NS3, NS5a and NS5b. The loaded fractions are in lanes 11, 12 and 13 and the lanes 1 to 10 represent the membrane floatation fractions.

C. Experiment as in B, but differs in that the cell lysate, which was loaded underneath the sucrose density gradient, contained 0.05% NP40.

Together, NS3 and NS4a are anchored to membranes by the N-terminal transmembrane domain of NS4a^{42, 43}. More recently, a direct interaction with membranes was identified within NS3, that is a hydrophobic helix at the N-terminus of NS3⁴².

To examine some properties of the NS3 purified material several experiments were carried out. First a membrane floatation gradient assay on replicon lysates was performed to determine membrane association of NS3 in the presence or absence of NP40 (Fig.6)⁴⁴. Replicon cells were lysed in isotonic buffer using a ball bearing homogeniser. Following centrifugation either no detergents or NP40, with a final concentration of 0.05%, were added to the 800g supernatant. These two samples were then adjusted to a sucrose density concentration of ~1.26 g/ml and loaded underneath a sucrose gradient with densities ranging from 1.23 to 1.09 g/ml (Fig.6A). After centrifugation, subsequent samples were taken from the top of the gradient and analysed by western blotting (Fig.6B and C). Fractions 1 to 10 represent floating membranes and their associated proteins. The last three samples (11 to 13) correspond to the loaded fractions; where all material resides that does not float. When no detergent is added to the lysate, Calnexin, an ER transmembrane protein, is mainly observed in fractions 6 to 10 (Fig.6B). Similarly, we predominantly find the HCV non-structural proteins, NS3, NS5a and NS5b, in the floating fractions 7 to 10. Yet addition of 0.05% NP40 relocates Calnexin, as well as the HCV NS proteins, towards the three bottom fractions corresponding to non-floating material (Fig.6C). Together this indicates that at a concentration of 0.05% NP40 most membranes are disrupted. Moreover, it suggests that the majority of non-structural proteins no longer reside on floating membranes.

Supplementary, a centrifugation assay of replicon cell lysates (800g Sup) containing NP40 illustrates that NS3 is present in the supernatant (100kg Sup) and in the pellet fraction (100kg Pellet) after 100,000 ×g centrifugation of these lysates (Fig.7A; middle and bottom panel); indicating NS3 could still be associated to non-floating large structures, which might be protein complexes and/ or membrane sheets. We therefore investigated the composition of the isolated NS3 during affinity purification (Fig.7B). Replicon cells with (+) and without (–) a His6-tag at NS3 were lysed in an isotonic buffer and following centrifugation at 800 ×g NP40 was added to the supernatant. After affinity purification, proteins associated to these beads were eluted and the eluate was subjected to centrifugation at 100,000 ×g. Figure 7B shows that some of the NS3 that is bound to the beads can be pelleted at 100,000 ×g (bottom panel) and suggests that a very small portion of NS3 might be in a complex or associated to membrane

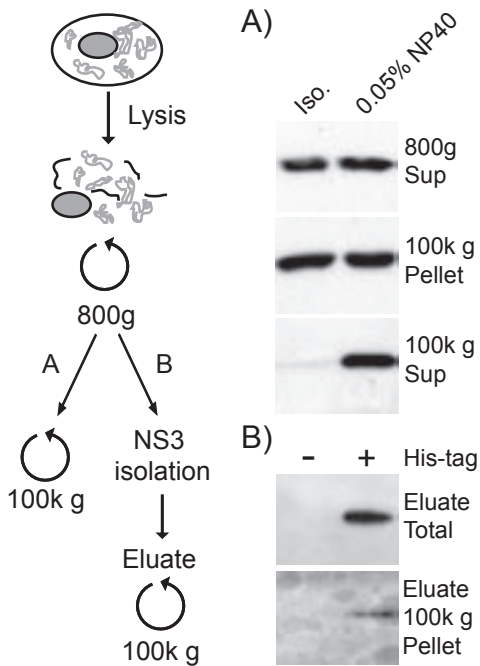


Figure 7 - Differential centrifugation of purified NS3

A. Using the ball-bearing homogeniser Huh7 cells containing replicon were lysed in isotonic buffer. The supernatant of an 800 xg spin was split and one part was supplemented with 0.05% NP40 (0.05% NP40), the other part was not (Iso.). Next, both lysates were subjected to 100,000 xg centrifugation. The 800g supernatant (top panel), the 100,000 g pellet (1/3 of total amount) (middle panel) and the 100,000g supernatant (1/60 of total volume) (bottom panel) were examined for NS3 using SDS-PAGE and western blot with an anti-NS3 antibody.

B. In the presence of 0.05% NP40, NS3 was isolated from the 800g supernatant of Huh7 cells harbouring replicons with (+) and without (-) a His6-tag at NS3. NS3 eluted from the beads was used for 100,000 xg centrifugation. The total eluate (top panel) and the 100,000g pellet of the eluate (bottom panel) were analysed by western blotting using an antibody against NS3.

sheets. Both the density gradient and the differential centrifugation assay results suggest that a part of purified NS3 is soluble and that a small portion might be in a large complex or membrane associated.

Co-purification of HCV RNA with His6-NS3, though lack of other HCV proteins

As NS3 contains helicase activity and can bind to RNA ⁴⁵, HCV RNA might still be associated to NS3. Therefore, affinity-purified material was examined on the presence of HCV RNA (Fig.8). Eluates from beads incubated with lysates of replicon cells with (+) or without (-) a His6-tag at NS3 were subjected to RNA isolation, followed by reverse transcriptase using a primer at the end of NS5b and PCR with an additional primer at the beginning of NS5b. The resulting PCR products were analysed on an agarose gel (Fig.8). The PCR product from HCV RNA is 1683 bp. At this size, bands are only visible in the lanes containing PCR products amplified from cDNA of the His6-tagged NS3 replicon eluate (+) (Fig.8; lanes 3 and 4).

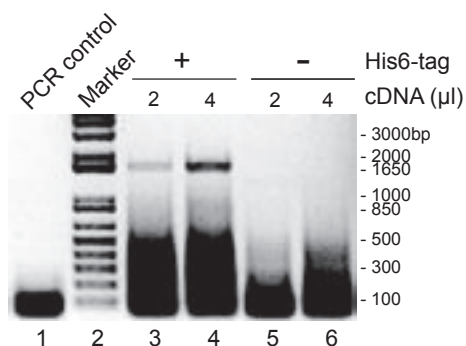


Figure 8 - Co-purification of HCV-RNA together with NS3

Huh7 cells harbouring replicons with (+) (lanes 3 and 4) and without (-) (lanes 5 and 6) a His6-tag at NS3 were subjected to NS3 purification using 0.05% NP40. This was followed by elution of the material from the beads. RNA was isolated from this eluate and subsequently reverse transcription was performed with a primer at the end of NS5b. With 2 or 4 µl cDNA a PCR was carried out, using primers resulting in a 1683 bp product (lanes 3 to 6). A negative PCR control was taken along, which is shown in lane 1. A marker was loaded on the agarose gel (lane 2) and its band sizes are indicated on the right.

Conversely, there are no PCR products observed in the cDNA from eluate of the control cell line (-, no His6-tag), nor in the PCR control (Fig.8; lanes 1, 5 and 6). This clearly shows HCV RNA is co-purified together with NS3 isolated from replicon cells, implying a putative protein-RNA complex.

The replicon cells contain the non-structural proteins 3 to 5b and interactions between NS3 and other NS proteins have been shown previously³⁶. For that reason we examined the presence of NS4b, NS5a and NS5b, three other HCV proteins, in NS3 purified material using western blot analysis. NS3 was purified from Huh7 replicon containing cells with (+) or without (-) a His6-tag at NS3. Proteins associated to the beads (AB) were separated by SDS-PAGE and HCV proteins were detected by western blotting. Figure 9 shows that His6-tagged NS3 is specifically associated to the beads (Fig. 9; NS3, lane 3 versus lane 4). To check for residual cellular proteins present in the beads fraction, GAPDH a cytosolic protein was used. Clearly, GAPDH wasn't found in the beads fraction, indicating that no remainder of the 800g supernatant was left (Fig.9; GAPDH, lanes 3 and 4).

The three bottom panels in Figure 9 show the co-purification results of the three other HCV proteins, NS4b, NS5a and NS5b. The proteins are present at a similar expression level in both cell lines, with and without a His6-tag at NS3 (Fig.9; lanes 1 and 2), confirming that there is no interference of the tag in these replicon cells. Some non-specific background binding to the beads is observed for NS5a and NS5b, but there is no increase in NS4b, NS5a and NS5b signal in the lanes of isolated NS3

(Fig.9; NS4b/NS5a/NS5b, lanes 3 and 4), indicating that the HCV replicon proteins NS4b, NS5a and NS5b were not co-purified together with NS3.

This could, on one hand, demonstrate dissociation of NS3 from the other NS proteins during the procedure. On the other hand, a specific sub-population of NS3, not containing other NS proteins, might have been isolated. In literature, different pools of NS3 are suggested. Only less than 5% of the non-structural proteins are protease resistant. In fact this small pool is accountable for all the RNA synthesis activity ⁴⁶. Additionally, immunofluorescence assays indicate slight differences in ER distribution of the NS proteins ^{47, 48}. NS3 seems to also localise at ER membranes more closely to mitochondria ^{38, 47, 49}, implying different pools of NS3. Both possibilities could explain why we do find an interaction between NS3-NS5a, using our crosslinking strategy. In that approach any transient interactions are fixed and possibly all pools of NS3 are captured due to the harsh denaturing conditions we used after crosslinking.

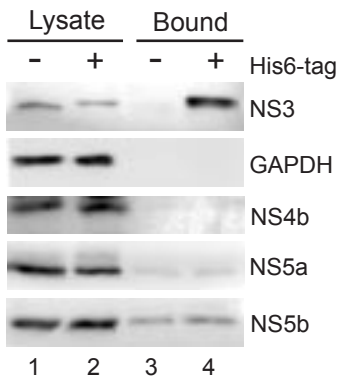


Figure 9 - Lack of co-purification of other HCV proteins together with NS3

The 800g supernatant (Lysate, lanes 1 and 2), from ball-bearing homogeniser lysed Huh7 cells that contain replicons with (+) and without (-) a His6-tag at NS3, was supplemented with 0.05% NP40. Subsequently, NS3 and associated proteins (Bound, lanes 3 and 4) were isolated and analysed by SDS-PAGE and western blotting using antibodies against NS3, Glyceraldehyde 3-phosphate dehydrogenase (GAPDH), NS4b, NS5a and NS5b.

Reducing non-specific binding of proteins to Cobalt bound-beads

To identify NS3 interacting proteins, reduction of background binding proteins to beads is required. Non-specific protein binding to positively charged metal-affinity beads can be reduced by low concentrations of imidazole, an organic compound competing for binding with histidine due to similar ring structures. Conversely, too high concentrations of imidazole will prevent specific binding of His6-tagged proteins to the Cobalt bound-beads. We therefore optimised imidazole concentrations during isolation (Fig.10). Replicon cells with (+) and without (-) a His6-tag at NS3

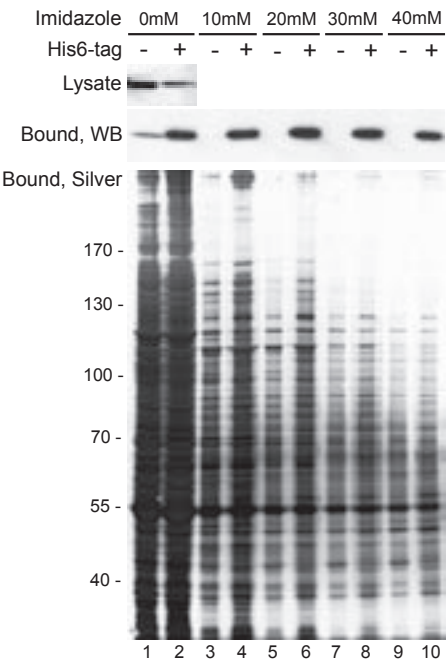


Figure 10 - Background reduction of NS3 isolation using Imidazole

Huh7 cells containing replicons with (+) and without (-) a His6-tag at NS3 were lysed. To the 800g supernatant (Lysate, top panel) 0.05% NP40 and increasing concentrations of imidazole (0mM – 40mM) were added. Under these conditions NS3 was purified. NS3 binding to the beads was examined by SDS-PAGE and western blot using anti-NS3 (Bound WB, middle panel). Proteins associated to the beads were separated on SDS-PAGE and visualised by silverstaining (Bound Silver, bottom panel).

were used for NS3 purification. Binding of proteins to the beads was performed in presence of increasing concentrations of imidazole, ranging from 0mM to 40mM. Proteins associated to the beads were subjected to SDS-PAGE, followed by western blot analysis with an antibody against NS3 (Fig.10; middle panel) or the gel was directly silver stained (Fig.10; bottom panel). Figure 10, bottom panel shows that increasing the concentration of imidazole decreases the binding of non-specific proteins to the beads. Without imidazole, some NS3 binds non-specifically to the metal-affinity beads (Fig.10. middle panel, lanes 1 and 2). Together this indicates that imidazole is necessary for background reduction and increased specificity of the assay. At this point, it should be noted that previous experiments shown here were performed with optimised imidazole concentrations. High concentrations of imidazole reduce association of NS3, which is observed at concentrations of 30 and 40 mM imidazole (Fig.10; middle panel, lanes 7 to 10). Therefore, the optimum in NS3 binding to the beads seems to be at 20mM imidazole (Fig.10; middle panel, lanes 5 and 6).

For specificity of the assay and maximum association of NS3 to the beads, other steps of the purification protocol, including pH during purification, elution of the proteins from the beads and concentration of the eluate, were optimised as well. Op-

timal conditions were used for the experiments to identify NS3 interacting proteins (see Methods for conditions).

Isolation of His6-NS3 interacting proteins in SDS-PAGE

In order to identify NS3 interacting proteins by mass spectrometry (MS) analysis a sufficient amount of associated proteins needs to be purified, typically a minimum amount of 5-10 ng. This would correspond to about 100 femtomol of NS3 (70kDa). In order to reach these requirements the purification was scaled up to 1.5×10^8 cells, proteins in the eluate from the Cobalt coated beads were concentrated using tri-chloroacetic acid (TCA). Subsequently, the proteins were separated on SDS-PAGE and visualised by silverstaining (Fig.11). Comparison of the lanes from the two cell lines uncovered several differences, which are indicated by arrows in Figure 11. These bands were excised and analysed by MS as described before, which resulted in the identification of NS3 with 8 peptides in band 3. In the other bands either no proteins could be identified or proteins with only one corresponding peptide. Within band 1, two peptides of collagen XVIII, a precursor of endostatin, were obtained. In band 2 two peptides of lysyl hydroxylase 3 (LH3) were found. These were good leads to start additional experiments. However, we sometimes identified more than one protein in a single band. Likewise, the intensities of the silver

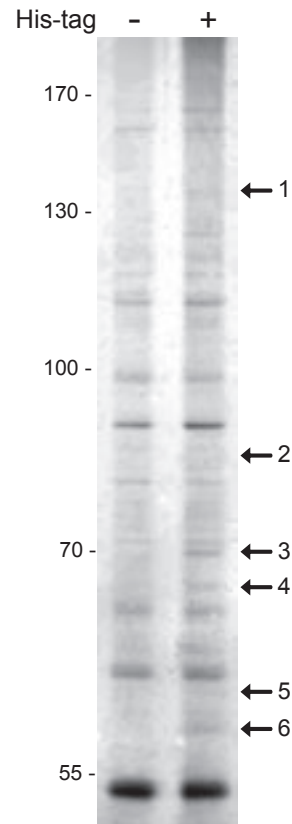


Figure 11 - Isolated NS3 and associated proteins after 0,05% NP40 detergent extraction in 1D SDS-PAGE

In an isotonic buffer and with the ball-bearing homogeniser Huh7 cells harbouring replicons with (+) (lane 2) and without (-) (lane 1) a His6-tag at NS3 were lysed. Following 800 xg centrifugation, 0.05% NP40 was added to the supernatant. Subsequently, NS3 was metal-affinity purified and after separation on 10% SDS-PAGE, proteins associated to the beads were coloured by silverstaining. Arrows point to band differences between the two lanes. On the left, the sizes of a molecular weight maker are indicated.

stained bands at identical molecular weight were sometimes more intense in the NS3 His6-tagged cell line compared to the non-His-tagged cell line, which could imply that associated proteins are underneath the non-specific background band. Therefore, more resolution was required for protein separation to unambiguously identify interacting proteins. This was accomplished by two-dimensional PAGE (2D-PAGE), which separates proteins on isoelectric point, as well as on molecular weight. Consequently, several proteins found within one band of SDS-PAGE can be observed in distinct spots with 2D-PAGE.

Isolation of His6-NS3 interacting proteins in 2D-PAGE

This time NS3 isolation was performed followed by 2D-PAGE (Fig.12). Again, like for the 1D SDS-PAGE experiments, the purification was scaled up to 2×10^8 cells. Pooled eluates were concentrated with ethanol, because this matches better with isoelectric focusing and yields a higher amount of proteins compared to TCA precipitation, which was assessed by silverstaining and western blot analysis of NS3 (data not shown). The concentrated protein samples were applied to isoelectric focusing, followed by SDS-PAGE and silver staining (see Methods for more detail) (Fig.12). The non-specific binding proteins are observed in the gel from the control cell line (Fig.12; left panel, no His-tag). Some spots in the 2D-PAGE gel from the NS3 His6-tagged replicon cell line (+) were clearly distinct from the control cell line (–, no His6-tag) and indicated by arrows (Fig.12; right panel, arrows). These spots could represent NS3 and NS3 interacting proteins. Hence, the indicated spots were excised and analysed by MS (see Methods). From spots 1 and spot 2, in total 12 NS3 peptides were obtained. In spot 3, which has a molecular weight of about 72 kDa, Glycosyl transferase 25 containing domain 1 (GLT25D1) was identified with about 10 peptides. No significant protein match could be found in spot 4 and only two peptides of eukaryotic initiation factor 4A-I (eIF4A-I) were observed in spot 5. Both NS3 and GLT25D1 could be reproduced in repeated experiments. In addition, LH3, a protein previously identified in 1D SDS-PAGE, was observed in repeated 2D-PAGE experiments.

Conversely, eIF4A-I and other RNA binding proteins, like DDX19A, DDX42 and polyRC-binding protein^{50, 51}, were observed in a single experiment. This could indicate some non-specific background of RNA binding proteins, possibly associating to HCV RNA, which we showed to co-purify with NS3 earlier (Fig.8). Furthermore, some spot differences seem to be at other molecular weights than observed

in the previous 1D SDS-PAGE experiment (Fig.11). One of the reasons might be the solubilisation of proteins that is important in the first step of 2D-PAGE, which is sometimes difficult to achieve especially for hydrophobic proteins like membrane proteins. The next step was to substantiate the proteins GLT25D1 and LH3, which were reproducibly found.

Specific co-purification of GLT25D1 and LH3 together with His6-NS3

To confirm the interaction of GLT25D1 and LH3 with His6-NS3 and verify their identification by mass spectrometry, samples with purified NS3 were investigated

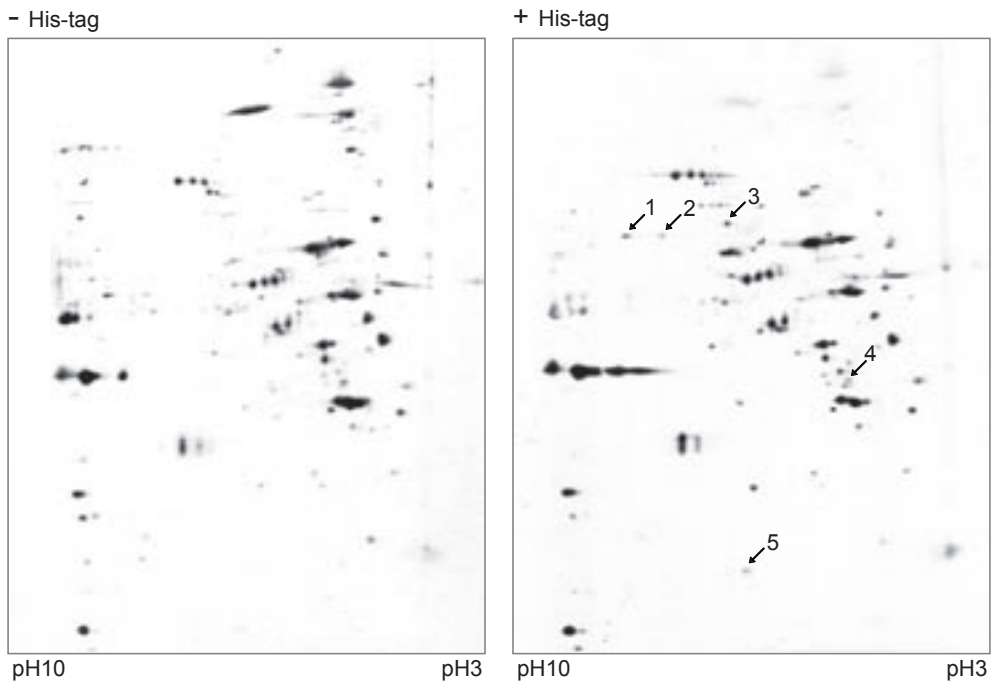


Figure 12 - Isolated NS3 and associated proteins in two-dimensional PAGE

From Huh7 cells, containing replicons with (+) (right panel) and without (-) (left panel) a His6-tag at NS3, NS3 and associated proteins were isolated in the presence of 0.05% NP40. Proteins associated to the beads were eluted, concentrated and applied to two-dimensional PAGE. The proteins were first focused on their isoelectric point in an immobilised pH gradient strip ranging from pH 3 to pH 10 and subsequently separated on a 9% SDS-PAGE gel. Proteins in the SDS-PAGE gel were visualised by silverstaining. Arrows indicate protein spots that differ from the other gel.

by western blotting using specific antibodies against GLT25D1 and LH3 (Fig.13). Since an antibody against GLT25D1 was not available, we raised one against a selected peptide of GLT25D1 (See Methods). This antibody recognises in western blot analysis a protein at 72 kDa, the expected molecular weight of GLT25D1. Additionally, this antibody recognised GLT25D1 expressed from a CMV driven construct that was overexpressed in Huh7 cells (data not shown)⁵².

NS3 was isolated from replicon cells with (+) and without (–) His6-tag at NS3 and subjected to western blot analysis. As shown in Figure 13, NS3 is specifically isolated (Fig.13; NS3, lanes 3 and 4) (same as Fig.9). Similar amounts of GLT25D1 and LH3 are present in the 800g supernatant (Fig.13; GLT25D1 and LH3, lanes 1 and 2). In the lanes loaded with proteins associated to the beads, a clear increase in signal for both GLT25D1 and LH3 is observed in the lanes representing isolated proteins

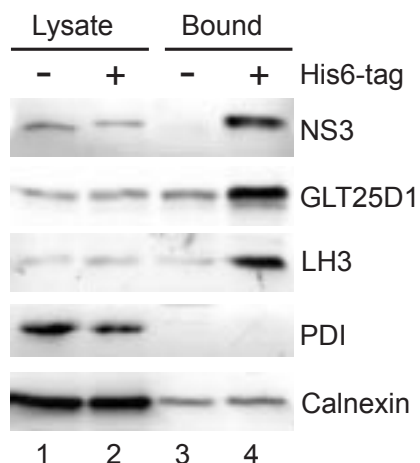


Figure 13 - Co-purification of GLT25D1 and LH3 together with NS3

Huh7 cells, containing replicons with (+) and without (–) a His6-tag at NS3, were lysed and subjected to 800 xg centrifugation. NP40 was added to the 800g supernatant, to a final concentration of 0.05%. Subsequently, NS3 was affinity purified from these lysates. The proteins associated to the beads (Bound, lanes 3 and 4) and 800g supernatant (Lysate, lanes 1 and 2) were analysed by SDS-PAGE and western blot using antibodies against NS3, glycosyltransferase 25 containing domain 1 (GLT25D1), lysylhydroxylase 3 (LH3), protein disulfide isomerase (PDI) and calnexin.

from replicons containing His6-tagged NS3 (Fig.13; GLT25D1 and LH3, compare lanes 3 and 4). This suggests there is specific co-purification of these two proteins with NS3.

To exclude non-specific binding of ER, especially since LH3 localises to the ER⁵³ and GLT25D1 is predicted to localise there⁵⁴, we incubated the western blot membranes with antibodies specific for the ER resident proteins, calnexin and protein disulfide isomerase (PDI). Although some non-specific association of calnexin to the beads is detected, there is no specific binding observed for both ER proteins in

the presence of NS3 (Fig.13; PDI and calnexin, lanes 3 and 4). Together these data clearly indicate specific association of LH3 and GLT25D1 with NS3.

After confirming the interaction of NS3 with GLT25D1 and LH3 in western blot analysis, further validation is necessary. For instance, in order to interact, two proteins need to be targeted to the same organelle. Since the cellular localisation of GLT25D1 is predicted, we first investigated this. The findings of that study are described in the next chapter.

Both LH3 and GLT25D1 are suggested to play a role in glycosylation of collagens and certain immune defence molecules (collectins)^{53,54}. New data is becoming available on the role of collectins in antiviral defence and it will be interesting to know how HCV affects these molecules. More specifically, GLT25D1 was shown to be able to galactosylate mannose binding lectin, a collectin capable of binding to viral glycoproteins⁵⁵⁻⁵⁷. We therefore continued our research in that direction.

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**The human collagen beta
(1-O)galactosyltransferase,
GLT25D1, is a soluble
endoplasmic reticulum-
localised protein**

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Abstract

Glycosyl transferases transfer glycosyl groups onto their substrate. Localisation partially defines their function. Glycosyl transferase 25 domain 1 (GLT25D1) was recently shown to have galactosyltransferase activity towards collagens and another well known substrate, mannose binding lectin (MBL). To gain more insight in the role of galactosylation of lysines in the Gly-X-Lys repeats of collagenous proteins, we investigated the subcellular localisation of GLT25D1. Immunofluorescence analysis of GLT25D1 expressed in the human hepatoma cell line (Huh7), revealed a perinuclear lattice-like staining, resembling localisation to the endoplasmic reticulum (ER). Possible targeting signals, an N-terminal signal sequence and a C-terminal ER-retention signal, were identified using prediction programs. These signals were then investigated by constructing a series of epitope-tagged forms of GLT25D1 that were analysed by immunofluorescence and western blotting. In agreement with the predictions, our results show that GLT25D1 is directed to the ER lumen as a soluble protein and retained there. Moreover, using two endoglycosidase enzymes EndoH and EndoF, we demonstrate that the putative bi-functional glycosyl transferase itself is a glycoprotein. Additionally we examined co-localisation of GLT25D1 with MBL and lysyl hydroxylase 3 (LH3, PLOD3), which is a protein able to catalyse hydroxylation of lysine residues before they can be glycosylated. We demonstrate overlapping localisation patterns of GLT25D1, MBL and LH3. Taken together our data indicate that galactosylation of collagenous proteins by the soluble GLT25D1 occurs in the early secretory pathway.

Introduction

Hydroxylation and subsequent glycosylation of lysine residues is a characteristic of collagens and proteins containing a collagen-like region (collectins) ¹⁻³. These proteins contain repeats of Gly-X-Y motifs, where lysines present at the Y-position can be galactosylated ^{4,5}.

Collagens and collectins are built up of three polypeptide chains, which wind together to form a triple helix. The Gly-X-Y repeats, where X is often a proline, allow tight coiling of the chains as the small glycines fit into sterically restricted spaces where the three chains come together ⁶.

The function of the lysine linked sugars is not fully understood, but this posttranslational event occurs before triple helix formation ⁷ and mutations in these residues clearly affect the oligomerisation state resulting in aberrant secretion ⁸. Mutation of two lysines to arginines in the Gly-X-Lys repeat of mannose binding lectin (MBL), which is involved in neutralisation of invading micro-organisms by triggering the complement cascade, resulted in inefficient complement activation ⁸. At the protein level glycosylated lysines are suggested to play a role in folding, stability and prevention of inter-chain cross-linking ^{4,8,9}.

Lysyl hydroxylase enzymes catalyse the hydroxylation reaction on lysines, after which the residue can be glycosylated ¹⁰. Recently, two new genes responsible for the galactosylation of Gly-X-Y repeats were described. Using affinity chromatography and tandem mass spectrometry, Schegg et al. identified two glycosyltransferase GT25 family members, GLT25D1 and GLT25D2, to encode galactosyltransferases involved in transfer of galactose to hydroxylysine residues in MBL and collagen ¹¹. Attachment of galactose occurs via the β (1-O) bond to the hydroxylated lysine (C δ), assigning these proteins to β (1-O) galactosyltransferases.

Most members of glycosyltransferases (GT) are localised to the Golgi, but GTs can also be observed in the cytosol, nucleus, mitochondria, endoplasmic reticulum (ER), on cellular membranes, secreted from the cell or widely distributed between all these ¹². Their distinct subcellular localisation most likely reflects their role in glycosylation pathways. For example, the subsequent arrangement in which glycan synthesis takes place in the Golgi apparatus can often be related to the location of the glycosyltransferases in the cisternae ¹³. Here we examined the cellular localisation of GLT25D1 relative to one of its reported substrates, MBL, which is produced in

liver cells¹⁴. We also identified ER-targeting signals within GLT25D1 causing it to localise to the early secretory pathway.

Methods

Signal predictions

Prediction of signal peptides: <http://www.cbs.dtu.dk/services/SignalP/>¹⁵. Prediction of N-glycosylation sites: <http://www.cbs.dtu.dk/services/NetNGlyc/>.

Plasmid construction

To construct epitope-tagged GLT25D1 expression plasmids; the sequence was amplified by PCR from GLT25D1 full-length clone, IRAKp961P01217Q (imaGenes, Berlin, Germany), using specific primers, see Table 1. The PCR products were digested with *KpnI* and *XbaI* and ligated into pCDNA3.1mychisB (Invitrogen) similarly digested with *KpnI* and *XbaI*. This resulted in the construction of expression vectors containing a 10-residue Myc-epitope-tag at its C-terminus. In order to construct C-terminal HA-epitope-tagged GLT25D1 expression constructs (GLT25D1-HA, Δsignal sequence-GLT25D1-HA), the Myc-epitope sequence was *XbaI* – *PmeI* cut and replaced with an *XbaI* – *PmeI* fragment coding for the HA-epitope. For construction of the RDEL at the extreme C-terminus (GLT25D1-MycRDEL, HA-GLT25D1-MycRDEL), the constructs were *AgeI* – *PmeI* cut and replaced with a fragment coding for RDEL. For construction of GLT25D1 with an internal Myc-epitope-tag (GLT25D1 FL Myc-int.) after the signal sequence, two annealed oligos with protruding TGCA nucleotides (see Table 1) were ligated into the GLT25D1 gene cut with restriction enzyme *SbfI*, which generates complementary ends (CCTGCA|GG).

Cell culture and transfection

Human hepatoma cell line Huh7 was grown in Dulbecco's Modified Eagle's Medium supplemented with non-essential amino acids, L-glutamate, Penicillin and Streptavadin. Trypsin was used to subculture the cells. Cells were transfected using Amaxa Cell line Nucleofactor kit T (Lonza), program T016 and 4μg of DNA.

Antibodies

The following antibodies were used: anti-PDI (Stressgen), anti-Giantin (Alexis Biochemicals), anti-ERGIC53 (Alexis Biochemicals), anti-LH3 (Abnova), anti-Myc

(mouse, immunofluorescence) (Invitrogen), anti-Myc (rabbit, immunofluorescence) (Abcam), anti-Myc (mouse, western blotting) (Roche) and anti-HA (mouse) (Abcam), Donkey-anti-mouse-cy3 (Jackson), Donkey-anti-rabbit-cy3 (Jackson), Goat-anti-mouse-Alexa488 (Invitrogen), Goat-anti-rabbit-Alexa488 (Invitrogen), Goat-anti-mouse-Alexa633 (Invitrogen) and Goat-anti-mouse-HRP (Dako).

Table 1 - Primers used to generate expression constructs

HA-GLT25D1-Myc (construct 1)
Forward primer, GTGGGTACCATGTACCCATACGATGTTCCAGATTACGCCGCGGCGGCCCCACGCGC
Reverse primer, TAGTCTAGAGAGAGTTCATCCCGGGCAGCAC
GLT25D1-Myc (construct 2)
Forward primer, GTGGGTACCATGGCGGCGGCCCCACGCGC
Reverse primer as for HA-GLT25D1-Myc
Δ signal sequence -GLT25D1-Myc (construct 3)
Forward primer, GTGGGTACCATGGGCGCCGACGCCTACTTC
Reverse primer as for HA-GLT25D1-Myc
HA-GLT25D1 (construct 8)
Forward primer as for HA-GLT25D1-Myc
Reverse primer, TAGTCTAGATCAGAGTTCATCCCGGGCAGCAC
HA-GLT25D1-ΔRDEL (construct 9)
Forward primer as for HA-GLT25D1-Myc
Reverse primer, TAGTCTAGATCAGGCAGCACTGTCCAGTGG
GLT25D1-Myc internal tag (construct 10)
Oligo 1, GGAACAAAACTCATCTCAGAAGAGGATCTGCA
Oligo 2, GATCCTCTTCTGAGATGAGTTTTTGTTCCTGCA

Immunofluorescence

Cells were fixed with 3% paraformaldehyde in PBS (154 mM NaCl, 1.4 mM phosphate, pH 7.5) 24 h post transfection. PFA was quenched using 50 mM NH₄Cl in blockbuffer, which contained 5% fetal calf serum (FCS) in PBS. The cells were permeabilised with 0.1% TritonX-100 in blockbuffer and stained with primary antibodies diluted in blockbuffer for 1 h. Next the coverslips were washed with blockbuffer and incubated with secondary antibody diluted in blockbuffer for 1 h. After washing with glycinebuffer (10 mM glycine in PBS), PBS and water, the coverslips were mounted with Prolong mounting medium (Invitrogen). Fluorescence images were captured using Leica TCS SL confocal microscope, 63x Plan Apo oil immersion objective (zoom approximately three times), appropriate filter settings

and sequential scan modes. Images were optimised with Adobe Photoshop CS2. Profile plots were generated using ImageJ (NIH).

SDS-PAGE and western blotting

After separation on SDS-PAGE gels, proteins were transferred to PVDF membranes (Hybond P, GE-Healthcare) using a Semi-Dry blot apparatus (Biorad). Membrane blocking and antibody incubations were performed using 0.5% Tween-20, 5% non-fat, dry milk (Campina) in PBS. Since all secondary antibodies were conjugated to horseradish peroxidase, the proteins were visualised using enzyme-catalysed chemiluminescence (ECL+, GE-Healthcare) and Typhoon Imager (GE-Healthcare).

De-glycosylation assay

Cells transfected with GLT25D1-FL-MycHis6 or GLT25D1-ΔSS-MycHis6 were lysed after 16 h in isotonic buffer, which contains 20 mM Tris pH 7, 1 mM MgCl₂, 15 mM NaCl, 240 mM sucrose and 10 mM imidazole, using a ball bearing homogeniser (Isobiotec, Heidelberg Germany). Following centrifugation of the cell lysates at 800 xg for 10 minutes, supernatants were incubated with cobalt-beads (Talon) for 2 h under continuous rotation. Proteins binding to the beads were eluted using the isotonic buffer now containing 190 mM imidazole. Eluates were split into three portions to de-glycosylate or not. One sample was boiled for 10 min in Laemmli buffer and subsequently treated with Endoglycosidase H (New England Biolabs) in 50 mM sodium citrate for 3.5 h or overnight at 37°C. Another sample was adjusted to 1% NP40, followed by boiling for 10 min. N-Glycosidase F (EndoF) (Roche) was added to this sample and incubated for 3.5 h or overnight at 37°C. The third sample was directly examined by SDS-PAGE.

Modular structures of GLT25D1

A multiple sequence alignment of the strongly similar sequences corresponding to GLT25D1 (Swiss-Prot:Q8NBJ5), GLT25D2 (Swiss-Prot:Q8IYK4) and GLT25D3 (Swiss-Prot: Q5T4B2) was created using AlignX (VectorNTI, invitrogen) with the ClustalW algorithm (default settings). This sequence alignment was used as input for the HHpred comparison tool (<http://toolkit.tuebingen.mpg.de/hhpred>¹⁶). Selected: database pdb70, Max. PSI-BLAST iterations: 0, other settings default.

The region corresponding to aa 48 - 299 of GLT25D1 produced a hit with the structure of pp-GalNAc-T10 (PDB ID: 2D7I¹⁷, belonging to the CAZY family GT-2/PF00535), probability 92.6, E-value-0.006, P-value 3E-7. The region corresponding

to aa 331 - 525 of GLT25D1 produced a hit with the structure of the catalytic Domain Of Mouse Manic Fringe (PDB ID: 2J0A ¹⁸, belonging to the CAZY family GT-31 ¹⁹, probability 43, E-value-0.15, P-value 0.00076.

The indicated sequences of GLT25D1, including gaps and insertions created by the HHpred, were structurally modelled onto the backbone of these structures, 2D7I and 2J0A, using Protein Homology/analogy Recognition Engine ²⁰.

Results

GLT25D1 localises mainly to the ER

To elucidate to which particular compartment (tagged-) GLT25D1 was targeted, we compared the localisation with subcellular markers by immunofluorescence in human hepatoma cell line Huh7 (Fig.1). Dual staining of the internally Myc-tagged GLT25D1 transfected cells using antibody to the endoplasmic reticulum (ER)-resident protein, protein disulphide isomerase (PDI) together with the Myc-antibody demonstrated virtually complete overlap (yellow colouring), showing clear ER localisation of GLT25D1 (Fig.1). Co-localisation can be quantified using Pearsons correlation, where -1 means no overlap and 1 complete co-localisation. The Pearsons correlation calculated for PDI compared to GLT25D1 was 0.58, meaning considerable overlapping patterns. We furthermore examined the ER-Golgi intermediate compartment (ERGIC) and Golgi, using ERGIC53 and giantin respectively. Figure 1, however shows only minor overlap between GLT25D1 and ERGIC/ Golgi with Pearsons correlations of 0.13 and 0.04 respectively. Detailed inspection of many transfected cells showed no altered ER or Golgi distribution in GLT25D1 transfected compared to non-transfected cells (Fig. 1 and data not shown). Together these data indicate GLT25D1 mainly localises to the early secretory pathway; particularly ER.

Predictions of GLT25D1

A first step towards identifying the mechanisms responsible for directing GLT25D1 to the ER is defining subcellular localisation signals by using prediction algorithms (See Methods). Consistent with our observed ER localisation, we noted that the N-terminal 40 amino acids encompass a potential ER signal sequence, including a hydrophobic core region (h-region), a positively charged N-terminus (n-region) and a potential cleavage site between position 36 and 37 (Fig.2). Furthermore, upon

examining the C-terminus of the protein we noticed that the four extreme C-terminal residues Arg-Asp-Glu-Leu (RDEL) strongly resemble the tetrapeptide sequence KDEL, which causes retention in the ER²¹. Both predictions point to possible insertion of GLT25D1 into the ER and, if cleavage occurs, release from the ER membrane into the ER lumen. In order to investigate these potential targeting signals a series of deletions from the N-terminus as well as the C-terminus, including epitope tags on the opposite side, were made to observe their effect on localisation (Fig.3A).

N-terminal signal sequence targets GLT25D1 to the ER

We first studied the localisation of GLT25D1 with the putative signal sequence deleted (GLT25D1- Δ SS-Myc-tagged) compared to full-length GLT25D1 (GLT25D1 FL-HA-tagged). As shown in Figure 3B little overlap was evident with the full-length protein and the Δ SS protein, the latter displaying a diffuse cytosolic staining. This strongly suggests that the extreme N-terminus is a genuine signal sequence and targets the protein to the ER. Furthermore, when the N-terminal signal sequence of GLT25D1 was fused to GFP (SS-GFP), a reticular expression pattern was observed in a few cells (data not shown).

Cleavage of the GLT25D1 signal sequence

Next to the signal sequence a potential cleavage site is predicted (c-region, Fig.2). Cleavage of this N-terminal signal of GLT25D1 was analysed by blotting total cell lysate of cells transfected with N-terminally HA-tagged GLT25D1 construct (Fig.3A: HA-FL-Myc, construct 1). As shown in Figure 3C, the N-terminal HA-tagged GLT25D1 protein (Fig.3C: lane1, Total, anti-HA) was not visible with an anti-HA antibody, suggesting that the N-terminus was cleaved off. This construct is also tagged at the C-terminus with a Myc-tag that was detected in the total cell lysate, showing that the protein was indeed correctly expressed (Fig.3C: lane1, Total, anti-Myc). When Huh7 cells were transfected with GLT25D1 only Myc-tagged at the C-terminus (Fig.3A: FL-Myc, construct 2) identical protein size was detected compared to GLT25D1 tagged on both sides (Fig.3C: lane1 and 2, Total, anti-Myc). This furthermore indicates that the N-terminal signal sequence must be cleaved, since both proteins have a distinct N-terminus, which should have resulted in a different size in the absence of cleavage. Note the size difference between the full-length GLT25D1 construct and the GLT25D1 construct with a deleted signal sequence (Fig.3C: compare lanes 2 and 3, Total, anti-Myc), which is caused by protein

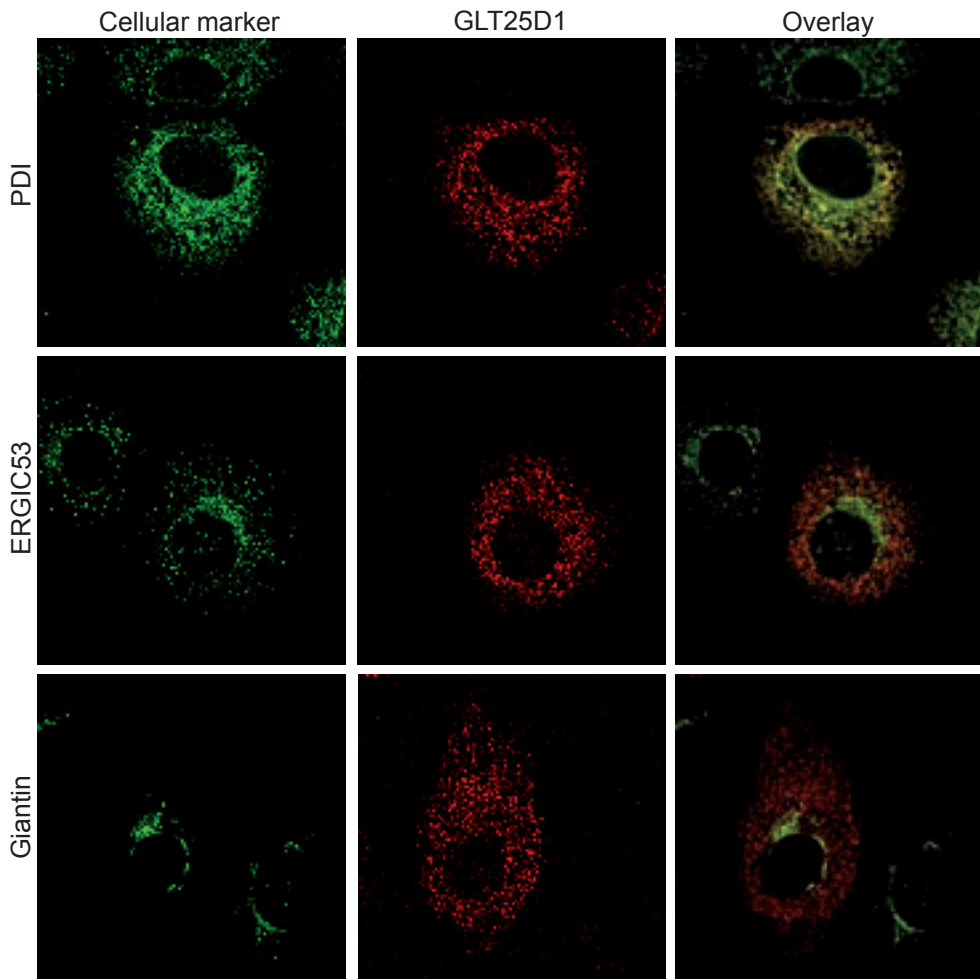


Figure 1 - Subcellular localization of Glycosyltransferase 25 domain 1 protein

Huh7 cells were transfected with full-length Glycosyltransferase 25 domain 1 containing an internal Myc-tag (GLT25D1) (See Methods for more details). After 24 h of expression, cells were fixed and subjected to immunofluorescence analysis. Cells were double labelled for GLT25D1 by anti-Myc (shown in red) and for the cellular markers (shown in green). ER is visualised by anti-protein disulfide isomerase (PDI) (top panels), ER-Golgi intermediate compartment by anti-ERGIC53 (middle panels) or Golgi by anti-giantin (bottom panels). Yellow observed in the overlay indicates overlap of red and green signal. Pearson's correlation of PDI and GLT25D1 is 0.58, ERGIC53 and GLT25D1 is 0.13, and giantin and GLT25D1 is 0.04.

glycosylation and will be explained below. Our findings are furthermore confirmed with other constructs, FL-MycRDEL, HA-FL-MycRDEL, HA-FL and HA- Δ RDEL (Fig.3C: lanes 6, 7, 8 and 9, Total, anti-Myc and anti-HA). The N-terminal HA-tag is not detected in lanes 7, 8 and 9 and a similar molecular weight of the constructs HA-FL-MycRDEL and FL-MycRDEL is observed. Hence, apparent by these results, the signal sequence of GLT25D1 is cleaved.

Carboxy terminal RDEL retains GLT25D1 in the ER

Proteins targeted to the ER that lack a specific retention signal are secreted or transported to the plasma membrane. Since the hydrophobic signal sequence of GLT25D1 is clearly cleaved off and its potential hydrophobic membrane anchor lost, the protein could be secreted in the cell culture medium. In contrast, if the C-terminal residues RDEL represent an ER retrieval signal, GLT25D1 would be

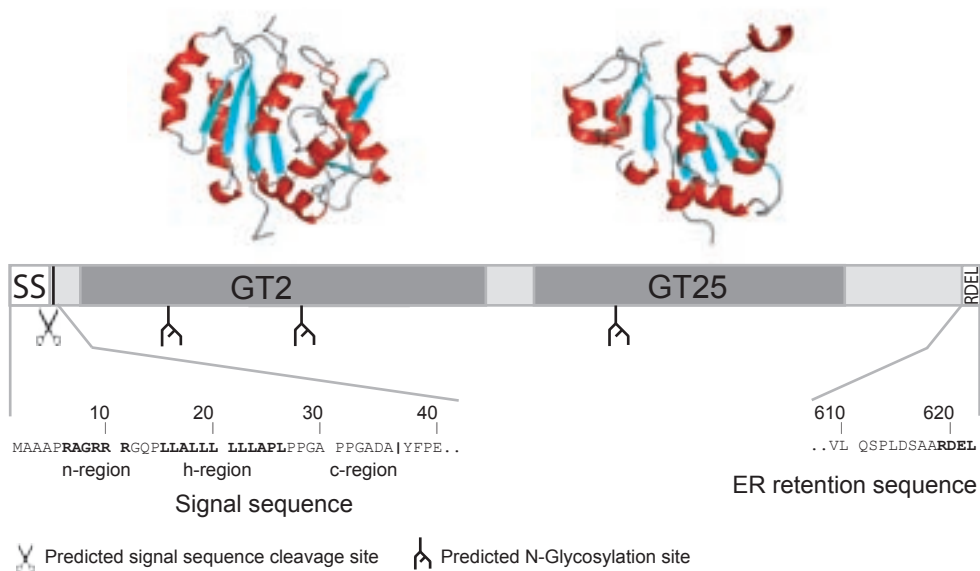


Figure 2 - Schematic representation of Glycosyltransferase 25 domain 1, including various predicted motifs

Top: Homology based structural models of the tandem glycosyltransferase domains. Bottom: Linear representation of GLT25D1 with possible signal sequence, ER retention signal and N-linked glycosylation sites indicated. Verification and discussion of these predictions are described in this article.

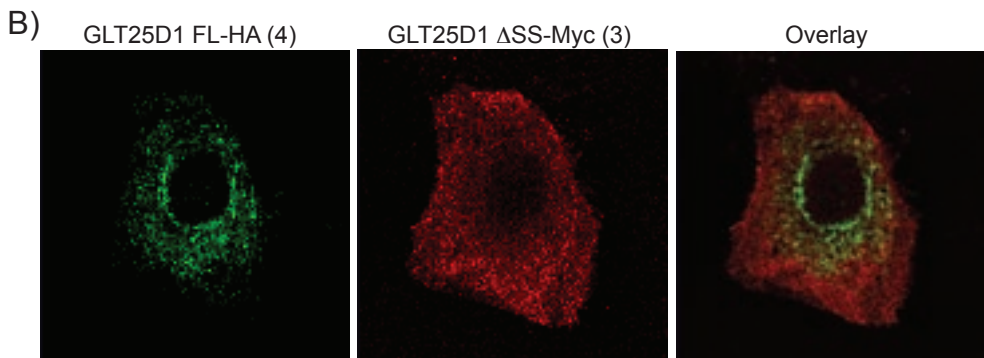
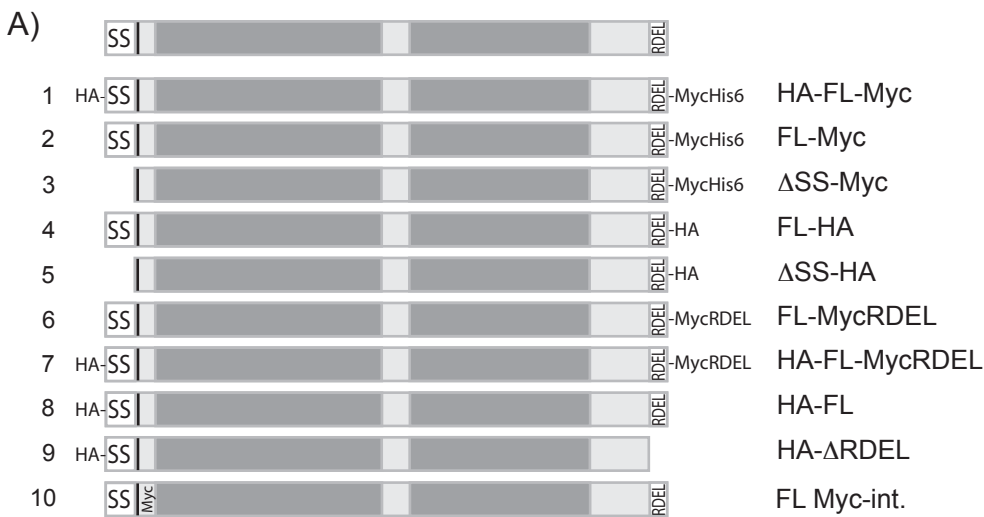
retained. We therefore examined the presence of the tagged GLT25D1 constructs in the cell culture medium. The constructs HA-FL and HA-FL with RDEL deleted (HA-FL- Δ RDEL) could not be shown to be present in the medium (Fig.3C: lanes 8 and 9, Medium, anti-HA), because the HA recognition epitope tag is cleaved off together with the signal sequence. Transfected HA-FL-Myc, FL-Myc and FL-HA could be detected with a C-terminal tag in the medium of Huh7 cells (Fig.3C: lanes 1, 2, and 4, Medium, anti-Myc and anti-HA). However, addition of an epitope tag (either Myc or HA) at the C-terminus of GLT25D1 could interfere with the RDEL retention function, resulting in (partial) secretion. When we compare localisation of GLT25D1-HA tagged and GLT25D1 internally Myc-tagged (GLT25D1 FL Myc-int.), we observe large overlapping patterns (Supplemental figure 1). Assuming that the internal tag reflects endogenous proteins best, the tagging seems to influence localisation only slightly.

In order to preserve the RDEL at the extreme C-terminus, we constructed RDEL at the end of two constructs, HA-FL-MycRDEL and FL-MycRDEL. In contrast to HA-FL-Myc and FL-Myc, both these constructs could not be detected in the medium (Fig.3C: lanes 6 and 7, Medium, anti-Myc), indicating that the RDEL is an ER-retention signal for GLT25D1. Additionally, as anticipated protein without the signal sequence Δ SS-Myc and Δ SS-HA, which are located in the cytosol, were not observed in the hepatocyte cell culture medium (Fig.3C: lanes 3 and 5, Medium, anti-Myc and anti-HA). Taken together these data clearly indicate that RDEL retrieves GLT25D1 to the ER.

Cleavage and retrieval of GLT25D1 could indicate that the protein is soluble. To confirm this we performed a membrane-floatation gradient experiment (Supplemental figure 2). As marker for integral membrane proteins we used calnexin and PDI was used as a type of ER soluble protein. As anticipated, calnexin is observed in the membrane-floating fractions with a peak signal present in fraction 7, while PDI is mainly found in the non-floating, bottom fractions. The bulk of GLT25D1 is observed in the bottom fractions of the gradient, indicating the protein is not an integral membrane protein.

GLT25D1 is N-linked glycosylated

Unexpectedly there is a substantial size difference between full-length GLT25D1 proteins, which are cleaved in the ER, and proteins with a deleted ER signal sequence (Δ SS) (Fig.4A: compare lane 1 with 2 and Fig.3C: compare lanes 1, 2 and 4 with lanes 3 and 5, Total, anti-Myc and anti-HA). The signal sequence deletion constructs



C)

Construct	1	2	3	4	5	6	7	8	9
N-tag	FL	FL	ΔSS	FL	ΔSS	FL	FL	FL	ΔRDEL
C-tag	HA	-	-	-	HA	-	HA	HA	-
Total anti-Myc									
anti-HA									
Medium anti-Myc									
anti-HA									

Figure 3 - Characterisation of signals in Glycosyl transferase 25 domain 1, suggesting ER residence

A. Summary of GLT25D1 expression constructs. HA indicates Hemagglutinin epitope tag; Myc refers to Myc epitope tag; FL denotes a full-length protein; RDEL represents carboxy-terminal Arg-Asp-Glu-Leu sequence; SS stands for signal sequence and Δ is followed by deleted part of the protein.

B. Co-transfected Huh7 cells with constructs GLT25D1 FL-HA and GLT25D1 Δ SS-Myc were subjected to immunofluorescence analysis. Signal of FL-HA is shown in green and of Δ SS-Myc in red.

C. Constructs 1 to 9, shown in A, were transfected into Huh7 cells. Total cell lysates (Total) and culture medium (Medium) from these cells were analysed by SDS-PAGE and western blot. Using antibodies against the N- and C-terminal tags of the constructs, anti-HA and anti-Myc, the proteins were detected.

were made starting at the predicted signal cleavage site and should therefore be of equal size. The higher molecular weight of the processed full-length proteins might be explained by posttranslational modifications occurring in the ER, which cannot take place on proteins with a deleted signal sequence contained in the cytosol. A potential modification increasing the molecular weight of a protein occurring in the ER is Asn-linked glycosylation. To assess glycosylation, we employed digestion with Endoglycosidase H and F (EndoH, EndoF), which cleave high mannose-containing oligosaccharides. GLT25D1 was purified from Huh7 cells transfected with FL-Myc (Fig.3A: construct 2) or Δ SS-Myc (Fig.3A: construct 3), then treated with EndoH/EndoF, separated by SDS-PAGE and subjected to western blot analysis (Fig.4A) (See for more details Methods). Figure 4A shows that after de-glycosylation the full-length protein decreases in molecular weight similar to the delta-signal sequence protein. This illustrates that the size difference is caused by glycosylation. When the full-length protein was only shortly incubated with EndoH or EndoF, we could discern, besides the full-length protein, three additional products (Fig.4B). These four bands likely correspond to three glycosylations of GLT25D1.

GLT25D1 co-localises with mannose binding lectin and lysyl hydroxylase

The report by Schegg et al., which elegantly demonstrated that GLT25D1 shows a strong galactosyltransferase activity towards mannose binding lectin (MBL), prompted us to compare the intracellular location of GLT25D1 and its substrate MBL¹¹. To correlate the expression of GLT25D1 with MBL in liver cells, where MBL is synthesised, we transfected GFP-tagged MBL and internal Myc-tagged GLT25D1 into Huh7 cells and performed immunofluorescence analysis. In Figure 5A GLT25D1 is shown in red and MBL in green (Fig.5A: top, first two panels), they both have a

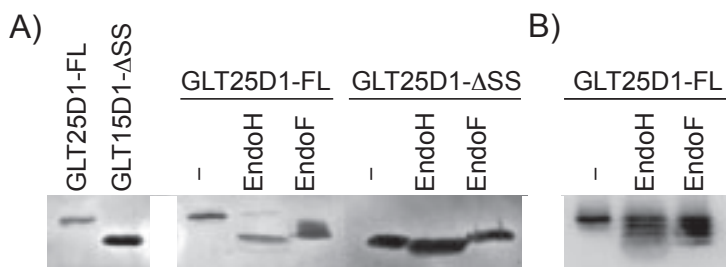


Figure 4: N-linked glycosylation of Glycosyl transferase 25 domain 1

A. Full-length GLT25D1 (FL) or GLT25D1 with the signal sequence deleted (Δ SS) constructs were transfected into Huh7 cells. Both constructs were carboxy terminal tagged with MycHis6, to purify proteins using Co^{2+} -beads and to detect in western blotting. Cell lysates are shown in the first two lanes after separation on SDS-PAGE and western blotting using an antibody against Myc. The purified proteins were subjected to either none (-) or Endoglycosidase H (EndoH) or F (EndoF) treatment (See for more details Methods). Results for FL protein are shown in lanes 3 to 5 and for Δ SS protein in lanes 6 to 8.

B. Partial de-glycosylation treatment of GLT25D1 full-length (FL).

perinuclear and reticular staining. MBL localises intracellular predominantly to the ER, though also accumulates in foci (Fig.5A: top, first panel) that were previously shown by Nonaka et al. to be ER exit sites ²². In accordance with ER localisation, substantial co-localisation of GLT25D1 with MBL is illustrated by yellow colouring in the overlay (Fig.5A: bottom, first panel).

Hydroxylation of lysines, to which galactosyltransferases transfer galactose, is carried out by lysyl hydroxylases (LH). These enzymes act upstream of glycosyltransferases. We therefore triple labelled the cells with LH3, which is one of the three LH isoforms in humans ¹⁰, depicted in blue (Fig.5A: top, third panel). The reticular and perinuclear staining of LH3 is similar to GLT25D1, which is also shown in the overlay of Figure 5A in purple (Pearson correlation: 0.42) (Fig.5A: bottom, second panel). In summary, there seem to be partially overlapping patterns of GLT25D1, MBL and LH3 (Fig.5A: bottom, third panel, white staining). To further substantiate co-localisation, we generated an intensity graph of the signals from each of these three proteins (Figure 5B). In the plot we observe similar profiles, indicating comparable sub-cellular localisation of GLT25D1, MBL and LH3, with exception of structures close to the nucleus containing MBL.

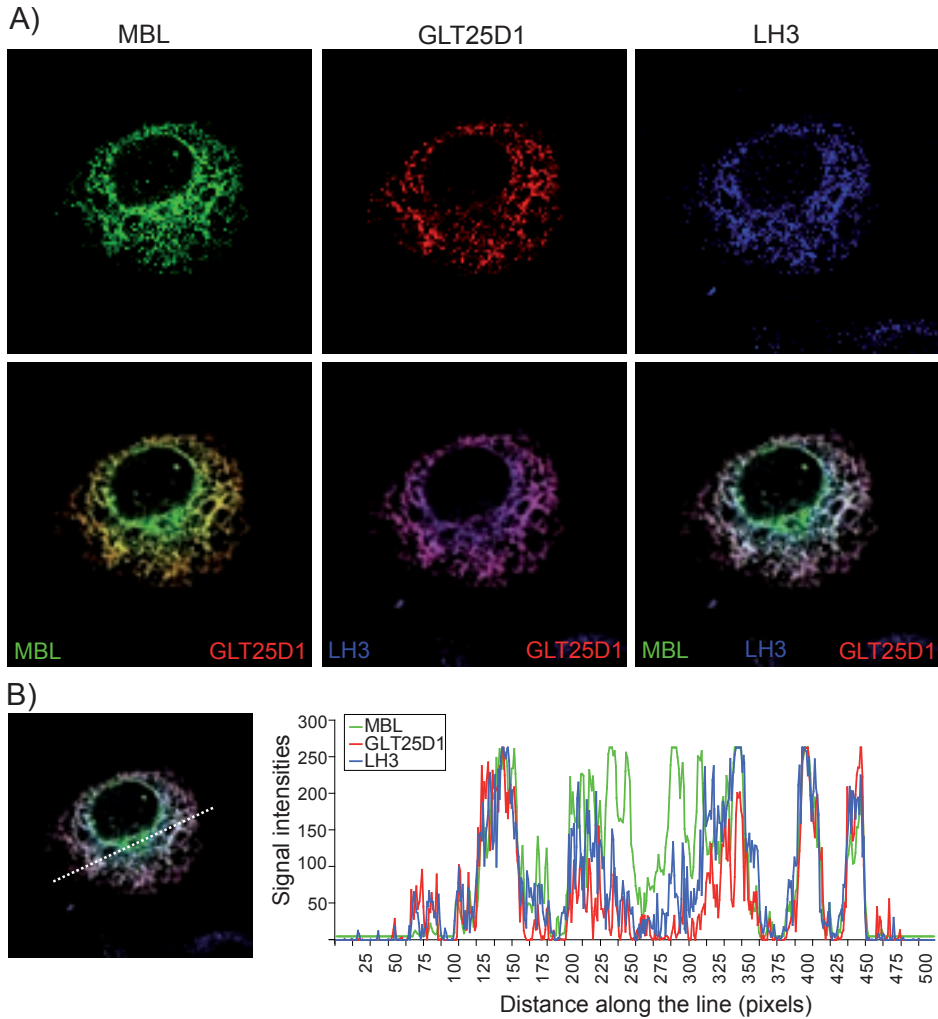


Figure 5: Co-localisation of Glycosyl transferase 25 domain 1 with Mannose binding lectin and Lysyl hydroxylase 3

A. Huh7 cells were co-transfected with Mannose binding lectin fused to GFP (MBL-GFP) and full-length GLT25D1 tagged with Myc internally (GLT25D1), and analysed by immunofluorescence after 24 h of expression. MBL was detected directly due to GFP and is shown in green. GLT25D1 and lysyl hydroxylase 3 (LH3) were visualised using antibodies against Myc or LH3 and are presented in red and blue respectively. When red and green signals overlap this is observed as yellow, red and blue as purple and red, blue and green as white. Pearson correlation of GLT25D1 and MBL is 0.53, GLT25D1 and LH3 is 0.42, and LH3 and MBL is 0.45.

B. A profile plot with signal intensities from MBL (green), GLT25D1 (red) and LH3 (blue) along the line drawn in the overlay on the left.

Discussion

The human protein GLT25D1 was reported to have galactosyltransferase activity towards mannose binding lectin (MBL), transferring galactose to hydroxylysine residues in the Gly-X-Lys repeats ¹¹. In this report, we examined the GLT25D1 localisation by immunofluorescence and made deletion mutants of GLT25D1 to ascertain whether the predicted subcellular targeting signal sequences were functional (Fig.2 and Fig.3A). It should be noted that exogenous over-expression might influence localisation due to saturation of the transport machinery. We performed our studies with a human hepatoma cell line, Huh7, because MBL is produced mainly by liver cells ¹⁴. Additionally, we confirmed by mass spectrometry analysis that GLT25D1 is normally expressed in these cells (Observed peptides are shown in Supplemental figure 3). Moreover, identical results were obtained in an additional cell line VERO, derived from monkey kidney epithelial cells. We found that GLT25D1, after being targeted to and cleaved in the ER (Fig.1 and Fig.3C), appears primarily in the ER most likely due to a functional ER retention signal, RDEL (Fig.3C). Although GLT25D1 gets secreted when a C-terminal tag is present, no secretion was found when the extreme four amino acids are RDEL, showing that these represent a functional ER retention signal (Fig.3C). This modified but related to KDEL carboxyl-terminal tetrapeptide has been shown to direct intracellular retention for several other proteins ²¹. Moreover GLT25D1 is highly sensitive to Endoglycosidase H, which is able to cleave non-complex N-linked oligosaccharides present in the ER ²³. This not only shows targeting to the ER lumen, but also ER-retention of the soluble GLT25D1 (Fig.4A).

We furthermore demonstrate by partial digestion with Endoglycosidase H or F that at least three asparagine residues become N-glycosylated (Fig.4B). In accordance with the number of glycosylated forms we distinguish, the NetNglyc server exactly predicted three asparagines to be N-glycosylated (residues 96, 184, 404) (Fig.2), suggesting that all these three residues are modified.

GLT25D1 is able to galactosylate MBL and other collagens ¹¹. Interestingly, we observe co-localisation between GLT25D1 and MBL. Intracellularly, MBL forms oligomers before it moves from the ER to the Golgi apparatus ⁸ and is secreted to serve as an activator of the lectin complement pathway. Binding of MBL to

carbohydrates on pathogens not only can mediate an innate immune response towards microbes, but more and more data indicate a potential defence against viruses ^{24, 25}. The envelope glycoproteins of human immunodeficiency virus (HIV), Ebola and Influenza A virus were demonstrated to attach to serum MBL ²⁶⁻²⁸. Additionally a subcellular interaction between HIV glycoprotein gp120 and MBL was shown ²². The presence of GLT25D1 mainly in the ER, where it co-localises with MBL and LH3, suggests that galactosylation by GLT25D1 occurs early in biosynthesis before being transported to the Golgi. This would be in line with the results of Heise et al., which demonstrate that glycosylation of MBL continues while transport to the Golgi complex is blocked by Brefeldin A treatment ⁸.

Before galactosylation of the lysine in the Gly-X-Lys repeat, the lysine is hydroxylated. LH3 has hydroxylase, galactosyltransferase and glucosyltransferase activity (Reviewed in ¹⁰). When performing domain prediction analysis of GLT25D1 (see Methods), we noticed that GLT25D1 is a modular glycosyltransferase (GT) composed of two GT family members, with the C-terminal one related to glycosyltransferase of family 25 (hence its name) and an additional N-terminal domain which displays distant relation to family GT2 transferases (Fig.2 and Supplemental figure 4). In both domains the conserved DxD motif, which binds one of the ribose hydroxyl groups, could be observed (See Supplemental figure 4; Bernard Henrissat personal communications). Other examples of modular GTs are Heparin synthase, Chondroitin synthase and hyaluronan synthase, each involved in addition of alternating sugars, and each containing an N-terminal GT2 domain ²⁹. Part of this GT2 domain of GLT25D1 surprisingly shows sequence homology to LH3, which suggests that they might function similarly either in substrate recognition or in glucosyltransferase activity.

It is interesting to note that in a search for tumor-specific markers both GLT25D1 and LH3 genes were up regulated in a large majority of human malignancies ³⁰, but the biological pathway involved is unknown. The location of a particular glycosyltransferase in the cell clearly defines its function and biological importance. Most glycosyltransferases reside in the Golgi, where glycan synthesis takes place in a sequential order ^{12, 13}. The presence of GLT25D1 early in the secretory pathway indicates its enzyme activity is displayed there, confirming that collagens and collectins are likely to be glycosylated in the ER before they move to the Golgi apparatus and are secreted. Yet, the exact role of glycosylated hydroxylysine residues

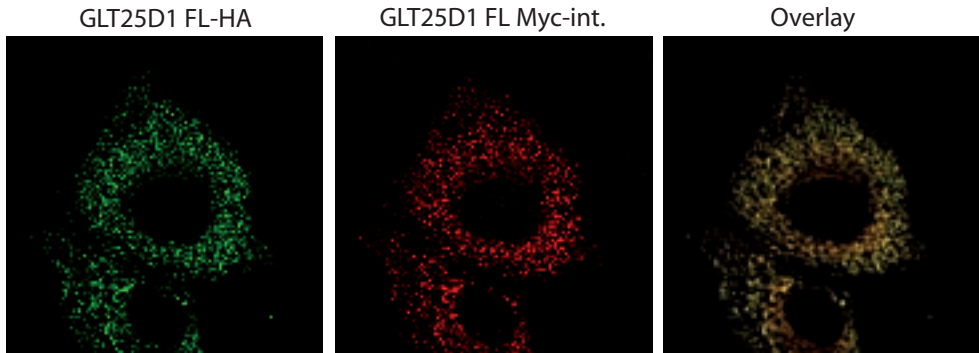
in collagenous proteins is still poorly understood. Nonetheless, our results strengthen the hypothesis that glycosylation of collagens and collectins occurs early in their biosynthesis.

Conclusions

Our experiments show that GLT25D1 is a soluble protein present in the lumen of the ER, is cleaved after its signal sequence and is N-glycosylated at three positions. The occurrence of GLT25D1 early in the secretory pathway, mainly ER, suggests that collagens and collectins are likely to be galactosylated before trafficking to the Golgi and are secreted.

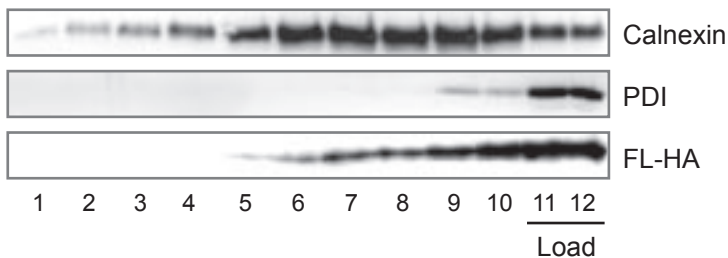
Acknowledgments

We thank Prof. Dr. T. Kawasaki for providing the pEGFP-MBL construct, P.A. van Veelen for mass spectrometry analysis and K. Knoops for critical reading of this manuscript.



Supplemental figure 1 - Localisation of GLT25D1-HA compared to internally Myc-tagged GLT25D1

Huh7 cells were co-transfected with full-length GLT25D1-HA (GLT25D1 FL-HA) and internally Myc-tagged full-length GLT25D1 (GLT25D1 FL Myc-int.). 24 h after transfection the cells were subjected to immunofluorescence analysis. GLT25D1 FL-HA and GLT25D1 FL Myc-int. were detected by antibodies against HA (green) and Myc (red), respectively. Pearson correlation of GLT25D1 FL-HA and GLT25D1 FL Myc-int. is 0.83.



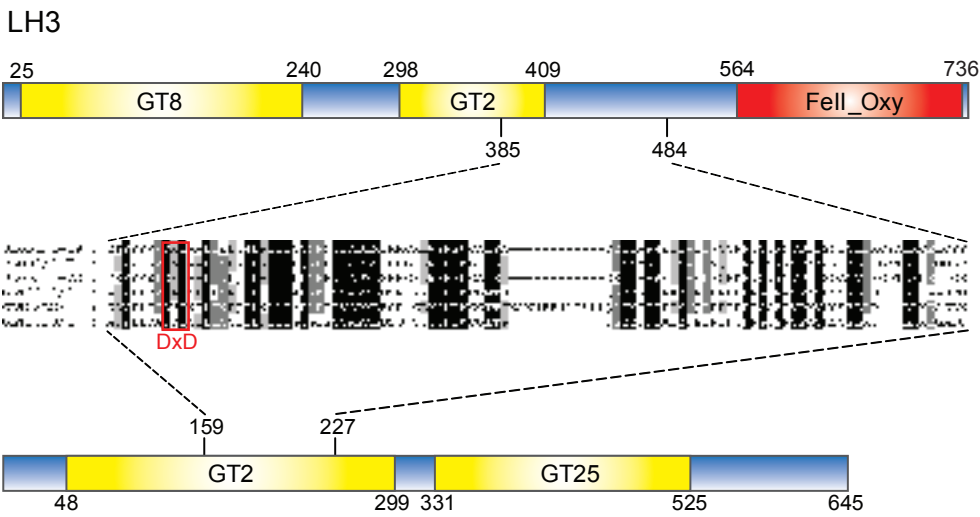
Supplemental figure 2 - GLT25D1 is a luminal ER protein

After 24 h, Huh7 cells transfected with GLT25D1 FL-HA were subjected to sucrose density gradient centrifugation. Cell lysates were loaded under a sucrose gradient from 10-80% w/v. Fractions were taken from top (fraction 1) to bottom (fraction 12) and separated by SDS-PAGE, followed by immunoblot analysis for calnexin and PDI (protein disulfide isomerase). GLT25D1 FL-HA was visualised using an antibody against HA-epitope.

MAAAPRAGRRRRGQPLLALLLLLLLAPLPFGAPPGADAYFPEER**WSPESPLQAPRVLIALLA**
RNAAHALPTTLGALERLRHPRERTALWVATDHNMDNTSTVLREWLVAVK**SLYHSVEWRPA**
EEPRSYPDEEGPKHWSDSRYEVMKLRQAALKSARDMWADYILFVDADNLIILNPDTLSLL
IAENK**TVVAPMLDSRA**AYSNFWCGMTSQGYKRT**PAYIPI**RKRDRRGCFAPVMVHSTFLI
DLRKAASRNLAFYPPHPDYTWSFDDIIVFAFSCKQAEVQMYVCNK**EEYGFLPVPLRAHST**
LQDEAESFMHVQLVVMVK**HPPAEP****S**R**FIS**APT**K**TPDKMGFDEVFMINLRRRQDRRERMLR
ALQAQEIECRLVEAVDGKAMNTSQVEALGIQMLPGYRDPYHGRPLTKGELGCFLSHYNIW
KEVVDRLGQKSLVFEDDLR**FEIFFKRR**LMNLMRDVEREGLD**WDLIYVGRKRMQVEHPEKA**
VPRVRNLVEADYSYWTLAYVISLQGARK**LLAAEPLSK**MLPVDEFPLVPMFDKHPVSEYKA**H**
FSLRNLHAFSVEPLLIYPHTYTGDGYSVSDTETSVVWNNEHVKTWDRAKSQ**KMREQQAL**
SREAKNSDVLQSP~~LD~~SAARDEL

Supplemental figure 3 - GLT25D1 peptide coverage

Huh7 cell lysates were subjected to two-dimensional PAGE, followed by silverstaining. Spots, corresponding to Mw 72 kDa and isoelectric point ~7 were digested with trypsin and analysed by ESI tandem MS. The obtained peptide sequences from GLT25D1 are shown in bold in the primary sequence of the protein.



GLT25D1

Supplemental figure 4 - Sequence alignment of human GLT25D1, LH3(PLOD3) and close homologues

Alignment was generated using the ClustalX program. DXD motif is indicated (red box). Yellow indicates putative glycosyltransferase folds. Fell_Oxy indicates a predicted Fe(II)-dependent oxygenase superfamily. Numbers indicate amino acid position.

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6

Characterisation of the interaction between Hepatitis C virus NS3 and GLT25D1



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Introduction

The interplay between a virus and its host is important for the replication of the virus. Hepatitis C virus, a positive single-stranded RNA virus, infects human hepatocytes, in which the virus life cycle depends on many cellular processes. In Chapter 4, we investigated protein-protein interactions between the HCV multifunctional non-structural protein 3 (NS3) and host-proteins. In that study we identified glycosyl-transferase 25 containing domain 1 (GLT25D1) as a novel NS3 associated protein. Since the cellular localisation of GLT25D1 was unknown, we determined in Chapter 5 that the protein is targeted to the lumen of the endoplasmic reticulum (ER). NS3, on the other hand, is localised on the cytosolic side of the ER-membrane¹, pointing out a membrane barrier hampering a direct interaction. Therefore, additional characterisation of the association mode of NS3 with GLT25D1 is required. This was achieved by performing experiments to determine domains within the protein important for association. In addition, the site of interaction was established at the ER, using co-localisation analysis. These characterisation studies of the NS3 interaction with GLT25D1 are shown here and discussed using interaction models.

Methods

Cell culture and transfection

Human hepatoma cell lines, Huh7, harbouring the HCV replicon were grown in Dulbecco's Modified Eagle's Medium without NaPyruvate, supplemented with non-essential amino acids, L-glutamate, Penicillin, Streptavadin and Geneticin. Cells were subcultured using Trypsin.

Cells were transfected using Amaxa nucleofactor Kit (Lonza). In brief, cells were trypsinised and spun down at 100 ×g for 5 min. Subsequently cell pellets were solved in solution T (Lonza) and mixed with 4 µg of plasmid DNA. Cells were placed in the electroporation cuvet and pulsed with program T016. Subsequently, the cells were directly transferred to pre-warmed and humidified cell culture medium.

Plasmid construction

The plasmid expressing EGFP targeted to the ER is from Clontech. GLT25D1-HA, deltaSS-GLT25D1-HA, GLT25D1-mycRDEL, HA-GLT25D1-mycRDEL

were used previously ². To construct GLT25D1 with the PDI signal sequence, the sequence was amplified by PCR from GLT25D1 full-length clone, IRAKp-961P01217Q (imaGenes, Berlin, Germany), using forward primer: GCTTGTAC-CATGCTGCGCCGCGCTCTGCTGTGCCTGGCCGTGGCCGCCCTGGT-GCGCGCCGACGCCCCCGAGGAGCGCTGGAGCCCCGGAGTCGC and reverse primer: CGCCAACAGCGCGATGAGCAC. The PCR product was digested with *KpnI* and *SbfI*-HF and ligated into GLT25D1-HA similarly digested with *KpnI* and *SbfI*-HF.

NS3 isolation

36 h post-transfection, 1.5×10^7 HCV replicon containing Huh7 cells were washed with PBS and scraped in isotonic buffer (50 mM Tris pH7.0, 15 mM NaCl, 1 mM $MgCl_2$, 240 mM sucrose and 20 mM imidazole). Cells were mechanically lysed in a ball-bearing homogeniser (Isobiotec, Heidelberg Germany), with a clearance of 18 micron. Following centrifugation at $800 \times g$ for 10 min, supernatant (volume around 2 ml) was collected and the two replicon cell lines with and without a His6-tag at NS3 were equalised for protein concentration using Bradford reagent (Biorad). Subsequently, NP40 was added to the cell lysate, to a final concentration of 0.05%. After 30 min on ice, cell lysate was incubated with 80 μ l 50% Cobalt²⁺ bound-beads (Clontech) for 2 h under continuous rotation. Next the beads were washed three times with 1 ml isotonic buffer containing 0.05% NP40, which was followed by heating the beads in Laemmli-buffer at 95°C for 10 min.

SDS-PAGE and western blotting

SDS-PAGE and western blotting was performed as described previously (e.g. Chapter 4)

Immunofluorescence microscopy

Cells were fixed 12-16 h post transfection with 3% paraformaldehyde in PBS. PFA was quenched using 50 mM NH_4Cl in blockbuffer, which contained 5% fetal calf serum (FCS) in PBS. After permeabilisation of the cell membranes in blockbuffer, supplemented with 0.1% Triton X-100, the cells were stained for 1 h with primary antibodies diluted in blockbuffer. The following washing steps were performed with blockbuffer or glycinebuffer (10 mM glycine in PBS). Next the coverslips were incubated with secondary antibodies diluted in blockbuffer for 1 h. Subsequent to washing with glycine buffer, PBS and H_2O , the coverslips were mounted with

Prolong (Invitrogen) mounting medium. Fluorescence images were captured using Leica TCS SL confocal microscope, 63x Plan Apo oil immersion objective (zoom two or four times), appropriate filter settings and sequential scan modes. Images were optimised with Adobe Photoshop CS2. PDM images, Pearson's correlation and Manders' coefficient were generated using WCIF_ImageJ (NIH).

Antibodies

The following antibodies were used: anti-NS3 (mouse) (NovaCastra), anti-Myc (rabbit, immunofluorescence) (Abcam), anti-Myc (mouse, western blotting) (Roche), anti-HA (mouse) (Abcam), Donkey-anti-mouse-cy3 (Jackson), Goat-anti-rabbit-Alexa488 (Invitrogen), Goat-anti-rabbit-Alexa633 (Invitrogen) and Goat-anti-mouse-HRP (Dako).

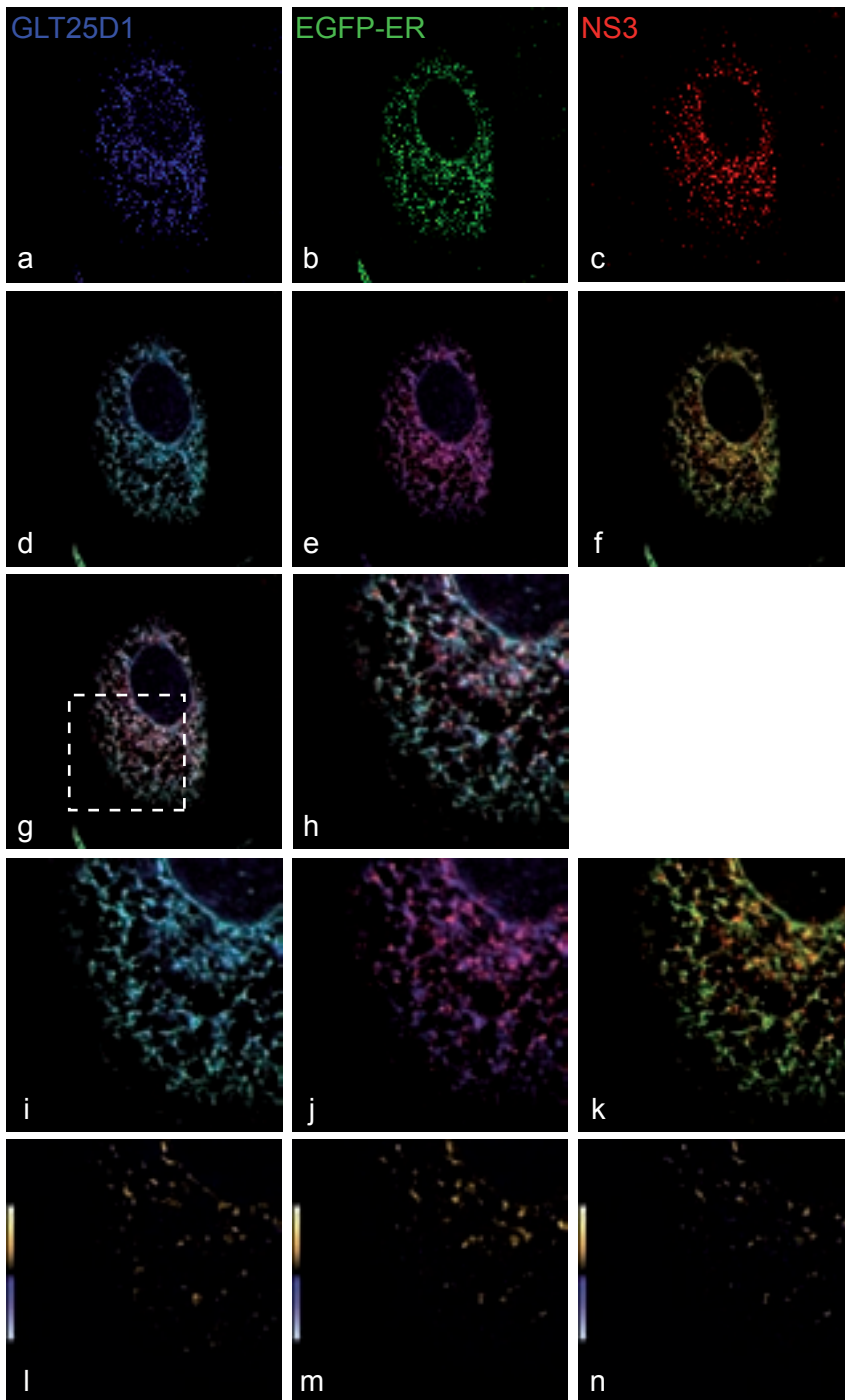
Results and discussion

Partial co-localisation of NS3 and GLT25D1

A first step required for protein-protein association is localisation to the same cellular compartment. NS3 is present on endoplasmic reticulum (ER) membranes ^{1, 3} and GLT25D1 resides within this compartment ², suggesting that the ER must be the place of interaction. Therefore, an immunofluorescence assay was set up to determine co-localisation of the two proteins at the ER (Fig.1). HCV replicon containing cells (pFK5.1Neo ⁴) were co-transfected with an EGFP expression construct containing ER targeting signals (ER-EGFP), to visualise the ER, and a construct expressing GLT25D1 with a mycRDEL-tag at the C-terminus for detection ². The cells were fixed 16 h post-transfection and subsequently analysed by immunofluorescence.

Figure 1 - Partial co-localisation of GLT25D1 and NS3 at the endoplasmic reticulum

Huh7 HCV subgenomic replicon containing cells were co-transfected with constructs expressing an endoplasmic reticulum localised enhanced-green-fluorescent protein (EGFP-ER) and a GLT25D1 fused with a C-terminal mycRDEL-tag. After 16 h of expression the cells were fixed, using 3% PFA. In immunofluorescence NS3 and GLT25D1 were detected with antibodies against NS3 or the myc-epitope. GLT25D1 (panel a), EGFP-ER (panel b) and NS3 (panel c) are labelled in blue, green and red, respectively. Panels d to f show merged images of two channels. Panel g illustrates all three signals together and the drawn square is the zoom of panels h to k. Panels l, m and n show pseudocolour PDM images of the panels above (h, i and k). High and low co-localisation is depicted in yellow and blue, respectively.



GLT25D1 was visualised with Alexa633 colouring blue and NS3 was labelled in red using Cy3 (Fig.1; panels a and c, respectively). Figure 1 shows a perinuclear staining for GLT25D1 (Fig.1; panel a). Apparent overlap of this pattern with ER-EGFP that is targeted to the ER (Fig.1; panel b) indicates ER localisation of GLT25D1 and confirms our previous results ² (Fig.1; panels d and i). Examining NS3 localisation in the same cell shows a perinuclear staining (Fig.1; panel c). Overlay images illustrate that there is partial co-localisation between NS3 and GLT25D1 (Fig.1; panels e and j), similar for NS3 and the ER targeted EGFP (Fig.1; panels f and k).

To corroborate these results, co-localisation was quantified by calculating Pearson's correlation coefficient for NS3 compared to GLT25D1 or ER targeted EGFP. A value of 1 indicates perfect overlap of patterns, while a value of -1 suggests no relation between the two signals. Determined from an average of 6 pictures, NS3 related to GLT25D1 with a coefficient of 0.35. The correlation between NS3 and ER targeted EGFP was 0.37. Both numbers verify our observation of partial co-localisation (Fig.1; panels e, f, j and k). Interestingly, the immunofluorescence images showing only partial co-localisation of NS3 with the ER (Fig.1; panel f and k), seem to substantiate our previous results on the presence of different pools of NS3 (Chapter 4). Likewise, other groups observed a wider distribution of HCV non-structural proteins compared to the ER or compared to HCV RNA ^{3,5,6}. Together these data suggest the presence of diverse localisation of NS proteins, which are most likely involved in different functions during the virus life cycle.

Using PDM (Product of the Differences from the Mean) images, which illustrate where signals do or do not overlap, we investigated the site of co-localisation between NS3 and GLT25D1. In the panels l, m and n of Figure 1 the coincidence of the three proteins is shown, using pseudo-colouring of yellow and blue indicating co-localisation or no co-localisation respectively (GLT25D1 to ER-EGFP in panel l, GLT25D1 to NS3 in panel m and NS3 to ER-EGFP in panel n). It is evident that these patterns of co-localisation resemble each other, indicating that co-localisation of NS3 with GLT25D1 is mainly at the ER (Fig.1; compare panels l to n).

The signal sequence of GLT25D1 is important for association with NS3

To further characterise the interaction between NS3 and GLT25D1 at the ER, the domain important for association can be determined. This can be accomplished by constructing deletion mutants of GLT25D1, transfecting them into Huh7 cells supporting a subgenomic RNA replicon and analyse their capability of binding to NS3.

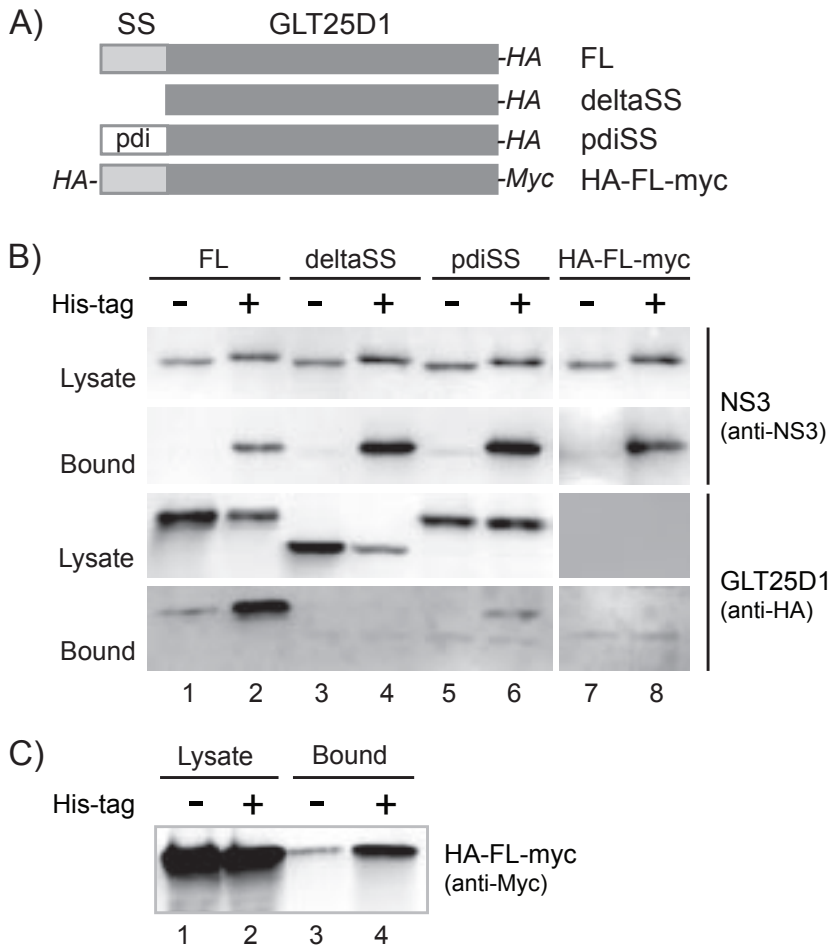


Figure 2 - Importance of the GLT25D1 signal sequence for association with NS3

A. Schematic drawing of the GLT25D1 constructs used in this figure. The constructs are full-length GLT25D1 (FL), GLT25D1 with a deleted signal sequence (deltaSS) and GLT25D1 with the signal sequence of protein disulfide isomerase (pdiSS) instead of its own signal sequence. These constructs are C-terminally tagged with HA for detection in western blot. The fourth construct is HA- and Myc-tagged at the N- and C-terminus, respectively (HA-FL-myc).

B. Huh7 cells, containing HCV replicon with (+) or without (-) a His6-tag at NS3, were transfected with the four different constructs of GLT25D1. After 36 h of expression cells were lysed and subjected to metal affinity purification of NS3 (See for more detail Methods). Lysates and the proteins associated to the beads (Bound) were analysed by SDS-PAGE and western blotting using antibodies against NS3 and the HA-tag.

C. Here the western blot results are shown from construct HA-FL-myc with anti-Myc antibody.

In our preceding study, where we identified GLT25D1 as an interacting protein of NS3, we used a replicon cell line containing a His6-tag at NS3 ⁷. This cell line is used in the assays described here. In the previous chapter we made a GLT25D1 construct lacking the signal sequence that localises to the cytosol (deltaSS) ². Since NS3 is localised on the cytosolic side of membranes ¹, we could envision binding of the two proteins NS3 and deltaSS. Replicon cells containing no tag (–) or a His6-tag (+) at NS3 were transfected with full-length GLT25D1 (FL) or with GLT25D1 lacking the signal sequence (deltaSS), both HA-tagged at the C-terminus (Fig.2A). After 36h, the cells were lysed and His6-NS3 was purified from these cells using Cobalt-coated beads. Proteins associated to the beads were separated on SDS-PAGE and subjected to western blot analysis. Figure 2B shows there is an equal amount of NS3 present in all the lysates (Fig.2B; top panel). After purification, NS3 is only present in the lanes representing the proteins bound to the beads isolated from lysates of the His6-tagged NS3 containing replicon cell line (Fig.2B; second panel, lanes 1 versus 2). In addition, full-length GLT25D1 (FL) was co-purified together with NS3 (Fig.2B; bottom panel, lanes 1 and 2). Surprisingly, GLT25D1 lacking the signal sequence (deltaSS) did not associate to NS3 (Fig.2B; bottom panel, lanes 3 and 4), suggesting that the signal sequence is important for binding to NS3. There could be several explanations for this observation. First, there might be direct binding of the signal sequence to NS3. Secondly, a correct localisation of GLT25D1 to the ER might be required for the association, which is a result of targeting by the signal sequence. To discriminate between these two possibilities, we exchanged this region of GLT25D1 with the signal sequence from the ER resident protein PDI (protein disulfide isomerase) (Fig.2A; pdiSS). This signal sequence differs substantially from the one of GLT25D1 and should exclude direct interaction with the signal sequence if binding is sequence specific (Table 1). Moreover, PDI was shown not to associate with NS3 (Chapter 4). As shown in Figure 2B, there is co-purification of this protein (pdiSS) together with NS3 (Fig.2B; bottom panel, lanes 5 and 6), indicating that this signal sequence enables co-purification with NS3. However, when comparing the intensity

Table 1

Protein	Signal sequence
GLT25D1	MAAAPRAGRRRGQPLLALLLLLLLAPLPPGAPPGADA / YFPEERWSPE
LH3	MTSSGPGPRFLLLLLPLLLPPAASA / SDRPRGRDPV
PDI	MLRRALLCLAVAALVRA / DAPEEEDHVL

of western blot signals from FL and pdiSS, isolation appears much less efficient (Fig.2B; bottom panel, lanes 2 and 6). This suggests that the signal sequence itself is needed for direct interaction.

To investigate binding to this domain, a GLT25D1-construct was used with an N-terminal HA-tag to detect the signal sequence. Since the signal sequence is cleaved off GLT25D1², a Myc-tag at the C-terminus was constructed to demonstrate protein expression (Fig.2A; HA-FL-myc). NS3 was specifically isolated (Fig.2B; second panel, lanes 7 and 8) and HA-FL-myc was co-purified with NS3, examined by western blotting using anti-Myc antibody (Fig.2C; lanes 3 versus 4). When looking at the results of the western blot incubated with anti-HA antibody, no HA signal is observed to co-purify with NS3, indicating that the signal sequence of NS3 associated GLT25D1 is cleaved off (Fig.2B; bottom panel, lanes 7 and 8). Additionally, uncleaved GLT25D1 runs slightly higher than the processed form (data not shown), yet the co-purified HA-FL-myc protein is detected at a similar molecular weight as the proteins in the lysate (Fig.2C). This points to association of processed GLT25D1 to NS3. Together these data show that, in order to interact with NS3, the signal sequence is essential for the targeting of GLT25D1. Additionally, some signal sequence specificity might be needed for initial binding, though when associated to NS3 the signal sequence is cleaved.

Co-localisation of GLT25D1 and pdiSS

The difference in co-purification with NS3 between full-length GLT25D1 and the GLT25D1 containing the alternative PDI signal sequence (pdiSS) might be due to an altered targeting to the ER (Table 1). To verify the localisation of this protein compared to full-length GLT25D1, immunofluorescence was performed on Huh7 cells co-transfected with these two constructs (Fig.3). For detection both constructs are C-terminally tagged, full-length GLT25D1 with a myc-RDEL (labelled in green, panel a and d) and an HA-tag on GLT25D1 with the PDI signal sequence (labelled in red, panel b and e). Figure 3 shows that the perinuclear staining of the two constructs substantially overlap (panel c and f). In addition, Pearson's correlation coefficient was calculated at 0.66 (average of 4 pictures), indicating considerable co-localisation. Moreover, the PDM images in Figure 3 illustrate in yellow that the staining pattern of the two proteins overlap (Fig.3; panel g and h). These data demonstrate that pdiSS and full-length GLT25D1 are targeted in a similar way to the same compartment, indicating that the distinction in co-isolation is not attributable to a changed localisation to the ER.

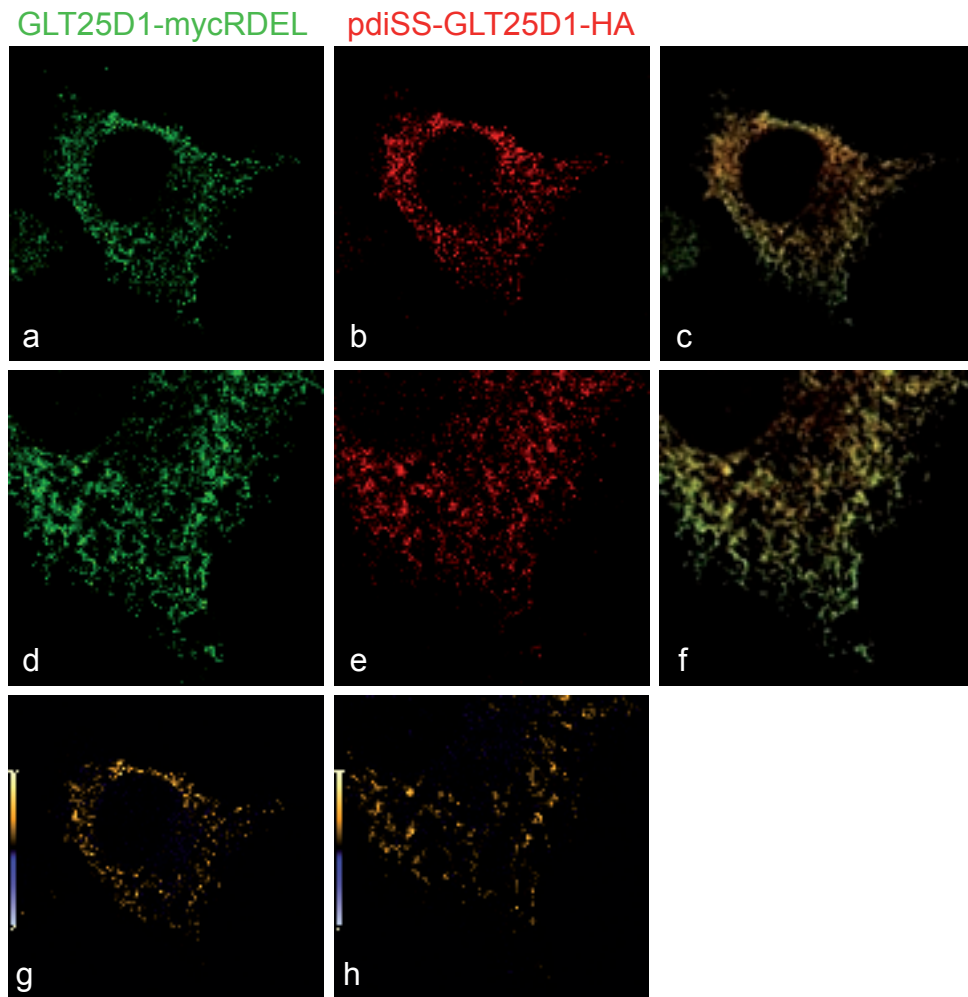


Figure 3 - Co-localisation of GLT25D1 and PDI signal sequence-GLT25D1

GLT25D1-mycRDEL and PDI signal sequence-GLT25D1-HA (pdiSS) were co-transfected into Huh7 cells. After 16 h, the cells were fixed and subjected to immunofluorescence using antibodies against the Myc-epitope and the HA-tag. GLT25D1-mycRDEL is coloured in green (panel a, d), red represents pdiSS-GLT25D1-HA (panel b, e) and merged pictures are shown in the right panels c and f. Panels g and h show pseudocolour PDM images. High and low co-localisation is depicted in yellow and blue, respectively.

Models for interaction

The experiments described above indicate the ER as the site of interaction (Fig.1 and 3) and an essential role for the signal sequence of GLT25D1 for association to NS3 (Fig.2). These results point out the membrane barrier between GLT25D1, which is in the ER lumen, and NS3 that associates via NS4a to the cytosolic side of the ER membranes (Fig.4; top image)^{1,2}. In Figure 4 several models for association of NS3 with GLT25D1 are shown that might overcome this membrane barrier. However, none of these models are completely in accordance with our experimental results as will be discussed.

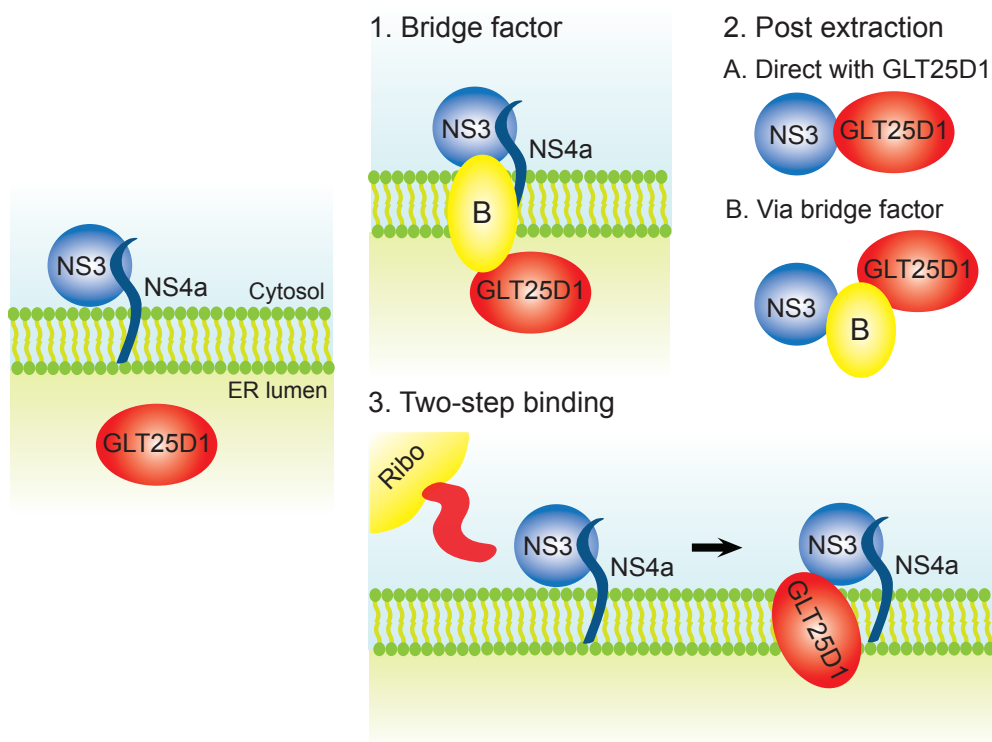


Figure 4 - Models for association of NS3 to GLT25D1

Top image indicates the localisation of NS3/NS4a relative to GLT25D1. Three models are proposed for interaction of NS3 with GLT25D1, these are discussed in the text.

Abbreviations: Non-structural protein (NS), Glycosyltransferase 25 containing domain 1 (GLT25D1), endoplasmic reticulum (ER), bridge factor (B), ribosome (Ribo).

Model 1: Bridge factor

The first model proposes an additional protein, spanning the membrane and interacting with both GLT25D1 and NS3 (Fig.4; model 1). In this model correct luminal localisation of GLT25D1 is important for interaction. Our data support that the targeting to the ER by the signal sequence is essential. The failure of GLT25D1 without a signal sequence to co-purify with NS3, illustrates the requirement of the signal sequence (Fig.2B). In addition, the signal sequence of NS3 associated GLT25D1 is cleaved off (Fig.2B and C). These data indicate that only the luminal localised GLT25D1 can interact with the bridge factor and form a complex with NS3. However, if the signal sequence itself is not important for binding, this model does not explain the results of GLT25D1 with the PDI signal sequence. That protein co-purifies to a much lower extent than full-length GLT25D1, while we would expect similar association efficiency of the same luminal protein (Fig.2B). This pdiSS construct implies that the signal sequence contains a specific function.

Besides GLT25D1, we have identified LH3 as an NS3 interacting protein. When performing sequence alignments, we observed a similar domain in both proteins, named GT2 domain (glycosyltransferase 2), implying a potential common association domain (Chapter 5, Supplemental figure 4). This domain could be involved in attachment to the bridge factor.

Initially NS4a was considered to be the bridge factor, since it binds directly to NS3 and is membrane associated. Brass et al., however, have shown that NS4a does not traverse the lipid bilayer and thus cannot contact GLT25D1 directly ⁸ (Fig.5). In Chapter 4, we investigated co-purification of the other HCV NS proteins and observed no co-purification with NS3, indicating that the bridge factor must be a host-protein. However, in 2D-PAGE we do not co-isolate the bridge factor. Likely because hydrophobic proteins are difficult to analyze in 2D-PAGE, owing to protein solubility, hence a membrane-spanning bridge factor might not be observed.

Model 2: Post extraction

Another possibility might be binding of the two proteins after cell lysis, during isolation of the NS3 protein. Two situations can be envisaged, the first is direct binding of GLT25D1 to NS3 and the second is via a bridge factor (Fig.4; model 2). In the latter condition, GLT25D1 should pre-form a complex with the bridge factor in the ER compartment in order to interact with NS3, later during isolation. This would explain why the ER targeting, due to the signal sequence, is essential for NS3 association

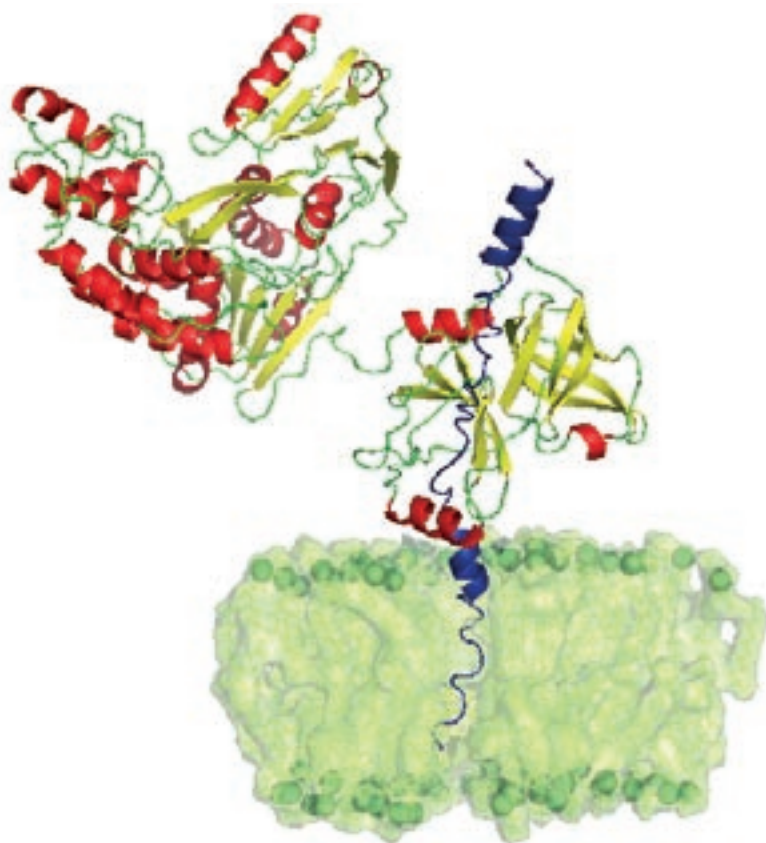


Figure 5 - Model for the binding of NS3-4a with the membrane

The tertiary structure of NS3 is shown in yellow and red, and in blue NS4a is depicted. The NS3-4a complex is positioned into a lipid bilayer (green). This model was kindly provided by Dr. H.C. van Leeuwen.

(Fig.2B). For the same reason, a direct interaction between NS3 and GLT25D1 might be excluded, because GLT25D1 lacking the signal sequence does not co-purify together with NS3 (Fig.2B). On the other hand, NS3 may possibly contain a binding site for GLT25D1 and LH3. GLT25D1 and LH3 are involved in lysyl-galactosylation of collagenous proteins, containing Gly-X-Y repeats with X often being a proline and Y a lysine^{9,10}. NS3 contains a small repeat, Gly-Pro-Lys-Gly-Pro-Ile (aa 1092 to 1097 in HCV genome). If this is a binding site, the association could only take place after disruption of the ER membrane, during NS3 isolation.

Model 3: Two-step binding

In the third model, at first the signal sequence is required for targeting to the ER, subsequently a binding site of GLT25D1 is close enough to interact with NS3 (Fig.4; model 3). Here the signal sequence is only required initially. For that reason the signal sequence of NS3 co-purified GLT25D1 can be cleaved (Fig.2B and C). This model should occur during translation of GLT25D1, because only then the GLT25D1 polypeptide is at the cytosolic side of the ER membrane. Since the molecular weight of associated GLT25D1 is identical to the mature form in the lysate (Fig.2C), this model furthermore includes that the protein is completely translated and fully glycosylated when bound to NS3.

The fact that each signal sequence has its own efficiency and specificity in translocation (Reviewed in ¹¹), might explain the difference in co-purification efficiency of the full-length protein and GLT25D1 with a PDI-signal sequence (Fig.2B). Therefore, the PDI signal sequence might interfere with the first step of association and consequently reduce co-purification. Otherwise co-isolation might be decreased, because specific amino acid contacts are lost as a consequence of the alternative signal sequence.

It is difficult to discriminate experimentally between these three models. A GT2 domain deletion mutant could give a clue to the possible interaction domain. Another option could be an *in vitro* binding assay. In such an experiment, NS3 and GLT25D1 are separately purified from cells, followed by analysis of their binding capabilities to each other *in vitro*. However, the bridge factor might be co-isolated and again one can only distinguish between the two-step binding mode (model 3) and the other two modes of interaction (model 1 and 2). It is very complicated to differentiate between the bridge factor (model 1) and the post extraction model (model 2), because a true protein-protein interaction occurs intracellularly as well as after cell lysis.

In the two-step binding model (model 3), GLT25D1 would change from a luminal protein to a membrane-associated protein. Consequently, a membrane-floatation assay could be used to reveal this difference, by showing a shift from the loaded fractions towards the floating membrane fractions. This third model also postulates association during translation and translocation. Therefore, an interaction competition experiment might be set up. In that assay, HCV replicon-containing cells are transfected with a myc-tagged GLT25D1 and mixed with cells expressing GLT25D1 that is HA-tagged, this is followed by lysis of the cells. Subsequently, NS3 is isolated and

co-purification of each GLT25D1 construct is examined. Competition could indicate complex formation after synthesis, whilst a lack of competition would indicate intracellular protein association. Another experiment could be analysis of association of a GLT25D1 construct without an RDEL. This protein would be translocated into the ER lumen and secreted; the only opportunity for NS3 to associate to this GLT25D1 would be during protein synthesis. In model 3 the absence of the RDEL should not result in a loss of association, whereas significantly less co-purification would be observed in models 1 and 2.

Taken together more experiments are required to examine the mode of interaction between GLT25D1 and NS3. It is clear from our experiments that the signal sequence of GLT25D1 is required for association, most likely for targeting to the ER. Additionally, there also seems to be some sequence specificity in the signal sequence for binding to NS3. Co-localisation is the first step towards an association and our experiments indicate that the ER is the main interaction compartment. The models proposed are testable and should enable us to elucidate the mode of interaction between GLT25D1 and NS3.

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7

General Discussion



The studies described in this thesis concern virus-host interactions, at the level of protein-membrane association, protein-protein interactions and viral-protein modifications. In Chapter 2, we show that the membrane-altering protein NS4b associates with membranes through its C-terminal domain via electrostatic interactions. This additional membrane binding seems to emphasise that the protein plays an important role in ER membrane shaping.

Using a replicon cell line, in which we His6-tagged the NS3 protein, we affinity purified NS3 from cell lysates in the context of active HCV RNA replication. With that cell system we determined in Chapter 3 that NS3 is modified by phosphorylation and in Chapter 4, we identified two new proteins associating with NS3, i.e. GLT25D1 and LH3. The interaction of NS3 with GLT25D1 was further investigated in Chapters 5 and 6. The results from those studies suggest that a specific pool of NS3 interacts with GLT25D1 at the ER. In this section, we place these observations in the broader perspective of the virus life cycle.

The interactions of NS4b with membranes

Full length NS4b localises to the ER ^{1,2} and is capable of modifying ER membranes ³. How exactly these membrane alterations are accomplished is still unknown. Therefore, it will be valuable to understand how the ER is shaped. In Chapter 2, we demonstrated membrane association of the C-terminal domain of NS4b. This membrane binding will be discussed in relation to the overall membrane-changing function of NS4b. Here new and more in-depth descriptions of membrane alteration models are given.

Shaping the ER

The ER membrane system has different domains with distinct shapes, however the luminal space is continuous. Generally, these domains are: membranes surrounding the nucleus (nuclear envelope), the peripheral ER sheets (where translation takes place) and the tubular structured ER that associates with other organelles (Reviewed in ⁴). The structures of these ER domains are shaped by three main mechanisms. The first is by mechanical force of proteins on the membrane bilayer, via association of these proteins with the cytoskeleton and the membrane. Secondly, proteins can

partition the lipid bilayer thereby moulding its shape and thirdly, by formation of protein complexes connecting ER membranes with other membranes including different organelles (Reviewed in ⁴⁻⁶).

Microtubules and membranes

In fluorescence microscopy, one can observe that ER tubules align with microtubules of the cytoskeleton and ER tubules. Actually, the membrane tubules of the ER track along the microtubules in three different ways (⁷ and reviewed in ⁸). They can be statically attached to the microtubules via ER membrane proteins, which contain a microtubule binding domain associating the ER membranes to the cytoskeleton ⁹.

Another possibility is movement along microtubules, which is mediated by motor proteins such as kinesin and dynein for transport from and to the cell nucleus, respectively ¹⁰. A different mechanism is that the ER tubules attach to the growing tip of a dynamic microtubule. Proteins bound to the microtubule's positive tip connect with integral membrane proteins that reside in the ER ¹¹.

A cDNA microarray analysis using NS4b-expressing cells shows altered gene expression profiles of proteins involved in the cytoskeleton, e.g. a protein that stabilises and reorganises microtubules ^{12, 13}, indicating that these are affected by NS4b. The participation of individual ER microtubule track mechanisms in HCV replication could be investigated by inhibiting each pathway separately using drugs or siRNA knockdown.

Additionally, if NS4b and the cytoskeleton are localised close to each other, confocal microscopy might reveal a connection between the two. In fact, using electron and immunofluorescence microscopy, Lai et al. suggest a role for the cytoskeleton in the cytoplasmic localisation of replication complexes ¹⁴. Possibly NS4b associates with proteins involved in one of the ER microtubule track mechanisms, or NS4b attaches to microtubules itself. In view of that, it is noteworthy that NS3 and NS5a were shown to associate with the cytoskeleton proteins tubulin and actin ¹⁴. Both the N- and C-terminal parts of the NS4b protein are at the cytosolic side of the ER membrane; therefore, these domains might be able to bind the cytoskeleton or the associated proteins. This can be investigated by protein-protein interaction studies.

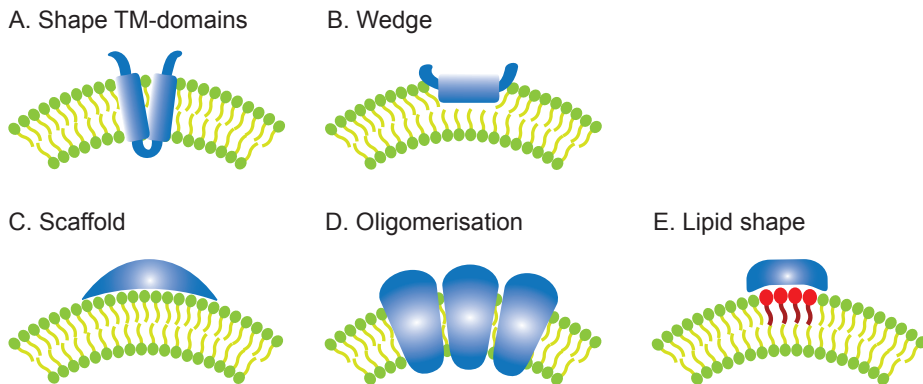


Figure 1 - Models for membrane curvature induced by proteins

Proteins (blue) can induce lipid bilayer asymmetry in various ways, which results in bending of the lipid bilayer (green). Several mechanisms are shown here. Red depicts specific lipids. See for more details the text.

Proteins in the lipid bilayer

Proteins shape the ER by being part of the lipid bilayer. Figure 1 shows models on how the lipid membrane can be bent. First, transmembrane domains can have a cone shape, which induces curvature (Figure 1A). Secondly, an amphipathic helix can wedge into one half of the lipid bilayer, generating asymmetry between the two layers and resulting in bending of the membrane (Figure 1B). Another mechanism is scaffolding, where proteins impose upon the membrane the same shape of the protein by interacting with the lipid bilayer (Figure 1C). Scaffolding proteins usually interact with the negative headgroups of the lipid bilayer by positive amino acid residues (Reviewed in ⁶). An example of a scaffolding protein domain is the BAR-domain, which is rigid and thereby forces membrane bending. Most of the BAR-domains induce positive curvature, but recently a BAR-domain generating negative curvature was identified ¹⁵. There are also scaffolding proteins that do not curve the membrane, but support the membranes into flat sheets. Additionally, large protein complexes, like the polyribosome-bound translocation complexes, can also flatten the ER membranes ^{16, 17}. A fourth mechanism to create membrane asymmetry is via oligomerisation of membrane proteins, which could bend the membrane depending on the shape of the protein (Reviewed in ⁵) (Figure 1D).

The last model is a combination of lipids and proteins (Figure 1E). Lipids themselves

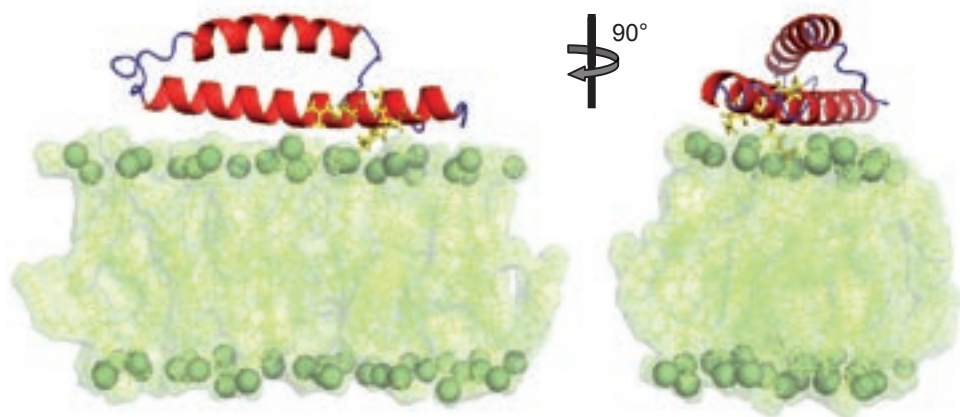


Figure 2 - Model on the association of the NS4b C-terminal domain with the membrane

The putative tertiary structure of the NS4b C-terminal domain is positioned onto a lipid bilayer (green). The domain interacts via positive residues, here depicted in yellow, with the negative headgroups of the lipids. Left panel shows a frontal view and right panel illustrates a side view. This model was kindly provided by Dr. H.C. van Leeuwen.

can influence the curvature of membranes via acyl chain group composition or the size of the lipid headgroup. In this way lipids can have a cylindrical or cone-like shape (Reviewed in ⁵). There are protein domains that bind to specific lipids, such as the lipid-binding domains C2, PH, FYVE, PX, ENTH ¹⁸. For example, the C2-domain binds to phosphatidylserine, whereas the ENTH-domain prefers certain phosphatidylinositols ¹⁹⁻²¹. In NS5b a V3 loop domain is observed, through which the RdRp is able to interact with sphingomyelin, a lipid localised in membrane rafts ²². Although not specific to lipids, electrostatic interactions are also effective in lipid-protein binding. Not every protein, however, exposes positive or negative residues and not all membranes are charged, indicating specificity to a certain extent. Only about 10-20% of the lipids are negatively charged. Most of the lipids in the bilayer are zwitterionic and have no overall charge ²³. The cytoplasmic side of the plasma membrane and the mitochondrial membranes are known to be more negatively charged ^{24,25}. The association of proteins to lipids suggests that asymmetry between two lipid layers of the membrane, thus possible curvature, can be stabilised by protein-lipid interactions.

Several characteristics of NS4b indicate that the protein itself might bend membranes. Therefore NS4b seems the ideal protein candidate for the induction of

the membranous web, which consists of highly curved membranes³. NS4b has four to five transmembrane domains²⁶, which possibly fold into a cone shape. Additionally, within these transmembrane domains a P-loop motif, common for NTPases, may be present^{27,28}. P-loop NTPases often regulate cellular membrane alterations and vesicle trafficking²⁹. However, the role in the viral life cycle has not been determined yet. The transmembrane domains could also oligomerise and be involved in membrane curvature of larger surfaces. Unpublished data of Gouttenoire et al. indicate that the second alpha helix in the N-terminus, which can traverse the membrane, is necessary to form NS4b multimers^{30,31}.

Not only the transmembrane domains, but also the N- and C-terminal domains might be involved in inducing membrane curvature. The first N-terminal alpha-helix was suggested to be an amphipathic helix that wedges into the membrane³², though the membrane-binding capacity of this amphipathic alpha-helix was recently challenged. The dissimilarity in association was ascribed to differences in expression constructs³¹.

Furthermore, mediated via an alpha helix formed by amino acids 229-253, others and we found that the C-terminal domain associates to membranes³³⁻³⁶. Gouttenoire et al. suggest this is a “twisted” amphipathic helix because two patches of hydrophobic residues are positioned on slightly different sides of the helix³⁴. We determined that the positive residues in the alpha-helix are responsible for membrane binding³⁶. These residues partially line up at the membrane surface with one of the hydrophobic patches, suggesting that in this helix both electrostatic and hydrophobic interactions are responsible for lipid bilayer association. As a result, the helix might wedge into the ER membrane and induce curvature. Figure 2 shows a possible model for membrane association of the C-terminal domain. The positive residues of the alpha-helix are positioned at the interface region, close to the lipid head groups, because this will cost the least solvation energy³⁷.

On the other hand, the C-terminus could act as a scaffold domain via the positive residues that can interact with the lipid head groups and thereby forcing the membrane into the shape of the domain. Alternatively, the domain might bind to specific lipids that by their shape mould the bilayer. Collectively, this strongly suggests that the NS4b protein itself can induce membrane curvature. Therefore membrane curvature by NS4b could be further investigated, either using the full-length protein or with separate domains like the C-terminal domain.

Connecting membranes

The third way of shaping the ER membrane is via connecting two membranes to each other, either with membranes of the same compartment or from another organelle. A possible mechanism to form ER sheets is via intraluminal bridges, where a protein dimerises with its ER luminal domain to a protein in the opposite membrane. This could also explain why membrane sheets have the same width⁶. It is well known that the ER is closely connected with other organelles in the cell. In fact, there seem to be special contact sites between the ER and each compartment, coordinated by proteins or protein complexes. For mitochondria the bridging protein to the ER might be mitofusin2³⁸ and the Golgi-ER contact sites are postulated to be stabilised by VAP-A and VAP-B³⁹. The latter are remarkable, because the HCV proteins NS5a and NS5b were shown to interact with VAP-A and -B^{40, 41}. Moreover the association of NS5a with VAP-A is affected by the phosphorylation state of the HCV protein, which is suggested to regulate replication⁴². HCV possibly modulates the contact sites between the Golgi and the ER via this interaction.

NS4b is a transmembrane protein with two cytosolically localised domains. These might therefore associate with membranes in trans instead of in cis and pull two membranes together. If NS4b is connecting membranes of the ER, one can imagine several models (Fig.3A and B). The first is a paper-ball like folding of the ER compartment, where ER membranes are randomly connected (Fig.3A). The second model shows linking of separate vesicles to each other (Fig.3B). EM pictures in the paper of Aligo et al. might hint towards the second model. Huh7 cells transfected with full-length NS4b show membrane structures packed closely together, whereas in cells, expressing NS4b lacking the C-terminal domain, large vesicles are dispersed throughout the cytoplasm. This could indicate that the C-terminal domain is involved in keeping vesicles together³³. Yet, pictures of the actual membranous web in 2D cannot distinguish these types of structures from one another, or from multiple invaginations (Fig.3C) or from vesicles inside a larger membrane (Fig.3D). To discriminate between these models, 3D images of the membranous web are required to see the connections of the membranes.

Alternatively, NS4b could associate with other organelles. Actually, the isolated C-terminal domain of NS4b has a preference for mitochondrial membranes^{34, 36} and also the N-terminal domain (aa 1-70) has been reported to occasionally localise to

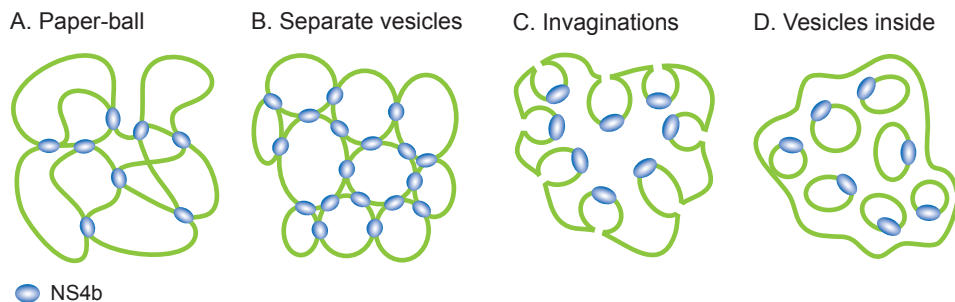


Figure 3 - Models for architecture of the membranous web

The hepatitis C virus induces a membranous web. Here several models on the structure of this membrane alteration are shown. See the text for more details.

mitochondria¹. This suggests a possible association of NS4b with mitochondria. Furthermore, HCV regulates several mitochondria related processes, for example NS3 cleaves IPS-1 and MAVS at mitochondria⁴³ and core partially localises to mitochondria to increase reactive oxygen species production^{44,45}. However, in the immunofluorescence experiments performed for Chapter 2, we were not able to find a correlation between the localisation of full-length NS4b and mitochondria. However, these figures show whole cell images, which could mask partial co-localisation. Therefore, confocal microscopy through (Z-)sections of the cell might reveal NS4b at ER-mitochondrial contact sites.

Inducing membrane alterations

It is unknown whether the membranous webs are concave (negative) or convex (positive) curved membranes of the ER. From the publication by Quinkert et al., it could be derived that a membrane protects the replication complexes⁴⁶, whether this is for example via invaginations or a paper ball-like folding of the ER is not known (Fig.3).

If HCV is along the lines of other *Flaviviridae*, concave curvature of the ER membrane is expected. The replication complexes of Dengue virus were recently shown to be invaginations into the ER membrane in which the Dengue viral proteins and dsRNA reside⁴⁷. Moreover electron tomography indicates a connection between the interior of the vesicles and the cytosol⁴⁷. This would be similar to model C in Figure 3. These structures are called vesicle packets and sometimes appear as double membrane vesicles, due to a double lipid bilayer^{47,48}. In addition, the replication complexes of Kunjin virus, an Australian variant of the West Nile flavivirus, are localised in such

vesicle packets^{49,50}. These membranes are derived from the ER as well⁵¹. How these double membrane structures are formed is still under investigation. Given that the structures look as if ER membranes surround many invaginated membranes (Fig.3C and D)^{51,52}, Kunjin virus seems to induce negative curvature. Taken together, these electron microscopy studies of other *Flaviviridae* indicate that the membranous web of HCV is formed by inducing negative membrane curvature.

Unpublished data from the group of Ralf Bartenschlager indicate that HCV infection generates multiple types of membrane alterations, which all seem to have a specific function in the virus life cycle⁵³. The membranous web is associated with replication and lipid droplets are related to virus particle assembly⁵⁴⁻⁵⁶. Possibly different mechanisms are used to generate each type of membrane arrangement. For example the host proteins of the ESCRT complexes are involved in the late stages of the HCV life cycle, probably scission and/or secretion^{57,58}. The ESCRT complexes in the host-cell are required for the formation of the inner vesicles of the multivesicular body, a late endosome (Reviewed in⁵⁹). In addition, more than one HCV protein might be involved in the modification of membranes. An example would be NS3-4a, which induces smooth-surfaced vesicles in the cytoplasm when overexpressed in U2OS cells³. Furthermore, NS4b might have more than one-way to alter membranes, as described above. Additionally, the second N-terminal alpha helix of NS4b is suggested to translocate across the membrane^{26,31}. This dual topology seems to be regulated by other HCV proteins, in particular NS5a⁶⁰. It is conceivable that these conformational changes of NS4b control different protein functions. Moreover, it would be interesting to know the difference between the ER-luminal and the cytosolic localisation of the N-terminus in relation to the function of NS4b. Perhaps one of the two topologies is required for replication.

NS3 protein modifications

In Chapter 3, phosphorylation of NS3 was described, although the exact site of phosphorylation could not be identified. In this section, several strategies will be discussed, to identify the exact site of phosphorylation and the kinase responsible. Additionally, some approaches will be suggested to determine the consequences of NS3 phosphorylation.

Identifying the site of phosphorylation and the responsible kinase

Tryptic digestion and MALDI-ToF analysis of NS3 spots resulted in the identification of multiple NS3 peptides, which together add up to 60% sequence coverage of NS3. Since a phosphate acceptor site might be in a non-covered part of the protein, obtaining complete sequence coverage of NS3 is a challenge. Incomplete coverage is often caused by protein regions that lack or have too many tryptic cleavage sites. As a result, either large or very small peptides are generated which complicate standard mass spectrometric analysis. Additionally, some peptides are inefficiently ionised. NS3 also contains both poor and rich lysine and arginine regions and the use of a different proteolytic enzyme, e.g. chymotrypsin, could be a possibility to increase the overall sequence coverage of NS3. Low coverage was also experienced by Tellinghuisen et al., complicating the identification of the exact CKII phosphorylation site in domain III of NS5a⁶¹.

The HCV phospho-protein NS5a has been extensively examined to determine the phosphate acceptor site. Deletion studies indicated two regions, aa 2200-2250 and aa 2350-2419, involved in the basal phosphorylation of NS5a⁶²⁻⁶⁴. Additionally, site-directed mutagenesis suggested three serines (aa 2197, 2201 and 2204) play a role in hyperphosphorylation of NS5a^{64, 65}. Mutational studies could also be performed to identify the phosphorylation sites in NS3. These experiments would be very laborious, because until now, our only readout for phosphorylation of NS3 was 2D-PAGE with western blot. Prior screening with antibodies that specifically recognise each phosphorylated serine, threonine and tyrosine residue, could limit the number of potential phosphorylation sites and as such limit the number of mutations which have to be analysed.

In NS5a two phosphate acceptor sites were identified at aa 2321 and aa 2194 either by two-dimensional peptide mapping of ³²P-labeled recombinant NS5a, combined with phospho amino acid analysis and Edman degradation, or with electrospray ionisation mass spectrometry^{66,67}. Similar techniques could be employed for NS3 overexpression or NS3 in the context of active replication. Although preliminary ³²P-orthophosphate labelling experiments within the replicon cell line were unsuccessful (data not shown). This could be due to the low amounts of phosphorylation⁶⁸.

An alternative method to confine the site of phosphorylation could be a peptide array (reviewed in⁶⁹). This technique has multiple applications and has for example been

used to define the consensus motif of kinases ^{70, 71}. For our purpose, we can use it in a slightly different way. Using the phosphorylation prediction program NetPhos ⁷², in total 17 serine, 13 threonine and 4 tyrosine potential phosphorylation sites are proposed. Interestingly, PhosNetK ⁷³ and KinasePhos ⁷⁴ predict PKC, PKA and ATM phosphorylation sites in NS3, three kinases that were demonstrated to associate with NS3 ⁷⁵⁻⁷⁷. Moreover, ATM was shown to be important for HCV replication ⁷⁸. A library of NS3 peptides containing these putative phosphorylation sites could be synthesised and put onto a support. Incubation with a Huh7 cell lysate and radioactively labelled ³²P-orthophosphate could then reveal which NS3 region can be phosphorylated. Obviously, this has to be confirmed using full-length NS3 and NS3 in the context of replication or the complete virus life cycle cell culture system. Possible experiments to investigate a predicted kinase are siRNA directed knockdown or over-expression of the enzyme, measuring the effect on phosphorylation of NS3. A similar strategy was also used to identify a basal phosphorylation site in NS5a and the responsible kinase CKII ⁶¹. Additional experiments showed that this kinase plays a role in virus production ⁶¹.

The advantage of an array is that it can subsequently be used to screen for the responsible kinase, either through the application of siRNA knockdown technologies or by the use of kinase inhibitors. In this manner, kinase inhibitor screening revealed that NS5a can be hyper-phosphorylated by casein kinase I ^{79, 80}.

Functional relevance of phosphorylation

It is certainly interesting to know the phosphorylation site and the kinase involved, but even more important is the effect on the functions of NS3 and the ensuing virus life cycle. Even without knowing the exact phosphorylation site, siRNA directed knockdown and kinase inhibitor approaches could be used to detect the kinase responsible for NS3 phosphorylation. As more than 500 putative protein kinase genes are identified in the human genome ⁸¹, functional high throughput assays need to be developed.

NS3 has multiple roles, such as RNA unwinding, proteolytic cleavage and several protein-protein interactions. The NS3 protease activity can be quickly assessed *in vivo* by a fluorescence assay ⁸². Human cells expressing NS3-4a can be co-transfected with a protease substrate containing a membrane anchor, a cleavage site and DsRed. Upon cleavage DsRed will be released from the membrane and will localise diffusely

throughout the cell ⁸². Using this assay, high-throughput siRNA directed knockdown or kinase inhibitors screens could be tested. In contrast to the protease assay, at the moment no cellular *in vitro* assays are available for measuring helicase activity (e.g. ⁸³), which would considerably complicate similar high throughput assays for kinase identification.

Assessment of the influence of a specific phosphorylation on NS3-protein interactions is even more difficult, because no phosphorylation dependent interaction is known. Such an interaction has been suggested for NS5a with VAP-A ⁴². In this case, VAP-A seems to dissociate from NS5a when the protein is hyperphosphorylated. Additionally, this correlates with replication inhibition ⁴².

Besides looking at the effect on NS3 functions specifically, the influence of phosphorylation on the virus life cycle can be investigated, using the replicon or the complete virus life cycle cell culture system ⁸⁴. Experiments, using a panel of siRNAs that target kinases in an HCV replicon system expressing a reporter gene, identified kinases Csk, Jak1 and Vrk1 to be required for replicon propagation ⁸⁴. The effect of Csk on replication was shown to be indirect via Fyn kinase, which is able to interact with NS5a via its Src-homology domain 3 ^{84, 85}.

Studying the HCV NS3 interactome

The approach

To identify NS3 interacting proteins we have chosen the strategy of a His6-tag-affinity purification because it can be used in multiple purification approaches. We applied it to discover modifications of NS3 (Chapter 3) and to identify NS3 interacting proteins (Chapter 4).

A tag may not interfere with protein activity or its function in the virus lifecycle; another limitation of using a tag is its accessibility. The tag should be exposed on the outside of the protein and on the surface of a protein complex (Reviewed in ⁸⁶). It is likely that we have encountered this constraint as well, since NS3 could only be purified using detergents. These results indicate that tag availability of NS3 is influenced by one of the properties of detergents. Several models on the availability of the His6-tag are illustrated in Figure 4. To begin with, the tag might fold back and

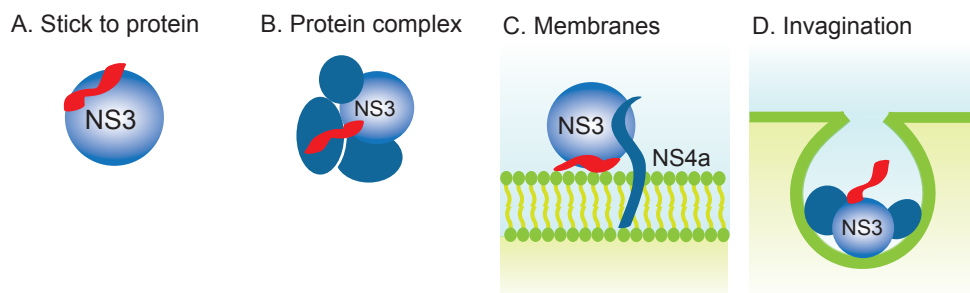


Figure 4 - Models for NS3 His6-tag unavailability

The His6-tag (red) on NS3 is not accessible without detergent treatment. Some possible models are illustrated here. More details can be read in the text.

adhere to the protein, but is released due to the detergents (Fig.4A). Alternatively, a protein complex might hide the tag inside (Fig.4B). Detergents could open up the complex to make the tag available. A third possibility is that membranes could shield the tag (Fig.4C). A tagged-protein could associate to the membrane thereby covering the tag, which would prevent affinity purification. A paper from Brass et al. on membrane association of the NS3-4a complex, suggests that the N-terminus of NS3 is in close proximity with the membrane⁸⁷. Therefore, detergents could make the tag available by dissolving the membranes. A fourth option is that a protein complex is in a membrane invagination (Fig.4D). The HCV replication complex seems to be surrounded by membranes, because HCV proteins involved in replication are proteinase resistant unless detergents are added⁴⁶.

When we isolated other NS proteins in the context of active replication, we noticed that some of the above mentioned models do occur. Besides NS3, we also have tried to purify NS5a from replicon containing cell lines. The NS5a protein was His6-tagged at different positions, at the C-terminus and internally. During His6-NS5a isolation experiments, we observed variation in NS5a binding to the beads between the differently tagged NS5a proteins, indicating that the tag position can influence purification. In addition, the properties of the protein itself seem to have an effect as well. Irrespective of the His6-tag position, association of NS5a to the Cobalt²⁺-bound beads was very weak compared to NS3. This indicates that also protein characteristics play a role in isolation.

The study in this thesis to His6-tag affinity purify the NS3 protein, illustrates the difficulty of non-specific binding proteins. These most likely are proteins containing negatively charged regions, which are sticking to the positively charged Cobalt²⁺-bound beads, or proteins with natural histidine-stretches. The presence of proteins with a histidine-stretch is shown in Figure 1 of Chapter 4, where cell lysates are subjected to western blot analysis using an antibody against His6. YY1 (GeneID: 7528), a transcription factor, is such a background binding protein. In one of our experiments this protein was identified by MS and it contains a stretch of eleven histidines. One of the possibilities to overcome this unwanted association could be the introduction of a second tag for isolation. Tandem affinity purification would allow for more stringent washing conditions (Reviewed in ^{86, 88}). We therefore constructed a His6-Myc-tag at the N-terminus of NS3 in the HCV subgenomic replicon. However, this HCV RNA was unable to replicate in Huh7 cells, indicating interference of this longer tag with the function of the NS3 protein (data not shown). For example, additional amino acids at the N-terminus of NS3 may affect hyperphosphorylation of NS5a, indicating the necessity of an authentic N-terminus ⁸⁹.

Another possibility to reduce background binding could be introducing an alternative tag, such as an epitope-tag or a *Strep*-tag that is only 8 amino acids long (Reviewed in ⁸⁶). A major drawback of epitope-tag immuno-affinity purification is the high concentration of immunoglobulins in the eluate, which will interfere in later analysis. The principle of the *Strep*-tag is similar to the His6-tag. First, association of a peptide to an affinity column, in this case a streptavidin-coated resin. This is followed by elution through competition for the biotin-binding pocket with a chemical compound, d-desthiobiotin. This chemical compound would not perturb subsequent gel-based techniques; therefore, the *Strep*-tag might be a good alternative. However there is no guarantee for successful isolation, for each tag has its own advantages and disadvantages (Reviewed in ^{86, 88}).

Before identifying proteins with MS, we separated the proteins on 1D- or 2D-PAGE and used silver staining for visualisation. PAGE is a visual method to spot differences, though more importantly it is used to simplify complex mixtures for MS analysis. Additionally, a PAGE gel is an effective interface between biochemistry and mass spectrometry, to remove mass spectrometry interfering compounds, such as salts and detergents. However one limitation of PAGE is loading capacity, a too high concentration of proteins can lead to poor separation of proteins in 1D PAGE (Chapter

4). In 2D-PAGE large amounts of proteins, lead to strip-overload and consequently proteins will not be focused at their isoelectric point. Moreover, some proteins can only focus when loaded at the correct pole of the strip, indicating a restricted protein representation. To separate NS3 and associated proteins, the samples were applied at the basic end (- pole) of the strip. In contrast, NS5a could only be separated in 2D-PAGE when loaded at the acidic side (data not shown). Another limitation is that low abundant and/ or small proteins can be missed due to the protein detection limit of the stain (Reviewed in ⁸⁶). Together this indicates that separation of proteins on an acrylamide gel has some drawbacks.

PAGE-based methods can be avoided when the total protein sample is directly subjected to digestion and subsequent MS analysis. For example with multi-dimensional liquid chromatography combined with tandem MS (MudPIT), where a pool of proteins is analysed ^{90, 91}. The proteins are digested in-solution, after that the peptides are separated by a cation exchange column on their charge and subsequently by reversed phase chromatography on their solubility or hydrophobicity ^{90, 91}. To identify NS3 and interacting proteins, the samples of the control cell line and the His6-NS3 tagged cell line could be compared on the exclusive presence of peptides in the His6-NS3 purified samples. However, these samples give complex MS data and are quantitatively difficult to compare.

An additional method, to identify NS3 interacting proteins more easily after MudPIT, is SILAC (stable isotope labelling by amino acids in cell culture) ⁹². One cell line will be metabolically labelled with ‘normal’ amino acids, while the other is labelled with a heavy variant amino acid, e.g. $^{12}\text{C}_6$ -Arg versus $^{13}\text{C}_6$ -Arg. Lysates from both cell lines are mixed and followed by affinity purification of His6-NS3. After that, the sample is analysed by MudPIT. The peptides from proteins that bind non-specifically to the beads will show a “twin” peak in the MS spectra, because these peptides will have a labelled equivalent that is slightly heavier. Peptides without a “twin”-peak are unique to the sample and could be from NS3 or interacting proteins. Since both conditions are measured in the same sample, there are fewer variables, which make it easier to discover differences between two samples.

Alternatively, the samples can be labelled post-trypsin digestion with tandem mass tags (also known as isobaric tags), circumventing difficulties of label incorporation

into proteins when using SILAC⁹³. Peptides from two (or maximally eight) samples are chemically labelled with these types of tags. With isobaric tags, the same peptides from different sample have the same mass (same peak in MS), but can be distinguished upon fragmentation in MS/MS. More samples can be analysed and compared at the same time with this technique.

In our approach, we tagged NS3 in the replicon, which is only part of the virus life cycle. So, the next step would be labelling of an HCV protein under the circumstances of a full virus life cycle. Arumugaswami et al. introduced randomly 15 nucleotides into the full-length HCV genome and analysed the mutant library for replication, infectivity and virus production, thereby creating a map of the genome, showing where the virus can tolerate sequence insertions⁹⁴. This map could be used as a starting point for introducing a tag into an HCV protein, now in the context of the full virus lifecycle. A great step forward would be to perform proteomic experiments under *in vivo* conditions, for instance infection of animal models with a tagged HCV virus. Under these circumstances the influences of the immune system on the HCV life cycle could also be investigated. A good animal model for HCV infection, however, is still not available⁹⁵.

Spatial separation

Mapping virus-host protein interactions is a start to unravel the virus life cycle. Although it should not be disregarded that interactions are dynamic. Cellular processes happen at a defined site and at a specific time in response to intra- or extra-cellular signals. Often, interactions are transient; complexes form to fulfil their function and disassemble again. For that reason not only the occurrence of an interaction is important, but also its spatial and temporal context.

This is most evident during virus entry, a process that seems to be spatially and temporally arranged. From experiments studying kinetics of entry, using compounds or antibodies blocking internalisation, it was deduced that there is a subsequent organisation of initial binding. First, there is binding to GAG and/or LDL-R, followed by SR-BI and CD81 interaction, and after that claudin and occludin association⁹⁶⁻¹⁰⁰. There are more hints that these interactions and the receptor complex formation are coordinated in time. SR-BI and CD81 act cooperatively^{100, 101}, but virus particle binding to the cells surface is via SR-BI and not CD81 dependent⁹⁸, indicating

sequential association of the virus particle. Moreover, CD81 interacts with occludin^{102, 103}, which probably takes place after virus particle movement to the tight junction¹⁰². The participation of the two tight junction proteins in entry also demonstrates the spatial separation in the process^{98, 104}, because initial attachment most likely is at the basolateral membrane of the hepatocyte (reviewed in¹⁰⁵). The most apparent example of spatial separation during entry is of course the translocation from the outside of cell to the inside of the cell.

Also during polyprotein processing a temporal separation is observed, for polyprotein cleavage by the NS3 serine-protease occurs in the preferred order of NS3/4a, NS5a/5b, NS4a/4b, NS4b/5a. Only the cleavages NS3/4a and NS4b/5a are dependent on NS4a as a co-factor, indicating a particular complex is required at a certain time¹⁰⁶⁻¹⁰⁸.

Our research suggests a spatial separation of the multifunctional NS3 protein. In the NS3 isolation experiments, we seem to purify a specific pool of NS3, which interacts with RNA, GLT25D1 and/or LH3, but not with the other HCV proteins (Chapter 4). In addition, we observed partial co-localisation of NS3 with GLT25D1 or with the ER (Chapter 6). Other groups also show partial co-localisation with organelle markers or show that only 5% of the non-structural proteins are involved in active replication^{46, 109, 110}, indicating different pools of NS3. It is possibly owe to this spatial separation that NS3 is able to execute its many roles. Detailed (co-)localisation studies help determine where which function is carried out. A fine example is the recent study of Horner et al. where they showed, by co-localisation immunofluorescence analysis, targeting of NS3 to special ER membranes that closely associate to mitochondria (also known as mitochondria-associated ER membranes, MAM). On account of this localisation, they propose, it is possible for NS3 to cleave IPS-1 on mitochondria¹¹¹. Such experiments could be valuable to discover how NS3 executes all its different functions.

Besides the spatial separation of interactions, another aspect is the temporal separation. These types of experiments have become easier with the complete viral life cycle cell culture system¹¹². A recent temporal study investigated the overall changes of the proteome in that system¹¹³. It revealed that in general the metabolic homeostasis was modified in time of infection. To identify these alterations in protein expression, they used liquid chromatography-mass spectrometry combined with trypsin-catalysed ¹⁶O/ ¹⁸O labelling of protein samples, which were obtained at different time points from cells infected with HCV. Interestingly, Diamond et al.

combined this study with a high throughput protein-protein interaction study of De Chassey et al., highlighting pathways known to be perturbed by HCV ^{113, 114}. This could display proteins important for the host response to HCV infection. In addition, detailed inspection of such studies might give an indication on when interactions take place. Moreover, a more multi-dimensional view on virus-host interactions could reveal new targets to perturb the virus life cycle.

Implication of NS3 interaction with LH3 and GLT25D1

We identified LH3 and GLT25D1 as NS3 associated proteins. An important question that arises is the consequence of this interaction for the virus. Possibly the substrates of these two proteins that are involved in lysine glycosylation are affected. Substrates are collagenous proteins, which have a collagen like domain (Gly-X-Y repeats). Proteins containing such a domain are collagens and several proteins involved in innate immunity that require galactosylation to form triple helixes ^{115, 116}. The possible role of these substrates in the HCV lifecycle and the pathogenicity of the virus will be discussed here.

Collagens

Collagens are the main constituents of the extra cellular matrix (ECM), giving structural support to the surroundings of the cells ¹¹⁷. Besides this scaffolding function, the ECM plays a role in cell proliferation, differentiation, survival and polarity (Reviewed in ¹¹⁸). An imbalance between production and degradation of the ECM will eventually result in fibrosis. Fibrogenesis can be induced by HCV and comprises excess deposition of collagen, which is produced by the fibrogenic cells in the liver. The main origin of these cells are the hepatic stellate cells (Reviewed in ¹¹⁹). Whether hepatocytes, in which HCV replicates, can become collagen-producing cells is still unclear, because the hepatocytes may or may not undergo transition into fibrogenic cells ¹²⁰⁻¹²².

One of the experiments would be to assay the GLT25D1 galactosyl transferase activity as described by Schegg et al. Here they use lysates of Sf9 insect cells expressing human GLT25D1 and mix that with purified collagen and radiolabelled UDP-[¹⁴C]Galactose. Following incubation the incorporation of [¹⁴C]Galactose into collagen is measured ¹²³. This assay could be carried out in the absence or presence

of NS3, determining differences in [^{14}C] Gal incorporation. For our experiments, it should be taken into account that NS4a and a bridge factor might be required for the association of NS3 to GLT25D1. With this experiment, the activity of GLT25D1 *in vitro* is studied, but not whether collagen synthesis *in vivo* is affected.

If the virus can influence the remodelling of the ECM, this could be beneficial for the infectivity of HCV. The ECM is not only composed of collagens, but also contains proteoglycans with glycosaminoglycan chains. Heparin is such a protein and has been suggested to be involved in the initial attachment of HCV virus particles to the cell surface ^{99, 124-126}. An altered ECM might increase primary binding of the virus to hepatocytes. The ECM is also important for cell adhesion ¹¹⁸. Using polarised hepatoma cell lines, Mee and colleagues found that HCV infection reduces cell polarity by the secretion of vascular endothelial growth factor (VEGF), which regulates tight junctions integrity and ultimately leads to increased HCV permissiveness ¹²⁷. These results show that moderating cell polarity, which could possibly also occur via altering the ECM, enhances HCV infectivity. Furthermore, HCV infection seems to affect the ECM via matrix metalloproteinases. These proteins are involved in ECM degradation and remodelling. Engagement of CD81, on hepatic stellate cells, with the HCV envelope glycoprotein E2 stimulates synthesis and activity of the metalloproteinase MMP-2 ¹²⁸. Taken together these links indicate that remodelling the ECM could be advantageous to HCV infectivity.

Using polarised hepatoma cell lines, cell polarisation can be investigated in the absence of GLT25D1, by siRNA knockdown. Alternatively, the influence of NS3 expression on the polarisation of these cells could be assessed. When polarisation is disturbed, the next step would be to investigate HCV infectivity.

Innate immune defence molecules

Another substrate of GLT25D1, as shown by Schegg et al., is mannose-binding lectin (MBL) ¹²³. MBL is an innate immune defence molecule, which is secreted into the plasma and recognises polysaccharide structures on pathogens. Binding of MBL to the microbial surface activates the complement system, leading to osmotic disruption of the bacteria by the membrane attack complex (Reviewed in ^{129, 130}). Opsonisation of the pathogen by MBL can also result in internalisation by phagocytes, as a consequence of receptor ligation on the cell surface that initiates engulfment (Reviewed in ^{129, 130}). In addition to controlling bacterial and fungal infections, MBL

can inhibit viral infection as well. Opsonisation of the virions can enhance uptake by phagocytes or have a neutralizing effect by obstructing attachment to entry receptors. Furthermore, due to the multiple carbohydrate binding domains on the MBL molecule, the virus particles can be aggregated and in that way, entry can be blocked. Finally, the membrane attack complex can lyse infected cells or enveloped virions (Reviewed in ¹³¹).

To abrogate the activation of the complement pathway, members of for example *Herpesviridae*, *Coronaviridae*, *Poxviridae*, *Retroviridae* and *Flaviviridae* families evolved evasion strategies (Reviewed in ¹³¹⁻¹³³). Since MBL is produced by hepatocytes, where HCV proliferates, it seems probable that HCV also developed some strategies to evade complement activation. Additionally, synthesis in the same cells implies that MBL might already associate to the HCV E1 and E2 intracellularly. Binding by MBL inside the cell was demonstrated for glycoprotein gp120 of human immunodeficiency virus (HIV), which could be co-immunoprecipitated from cell lysates together with intracellular MBL ¹³⁴. Most likely binding occurs in the ER where intracellular MBL plays a role in glycoprotein quality control ¹³⁴. The HCV glycoproteins E1 and E2 also reside in the ER ^{135, 136}, indicating MBL attachment could already take place in the ER.

In Figure 5, a model is shown on how HCV might escape binding by the innate immune defence molecules in the ER. In the top panel, a situation is illustrated where no NS3 is present (Fig.5. top panel). Under these circumstances, MBL is able to form a triple helix structure, due to galactosylation by GLT25D1. Moreover, the carbohydrates on E1 and E2 are recognised by MBL. Attachment of MBL to E1 and E2 might either prevent virus particle formation or the virions are released coated with MBL, blocking entry receptor binding or activating the complement pathway. In our experiments, GLT25D1 was associated to NS3. This in turn might decrease galactosyl transferase activity of GLT25D1 and subsequently reduces MBL triple helix formation. As a result, MBL will not be capable of binding to the HCV glycoproteins, allowing assembly of functional virus particles (Fig.5. bottom panel).

This model could be tested as follows. First the galactosyl transferase activity of GLT25D1, in the presence or absence of NS3, needs to be determined (see above). In this experiment MBL is used as a substrate, instead of collagen, to measure [¹⁴C] Gal incorporation ¹²³. A more functional assay would be siRNA knockdown of GLT25D1

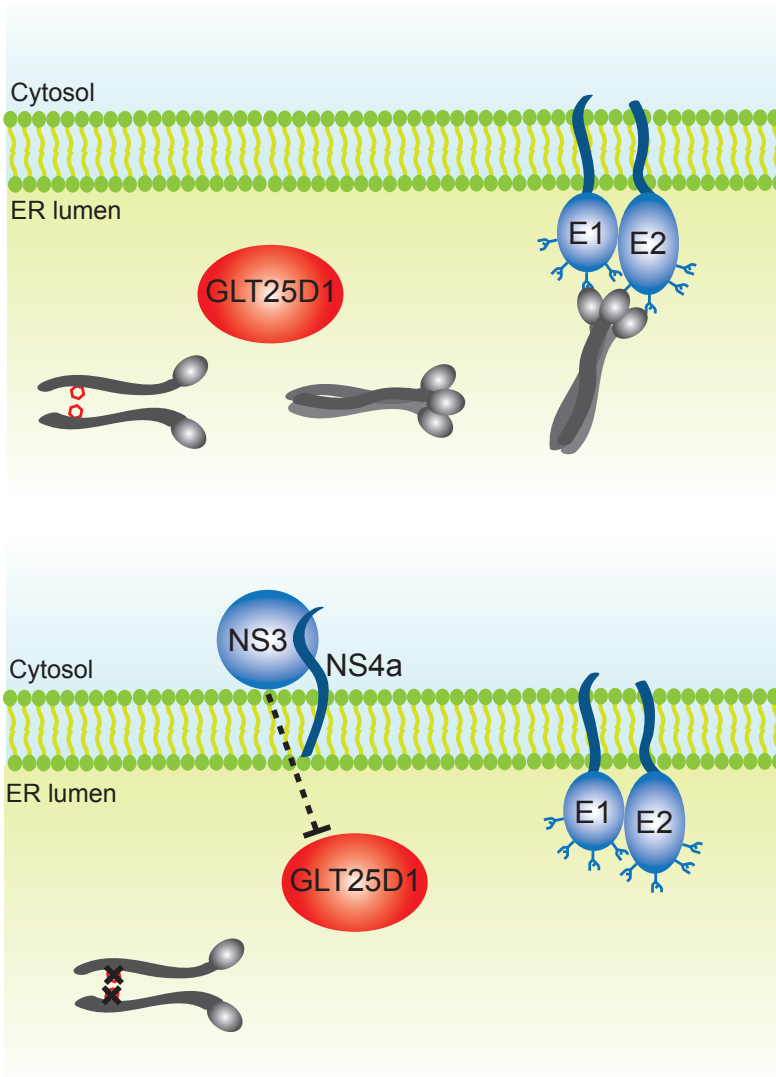


Figure 5 - Model on consequence of GLT25D1 and NS3 association

Top panel shows a situation in the absence of NS3. GLT25D1 (red) is able to galactosylate MBL (gray), which facilitates triple helix formation. Subsequently, the collectin can bind to the HCV glycoproteins E1 and E2 (blue). This might hinder virus particle assembly or reduce infectivity of the virus. In the bottom panel a setting is illustrated in the presence of NS3. There GLT25D1 is not active, leading to disturbed triple helices formation, which prevents recognition of E1 and E2 by MBL. NS3 might affect the function of GLT25D1 through association with the protein. However, the exact mechanism is not known, possible models of association are discussed in Chapter 6, Figure 4.

and its effect on HCV virus particle assembly or infectivity. Alternatively, ectopic overexpression of GLT25D1 could be studied. Furthermore, an experiment that is essential to prove our model is direct binding of MBL to HCV E1 and E2. MBL was previously shown to attach to the envelope proteins of influenza A, HIV and Ebola¹³⁷⁻¹³⁹. This has not yet been determined for the HCV glycoproteins.

Ficolin, another complement activation factor, however, has been shown to bind to HCV glycoproteins¹⁴⁰. Similar to MBL, ficolin is synthesised by hepatocytes and secreted into the blood. Moreover, ficolin can form triple-helices, because of a collagen-like domain in the N-terminus. The C-terminus contains the carbohydrate binding activity through a fibrinogen-like (FBG) domain. There is subtle difference in sugar specificity between MBL and ficolin. Both can bind to N-acetyl-glucosamine (GlcNAc), but for example MBL recognises mannose, while ficolin recognises N-acetyl-galactosamine (GalNAc) (Reviewed in¹⁴¹⁻¹⁴³). In addition to HCV glycoprotein binding, ficolin levels are significantly increased in HCV-infected patients¹⁴⁰. Several studies also indicate a link between MBL and HCV infection, though others do not find a correlation¹⁴⁴⁻¹⁴⁶. Furthermore, activation of the complement pathway by E1 or E2 cell surface expressing cells can partially be assigned to ficolin. This was measured by complement deposition of serum on these cells. Additionally, serum of HCV patients has a higher cytolytic activity than sera from healthy donors, which was attributable to ficolin. This enhanced activity is possibly a result of increased ficolin expression by HCV infected cells¹⁴⁰. Together these studies indicate that HCV should counteract ficolin, in order to prevent complete block of HCV assembly.

MBL is representative for the collectins, which all contain a collagen-like domain and have a lectin binding domain, hence their name. Therefore other collectins produced by the hepatocytes, such as collectin-10 (CL-L1) and collectin-11 (CL-K1), could also be potential substrates for GLT25D1 and LH3^{147, 148}. Both proteins are able to bind to sugars and probably play a role in the innate immune defence by activation of the complement system, like MBL^{147, 148}. Moreover, predicted signal sequences in these proteins suggest they are secreted. Collectin-11 is observed in blood¹⁴⁸, though collectin-10 was suggested to be cytoplasmic¹⁴⁷.

Overall, the liver produces many innate immune defence molecules, which could potentially bind to the glycoproteins of HCV thereby affecting HCV infectivity.

Reducing the activity of collectins via inhibiting a general mechanism in their production, such as the formation of the triple helix, could be an original tactic of the virus.

Future directions HCV research

This thesis exemplifies that the interactions between the HCV-proteins and the host cell are complex. Firstly, we show that the NS4b protein associates with the cellular host membranes in multiple ways, with each domain having a specific function creating a balanced interplay with the host's intracellular structures. Secondly, NS3 localises to different ER sub-compartments that might direct the different functions of NS3. More and more data are published on multiple roles of each separate HCV protein in the virus life cycle. Furthermore, HCV proteins are reported to interact with each other and with more than one host-protein. How these multiple functions of each HCV protein are regulated is not clear. Time and space seem to be crucial, as the different steps of the virus life cycle appear to be physically separated from each other. Various mechanisms could be envisaged to regulate these dimensions, such as cellular localisation signals, protein modifications and sequential protein-protein interactions.

Virus-host interactions can be further investigated by dissecting the specific protein characteristics of each HCV protein. Helpful tools are prediction programs for protein motifs, such as Expasy PROSITE and NetPhosK, in combination with biochemical assays. Examining protein modifications or the interaction domains, could reveal a particular mechanism that might be a new drug target.

The importance of the sequence of virus-host interactions in the virus life cycle can be best exemplified during entry. These interactions, forming diverse protein-complexes, are dictated in localisation and seem to be correlated to time. To get a better picture of sequential protein-protein interaction, virus-host protein complexes can be isolated, if possible under conditions that preserve these interactions. This might lead to key interactions within the virus life cycle that can be used to tackle HCV.

Alternatively, one can look more broadly at interactions, to the effect on processes inside or even outside the virus-producing cell. Perhaps the interaction of NS3 with GLT25D1 and LH3 illustrates this, because of the putative involvement in collagen

and collectin production. For that reason the replicon and complete virus life cycle cell culture system will be very useful in studying the consequence of an interaction. Additionally, *in vivo* animal models are required to investigate the roles of an interaction in for example the immune system.

More and more details of the virus life cycle are being uncovered. Small changes at the amino acid level, such as phosphorylation, can have big consequences. With a total of ~3000 aa in the HCV genome a huge potential for regulation of functions is possible. Elucidation at the ultrastructural level via crystallography of 3D protein structures or 3D tomography of membrane structures could be useful to understand the control of protein activities, protein-protein interactions and protein-membrane association. These details on interactions between the virus and the host-cell should be put into context with the viral lifecycle, regarding time and space. Unravelling these intricate details might lead to an effective antiviral strategy.

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8

Summary
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Summary

We all know that viruses are one of the causes why we get sick. There are many different viruses and therefore many different infectious diseases. Once a virus has entered a host-cell, it utilises the processes occurring in the cell to multiply itself, in the end leading to further spread of the virus.

The hepatitis C virus infects cells of the liver, called hepatocytes, causing inflammation and eventually dis-functioning of the liver. This process goes very slow and insidious and often takes 10 to 30 years before clinical symptoms are presented. Worldwide around 130-170 million people are infected with HCV, that is 2-3% of the world population. Unfortunately, there is no vaccine available against HCV and current treatments are for some category patients far from successful. Therefore, it is important to develop new medication. Yet, one first needs to understand the HCV lifecycle to discover possible drug-targets.

The lifecycle of HCV is illustrated in Chapter 1 of this thesis and starts with the entry of the virus into the hepatocyte. To enter, the virus particle binds to proteins on the cell surface. After that, the virus particle releases its genome content, which will be used to produce new viral proteins (translation). Subsequently, these proteins will start making new viral genome copies (replication). This occurs on a site in the cell that is specially created by the virus. The viral non-structural protein NS4b plays an important role in creating this special site by inducing membrane rearrangements of the cell. The other non-structural proteins of the virus are important in copying the genome, including the protein NS3. After completion, the genome is packaged into new virus particles, which leave the cell to infect other hepatocytes. All these steps happen with assistance of host proteins and processes. Within this thesis, I investigated the interactions of the viral proteins with the host cell at several levels.

All the viral proteins are associated with membranes of the host cell, which are double-layered sheets of lipids. NS4b is associated with the membrane in a number of ways, through hydrophobic interactions and by domains that span the membrane. We examined the carboxy-terminal domain of the protein and found that this modular domain binds to membranes. When comparing this domain to other proteins, we

observed similarity with a domain of a bacterial protein that also associates to membranes. Their mode of membrane binding is electrostatic: positively charged amino acids that interact with the negative headgroups of lipids. Experiments in which we mutated positively charged amino acids indicated the same mechanism of association for the carboxy-terminal domain of NS4b. In addition to that, we show that these amino acids, required for membrane interaction, are necessary for replication of the virus.

Several viral proteins have multiple roles in the life cycle, as is the case for NS3. Separate functions need to be regulated and a way to organise this is by protein modifications, these are moieties put onto a protein that modulate a particular function. Not much is known about modifications for NS3. We therefore set up experiments to investigate modifications of NS3 in the context of HCV RNA translation and replication in cells. We made cell lines where we constructed a label to NS3. Using a 'chemical magnet' for this label, we pulled NS3 out of these cells. After that, the isolated proteins were examined for protein modifications by several means, including two-dimensional PAGE (poly acrylamide gel electrophoresis) and mass spectrometry techniques. Two-dimensional PAGE showed that multiple forms of NS3 are present and additional experiments showed phosphorylation of the protein. Mass spectrometry furthermore indicated acetylation at the amino terminal end of the protein. These results show that NS3 is post-translationally modified and we postulate that these could be involved in the regulation of its functions.

A third subject of this thesis concerns viral and host protein-protein interactions. The same cell culture system as in the NS3 modification study was used to isolate the tagged version of NS3. To co-purify NS3 interacting proteins with NS3, several methods were employed. We identified two associating partners, GLT25D1 and LH3; both are engaged in the modification (galactosylation) of collagens and collectins. Collagens play a role in strengthening the cells and collectins are involved in the body's defence mechanism against pathogens. The interactions were further validated, especially as to where the two proteins inside the cell could interact. Since the subcellular localisation was unknown for GLT25D1, we investigated that first. GLT25D1 was shown to contain several signals, such as a signal sequence and a KDEL, that target the protein to the lumen of the endoplasmic reticulum, an organelle in the cell to which several HCV proteins localise. Our data illustrates

that NS3 and GLT25D1 co-localise partially at a specific part of the endoplasmic reticulum. Additional experiments could not reveal the exact protein domain of GLT25D1 required for interaction with NS3, but the signal sequence of GLT25D1 seems to be crucial for the association.

Together these data point out that there is a very complex interplay between HCV and the host-cell. The viral lifecycle is regulated at different levels and this thesis describes several of them, including protein-membrane association, protein modifications, subcellular localisation and protein-protein interactions. To fully understand these interactions and their functions, further investigations of these four new topics in the HCV lifecycle are needed.

Samenvatting

We weten allemaal dat een virus een van de oorzaken is waarom we ziek worden. Er zijn vele diverse soorten virussen, vandaar dat er ook menig verschillende infectieziekten zijn. Een virus komt in ons lichaam en dringt daar de cellen binnen. Op het moment dat een virus een cel is binnengedrongen maakt het gebruik van al de processen die normaal in de cel plaatsvinden om zich te vermeerderen. Dit heeft als uiteindelijk doel dat het virus zich verder kan verspreiden.

Hepatitis C virus (HCV) infecteert de cellen van de lever, hierdoor raakt deze ontstoken en dit leidt uiteindelijk tot het disfunctioneren van het orgaan. Dit proces gaat echter heel langzaam en sluipend, waardoor er vaak pas na 10 tot 30 jaar klachten optreden van een falende werking van de lever. Wereldwijd zijn ongeveer 130-170 miljoen mensen geïnfecteerd met HCV, dat is ongeveer 2-3% van de wereldbevolking. Helaas is er geen werkbaar vaccin tegen HCV beschikbaar en ook de behandeling laat bij sommige categorieën patiënten te wensen over. Het is daarom belangrijk om nieuwe medicatie te ontwikkelen, maar men moet daarvoor eerst begrijpen hoe de levenscyclus van HCV werkt.

De levenscyclus van een virus is geïllustreerd in het eerste hoofdstuk van dit proefschrift en start met het binnendringen van de levercel. Door middel van binding van het virus met eiwitten aan de buitenkant van de cel komt het virus binnen. Als het virus deeltje in de cel is dan laat het zijn genomisch materiaal vrij (RNA) en dit wordt gebruikt om de virale eiwitten te produceren (translatie). Daarna gaan de virale eiwitten aan de slag om het virale genoom te vermenigvuldigen (replicatie). Dit gebeurt op een speciaal door het virus gecreëerde plaats en het niet-structurele virus eiwit NS4b speelt daarbij een belangrijke rol, namelijk door het veranderen van de membranen in de cel. Andere niet-structurele eiwitten van het virus zijn belangrijk voor het kopiëren van het genoom, waaronder ook het eiwit NS3. Nadat het genoom gekopieerd is, zal het worden ingepakt in een nieuw virusdeeltje, ook bij dit proces is NS3 betrokken. De nieuwe virusdeeltjes gaan de cel uit en kunnen andere cellen binnendringen. Al deze stappen gebeuren met behulp van gastheereiwitten en -processen. In dit proefschrift heb ik op verschillende manieren gekeken naar de interacties tussen het hepatitis C virus en de gastheercel en deze onderzocht.

Alle virale eiwitten zijn geassocieerd met het membraan van de gastheercel, dit is een dubbele laag van lipiden (vetachtige stoffen). NS4b bindt op verschillende manieren aan het membraan, door hydrofobe-interacties en met eiwitdomeinen die door het membraan heen gaan. Wij hebben het carboxy-terminale domein van het eiwit onderzocht en zagen dat dit domein aan het membraan bindt. Vervolgens hebben we dit domein vergeleken met andere eiwitten, waardoor we een overeenkomst vonden met een domein van een bacterie-eiwit dat ook met het membraan associeert. De manier waarop dit eiwit bindt is elektrostatisch, dus de positief geladen aminozuren van het eiwit binden aan de negatief geladen kopjes van de lipiden. Vervolg experimenten, waarin we deze positief geladen aminozuren veranderen, wijzen op eenzelfde mechanisme voor het carboxy-terminale domein van NS4b. Daarnaast laten we zien dat deze aminozuren, die betrokken zijn bij membraaninteractie, belangrijk zijn voor de replicatie van het virus.

De virale eiwitten hebben allemaal meerdere functies in de levenscyclus, dit geldt ook voor NS3. Het is nodig dat deze functies gereguleerd worden en dit kan door eiwitmodificaties, dat zijn chemische structuren die aan een eiwit gekoppeld worden en een specifieke functie kunnen beïnvloeden. Er is nog niet veel bekend over deze modificaties van NS3. Wij hebben daarom de modificaties van NS3 onderzocht in de context van HCV RNA translatie en replicatie in cellen. Hiervoor hebben we cellijnen gemaakt waarbij we aan NS3 een label hebben gemaakt. Door middel van een soort 'chemische magneet' voor dit label hebben we vervolgens NS3 geïsoleerd uit deze cellen. Hierna werden de geïsoleerde eiwitten verder onderzocht met behulp van tweedimensionale poly-acrylamide gel electroforese (PAGE) gevolgd door massa spectrometrie. Tweedimensionale PAGE toonde aan dat er meerdere vormen van NS3 zijn en verdere experimenten lieten fosforylatie van het eiwit zien. Massa spectrometrie wees uit dat de amino-terminus van NS3 geacetyleerd wordt. Deze resultaten demonstreren dat NS3 gemodificeerd wordt. Mogelijk heeft dit betrekking op de regulatie van de verschillende functies van het eiwit, maar omdat we niet de plaats(en) waar NS3 wordt gefosforyleerd hebben kunnen identificeren, was een functionele analyse van de rol van deze modificatie in de levenscyclus van het virus niet mogelijk.

Het derde onderwerp van dit proefschrift omvat eiwit-eiwit interacties tussen de virus- en de gastheereiwitten. Hetzelfde celcultuur systeem als bij de NS3 modificaties

werd gebruikt om de gelabelde NS3 te isoleren. Om de interacterende eiwitten van NS3 samen met NS3 te zuiveren zijn er zeer uitgebreid verschillende methodes getest. We hebben uiteindelijk met de meest geschikte methode twee NS3-bindende eiwitten geïdentificeerd, namelijk GLT25D1 en LH3. Beiden zijn betrokken bij de modificatie (galactosylering) van collagenen en collectines. Collagenen spelen een rol bij het verstevigen van de cellen en collectines maken deel uit van het lichaamsafweersysteem tegen pathogenen. De interacties werden verder gevalideerd en tevens hebben we onderzocht op welke plek in de cel de eiwitten een interactie aangaan. Omdat de subcellulaire lokalisatie van GLT25D1 nog niet bekend was hebben wij dat eerst onderzocht. Aangetoond werd dat GLT25D1 een aantal signalen bevat, zoals een signaal sequentie en een KDEL-sigitaal, die het eiwit naar het lumen (holte) van het endoplasmatisch reticulum dirigeren. Het endoplasmatisch reticulum is een organel in de cel waar ook de HCV-eiwitten lokaliseren. Onze data laten zien dat NS3 en GLT25D1 deels co-lokaliseren in een specifiek deel van het endoplasmatisch reticulum. Vervolgexperimenten konden niet uitwijzen waar precies het domein in GLT25D1 zit dat nodig is voor een interactie met NS3, maar de signaal-sequentie van GLT25D1 lijkt cruciaal te zijn voor de associatie van de twee eiwitten.

Tezamen geven deze resultaten aan dat er een complexe interactie is tussen HCV en de gastheercel. De virale levenscyclus is op verschillende niveaus gereguleerd en in dit proefschrift worden er een aantal beschreven, zoals eiwit-membraan associatie, eiwit modificaties, subcellulaire lokalisatie en eiwit-eiwit interacties. Om deze interacties en hun functie volledig te begrijpen zullen deze vier nieuwe onderwerpen verder moeten worden onderzocht in een gastheer die een volledige virale levenscyclus ondersteunt, inclusief een werkende lever en een functioneel afweersysteem.

Curriculum Vitae

Johanna Maaïke Pieternella Liefhebber, met als roepnaam Jolanda, werd op de negende dag van de negende maand in 1981 te Hagestein geboren. Zij behaalde in 1999 haar Atheneum diploma aan het Koningin Wilhelmina College te Culemborg in de vakken nederlands, engels, wiskunde A en B, natuurkunde, scheikunde en biologie. Vervolgens koos zij als universitaire opleiding voor biomedische wetenschappen aan de universiteit van Utrecht, met de Masters biomoleculaire wetenschappen. Binnen deze studie liep zij stages bij het Rudolf Magnus instituut van neurowetenschappen (onder begeleiding van Corine E. de Rijke en Prof. Dr. Roger A.H. Adan) en bij de afdeling cel biologie van het Universitair Medisch Centrum Utrecht (onder begeleiding van Sonja I. Buschow en Prof. Dr. Willem Stoorvogel). Na afronding van haar Masters in oktober 2004, startte zij in januari 2005 haar promotie onderzoek bij de afdeling medische microbiologie in het Leids Universitair Medisch Centrum onder de supervisie van Dr. Hans C. van Leeuwen en Prof. Dr. Willy J.M. Spaan. Hier verrichtte zij onderzoek naar de virus-gastheer interacties van het Hepatitis C virus, waarvan de resultaten zijn beschreven in dit proefschrift. In mei 2010 is zij begonnen als post-doctoral fellow in de groep van Dr. John McLauchlan bij de MRC - Centre for Virus Research in Glasgow. Daar verricht zij onderzoek naar de rol van 'lipid droplets' in de levenscyclus van het Hepatitis C virus.

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