THÈSE DE DOCTORAT DE L'UNIVERSITÉ LILLE I

École Doctorale Biologie et Santé de LILLE

Pour l'obtention du grade de
Docteur de L'UNIVERSITE LILLE I

Etude de l'entrée cellulaire du virus de l'hépatite C : rôle du récepteur aux LDL et identification de régions fonctionnelles des protéines de l'enveloppe virale

Hepatitis C virus entry process : role of LDL receptor and identification of functional regions in viral envelope proteins

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Le 21 Décembre 2010

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Thanks

At the beginning, I would like to thank the members of the jury: president - Jean-Claude Michalski, examiner - Philip Meuleman and referees - Agata Budkowska and Dimitri Lavillette for agreeing to participate in this important day and for their interest in my work.

These three years were an amazing experience for me and several people contributed to that.
Firstly Jean, for accepting my application for PhD in his lab, his supervision and help during all this time. I hope, that finally our collaboration was not so hard for him and that I am not the last polish student he took in the lab (as he once said...).
Pauline, Caroline and Lucie for helping me to adapt to new french environment.
Birke, for all her help and advise in the lab, for helpful comments about my thesis and all nice time we spent together.
Girls from my office: Nathalie, Karin and Claire for all scientific and less scientific conversations. Good to have someone who knows when the sales start.
Moving team: Lucie, Birke and Noemie for helping me staying fit and cooking team: Gabi, Noemie and Birke for preventing me from loosing too many calories... moelleux au chocolat - 2000 kCal...
Yves, for his scientific advise and his amazing sense of humor.
Czesław, for polish jokes: 'les polonaises sont dures..." and his helpful tips about HCVcc.
Francois and Yann, for their help at the beginning of my lab life and for making the atmosphere in the lab really cool. It was never the same after you guys left.
Ioan, for all the beers and his never-ending questions... and answers.
PYD, for his funny behavior and Khaled, for his serious political lectures, who would guess that pigeon and rabbit could be friends.
Sophana, for all technical help and making beautiful models.
Laurence and Julie, for all their advise and help with FACS.

Sandrine and Anne, for their participation and help in my project.
Sumedha and Bhavna, for making me laugh a lot.
All the people that make or made the work in our lab really nice.

EIHCV network, especially Jonathan, Felix, Alex and Thomas for all scientific input and Natalia, Kuba and Vasco, for all the meetings, congresses and parties together.

Rodzicom i rodzinie, za wieloletnie wsparcie w tym co robię. No cóż, dzieci z tego nie ma, ale może niedługo...

Finally, I would like to thank Kevin, for being with me all this time (mostly on Skype...), for all our scientific conversations, for helping me believe in myself and showing me the real France. It was supposed to be a short professional adventure, but with Kevin, in France I found my place in the world.

Soon from Anna Albecka to Dr Anna Albecka-Moreau....

France changes my life.

Contents

Résumé

Le virus de l'hépatite C (HCV) est un agent pathogène majeur qui infecte environ 170 millions de personnes à travers le monde. Ce virus à ARN de polarité positive appartient à la famille des *Flaviviridae.* Il est constitué d'une nucléocapside entourée d'une enveloppe lipidique dans laquelle sont ancrées deux glycoprotéines d'enveloppe, E1 et E2. L'initiation du cycle infectieux viral nécessite la traversée de la membrane cellulaire. Ce processus d'entrée virale peut être divisé en plusieurs étapes: l'attachement du virus à la surface des cellules, l'interaction avec le(s) récepteur(s) spécifique(s) et finalement la fusion de l'enveloppe lipidique virale avec une membrane cellulaire. Ces étapes du cycle viral mettent en jeu deux acteurs majeurs: les protéines d'enveloppe virale et les récepteurs et co-récepteurs à la surface des cellules. Au cours de cette thèse, nous nous sommes intéressés à ces deux aspects. Nous avons d'une part cherché à identifier de nouveaux déterminants fonctionnels sur la glycoprotéine d'enveloppe E2 et d'autre part, nous avons étudié le rôle du récepteur aux LDL (LDLR) au cours du cycle viral.

Les protéines d'enveloppe du virus HCV, E1 et E2, s'assemblent en un hétérodimère non covalent pour former une unité fonctionnelle. Partant de l'hypothèse que les glycoprotéines E1 et E2 ont co-évolué au sein de chaque génotype, nous avons mis en évidence des incompatibilités fonctionnelles intergénotypiques entre ces protéines. Nous avons ensuite construit plusieurs séries de chimères intergénotypiques de E2 en nous basant sur un modèle structural. Ces chimères ont ensuite été étudiées d'un point de vue fonctionnel dans un système infectieux ainsi qu'à l'aide de pseudotypes rétroviraux. Ces travaux nous ont permis d'identifier plusieurs déterminants de E2 impliqués dans l'assemblage de la particule virale (HVR2, IgVR et une région du Domaine II) ainsi qu'une région juxtamembranaire prenant part au processus d'entrée virale. Cette dernière a également été caractérisée d'un point de vue structural pour mieux comprendre son rôle.

Du fait de l'association potentielle entre le virus HCV et des lipoprotéines de faible densité, le LDLR a été proposé comme facteur d'entrée pour ce virus. Cependant, son rôle précis dans l'entrée du virus HCV reste mal compris. Nous avons étudié l'implication de ce récepteur en comparant les mécanismes d'internalisation du virus HCV et des lipoprotéines. Nous avons montré que la particule virale interagit avec le LDLR. Cependant, cette interaction ne semble pas conduire à une infection productive. De plus, nos données suggèrent que par ses fonctions de transport lipidique, le LDLR module la réplication génomique du virus HCV.

En conclusion, ce travail a permis d'identifier de nouvelles régions fonctionnelles de la glycoprotéine d'enveloppe E2. De plus, il nous a conduit à mieux comprendre le rôle du LDLR au cours du cycle viral.

Abstract

Hepatitis C virus (HCV) is a major pathogen that infects approximately 170 million people around the world. This positive stranded RNA virus belongs to the *Flaviviridae* family. The viral particle is made of a nucleocapsid surrounded by a lipid envelope in which two envelope glycoproteins, E1 and E2, are anchored. To initiate its life cycle, a virus needs to cross the cellular membrane. This process can be divided into several steps: virus attachment to the cell surface, interaction with specific receptor(s) and finally fusion of the viral lipid membrane with a cellular membrane. These early steps of the viral life cycle need two major actors: the envelope proteins at the viral surface and receptor(s) and co-receptor(s) on the cell surface. During this thesis, we studied these two aspects. Our objectives were to identify new functional determinants in HCV glycoprotein E2 and to investigate the role of the LDL receptor (LDLR) during the HCV life cycle.

HCV envelope glycoproteins, E1 and E2, assemble as a non-covalent heterodimer, which forms a functional unit. With the hypothesis that E1 and E2 glycoproteins have co-evolved within the different genotypes, we identified functional intergenotypic incompatibilities between these two proteins. Based on a structural model, we then constructed several series of intergenotypic E2 chimeras. The functionality of these chimeras was analyzed in an infectious system and with the help of retroviral pseudotypes. This work led us to identify several E2 determinants involved in viral particle assembly (HVR2, IgVR and a region in Domain II) as well as a juxtamembrane region taking part in virus entry. This latter has also been characterized at a structural level to better understand its role.

Due to the potential interaction between HCV particle and low-density lipoproteins, the LDLR has been proposed as an entry factor for this virus. However, its exact role in HCV entry remains poorly understood. In this thesis, we investigated the role of this receptor in the HCV life cycle by comparing virus entry to the mechanism of lipoprotein uptake. We showed that the viral particle interacts with the LDLR. However, this interaction does not seem to lead to a productive infection. Furthermore, our data are in favour for a role of the LDLR as a lipid providing receptor which modules viral RNA replication.

In conclusion, this work allowed the identification of new functional regions in E2 envelope glycoprotein. Furthermore, it also led to better understand the role of the LDLR during the HCV life cycle.

List of figures

List of abbreviations

ACAT	acyl coenzyme-A: cholesterol acyltransferase
AIDS	acute immunodeficiency syndrome
ALT	alanine aminotransferase
AP	adaptor protein
Apo	Apolipoprotein
ARF	alternate reading frame
ARH	autosomal recessive hypercholesterolemia
ATG	autophagy protein
ATP	adenosine-5'-triphosphate
BVDV	bovine diarrhea virus
CIDE-B	cell death-inducing DFF45-like effector
CLDN	claudin
CMV	cytomegalovirus
CTL	cytotoxic T-lymphocytes
DC	dendritic cells
DGAT	diacylglycerol acyltransferase
DNA	deoxyribonucleic acid
EGF	endothelial growth factor
eIF	eukaryotic initial factor
EMCV	encephalomyocarditis virus
ER	endoplasmic reticulum
EWI-2wint	EWI-2 without its N-terminus
F	frame-shift protein
FRET	fluorescence resonance energy transfer
GAG	glycosaminoglycan
GFP	green fluorescent protein
Grb2	growth factor receptor-bound protein 2
GTP	guanosine-5'-triphosphate
HAART	highly active anti-retroviral therapy
HBV	hepatitis B virus
HCC	hepatocellular carcinoma
HCV	hepatitis C virus
HCVcc	hepatitis C virus cell culture
HCV-LPs	HCV-like particles
HCVpp	hepatitis C virus pseudoparticles
HDL	high-density lipoproteins
HVR	hyper-variable region
HIV	human immunodeficiency virus
HL	hepatic lipase
HLA	human leukocyte antigen
HMG CoA reductase	3-hydroxy-3-methylglutaryl coenzyme A reductase
HSPG	heparan sulfate proteoglycan
hVAP-A	human vesicle-associated membrane protein-associated protein A
IDL	intermediate-density lipoproteins
IFN	interferon
IgVR	intragenotypic variable region
IL	interleukin
IMPDH	inosine monophosphate dehydrogenase
IRES	internal ribosome entry site
IRF	interferon regulatory factor
JFH-1	Japanese fulminant hepatitis

LDL	low-density lipoproteins
LDLR	low-density lipoprotein receptor
LEL	large extracellular loop
LPL	lipoprotein lipase
LVP	lipoviroparticles
MAVS	mitochondrial antiviral signaling
MLV	murine leukemia virus
MTP	microsomal triacylglycerol transfer protein
MVA	modified virus of Ankara
NFκB	nuclear factor kappa-light-chain-enhancer of activated B cells
NK	natural killer
NKT	natural killer T-cells
NLS	nuclear localization signal
NMR	nuclear magnetic resonance
NS	non-structural protein
NTPase	nucleotide triphosphatase
NTR	non-translated RNA
OCLN	occludin
PC	phosphatidylcholine
PE	phosphatidylethanolamine
Peg-IFN	pegylated interferon
PKR	protein kinase R
PROVE	Protease Inhibition for Viral Evaluation
PTB	phosphotyrosine-binding domain
PDZK1	postsynaptic density protein K1
SCID	severe combine immuno deficiency
SEL	small extracellular loop
SNP	single nucleotide polymorphism
SPRINT	Serine Protease Inhibitor Therapy
SRBI	scavenger receptor BI
SREBPs	sterol regulatory element-binding proteins
ST	stem
STAT-C	specifically targeted antiviral therapy for hepatitis C
SVR	sustained virological response
RNA	ribonucleic acid
RIG-I	retinoic acid inducible–gene I
TEM	tetraspanin-enriched microdomains
Th1	helper 1 T cells
TLR	toll-like receptor
TM	transmembrane
TNF	tumor necrosis factor
TRIF	Toll-IL-1 receptor domain-containing adaptor inducing IFN-β
uPA	urinokinase-type plasminogen activator
VLDL	very low-density lipoproteins
VSV	vesicular stomatitis viruses

Introduction

I. Hepatitis C - disease

Hepatitis C virus (HCV) was discovered in 1989. Before that time, the major cause of acute viral hepatitis was referred to non-A non-B hepatitis. Extensive testing of serum of experimentally infected animals using molecular biology methods enabled identification of the infectious agent responsible for this new hepatitis by cloning the viral genome (Choo et al., 1989).

1. Hepatitis C around the world - epidemiology

170 million of people, 3% of the world population, are estimated to be infected with HCV (Wasley and Alter, 2000). The HCV prevalence is an estimated value because precise surveys from most countries are lacking. Often surveys focus on specific groups like blood-donors and drug users, however data concerning the general population is necessary to correctly estimate the number of infected people worldwide (Lavanchy, 2009).

In developed countries HCV seroprevalence is rather low with 0.6% in Germany, 0.8% in Canada, 1.1% in Australia and France. Higher rates are reported in USA-1.8%, Japan- 1.5-2.3% and Italy-2.2%. Among the developing countries the highest prevalence occurs in Egypt (up to 22%) (Shepard et al., 2005).

A map with the estimated prevalence of HCV in 2008 by the World Health Organization is shown in Figure 1.

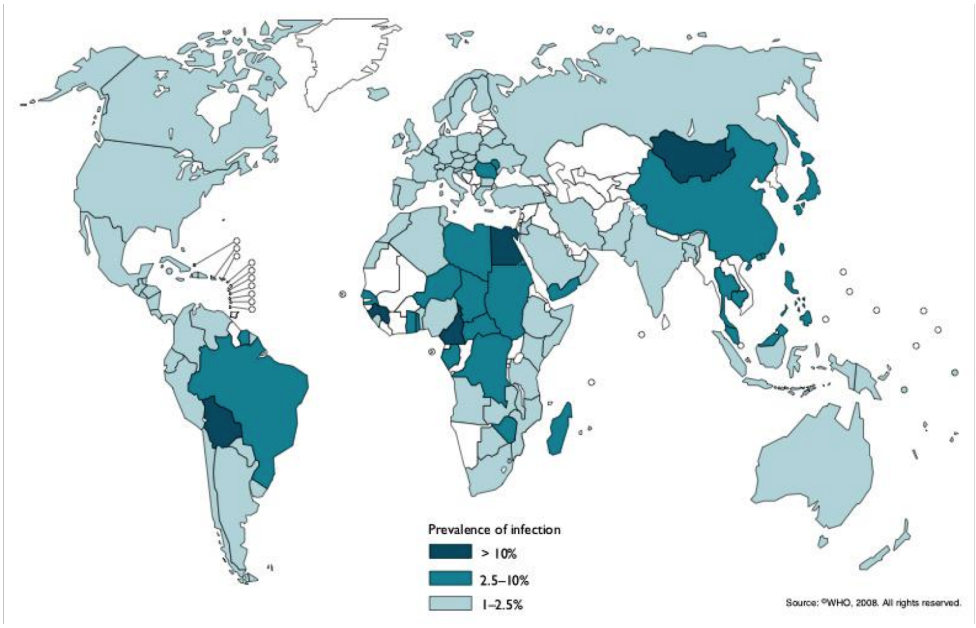

Figure 1. Estimated prevalence of HCV. World Health Organization. International travel and health, 2008.

2. From patient to patient – transmission

Hepatitis C is a blood-borne virus. After the Second World War and before the 1980, the major route of transmission was parenteral exposure to contaminated blood or blood products. After establishing the screening of blood samples for HCV before transfusions, this route of transmission has been practically eliminated in developed countries. At present, the major route of HCV contamination both in developed and developing countries is transmission among intravenous drug users (Lavanchy, 2009). For example in the United States, injection drug use accounts for 68% of current infections (Shepard et al., 2005).

In developing countries, the problem also lies in the re-use of syringes during standard medical procedures. Before 1980, mass treatment to schistsomiasis in Egypt and re-use of non-sterilized syringes led to massive HCV infection in children. The prevalence of antibodies to HCV in the general population in Egypt is now estimated to 15-20%, highly exceeding the world average value (Frank et al., 2000). Unsafe injections are still carried on in the Middle East, South-East Asia, the Western Pacific and Africa. In some countries at least 50% of injections is unsafe, with a clear link to hepatitis B, C, HIV, Ebola and Lassa viruses as well as malaria transmission. Moreover, most of these injections could easily be avoided by administration of oral drugs (Simonsen et al., 1999).

Interestingly, high occurrence of HCV hepatitis is observed in chronic hemodialysis patients, with a prevalence of 10-33%, varying between countries. This has been connected to nosocomial patient to patient transmission caused by insufficient hygienic precautions applied by institution staff. Therefore, hemodialysis patients are considered as a high-risk group for HCV infection (Alavian, 2009; Lavanchy, 2009).

HCV can also be transmitted perinatally from infected mother to child. Some studies suggest higher transmission risk in mothers with high HCV-RNA blood titers. To avoid child infection, special safety procedures must be employed especially during the delivery (Indolfi and Resti, 2009).

Finally, evidences exist to support the hypothesis that HCV can be transmitted by sexual route, however, this occurs seldom in comparison to other sexually-transmitted viruses like HBV and HIV (Terrault, 2002).

3. From infection to liver cancer - disease progression

HCV disease progresses during 10-25 years depending on additional factors described below (paragraph I.4.). As shown in Figure 2, of all patients infected with HCV, 80% progress into chronic hepatitis, which can develop into liver cirrhosis and in few cases into cancer.

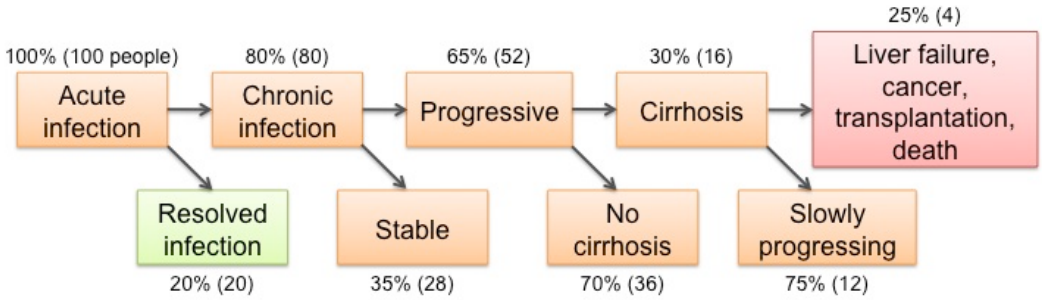

Figure 2. Estimated progression of liver disease in 100 HCV monoinfected patients. Adapted from (Kaminsky, 2007).

a. Acute hepatitis

Usually viral RNA can be detected 1-3 weeks after exposure to HCV. Symptoms of acute hepatitis can develop between 2-12 weeks after infection, however most acute HCV infections are asymptomatic. Non-specific symptoms like fatigue, jaundice, indigestion and abdominal pain are often difficult to diagnose. A high alanine aminotransferase (ALT) level is the first indication of liver injury. Diagnosis of HCV is based on RNA detection and anti-HCV antibody seroconversion (Santantonio et al., 2008), but acute hepatitis C is rarely diagnosed due to lack of symptoms.

During the first 3 months, 20-26% of patients spontaneously clear the virus, whereas the rest progress into chronic hepatitis. This is associated to viral factors like co-infections, HCV genotype and host factors like age, race and HLA (human leukocyte antigen). Interestingly, patients with symptomatic acute hepatitis more often overcome the disease, which is correlated to a strong cellular immune response (Santantonio et al., 2008).

No gold-standard therapy exists for the treatment of acute HCV hepatitis. The reason lies in the lack of proper clinical trials and most of all in difficulties in diagnosis of acute infection. Additionally, it is controversial when the therapy should be initiated. Remembering that approximately 20-26% of patients resolve the infection spontaneously, administering therapy to them brings in unnecessary health inconveniences and costs. Different strategies are tested but no clear answer exists for the moment. More predicting factors need to be studied to solve this issue (Santantonio et al., 2008; Wiegand et al., 2006).

Some reports suggest that peginterferon (Peg-IFN) alfa-2b therapy during the acute hepatitis effectively induces sustained virological response (SVR). The efficacy depends on HCV-genotype and treatment duration. Administration of Peg-IFN can thus prevent progression to chronic liver HCV infection (Kamal et al., 2006; Wiegand et al., 2006).

b. Chronic hepatitis

Failure in virus clearance during the acute phase leads to chronic HCV infection. This has been associated to the evolution of viral quasispecies that escape immune system reaction (Farci et al.,

2000) and insufficient cellular response (Bowen and Walker, 2005). Liver damage is mostly caused by the immune response that is enough to induce destruction of infected cells and fibrosis of the liver but not enough to eliminate the virus (Heydtmann et al., 2001). Liver fibrosis is an effect of scaring induced by the death of infected hepatocytes. During chronic infection, progressing fibrosis leads to cirrhosis. In compensated forms of cirrhosis, liver functions are preserved, whereas in decompensated disease, functionality of the liver is completely lost. Both forms of cirrhosis are preconditions for hepatocellular carcinoma (HCC) development (Schuppan et al., 2003). The effect of cirrhosis on liver appearance is shown in Figure 3.

Chronic HCV disease is linked to metabolic conditions like insulin resistance, type II diabetes and steatosis. Infection with genotype 1 viruses has been associated to insulin resistance and type II diabetes, which accelerates progression to fibrosis, cirrhosis and increases HCC incidence. HCV can directly induce insulin resistance and it is thought that core and NS5A proteins are involved in this process (Douglas and George, 2009). Chronic infection with genotype 3 increases the risk of liver steatosis - lipid accumulation in hepatocytes (Negro, 2010). This is probably induced by core-mediated changes in cellular lipid metabolism (Roingeard and Hourioux, 2008)(Clement-Leboube et al., 17th International Meeting on Hepatitis C Virus and Releated Viruses, Yokohama, Japan, September 9-14, 2010).

In spite of HCV marked hepatotropism, extrahepatic manifestations of the disease are also observed, among them mixed cryoglobulinemia, limphoproliferative disorders, renal failure and cognitive disorders (Jacobson et al., 2010).

Figure 3. Liver damage caused by chronic HCV infection. Adapted from www.healingdaily.com/conditions/hepatitis.htm

c. Hepatocellular carcinoma

Liver cancer is one of the most common and most fatal cancer worldwide. HCV and HBV infections are implicated in more than 70% of hepatocellular carcinoma (HCC) cases (Castello et al., 2010). As shown in Figure 2, according to estimations, four out of one hundred HCV patients can develop HCC. Making a short calculation reveals the scale of this problem. Out of 170 millions

HCV-infected people, 680,000 can develop HCC. This places HCV-associated liver cancer as a major health problem worldwide. Moreover, liver transplantation is the only efficient treatment currently available. For obvious reasons, as for example the scarcity of organ donors, this medical procedure cannot be performed on all patients requiring a new liver. Finally, even transplantation does not cure the disease, as the transplanted liver gets reinfected very rapidly. Therefore new therapeutic strategies are necessary to face this issue.

HCV triggers HCC development in an indirect way. According to a recent hypothesis, malignant transformation of hepatocytes is caused by chronic injury-mediated cell turnover and liver regeneration upon inflammation and oxidative DNA damage. Viral proteins like core, E2, NS3 and NS5A affect processes like cell signaling, transcription, apoptosis, membrane trafficking and production of cytokines and chemokines by interacting with cellular factors. This has a strong influence on the extracellular environment, modulating the immune response and often promoting tumor initiation and progression (Castello et al., 2010).

4. Co-factors accelerating disease progression

Several factors have been associated to rapid disease progression, among them male gender, older age and obesity. The most important accelerating factors are hepatitis B virus (HBV) and human immunodeficiency virus (HIV) co-infections as well as alcohol consumption (Shepard et al., 2005).

a. HBV co-infection

The effect of HBV-HCV co-infection on liver disease progression is controversial. For both viruses, the liver is the major site of infection. Some studies suggest that the risk of liver cancer development is higher in co-infection than in HCV or HBV mono-infection (Donato et al., 1998; Shi et al., 2005; Tanaka et al., 2004). However, superinfection with one virus may inhibit infection with the other, therefore a subadditive effect would be expected (Chu et al., 1998; Sheen et al., 1992). In this case one virus would be dominant, decreasing the replication of the second one. In consequence co-infection would not increase the risk of development of hepatocellular carcinoma, as other reports suggest (Cho et al., 2010). *In vitro* studies showed that HCV and HBV can replicate in the same cell, therefore interference observed in co-infected patients may be an effect of anti-viral immune response (Bellecave et al., 2009).

b. HIV co-infection

It is thought that HIV accelerates liver disease and lowers survival rate in HCV-HIV co-infection (Mohsen et al., 2002; Monga et al., 2001). In contrast, the role of HCV in HIV infection outcome is not clear. A few reports suggest that HCV accelerates HIV progression (Greub et al., 2000; Piroth

et al., 1998); others claim that HCV does not influence AIDS development (acute immuno-deficiency syndrome) nor response to HAART (highly active anti-retroviral therapy) (Sulkowski et al., 2002).

Although HAART may slow down liver disease progression, chronic liver damage is a major cause of death among HCV-HIV co-infected patients (Benhamou et al., 1999; Macias et al., 2002; Qurishi et al., 2003). Additionally, HAART correlated hepatotoxicity has also been reported; thus antiretroviral therapy can increase liver damage in HCV patients (Law et al., 2003). Clearly new therapeutic strategies should be developed concerning HCV-HIV co-infections, especially that important part of HIV positive patients is also positive for HCV.

c. Alcohol consumption

High intake of alcohol is associated with acceleration of chronic hepatitis in HCV patients. Consumption of more that 50g of alcohol per day increases liver fibrosis progression (Poynard et al., 1997). Alcohol consumption has also been correlated to cirrhosis and death in chronic HCV patients (Peters and Terrault, 2002). The molecular mechanism of the alcohol-HCV cumulative effect is not well understood, however a few reports shed light on this issue. Firstly, apoptosis is negatively regulated in cirrhotic tissues, whereas proliferation is upregulated (Mas et al., 2010). Both alcohol and HCV mediate oxidative stress in cells, so that a synergistic effect of both factors strongly increases inflammation, fibrosis and hepatocellular carcinoma development (McCartney and Beard, 2010). Finally alcohol uptake reduces the efficacy of interferon-α treatment probably by directly interfering with the IFN signaling pathway (McCartney and Beard, 2010). Indeed, a recent study demonstrates that alcohol suppresses type I interferon production by interfering with IFN signal transduction, thereby facilitating HCV replication cycle (Ye et al., 2010).

5. Counter-attack – immunity, therapies and vaccines

a. Innate immune response

Innate immunity is the first line of defense against pathogens. It is based on the production of type I interferons (IFN) and inflammatory cytokines that have antiviral, anti-proliferative and immunomodulatory effects. IFN and cytokines lead to activation of cytotoxic cells. Dendritic cells (DC) sense HCV infection by two signaling pathways: toll-like receptors (TLR) that recognize viral structures and retinoic acid inducible gene I (RIG-I) that detects double-stranded viral RNA. Recognition of a pathogen by these proteins leads to a signaling cascade that activates transcription factors like NFκB (nuclear factor kappa-light-chain-enhancer of activated B cells) and different IRF (interferon regulatory factor) (Kanto and Hayashi, 2007; Seth et al., 2006). Type I IFN produced by

stimulated DC activates natural killer (NK) and cytotoxic T-lymphocytes (CTL) that kill infected hepatocytes, inducing hepatitis. Liver damage activates myeloid DC that stimulate NK and NKT (natural killer T-cells) to secrete IFN-γ, leading to further increase in inflammation (Hiroishi et al., 2008).

In HCV-infected patients, TLR2 (recognizes core and NS3 proteins (Dolganiuc et al., 2004)), TLR4 and RIG-I expression levels are higher compared to the controls. Nevertheless, the levels of IFNβ and TNF-α (tumor necrosis factor) are lower than in uninfected patients. This suggests that HCV infection impairs the signaling and the cross-talk between immune cells. Moreover dendritic cells do not respond properly to exogenous IFNα and fail to activate NK cells (Kanto and Hayashi, 2007).

RIG-I, which is an interferon-inducible RNA helicase, binds HCV RNA and activates IRF-3. RIG-I recognizes the polyuridine motif on the 3' non-translated region of HCV RNA. This pathogen-associated molecular pattern is very characteristic for RNA viruses, and its ability to interact with RIG-I depends on homopolymeric ribonucleotide composition, linear structure and length of RNA (Saito et al., 2008). Upon virus recognition, RIG-I interacts with MAVS (mitochondrial antiviral signaling) also known as IPS-1, Cardif and VISA, whose oligomerization also leads to signaling cascade and activation of IRF3 and NF-κB (Baril et al., 2009).

HCV interferes with IFN production by blocking activation of signaling and transcription factors downstream of RIG-I signaling. One the most studied HCV proteins that has immunomodulatory effect is the NS3/4A protease. NS3/4A is able to proteolytically cleave MAVS, preventing its signaling cascade (Cheng et al., 2006). Another NS3/4A substrate is TRIF (Toll-IL-1 receptor domain-containing adaptor inducing IFN-β) an adaptor protein linking TLR3 to kinases responsible for activating IRF3 (Li et al., 2005). Finally, NS3/4A also inhibits phosphorylation of IRF-3 preventing NFκB activation and IFN expression (Breiman et al., 2005; Foy et al., 2003). Wide NS3/4A activity may support HCV persistence in the liver by constant blocking of RIG-I and TLR induced antiviral responses (Foy et al., 2005).

b. Adaptive immune response

In later stages of infection, a specific anti-HCV response is developed. The battle is played on two levels: HCV-infected hepatocytes are attacked by specific CTL and neutralizing antibodies directly recognize and inactivate virus circulating in the serum.

➢ Cellular reaction

The specific cellular anti-HCV reaction is stimulated by myeloid DC that move to lymph nodes to present viral antigens to naïve T-cells, which upon cross-stimulation differentiate into helper 1 T cells (Th1, $CD4^+$). Secretion of Il-2 (interleukin) and IFN-γ by these cells induce CTL ($CD8^+$) and NK cells activation and proliferation. Naïve $CD8^+$ recognize HCV antigens on DC and after maturation leave the lymph node to recognize and kill infected hepatocytes (Hiroishi et al., 2008). They also secrete IFN-γ that contributes to viral clearance (Lechner et al., 2000).

Insufficient cellular response is often a reason for HCV persistence. As shown in Figure 4 weak $CD4^+$ and $CD8^+$ responses related to low liver damage monitored by transaminases levels in the serum, lead to chronic hepatitis. The drop in viral titer after the initial response is usually not enough to successfully eradicate the virus. Only a prolonged cellular reaction can significantly reduce viral levels and prevent chronic infection (Bowen and Walker, 2005). The lack of proper cellular reaction may be due to failed antigen presentation by infected DC (Pawlotsky, 2004).

A high number of HCV-specific CTL is observed during the first 6 months of infection in patients with a self-limited course of disease. In patients that progress to chronic infection the level of HCV-specific CTL is significantly lower (Gruner et al., 2000; Lechner et al., 2000).

HCV-specific CTL can indeed restrict viral replication and liver damage. Low-titers of HCV-RNA are observed in patients with a good CTL-response suggesting a role in controling HCV replication. In contrast, infection with high-titer HCV may suppress CTL response (Hiroishi et al., 1997). Viral persistence has been associated with CTL exhaustion. HCV-specific CTL from the liver overexpress programmed death-1 factor, which leads to functional impairement. Additionaly, inhibitory receptor - cytotoxic T lymphocyte-associated antigen-4 is also upregulated in these cells contributing to CTL exhaustion (Nakamoto et al., 2009).

The major epitopes recognized by CTL are located in the non-structural region of HCV polyprotein, especially in the NS3 protein. Mutations within these regions may contribute to viral escape (Diepolder et al., 1997; Imawari et al., 1995).

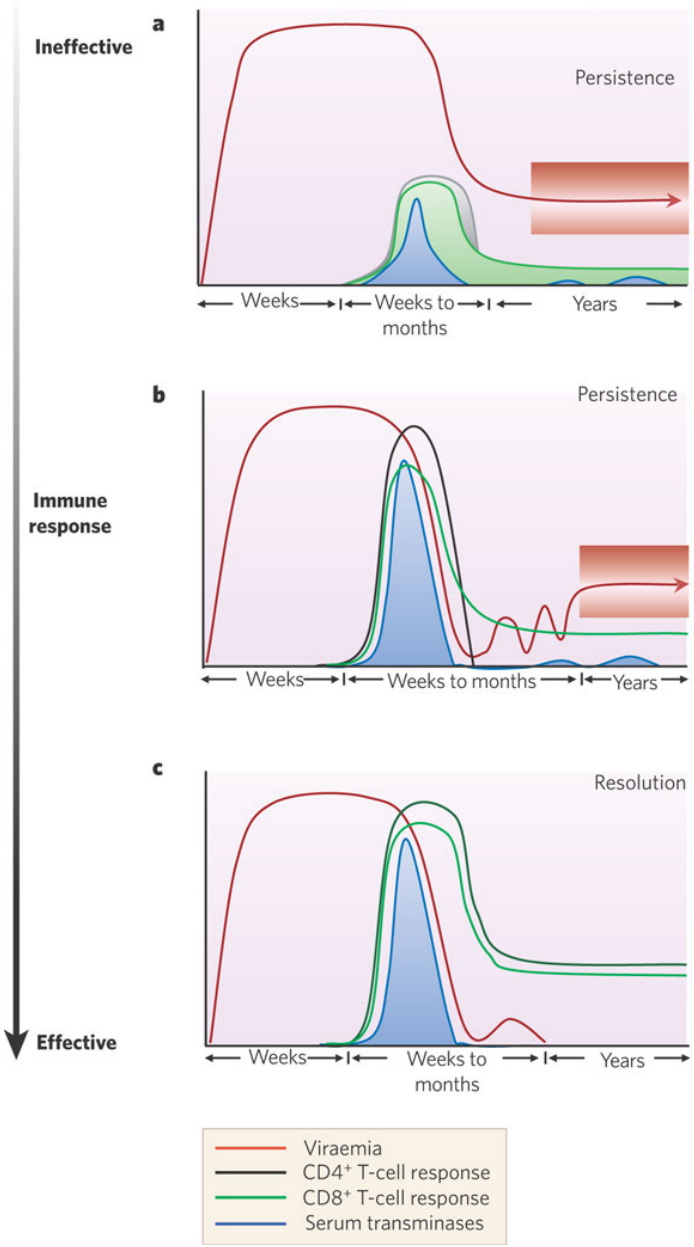

Figure 4. Patterns of acute replication and immunological response. a) Ineffective immune reaction leading to virus persistence characterized by poor $CD4^+$ and $CD8^+$ T-cells response. Also low serum transaminases levels indicate weak immune response in the liver. b) Delayed onset of $CD4^+$ and $CD8^+$ T-cells response controls infection. Contraction of $CD4^+$ response leads to virus rebound and persistent infection. Transaminases levels rise during the immune reaction. c) Strong and prolonged $CD4^+$ and $CD8^+$ reactions manage to control viraemia. Virus becomes undetectable, however rebound in viraemia may occur. Transaminases levels rise during the immune reaction. Shading indicates the variability between individuals. Adapted from (Bowen and Walker, 2005).

➢ Humoral reaction

HCV specific antibodies are detectable within 7-8 weeks after infection (Pawlotsky, 1999). Neutralizing antibodies usually recognize epitopes on structural proteins like E1 and E2 interfering with interactions with cellular receptors or blocking post-entry steps like the fusion process (Haberstroh et al., 2008). Antibodies generated against HVR1 (hyper-variable region) on E2 glycoprotein correlate with disease progression. The production of genetic variants that escape

neutralization (quasispecies) is associated with disease progression and virus persistence. Low genetic diversity of virus during the acute phase of infection can indicate a resolving of HCV infection (Farci et al., 2000).

Studies based on cohorts of patients infected with the same HCV strain showed that during acute infection, rapid induction of neutralizing antibodies could be associated with viral clearance, whereas poor antibodies titers during early phase of infection were observed in patients that later developed chronic infection. This indicates that a fast humoral response at the early stages of infection can eliminate the virus and prevent chronic disease (Lavillette et al., 2005a; Pestka et al., 2007).

In chronically infected patients, the presence of antibodies against both structural and non-structural HCV proteins is common (Bartosch et al., 2003a; Lavillette et al., 2005b; Logvinoff et al., 2004). In spite of that, the chronic infection cannot be defeated. The most probable scenario is that constant selection pressure from the immune system leads to continuous generation of escape variants (von Hahn et al., 2007). New variants of envelope proteins are no longer recognized by circulating antibodies. When new specific neutralizing antibodies are produced, further quasispecies appear, escaping neutralization.

It has been demonstrated that neutralizing antibodies can reduce HCV reinfection in patients after liver transplantation (Feray et al., 1998).

Interestingly, some of the HCV-specific antibodies may prevent the neutralization process. So called interfering, non-neutralizing antibody can bind to one epitope what disrupts binding of neutralizing antibody to a closely located epitope. Such interfering antibodies may play a role in HCV persistence (Zhang et al., 2009).

c. Therapy

Currently, standard anti-HCV therapy is composed of pegylated interferon alfa (Peg-IFN-α) and ribavirin. Unfortunately this therapy is effective in only 50% of the cases, depending on genotype and host factors. Other potential drugs are now under development, making the future of HCV patients brighter.

➢ Gold standard

Interferons are a group of endogenous glycoproteins that have antiviral and immunomodulatory effect. The potential effect of IFN-α in HCV patients has been first shown in 1986 (Hoofnagle et al., 1986). Later, this hypothesis was confirmed by other groups that proved that IFN-α could be used as antiviral drug in chronic HCV hepatitis. Indeed, long term and low-dose treatment

decreased serum aminotransferases levels and improved liver histology (Di Bisceglie et al., 1989; Schvarcz et al., 1989).

Nevertheless, only 15 to 20% of the patients have SVR to IFN-α treatment alone. Increased efficacy of this treatment was achieved by the combination of INF-α with ribavirin, which allowed an increase of the SVR rate to approximately 35%. The efficacy of treatment depends on HCV genotype, viral titer, age, sex and fibrosis stage. Patients with genotype 2 or 3 infection respond better to therapy than patients with genotype 1 (McHutchison et al., 1998; Poynard et al., 1998).

Ribavirin (1-β-D-ribofuranosyl-1,2,4-triazole-3-carboxamide) is a purine nucleoside analogue that was synthesized for the first time in 1972 (Witkowski et al., 1972). Its wide anti-viral properties have been first demonstrated on herpes, vaccinia and vesicular stomatitis viruses (VSV), it can also block respiratory infections caused by influenza and parainfluenza viruses and prevent leukemia during Friend leukemia virus infection in mice (Sidwell et al., 1972).

Ribavirin monotherapy does not reduce HCV titers, suggesting that its therapeutic effect can be achieved only in combination therapy with IFN-α (Bodenheimer et al., 1997; Dusheiko et al., 1996). The mechanism of ribavirin action can be indirect by modulation of immune response in favor of virus eradication (Tam et al., 1999). Another hypothesis suggests that ribavirin inhibits the host enzyme inosine monophosphate dehydrogenase (IMPDH) that is necessary during synthesis of guanosine triphosphate (Markland et al., 2000). Ribavirin is a mutagen that interferes with viral polymerases. Its incorporation into RNA leads to accumulation of mistakes, resulting in the so called ‘error catastrophe’ and prevents efficient replication (Crotty et al., 2000; Vo et al., 2003). Whether such a mechanism also contributes to its anti-HCV activity remains however to be demonstrated.

Further improvement in HCV therapy was achieved by pegylation of IFN-α. This modification decreases its clearance, reducing applications to once per week. It also improves drug tolerance and response to treatment (Fried et al., 2002; Manns et al., 2001). Two kinds of Peg-IFN-α are commercially available: 2a and 2b. The difference between them lies in their pharmacokinetic properties as they contain distinct polyethylene glycol moieties. For Peg-IFN-α 2b the dose is adjusted to body weight, while the Peg-IFN-α 2a the dose is a fixed quantity for all the patients. It is not yet clear whether the type of Peg-IFN-α used in treatment has a significant influence on SVR (Foster, 2010).

As discussed above, the efficacy of standard of care treatment with Peg-INF-α and ribavirin has its limitations since only a fraction of the patients respond to treatment. Moreover the therapy often leads to multiple side effects that may require dose modification, leading to reduced SVR. The most common IFN-α associated side effects are flu-like symptoms, bone-marrow depression,

neuropsychiatric disorders and autoimmune syndromes, whereas ribavirin may lead to haemolytic anaemia (Manns et al., 2006).

Gold standard anti-HCV therapy is currently the only option for HCV chronic patients. Costs, side effects and limited efficacy of the treatment are major concerns for HCV patients and health authorities. For these reasons the knowledge about factors that can predict the response to treatment is now highly desired. A milestone in this field came very recently, in 2009. Several groups reported at the same time that single nucleotide polymorphisms (SNP) near the IL28B (interleukin 28B) gene encoding IFN-lambda 3 are strongly associated with response to treatment (Ge et al., 2009; Suppiah et al., 2009; Tanaka et al., 2009). Moreover, IL28B polymorphism has its reflection in the immune system reaction to infection. Allel rs12979860 C/C is strongly associated with spontaneous viral clearance (Thomas et al., 2009). In conclusion, genotyping IL28B in patients can provide substantial information about the natural course of infection and predict the treatment outcome. A more individual treatment may prevent unnecessary discomfort for the patients that can naturally control the infection or in whom SVR is predicted to be poor.

- **New perspectives**

The limitations of standard of care treatment are the reason for constant research on novel anti-HCV drugs. To prevent generalized side effects, direct-acting antiviral agents that interfere with HCV proteins can be used. These types of drugs are named specifically targeted antiviral therapy for hepatitis C (STAT-C). The major problem of this strategy is selective pressure on the virus, caused by the drug, which leads to appearance of resistant variants. This problem can be overcome by combination therapy of several STAT-C molecules. STAT-C are the future perspective for effective HCV therapy.

The most promising and advanced drugs are the NS3/4A serine protease inhibitors especially Boceprevir (Serine Protease Inhibitor Therapy - SPRINT trial) and Telaprevir (Protease Inhibition for Viral Evaluation - PROVE trial), which are now in phase 3 clinical trials and might be approved for the treatment in 2011. These studies show that only combining standard Peg-IFN-α/ribavirin therapy with novel STAT-C compounds could bring improved results. In genotype 1 patients SVR approaches 70-75% (reviewed in (David and Nelson, 2009)).

Many molecules that target HCV replication by interfering with the NS5B polymerase are now under clinical trials. NS5B inhibitors can belong into nucleoside/nucleotide analogues. Among them R-7128 has been reported to have significant short-term antiviral activity and proved to be safe and well tolerated. Another anti-NS5B molecule is IDX-184 that is liver-targeted purine analogue. These types of drugs have a higher barrier to resistance in comparison to other STAT-C compounds. Non-nucleoside polymerase inhibitors usually target non-conserved allosteric sites on

NS5B, among them filibuvir (hydroxydihydropyranone derivative) that is now under phase 2 clinical trials (reviewed in (Legrand-Abravanel et al., 2010)).

Many promising molecules target other intracellular stages of HCV cell cycle. NS5A inhibitor BMS-790052 was shown to be efficacious against both HCV replicons *in vitro* and in phase 1 clinical trials, now the drug is in phase 2 investigation (Gao et al., 2010). HCV translation can be inhibited by changing conformation of viral internal ribosome entry site (IRES) by benzimidazole inhibitors (Parsons et al., 2009). The NS4B-RNA interaction can be blocked by clemizole that leads to replication inhibition at non-cytotoxic concentrations (Einav et al., 2008a). P7 channel activity can be blocked by BIT225, a compound that is now under phase 1 clinical trial and proved its efficiency against HIV and BVDV (bovine diarrhea virus) (Khoury et al., 2010; Luscombe et al., 2010).

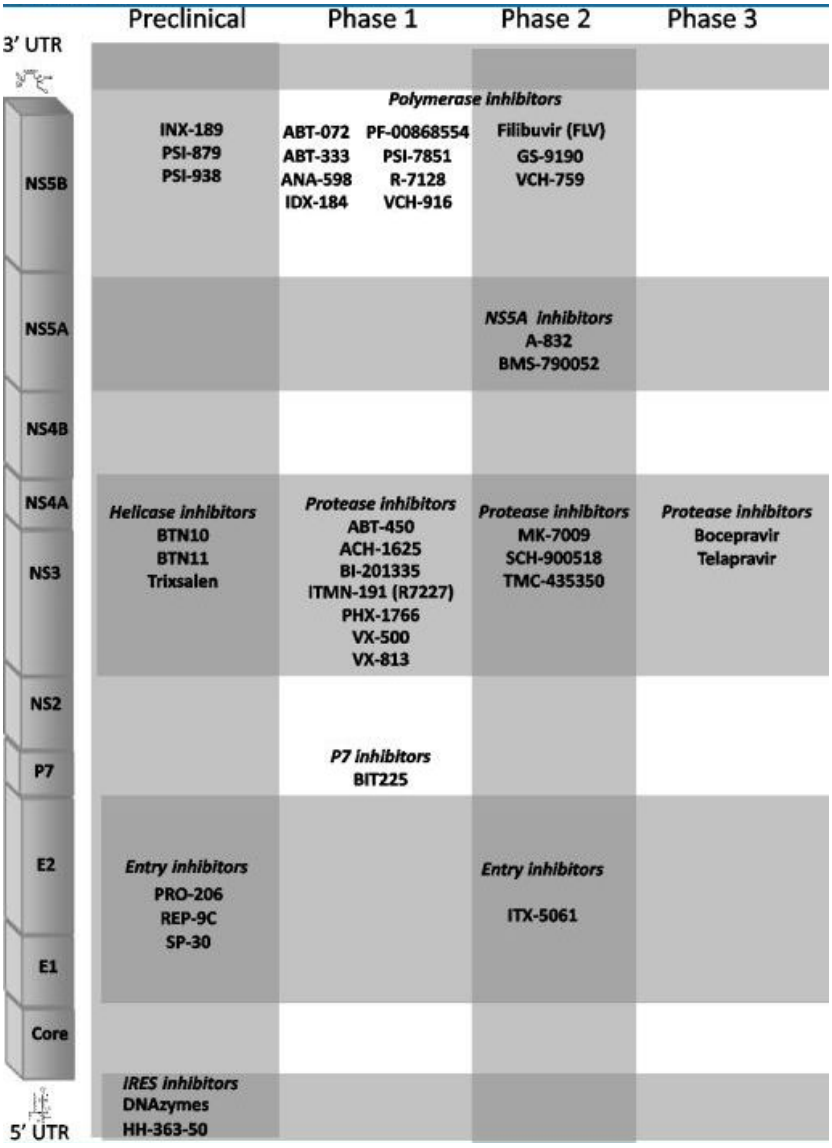

Figure 5. Anti-HCV STAT-C drugs under clinical investigation arranged by phase of clinical investigation and HCV protein targeted. Adapted from (Schinazi et al., 2010).

Several reports suggest targeting the HCV entry process, for example by preventing the interaction between viral particles and tetraspanin CD81, which is a major HCV receptor (Holzer et al., 2008; Ziegler et al., 2009) or by direct interaction with HCV glycoproteins (Baldick et al., 2010). Another molecule, ITX-5061, which is an SRBI antagonist, efficiently inhibits HCVcc and HCVpp entry and is now in phase 2 clinical trials (Syder et al., 2010).

Finally, therapy can target host factors required for the HCV life cycle. One of these targets is microRNA-122 that is essential for the regulation of HCV RNA levels (Jopling et al., 2008; Jopling et al., 2005). Treatment of infected chimpanzees with locked nucleic acid modified oligonucleotide (SPC3649) complementary to microRNA-122 was shown to suppress HCV viremia without signs of resistance (Lanford et al., 2010).

Viral replication can also be blocked by the non-immunosupressive cyclosporin Debio-025 that is effective both *in vitro* and *in vivo* in HCV and HIV patients (Flisiak et al., 2009; Flisiak et al., 2008; Paeshuyse et al., 2006). Debio-025 inhibits cellular cyclophilin A, which is essential during HCV replication (Chatterji et al., 2009; Kaul et al., 2009).

d. Vaccines

The replication of viral RNA is a mutation-prone process because of the low-fidelity of RNA-dependent RNA polymerases. The consequence is a rapid development of variants called quasispecies that may escape the immune system. This makes development of an anti-HCV vaccine very difficult. At present, no vaccine is available, however several groups around the world work to develope HCV vaccine. The strategies used are discussed in this paragraph. Because of the number of HCV chronic patients, therapeutic vaccines are now the major area of interest.

For a long time, the development of HCV vaccines had been hindered by the lack of a proper cell culture system and HCV tropism. Many vaccines are now tested in chimpanzees and humans, but hopes are set in small animal models that can accelerate HCV vaccine research.

The major challenge for a therapeutic vaccine would be to rescue impaired T cells in HCV chronic patients. The final goal of a vaccine would be to generate broad and multi-specific $CD4^+$ cells, to activate cytotoxic $CD8^+$, and to generate cross-genotype neutralizing antibodies.

Different kinds of vaccines are currently under development. Among these are peptide-based vaccines, as for example the IC41 vaccine that contains 5 conserved peptides from core, NS3 and NS4 proteins. This candidate proved to be safe and to induce a T cell response (Firbas et al., 2006; Klade et al., 2008). However, the reaction was not strong enough to be used as monotherapy. Nevertheless, in combination with standard therapy, it can lead to substantial increase in SVR rate (Wedemeyer et al., 2009).

Other types of vaccines are vector vaccines where viral antigens are delivered by attenuated viruses like Vaccinia modified Virus of Ankara (MVA), which belongs to the *Poxviridae* family. MVA expressing NS3, NS4 and NS5B activates both $CD4^+$ and $CD8^+$ T cells to produce IFN-γ and to kill infected cells when tested in transgenic mice (Fournillier et al., 2007). In phase 1 clinical trials, the vaccine was safe and in some cases it decreased viral titers (Habersetzer et al., 2009b). Currently phase 2 trials have been proposed in combination with standard of care treatment.

Another strategy is to use recombinant HCV glycoproteins, like the E1 alum adjuvanted vaccine, which unfortunately failed in phase 2 clinical trials (Leroux-Roels et al., 2005). Still another vaccine GI-5005 contains heat-killed yeast cells expressing a conserved NS3-core fusion protein. Because of the yeast components this vaccine induces a strong T-cell response, which was demonstrated in vaccinated mice (Haller et al., 2007) and in chronic HCV patients during phase 1 trials (Habersetzer et al., 2009a). In phase 2 trials GI-5005 in combination with standard therapy was more efficient than standard therapy alone. Combined therapy also improved liver functions as aminotransferases levels returned to the more normal levels (McHutchison et al., 2009).

Finally, DNA vaccines that contain the most conserved HCV regions including NS3 and NS4 are also under investigation. The DNA is delivered through intramuscular electroporation, which leads to expression of proteins and induction of both $CD4^+$ and $CD8^+$ responses. ChronVac-c led to elimination of NS3/NS4 expressing hepatocytes in a mouse model (Ahlen et al., 2005; Frelin et al., 2004). This vaccine gave also promising results in human studies, so that it was proposed as a treatment of chronic HCV patients in combination with standard therapy. Another DNA vaccine, based on adenoviral vectors or electroporated plasmids coding for the HCV nonstructural region, proved to be effective in chimpanzees. Animals developed a cross-reactive T-cell response and were protected from acute hepatitis (Folgori et al., 2006).

As discussed, many HCV vaccines are now under clinical trials and their future use in combination with standard Peg-INF-α and ribavirin may improve the disease outcome of many HCV chronic patients.

II. Hepatitis C virus

1. Virus classification

The Hepatitis C virus is a positive stranded RNA virus that belongs to the *Flaviviridae* family and the *Hepacivirus* genus (Table 1) (Lindenbach, 2007).

Table 1.

Family:	*Flaviviridae*	
Genus:	*Flavivirus*:	Tick-borne encephalitis virus West Nile virus Yellow fever virus
	Pestivirus:	Bovine viral diarrhea virus 1 and 2 Classical swine fever virus
	Hepacivirus:	GBV-B Hepatitis C virus
	unclassified:	GBV-A GBV-C GBV-D

HCV is currently classified into seven genotypes and several subtypes that at the nucleotide level differ from each other by 31-33% and 20-25%, respectively (Kuiken and Simmonds, 2009; Simmonds et al., 2005). The incidence of different genotypes has its reflection in the geographic distribution (Figure 6). Genotypes 1, 2 and 3 are distributed worldwide, however with different prevalence. Genotype 1a is the most common in North America, genotype 1b in Europe and Asia. Some genotypes are specific for a region, like genotype 4 in Egypt, 5 in South Africa and 6 on the Indochinese peninsula (Zein, 2000).

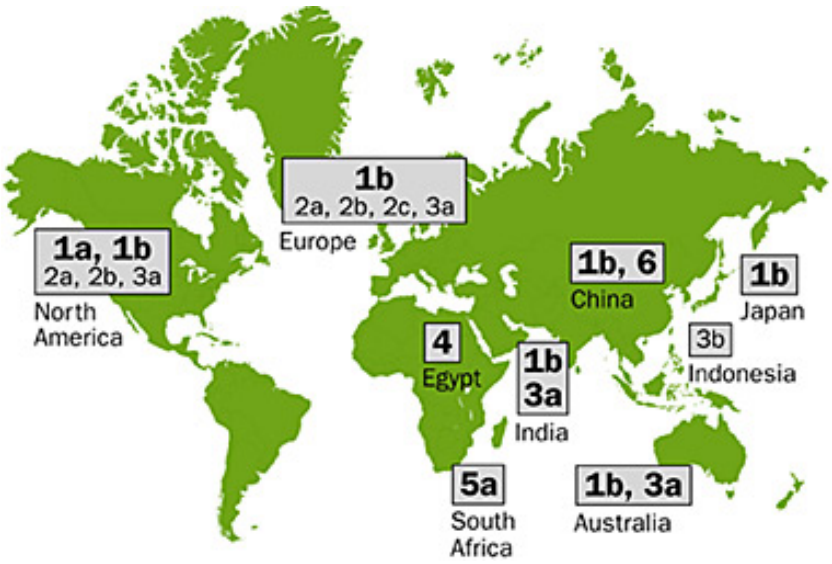

Figure 6. Geographic distribution of genotypes.

The reason for HCV variability originates in the RNA-dependent RNA polymerase, which is used by RNA viruses in the replication process. The frequency of error per nucleotide varies from 10^{-4} to 10^{-5} for HCV (Pawlotsky, 2003). The variability of HCV is not limited to genotypes and subtypes. High mutation incidence leads to generation of quasispecies that represent genome heterogeneity in the patients' sera (Martell et al., 1992). Generation of HCV quasispecies leads to escape from humoral and cellular immunity (Cox et al., 2005; Kato et al., 1993; von Hahn et al., 2007). It is also correlated to progression of liver disease (Honda et al., 1994).

2. Viral particle

Hepatitis C virus in an enveloped virus (Feinstone et al., 1983). Electron microscopy studies demonstrated that viral particles are spherical, 55 to 65 nm in diameter (Gastaminza et al., 2010; Kaito et al., 1994; Wakita et al., 2005).

Gradient density studies revealed that virus present in patients' sera has heterogeneous density and composition (Andre et al., 2002). During the infectious life cycle in hepatocytes, HCV uses VLDL assembly and secretion pathway (Huang et al., 2007). Therefore, the virus association to lipoproteins can explain differences in density. Particles containing both viral and lipoprotein components have been named lipoviroparticles (LVP). In iodixanol gradients, most of viral RNA sediments below 1.08 g/cm^3. In contrast, sucrose gradient ultracentrifugations show two RNA peaks, one at low density 1.06 g/cm^3 and a second between 1.13 and 1.16 g/cm^3. Furthermore, low-density fractions were precipitated with polyclonal anti-ApoE and anti-ApoB antibodies, confirming the lipoprotein content of LVP (Nielsen et al., 2006). Analysis of infectivity of different density fractions revealed, that low-density fractions are indeed the most infectious (Gastaminza et al., 2006; Lindenbach et al., 2006; Vieyres et al., 2010), suggesting that lipoprotein association of HCV plays a role during the entry process. Indeed, recent studies demonstrate that low-density virus has higher fusogenic properties (Haid et al., 2009). Virus hidden in lipoprotein form has also been suggested to avoid antibody-mediated neutralization. Part of viral RNA has been found in high-density fractions, which was explained by virus association with immunoglobulins (Thomssen et al., 1993). Such immune-complexes are often found in patients with chronic hepatitis (Hijikata et al., 1993).

HCV produced in cell culture has similar properties as serum derived virus, however the densities of different fractions of virus are higher after cell culture production (Lindenbach et al., 2006), suggesting some differences in viral properties.

HCV particle consists of a viral membrane envelope, in which E1 and E2 glycoproteins are anchored by their transmembrane domains, a nucleocapsid formed by core protein and a positive single-stranded RNA (Figure 7). The character of virus association to lipoproteins remains

unsolved, however recent visualization of viral particles by cryo-electron microscopy and co-localization of spherical viral particles with E2 and ApoE, suggest that LVP are integrative structures containing both viral and lipoprotein elements (Gastaminza et al., 2010).

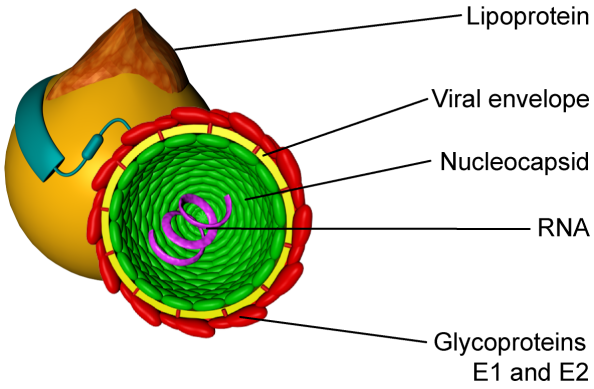

Figure 7. Schematic representation of HCV particle.

3. Genome organization

HCV genome is in the form of single-stranded, positive-sense RNA containing approximately 9.6 kb. A single open reading frame encodes one long polyprotein of about 3000 residues. The polyprotein encoding region is flanked by non-translated RNA (NTR) on both 5' and 3' ends.

Host and viral proteases are responsible for cleaving the polyprotein into ten mature proteins. The protein organization in the polyprotein is shown in Figure 8. From the NH_2- terminus, the polyprotein contains the structural proteins: core, E1 and E2, followed by the non-structural proteins: p7, NS2, NS3, NS4A, NS4B, NS5A and NS5B (Lemon, 2007).

Ribosomal frame shift during polyprotein synthesis leads to the production of an additional protein called ARF (alternate reading frame) (Xu et al., 2001).

a. Non-coding regions

The 5'NTR is strongly conserved among different HCV genomes. It is essential not only for polyprotein translation but also for replication. It consists of 341 nucleotides forming four secondary structured domains (Honda et al., 1999; Lukavsky et al., 2003). The complete 5'NTR is required for RNA replication, however critical signals are located within domains I and II (Friebe et al., 2001). 5'NTR binds miR-122, a micro-RNA specific for liver tissue that is also essential for replication (Jopling et al., 2005) and translation (Henke et al., 2008) of HCV.

Overlapping elements of domains II, III and IV function as IRES (internal ribosome entry site) and bind the 40S ribosome subunit (Pestova et al., 1998). The interaction leads to ribosome placing on start codon and initiation of translation process (Honda et al., 1996). High sequence conservation makes 5'NTR a candidate target for therapeutic molecules and anti-sense oligonucleotides.

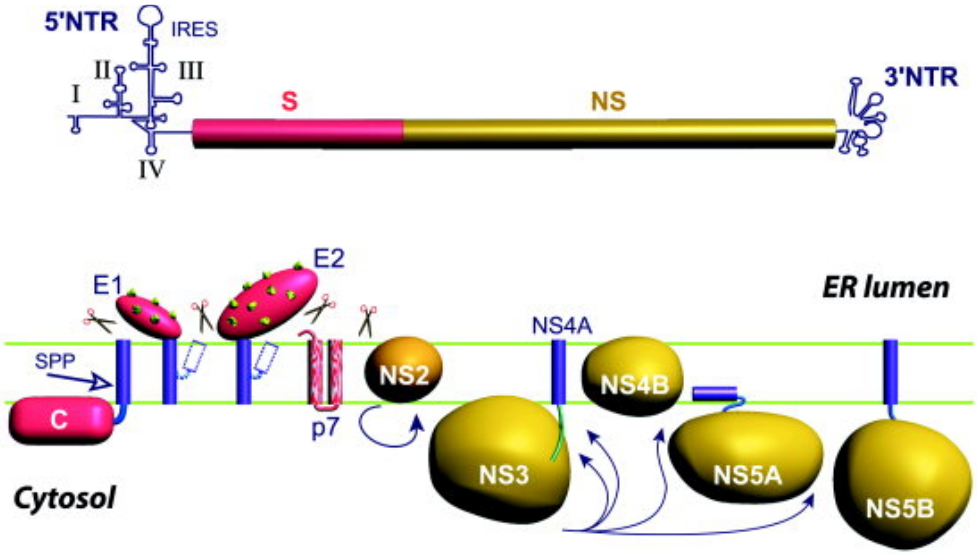

Figure 8. Schematic representation of HCV genome (top panel) and polyprotein (bottom panel) (Penin et al., 2004b). 5'NTR contains IRES sequence (internal ribosome entry site). RNA encodes structural (S) and non-structural (NS) proteins. Polyprotein processing in shown in the context of ER membrane. SPP and big arrow correspond to signal peptide peptidase cleavage site, scissors point ER signal peptidase cleavage sites, cyclic arrow shows autocatalytic NS2/NS3 cleavage site, black arrows point NS3/NS4A protease complex cleavage sites.

The 3'NTR region consists of a variable region, a U/C-rich region and a conserved 3' terminal domain called 3'X tail (Kolykhalov et al., 1996). Part of the U/C-rich region and the 3'X tail are essential for the replication process (Kolykhalov et al., 2000; Murayama et al., 2010; Yi and Lemon, 2003a; Yi and Lemon, 2003b). The U/C-rich tract and terminal stem loop of 3'X tail also take part in translation process by stimulating IRES-dependent translation in hepatocytes (Bung et al.; Song et al., 2006). Interestingly, stem-loop 2 within 3'NTR interacts with loop structure called 5BSL3.2 in NS5B region. This kissing-loop interaction is essential for HCV replication (Friebe et al., 2005).

b. Viral proteins

The organization of HCV proteins on the polyprotein is shown in Figure 8.

➢ Core

Core is a basic protein cleaved from the polyprotein as an immature 23 kDa form (191 residues). Further processing by signal peptide peptidase leads to release of a mature 21 kDa form (173-179 residues) (Yasui et al., 1998). The immature 23 kDa form of core contains approximately 20 residues that serve as signal peptide for Core/E1 cleavage by signal peptidase and mediate E1 ectodomain translocation in the ER lumen (Grakoui et al., 1993b; Santolini et al., 1994).

Mature core protein contains two distinct regions. The hydrophilic domain (D1) (residues 1-118) on the N-terminus contains positive charges and is responsible for RNA binding and nucleocapsid formation (Klein et al., 2005). D1 can also interact with tubulin, leading to its polymerization and formation of microtubules that are important for establishing efficient HCV infection (Roohvand et al., 2009). The C-terminal hydrophobic domain of core (D2) (119-174) consists of two α-helices separated by a short hydrophilic region. The hydrophobic and membrane interacting features of this region have been shown to play a role in D1 folding (Boulant et al., 2005). D2 promotes core association with ER membranes and mitochondria as well as with lipid droplets (Rouille et al., 2006; Schwer et al., 2004; Suzuki et al., 2005).

The role of core protein in the HCV life cycle is not limited to nucleocapsid formation. Indeed, core is also suggested to interact with a number of cellular proteins. It is able to regulate many cellular processes like apoptosis (Chou et al., 2005), growth (Fukutomi et al., 2005) or lipid droplet formation and steatosis (Barba et al., 1997). It has also been implicated in liver damage progression and fibrosis (Nunez et al., 2004). Finally, in a transgenic mouse model, it induces hepatocellular carcinoma (Moriya et al., 1998).

- **ARF proteins**

ARF proteins (ARFP) are the products of ribosome frame shift during the translation process. First frame shift was localized at the 11th codon of the core protein (Walewski et al., 2001). An open ARF in the +1 reading frame results in the expression of proteins, which presence in HCV-positive patients' sera has been confirmed by detection of ARFP-specific antibodies (Walewski et al., 2001; Xu et al., 2001).

The mechanism of ARFP translation is not fully understood. ARF are bounded by stop codons, however they do not contain AUG start codon. Few mechanisms have been proposed: ribosomal frameshifting and/or hopping, a novel initiation mechanism or polymerase stuttering. Most evidences suggest ribosome frameshifting, however novel internal initiation mechanism cannot be excluded (reviewed in (Branch et al., 2005)). Xu et al. proposed a mechanism where ribosome initiates translation at AUG codon, however AAA-rich sequence at codons 9 to 10 stimulates a frame shift that moves ribosome into +1 reading frame. A 17 kDa product was named F-protein (Xu et al., 2001). -1 and double frameshifts that give rise to several ARFP were also described (reviewed in (Branch et al., 2005)). The size of F varies between genotypes, however it does not exceed 160 aminoacids (Boulant et al., 2003).

The function of ARF proteins has not been described, however it seems that they do not participate in HCV replication (McMullan et al., 2007). Some studies suggest the role of F in immuno-modulation and HCV pathogenesis (Fiorucci et al., 2007). The ability of F to interact with the

proteasome subunit alpha3 can potentially reveal its role in regulation of protein degradation (Yuksek et al., 2009).

- **Envelope glycoproteins E1 and E2**

Detailed description of E1 and E2 glycoproteins is placed in paragraph III.1.

- **P7**

P7 is a small hydrophobic protein composed of 63 residues, which has been shown to be essential for HCV infectivity (Jones et al., 2007b; Sakai et al., 2003). It contains two transmembrane domains separated by a short hydrophilic segment (Carrere-Kremer et al., 2002). P7 is cleaved from E2 and NS2 by host signal peptidase. The cleavage was shown to be inefficient and the precursor product E2/p7/NS2 is often observed in infected cells (Carrere-Kremer et al., 2004). P7 forms hexamers or heptamers both *in vitro* and *in vivo* and functions as an ion channel. This function can be blocked by amantadine and alkylated iminosugar derivatives (Griffin et al., 2003; Pavlovic et al., 2003). Structure of p7 and its ion channel activity have been confirmed by NMR (nuclear magnetic resonance) studies and electron microscopy analysis (Luik et al., 2009; Montserret et al., 2010; Steinmann et al., 2007). The subcellular localisation of p7 reveals two populations of p7, one associated with the ER and second with mitochondria (Griffin et al., 2005). The exact role of p7 protein is not yet established, however it is suggested to play an important role during virion assembly and morphogenesis. Moreover, the genotype specific activity of p7 clearly points that p7 interacts with other viral proteins (Jones et al., 2007b; Steinmann et al., 2007).

- **NS2**

NS2 is a viral encoded proteinase of 23 kDa. NS2 and the N-terminal part of NS3 function as a zinc-dependent metalloprotease that cleaves the NS2/NS3 junction (Grakoui et al., 1993a). The mature NS2 is a transmembrane protein containing several transmembrane segments (Jirasko et al., 2008; Santolini et al., 1995; Yamaga and Ou, 2002).

NS2 is an essential factor suggested to take part in HCV assembly (Jones et al., 2007b; Ma et al., 2010). Trans-complementation studies demonstrated that NS2 could take part in a late post-assembly step, probably in collaboration with NS5A (Yi et al., 2009). The protease domain, but not its enzymatic activity is required for the production of infectious particles (Jirasko et al., 2008). Recent studies revealed that NS2 is in fact a cysteine protease itself and NS3 protein plays a role of regulatory cofactor (Schregel et al., 2009). The enzymatic activity of NS2 and NS2/NS3 cleavage is essential for viral replication (Dentzer et al., 2009). HCV replication can be blocked by Cyclosporine A that targets NS2/NS3 cleavage site (Ciesek et al., 2009).

NS2 contains a highly conserved casein kinase recognition site and its phosphorylation leads to NS2 degradation by the proteasome. This presents NS2 as short-lived protein (Franck et al., 2005).
NS2 has a general impact on regulation of cellular processes. When express alone, it modifies gene expression from different cellular and viral promoters, for example it inhibits HBV gene expression and replication (Dumoulin et al., 2003). It also interacts with some cellular proteins like CIDE-B (cell death-inducing DFF45-like effector) and inhibits CIDE-B mediated apoptosis (Erdtmann et al., 2003).

- **NS3/NS4A**

NS3 and NS4 are released from the polyprotein as 70 kDa and 8 kDa proteins, respectively (Grakoui et al., 1993b). The N-terminal third of NS3 is a chymotrypsin-like serine protease that cleaves the polyprotein at NS3/4A, NS4A/4B, NS4B/5A, and NS5A/5B junctions (Hahm et al., 1995). NS4A acts as a cofactor required for NS3 protease activity (Failla et al., 1994). The C-terminal two-thirds of the NS3 protein act as a RNA helicase (Tai et al., 1996). NS3/4A contains two membrane-binding determinants in α-helix form. The first α-helix in NS4A may play a role in intramembrane protein-protein interactions during formation of replication complex. The second, amphipathic α-helix in NS3 allows proper positioning of enzyme on the membrane (Brass et al., 2008).
Expression of NS3 protein, without NS4A cofactor results in diffused distribution of NS3 in the cytoplasm and nucleus. Co-expression of both NS3 and NS4A stabilizes NS3 and targets it to ER. Directing NS3 to the ER is mediated by the N-terminal, hydrophobic part of NS4A (Wolk et al., 2000). NS3/4A protease activity is enhanced by NS3 helicase (Beran and Pyle, 2008). In addition to its role in HCV polyprotein precessing, NS3/4A has an ability to interfere with cellular anti-viral response, by blocking IRF-3 (interferon regulatory factor-3) phosphorylation. This prevents activation of genes responsible for interferon production (Foy et al., 2003). NS3/4A is able to proteolytically cleave MAVS preventing signaling cascade induced by MAVS (Cheng et al., 2006). Another NS3/4A substract is TRIF (Toll-IL-1 receptor domain-containing adaptor inducing IFN-β). Its cleavage hinders double-stranded RNA signaling through Toll-like receptor 3 (TLR3). TLR3 signaling would normally induce an IRF-3 dependent cascade leading to interferon production. Thus, NS3/4A targets several proteins to block the IRF3 pathway. It equally affects production of co-stimulatory immune-molecules regulated by NF-κB (Ferreon et al., 2005; Li et al., 2005).
NS3 nucleotide triphosphatase (NTPase) activity is mediated by both RNA and DNA (Suzich et al., 1993). NTPase provides energy from ATP (adenosine-5'-triphosphate) required for the helicase activity and unwinding duplex RNA. The helicase activity of NS3 is dependent on pH and the

presence of divalent Mg^{2+}or Mn^{2+} cations (Kim et al., 1995). NS3 helicase (NS3H) is a unique enzyme, because it also possesses DNA helicase activity (Tai et al., 1996). The role of NS3H/NTPase has not been clearly demonstrated, however it is likely that unwinding double-stranded structures might be important during HCV replication. Inactivation of the enzyme blocks viral production (Kolykhalov et al., 2000). NS3 protease stimulates NS3H RNA binding activity (Beran et al., 2007). Recently NS4A has also been shown to promote ATP-dependent RNA binding by NS3H (Beran et al., 2009). Interestingly, NS3H may also play a role in HCV assembly, as mutations of some conserved residues had no effect on replication but on the production of virions (Ma et al., 2008).

Both NS3/4A serine protease and NS3 helicase/NTPase represent a common target in anti-HCV therapies.

- **NS4B**

NS4B is a 27 kDa, ER-membrane-associated protein oriented towards the cytoplasm. Co-localization with other non-structural proteins confirmed that NS4B is a component of the replication complex (Hugle et al., 2001). It consists of four transmembrane segments and cytoplasmic tails on N and C-terminuses. However, the N-terminal tail can be post-translationally translocated into the ER lumen. This process generates an additional, fifth transmembrane segment (Lundin et al., 2003). The amphipathic character of this helix mediates membrane association and plays a crucial role in HCV RNA replication (Elazar et al., 2004; Gouttenoire et al., 2009a; Gouttenoire et al., 2009b).

Interestingly NS4B has been shown to negatively regulate HCV and cellular protein translation (Kato et al., 2002). Other reports suggest that NS4B plays a role in HCV RNA replication. A nucleotide-binding motif conserved among HCV genotypes has been identified in NS4B. It enables GTP (guanosine-5'-triphosphate) binding and hydrolysis, which mediates replication by an unknown mechanism (Einav et al., 2004). The same GTP binding motif mediates cellular transformation both *in vitro* and *in vivo* (Einav et al., 2008b). NS4B may also play a role during virus assembly and release (Jones et al., 2009). Finally, NS4B probably plays a role in HCV-associated liver pathogenesis, because it has been shown to increase the transcriptional activity of SREBPs (sterol regulatory element-binding proteins), which leads to increase in lipogenic gene expression and lipid accumulation (Park et al., 2009).

- **NS5A**

NS5A is a 56 to 58 kDa protein (Grakoui et al., 1993b). It co-localizes with other NS proteins and viral RNA forming replication complexes in membranous webs (Moradpour et al., 2004b). It is also

engaged in the assembly process by interactions with structural core protein in the close association to lipid droplets (Miyanari et al., 2007).

NS5A is a membrane-associated protein. The N-terminal part of it forms a highly conserved amphipathic α-helix that enables post-translational membrane anchorage in the ER membrane (Brass et al., 2002). This region is not only important for NS5A localization but it is also required during RNA replication (Elazar et al., 2003; Penin et al., 2004a). NS5A consists of three domains. DI (a.a. 1-213) contains a conserved zinc-binding motif, meaning that NS5A is in fact a zinc metalloprotein. Zinc coordination is required for NS5A implication in RNA replication (Tellinghuisen et al., 2004; Tellinghuisen et al., 2005). On the C-terminal part, NS5A contains a nuclear localization signal (NLS). Truncated forms of NS5A containing no membrane anchor, as for example caspase-like protease cleaved products of NS5A can be found in the nucleus. This suggests a potential role of NS5A in the regulation of host-cell genes expression (Satoh et al., 2000).

NS5A is a phosphorylated protein and a hyperphosphorylated form of 58 kDa mass was also observed (Tanji et al., 1995). NS5A hyperphosphorylation has been proposed to act as a switch between viral replication and particle assembly. The enzyme responsible for the phosporylation has been identified as casein kinase II (Kim et al., 1999). The other NS proteins like NS3, NS4A and NS4B are implicated in the regulation of NS5A hyperphosphorylation (Koch and Bartenschlager, 1999). NS5A phosphorylation is also important for interaction with human vesicle-associated membrane protein-associated protein A (hVAP-A). Hyperphosphorylation inhibits this interaction and disrupts RNA replication (Evans et al., 2004). An electrostatic switch has been discovered on the C-terminus of NS4A. It plays a role in regulation of NS5A hyperphosphorylation that regulates viral RNA replication (Lindenbach et al., 2007). The role of NS5A in replication is not clear, however it has been shown that NS5A binds HCV RNA (Huang et al., 2005). Phosphorylation of NS5A may also play a role in the virion assembly process, because mutation of a serine in the casein kinase II consensus motif disrupts an early stage of the assembly process (Appel et al., 2008; Tellinghuisen et al., 2008). The explanation may lie in the fact that serine mutations prevent phosphorylation that results in lack of NS5A-core interaction (Masaki et al., 2008).

NS5A interacts with numerous cellular proteins, and thus may participate in the regulation of different cellular pathways and immune system evasion. A serine-rich motif in NS5A binds growth factor receptor-bound protein 2 (Grb2) adaptor protein, which may have implications in the regulation of cellular signaling and perturbation of the cell cycle (Tan et al., 1999). NS5A also interferes with the antiviral immune response by inducing interleukin-8, which leads to inhibition of interferon production (Polyak et al., 2001). It equally blocks the dsRNA TLR-3 induced antiviral

response by interacting with protein kinase R (PKR), which normally induces IRF-1 dependent signaling pathway (Pflugheber et al., 2002).

Antiviral drugs targeting NS5A are under investigation (paragraph I.5.c) (Gao et al., 2010).

- **NS5B**

NS5B is a 66 kDa protein that contains the characteristic Gly-Asp-Asp (GDD) motif specific for RNA-dependent RNA polymerases (Grakoui et al., 1993b). It is tail-anchored to membrane by a α-helical transmembrane domain at the C-terminus of the protein. Binding to the cytosolic-site of the ER takes place after translation (Schmidt-Mende et al., 2001). The transmembrane domain, called insertion sequence, not only localizes NS5B to the membranes, but it also provides intramembrane protein-protein interactions between NS5B and other viral and/or cellular components of replication complex (Moradpour et al., 2004a). The crystal structure of NS5B reveals characteristic "fingers, palm and thumb" organization of other polymerases (Figure 9)(Bressanelli et al., 1999). However, in contrast to other polymerases, the active site of the enzyme is encircled by the interacting fingers and thumb (Lesburg et al., 1999). The mechanism of NS5B polymerase activity has been described recently. During the first half-reaction – substrate binding, the polymerase binds template single-stranded RNA with high affinity. NTP(s) binding causes a tertiary structural change into an active conformation. The second half-reaction – turnover is divided into sequential polymerization and the rate-limiting product release (Reich et al., 2010).

Interestingly, HCV polymerase is the major factor responsible for the JFH-1 strain's ability to replicate in cell culture. It seems that JFH-1 polymerase has a higher replication initiation efficiency than J6 strain, antoher genotype 2a isolate, which shows no detectable replication in cell culture. This phenomenon can be explained by extra hydrophobic interactions between fingers and thumb that results in a closed conformation of the active site and easier initiation of replication of JFH-1 polymerase (Simister et al., 2009). Mutations that increase thumb-fingers interaction, thus increasing RNA binding have been associated to cyclosporine A resistance (Liu et al., 2009).

NS5B has been shown to interact with other non-structural HCV protein like NS3 helicase and NS4A (Ishido et al., 1998; Jennings et al., 2008). The non-structural proteins form a membrane-associated complex responsible for RNA replication (Dimitrova et al., 2003).

NS5B may also play some regulatory functions as it interacts with ATG5, an autophagy protein required for double-membrane vesicles formation. This interaction may promote viral replication, as autophagy is suggested to play a role in initial steps of replication (Guevin et al., 2010). NS5B is an important target for the development of anti-HCV drugs (Watkins et al., 2010).

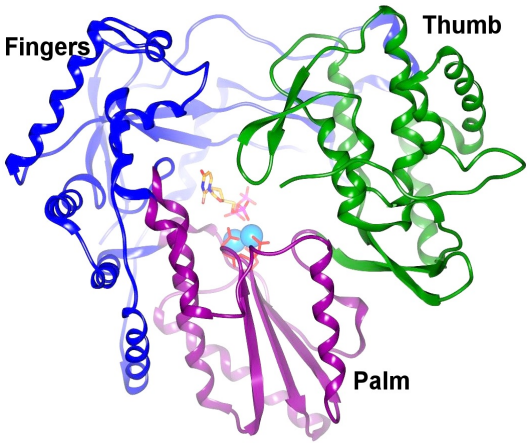

Figure 9. Ribbon diagram of NS5B polymerase complexed with UTP (O'Farrell et al., 2000)

4. Models of study

Since the isolation of HCV in 1989, researchers around the world have been trying to reveal its secrets. This process has been very difficult due to the lack of proper cell culture system to amplify the virus under laboratory conditions. However, surrogate models have been developed to study HCV genome organization, polyprotein processing, protein properties and single stages of the viral life cycle. Finally in 2005, a single HCV genome from a Japanese patient has been isolated, which surprisingly turned out to be able to replicate in cell culture. This discovery importantly accelerated the research focused on HCV and enabled uncovering of many HCV mysteries.

The following paragraph aims to explain the different models of HCV studies.

a. Infections with HCV isolated from patients

First attempts to establish cell culture systems supporting HCV replication were based on primary hepatocytes isolated from patients chronically infected with hepatitis C. Infectious particles were released, meaning that HCV can replicate in cultured hepatocytes (Ito et al., 1996). Equally, non-infected human or chimpanzee primary hepatocytes can be infected with HCV that leads to viral replication (Castet et al., 2002; Fournier et al., 1998; Lanford et al., 1994; Rumin et al., 1999). These methods faithfully reflect natural HCV infection, however the virus production remains at the limit of detection. Moreover, primary hepatocytes studies are not widely used due to difficulties in cultivating these cells and interindividual variability in the quality of the hepatocytes. This system is also very expensive. Finally, patient-derived virus accessibility is often limited to clinical institutions.

b. Stable and transient expression systems

Expression of HCV glycoproteins in stable or transient cell lines provided a lot of information about the localization, properties and structure of E1 and E2 (Dubuisson et al., 2000; Dubuisson et al., 1994; Patel et al., 2000). Equally, these systems enabled the studies on envelope glycoprotein interaction with HCV putative receptors like CD81 and SR-BI (Pileri et al., 1998; Scarselli et al., 2002). However, this system only allows studying limited stages of the HCV life cycle and do not reflect the complexity of the process.

c. Subgenomic replicons

The first milestone in the studies of HCV translation, replication and maturation of viral proteins was the development of HCV replicon systems. The full-length genome was cloned from RNA isolated from the liver of chronically HCV-infected patient. Constructed selective subgenomic replicons were able to replicate in cell culture to high levels (Lohmann et al., 1999).

The first subgenomic replicon was based on con1 clone of 1b genotype. The region coding for the structural genes was removed in order to allow more efficient replication, since shorter RNA are known to replicate better than longer ones. Two heterologous elements have been inserted. The neomycin phosphotransferase gene (neo), which provides resistance to G418 antibiotic and an EMCV (encephalomyocarditis virus) IRES. The construct thus obtained is bicistronic as it contains two IRES sequences, the first from HCV controls neo expression and the second from EMCV controlling expression of non-structural HCV proteins. The schematic structure of different replicons is presented in Figure 10. The resulting construct is transfected into human hepatoma cells (Huh-7) and selection upon G418 treatment enables selection of clones with high replication levels. Replicons containing NS3 to NS5 non-structural proteins replicate with high efficiency, indicating that structural proteins and NS2 are not required for the replication process (Lohmann et al., 1999).

After successive passages in cell culture, several mutations have been identified in replicating clones, mostly in the NS5A region (Blight et al., 2000). Some of these adaptive mutations can increase replication 500-fold when introduces into wild-type replicons (Krieger et al., 2001).

Generation of replicons led to the prospect of producing viral particles in a full-length genome replicon, however attempts were not successful (Pietschmann et al., 2002). The lack of effective assembly process in replicon system might be due to adaptive mutations likely changing interactions with some cellular or viral proteins (Blight et al., 2000).

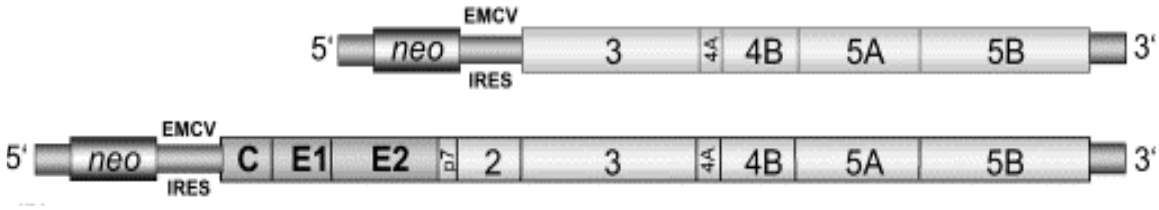

Figure 10. The structure of the subgenomic HCV replicon (on the top) and HCV full length genome (below). Neomycin (neo) selection marker is under control of HCV IRES. Viral proteins are under control of EMCV IRES.

d. HCV pseudovirions and virosomes

A promising tool to describe HCV assembly process has been introduced in 1998. Using baculovirus system, HCV-like particles (HCV-LPs) were produced in insect cells. A recombinant baculovirus bearing cDNA coding for structural HCV proteins was used to infect insect cells. It resulted in production of enveloped particles containing core and glycoproteins E1 and E2 (Baumert et al., 1998). HCV-LPs were suggested to be a potential candidate for the development of an anti-HCV vaccine as they induced humoral and cellular immune response in mice (Baumert et al., 1999; Lechmann et al., 2001). Anti-HCV structural protein antibodies from HCV patients reacted with HCV-LPs, which might be useful as a diagnostic tool (Baumert et al., 2000).

Envelope glycoproteins of HCV have also been produced with VSV resulting in chimeric HCV-pseudotyped VSV virus. Viral particles containing TM domains of G protein in fusion with the ectodomains of E1 and E2 were able to infect mammalian cells (Lagging et al., 1998; Matsuura et al., 2001) However, another study did not confirm the realibility of this system (Buonocore et al., 2002). Semliki forest virus containing HCV structural proteins has also been described. However, despite efficient assembly process, the chimeric viruses were poorly secreted from cells (Blanchard et al., 2002).

Finally, liposomes containing E1 and E2 glycoproteins have been described (virosomes). These particles were recognized by conformational-dependent antibodies and CD81 being a potential tool to study HCV glycoproteins and receptor interaction (Lambot et al., 2002).

e. HCV pseudoparticles

The development of retroviral and lentiviral particles pseudotyped with HCV glycoproteins enabled better understanding of HCV entry process (Bartosch et al., 2003b; Drummer et al., 2003; Hsu et al., 2003). HCV pseudoparticles (HCVpps) are recombinant viral particles that consist of retro or lentiviral core surrounded by an envelope containing HCV glycoproteins (Bartosch et al., 2003b).

Three plasmids are used to co-transfect 293T cells and generate HCVpp (Figure 11). The first plasmid contains MLV (murine leukemia virus) or HIV gag-pol genes responsible for capsid

formation under CMV immediate early promoter control. The second plasmid contains packaging-competent MLV–derived genome encoding the luciferase or green fluorescent protein (GFP) marker gene. The third plasmid contains E1 and E2 genes under CMV promoter. The C-terminal part of core protein has also been added to the construct to serve as a signal peptide for E1 protein. Using retroviruses as assembly platforms for HCV glycoproteins was dictated by retroviruses ability to incorporate different viral and cellular glycoproteins and to pack and deliver genetic markers into DNA of infected cells (Bartosch et al., 2003b).

HCVpps are safe, as defective viral genome cannot replicate in infected cells. The only manifestation of infection is the activity of reporter luciferase or GFP genes.

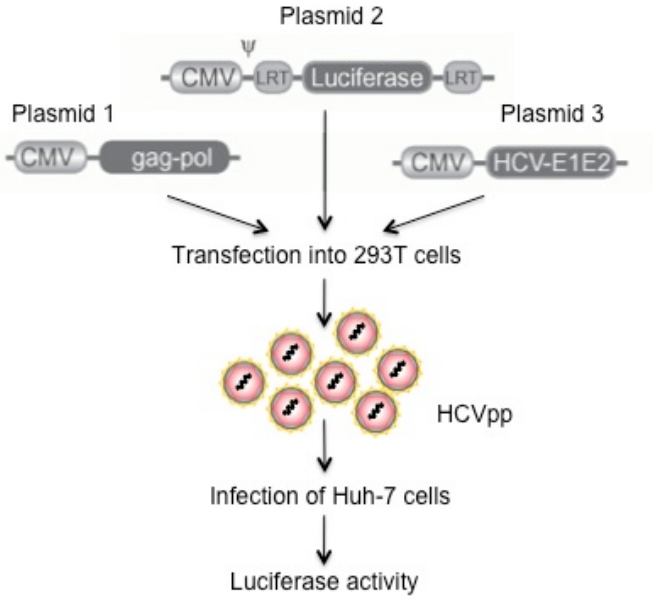

Figure 11. Schematic explanation of HCVpp assay. 293T cells are transfected with three plasmids, 1- containing gag-pol genes, 2- containing luciferase gene, packing signal ψ and LRT-long terminal repeats enabling retrotranscription and integration with host genome, 3- containing E1-E2 genes. All plasmids are under CMV promoter control. HCV pseudoparticules (HCVpp) released into the supernatant are used in Huh-7 cells infection. Quantification of luciferase activity in Huh-7 cells enables measurement of infectivity (adapted from (Lavie et al., 2006)).

HCVpps are used to infect Huh-7 cells as well as other cell lines expressing HCV entry factors. The infectivity is measured by the activity of the reporter gene. The infection can be neutralized by anti-E2 monoclonal antibodies and infected patient-derived sera (Bartosch et al., 2003a; Hsu et al., 2003). HCVpp entry has been shown to rely on the presence of CD81 and SR-BI on the permissive hepatocytes or hepatocytes-derived cell lines (Bartosch et al., 2003c; Hsu et al., 2003). Entry of HCVpps also depends on pH, suggesting pH-dependent conformational changes in glycoproteins during the fusion process (Op De Beeck et al., 2004).

Establishing HCVpp assay opened a wide range of possibilities to study HCV entry, however the system is imperfect due to the lack of VLDL-assembly pathway in 293T cells. HCV has been

shown to depend on VLDL-assembly and secretion during its infectious cell cycle (Huang et al., 2007). It results in HCV association to lipoproteins (Nielsen et al., 2006).

f. HCV cell culture system

The first HCV produced in cell culture (HCVcc) has been established in 2005 (Lindenbach et al., 2005; Wakita et al., 2005; Zhong et al., 2005). Interestingly, RNA isolated from a Japanese patient suffering from fulminant hepatitis gave rise to the JFH-1 strain (Japanese fulminant hepatitis) that is able to replicate in cell culture. This strain had been shown before to replicate as a subgenomic replicon without adaptive mutations (Kato et al., 2003). Wakita and his collaborators have cloned JFH-1 into a plasmid vector enabling transcription from the T7 promoter. *In vitro* transcribed RNA is electroporated into Huh-7 cells, which leads to production of high titers of infectious virus able to reinfect naïve cells. Higher titers of virus can be obtained in Huh-7 cells defficient in innate immune responses (Huh-7.5 and Huh-7.5.1) (Lindenbach et al., 2005; Zhong et al., 2005). Other genotypes can also be used in HCVcc system, however titers are much lower than the one observed for JFH-1 (Kato et al., 2007; Yi and Lemon, 2009; Yi et al., 2006). Many chimeric viruses containing part of NS2 and NS3-NS5 region from JFH-1 and the rest of the genome from another genotype have been constructed (Gottwein et al., 2009; Lindenbach et al., 2005; Pietschmann et al., 2006) and been shown to work reasonably well. The other advantage of the system is that luciferase gene can be used as an easy marker for HCV replication. The bicistronic constructs containing luciferase gene under HCV IRES and HCV proteins under EMCV IRES are less replicative than wild type viruses, however titers produced are still efficient for quantitative analyses of HCV infection (Koutsoudakis et al., 2006). Monocistronic reporter systems have also been reported (Jones et al., 2007b; Tscherne et al., 2006).

HCVcc is able to infect Huh-7 cells, primary human hepatocytes, chimpanzees and uPA SCID mice (Lindenbach et al., 2006). HCVcc system also enables observation of persistent *in vitro* infection. Interestingly, chronic infection leads to evolution of more aggressive viral variants able to enter/replicate better than wild type virus. Cells resistant to infection have also been observed, highlighting that during persistent infection both cellular and viral evolutionary mechanisms are operating in order to assure the survival (Zhong et al., 2006). HCVcc assays allowed answering the question whether superinfection takes place during HCV life cycle. It was shown that re-infection is inhibited during post-entry steps, most likely translation or replication initiation (Schaller et al., 2007; Tscherne et al., 2007). HCV produced in cell culture has similar properties as serum-derived virus, however the densities of different fractions of virus are higher after cell culture production (Gastaminza et al., 2006; Lindenbach et al., 2006), suggesting some differences in viral properties.

Nevertheless, the lipoprotein character of HCVcc virus has been confirmed (Gastaminza et al., 2008; Gastaminza et al., 2010).

At the moment, HCVcc represents the best available system to study all steps of the HCV life cycle and it is widely used in HCV-focused laboratories.

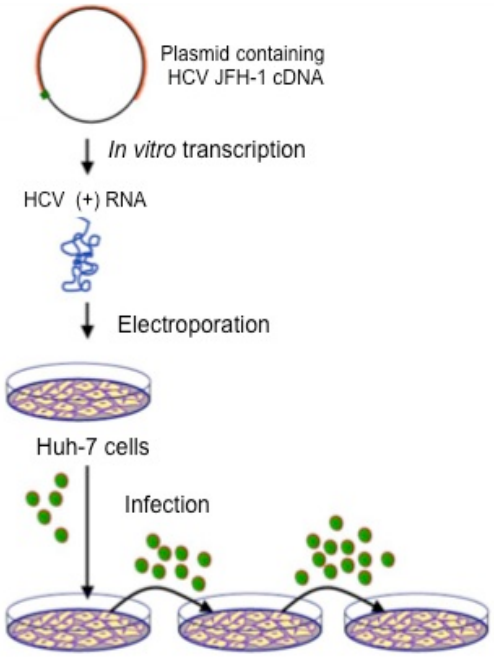

Figure 12. Schematic explanation of HCVcc assay. Plasmid containing HCV JFH-1 cDNA is transcribed *in vitro* to produce single-stranded positive RNA. Viral RNA is electroporated into Huh-7 cells and produced virions are used to infect naïve cells. More precise description is placed in the text.

g. Animal models

HCV in natural conditions may infect only humans and chimpanzees. However, studies involving these latter pose an ethical problem since these animals are indeed an endangered species. Nevertheless, chimpanzees are used in studies focusing on antiviral drugs and antiviral immunity. An interesting example has been published recently by Lanford et al., where they showed that silencing microRNA-122 in the liver has a therapeutic effect in chronically infected animals (Lanford et al., 2010). Other recent studies in chimpanzees showed that failure of CD4 T cell response during persistent infection is not only due to mutational escape of the virus, but additional mechanism must be involved (Fuller et al., 2010). Finally, chimpanzees are a powerful tool in vaccine research, as they develop an anti-viral immunity similar to what is observed in humans (Elmowalid et al., 2007; Youn et al., 2008; Zubkova et al., 2009).

Small animal models are very useful in preclinical studies on antiviral drugs and vaccines. However limited species tropism of HCV strongly hampers the development of such models. Two strategies can be used to overcome this problem. Firstly, the virus can be adapted to infect rodent cells or rodent liver could be humanized. The first strategy remains ineffective, because HCV not only

needs species-specific entry factors like CD81 and occludin (Ploss et al., 2009), but also specific intracellular factors (McCaffrey et al., 2002) required during replication. Some reports show that entry and replication are possible in murine cells, however no assembly and release was observed (Bitzegeio et al., 2010; Flint et al., 2006; Uprichard et al., 2006; Zhu et al., 2003). The second strategy was shown to be more effective. The best-described model is based on uPA (urinokinase-type plasminogen activator), immunodeficient mice. Transgenic SCID (severe combine immuno deficiency) mice express uPA transgene, which leads to severe liver destruction. These mice can be rescued by transplanting human hepatocytes (xenotransplantation). uPA SCID human chimeric liver mice were shown to support HCV and HBV infections (Mercer et al., 2001; Meuleman et al., 2005; Vanwolleghem et al., 2010). uPA SCID mice have already proven to be a useful tool in evaluation of potential antiviral drugs (Matsumura et al., 2009), neutralizing antibodies studies (Meuleman et al., 2008) and characterization of intracellular mechanisms induced upon infection (Joyce et al., 2009). Unfortunately immunodeficient mice cannot be used to study neither pathogen nor vaccine-induced immune responses. Moreover, very low throughput of chimeric mice generation, variability and the requirement for advanced surgical techniques are the reasons for limited uPA SCID mice utilization (Legrand et al., 2009).

To overcome the problem of immunity deficiency in chimeric mice, the attempts to combine humanized liver models with mice harboring a human haematolymphoid system are under way. Engraftment of suspension of human haematopoietic progenitor stem cells into SCID mice may lead to reconstitution of human immune responses. This model could be used to study all aspects of HCV infection, however the difficulties in using this system are the same as in the case of uPA SCID mice (reviewed in (Legrand et al., 2009).

Recently, another interesting chimeric mouse model has been described (Bissig et al., 2010). These immunodeficient mice lack three genes: fumaryl acetoacetate hydrolase [Fah], recombination activating gene 2 [Rag2], and the gamma-chain of the receptor for IL-2 [Il-2rgamma]. Transplantation of human hepatocytes into such mice results in up to 95% human hepatocyte chimerism. HBV and HCV infections are well propagated in these animals.

Xenotransplantation model difficulties can be overcame by an inbred mouse model. The natural susceptibility to HCV could be achieved by genetic adaptation of host. However, the complexity of HCV life cycle requires multiple adaptations. Moreover these genetic changes should be combined with downregulation of molecules responsible for antiviral response. This strategy remains a challenging task in HCV-focused research (Ploss and Rice, 2009).

5. Viral life cycle

Several stages of the HCV life cycle have been described (reverwed in (Pawlotsky et al., 2007)). Firstly, viral particles are non-specifically attached to the target cells. Then, upon interactions with specific receptors, virions are internalized through clathrin-dependent endocytosis. Acidic pH in early endosomes enables conformational changes in viral glycoproteins mediating fusion with a cellular membrane. In the cytoplasm, the released RNA is translated into a polyprotein. Processing of the polyprotein results in the formation of mature viral proteins. Non-structural proteins form complexes localized to membranous webs, responsible for viral RNA replication. Finally, in close association to lipid droplets, HCV is assembled and released following VLDL-secretion pathway. A schematic life cycle of HCV is shown in Figure 13. The following paragraph aims to describe the HCV life cycle in the hepatocytes.

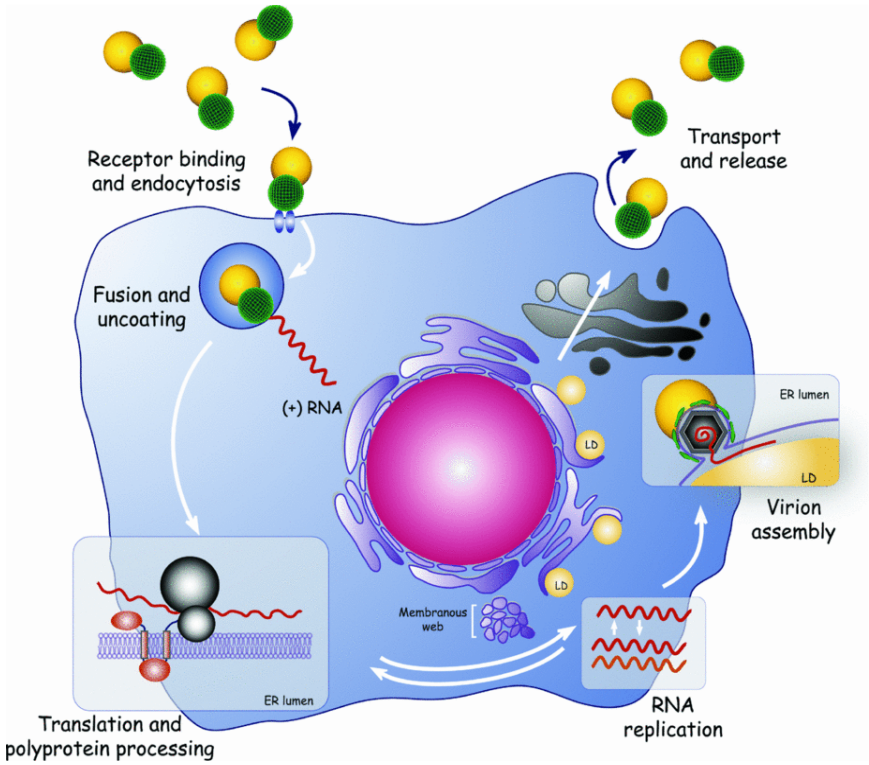

Figure 13. Putative HCV life cycle in non-differentiated hepatoma cells. Detailed explication is placed in the text. (Popescu and Dubuisson, 2009)

a. Attachment, endocytosis and fusion

HCV entry process is dependent on specific and non-specific interactions between viral glycoproteins and associated lipoproteins with cellular receptors and attachment factors. Advanced characterisation of the viral particle structure and cellular factor properties is required to fully understand this process. Since my thesis focuses on the entry process, a separate paragraph is dedicated to describe this stage of the HCV life cycle (paragraph III).

b. Translation and processing of the viral proteins

RNA liberated into the cytoplasm upon fusion, serves as messenger RNA for the synthesis of HCV polyprotein. 5'NTR on HCV sequence contains the IRES that is responsible for initiation of translation process (Tsukiyama-Kohara et al., 1992; Wang et al., 1993). The IRES consists of a pseudoknot structure that is conserved among the pestivirus family (Wang et al., 1995). The 5'NTR is structured into four domains and domain II has been shown to be required during translation initiation (Honda et al., 1999; Lukavsky et al., 2003). Domain III has been identified to interact with eukaryotic initiation factor eIF3 (Buratti et al., 1998). In addition, eIF2 has been shown to take part in HCV translation (Ji et al., 2004). In the first step, the IRES binds to the 40S ribosome subunit at the AUG initiation codon. Then, eIF3 and ternary complex (eIF2·Met-tRNA$_i^{Met}$·GTP) assemble to form a 48S complex. The formation of 48S depends on proper sequence in domain III and proper initiation codon. GTP hydrolysis enables 60S subunit binding and results in formation of the 80S complex, which is able to initiate translation. Binding of 60S is regulated by domains II and III of IRES (Otto and Puglisi, 2004). Dissociation of eIF2 during 80S assembly is mediated by IRES domain II (Locker et al., 2007).

The translation process is regulated by numerous viral and host factors. Probably sequential acting of different factors regulates switching from translation to replication during HCV life cycle (Lourenco et al., 2008).

A celullar factor implicated in HCV translation is the La autoantigen. La binds to RNA via its RNA-binding motif and stimulates IRES-mediated translation. La plays a role in translation of many RNA viruses (Ali and Siddiqui, 1997).

The polyprotein produced upon translation is targeted to the ER membrane where E1 ectodomain is translocated into the lumen of ER. This process is mediated by the signal sequence on the C-terminus of core protein. Host signal peptidase cuts the junction between core and E1, releasing the immature core form (McLauchlan et al., 2002). A host signal peptide peptidase further processes the signal peptide at the C-terminus of core, which results in the formation of the mature core protein. A host signal peptidase also cleaves E1-E2, E2-p7 and p7-NS2 junctions. In the ER lumen, E1 and E2 undergo further maturation steps like N-glycosylation (reviewed in (Penin et al., 2004b)). The cleavage between non-structural proteins is dependent on viral proteases. The NS2/3 zinc-dependent autoprotease cuts the junction between NS2 and NS3. Finally, the NS3/4A serine protease cleaves NS3/4A junction and downstream junctions of NS4A-NS4B, NS4B-NS5A, and NS5A-NS5B (Lindenbach and Rice, 2005).

c. Replication of viral HCV

Replication complexes are composed of viral and cellular proteins as well as RNA molecules. These complexes localize to specific membrane structures formed from ER membranes called membranous webs. The properties of membranous webs like cholesterol and fatty acid composition modulate the replication process (Gosert et al., 2003; Kapadia and Chisari, 2005). Membranes associated to replication complexes resemble lipid rafts domains as caveolin-2 co-localization has been observed (Shi et al., 2003). Moreover, NS5A and NS5B do interact with hVAP-33 (human vesicle-associated membrane protein-associated protein) that promotes replication complexes formation (Gao et al., 2004; Tu et al., 1999).

Replication of HCV RNA can be divided into two steps. In the first step positive-strand RNA serves as a template for negative-strand RNA production. Negative-strand RNA is then used as a template to produce new positive strands that will be used for translation, production of new replication intermediates and the formation of new virions. NS5B polymerase is responsible for the synthesis of both negative and positive strands (reviewed in (Bartenschlager et al., 2004). It is worth noting that RNA replication is asymmetric. Indeed 5 to 10 fold more positive strands is synthesized (Lohmann et al., 1999).

The replication is initiated by interaction between replication complex, 3'X tail at 3'NTR and 5BSL3.2- kissing loop RNA structure in NS5B region (Astier-Gin et al., 2005; Friebe et al., 2005; You et al., 2004). The mechanism of NS5B action has been described above (paragraph II.3).

d. Assembly and release

In 2005, the HCVcc system enabled appropriate investigation of assembly of infectious viral particles (Lindenbach et al., 2005; Wakita et al., 2005; Zhong et al., 2005). The association of core protein to lipid droplets seems to be essential for the production of HCV (Boulant et al., 2006; Boulant et al., 2007; Shavinskaya et al., 2007), however other viral proteins like NS5A and NS3 are also localized to lipid droplets in infected cells (Miyanari et al., 2007). The current model for the assembly process suggests that core interacts with lipid droplets through its D2 domain and further recruits NS5A to the complex (Appel et al., 2008; Masaki et al., 2008). The exact mechanism for the assembly process has not been described yet, however increasing evidences indicate that HCV uses VLDL-assembly and secretion pathway. Firstly, NS5A associated membranes contain a number of proteins related to lipid metabolism. Moreover, down-regulation of Apolipoprotein B (ApoB) production inhibited virus production. Inhibition of MTP (microsomal triacylglycerol transfer protein), the protein required for VLDL production, has also an inhibitory effect on HCV production (Huang et al., 2007). The similarities between HCV and VLDL production pathways have further been described by Gastaminza et al. (Gastaminza et al., 2008). Finally, apolipoprotein

E (ApoE) and ApoC1 have also been shown to be associated with HCV particles (Benga et al., 2010; Chang et al., 2007; Cun et al., 2010; Meunier et al., 2008). The model of HCV VLDL-associated assembly is shown in Figure 14. VLDL-assembly pathway will be described later (paragraph IV.1).

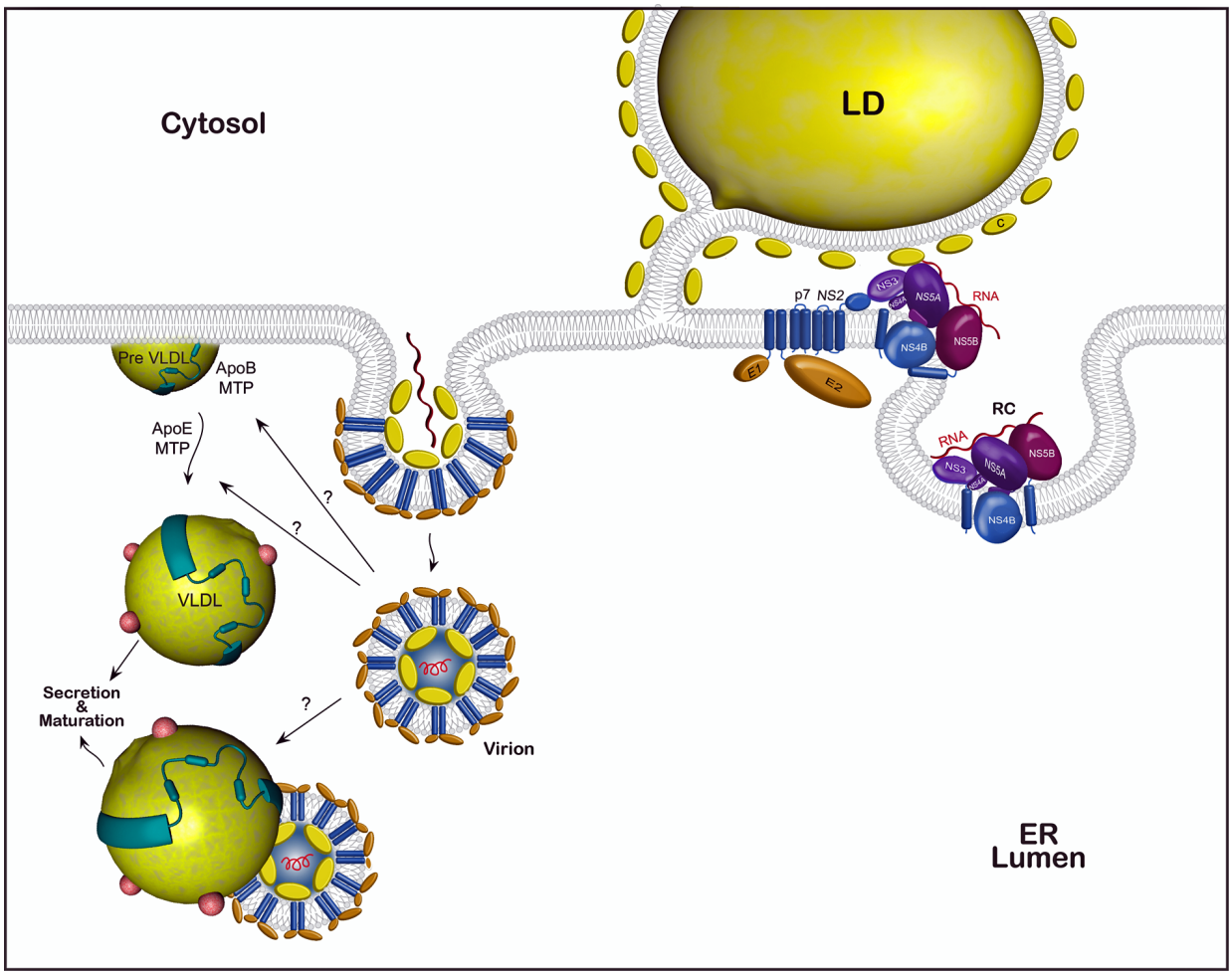

Figure 14. Current model of HCV assembly. RNA replication complexes are localized in close association to lipid droplet. Virus assembly and secretion follows VLDL pathway, however the exact relations between these two processes are not yet known (question marks). (Tews et al., 2010).

Characterisation of HCV glycoproteins using heterolous expression systems suggested that E1E2 non-covalent heterodimer would be the functional complex for HCV entry (Op De Beeck et al., 2004). However, in the context of the HCVcc system, virions-associated E1 and E2 envelope glycoproteins form large complexes stabilized by disulfide bridges, whereas the intracellular forms of these proteins assemble as non-covalent heterodimers (Vieyres et al., 2010). The presence of disulfide bridges between HCV envelope glycoproteins suggests that lateral protein-protein interactions assisted by disulfide-bonds formation might play an active role in the budding of HCV particles. Interestingly, subviral HCV particles can be only produced when the HCV envelope glycoproteins are expressed in lipoprotein-producing cell lines (Icard et al., 2009; Pecheur et al., 2010), also suggesting that E1 and E2 play an active role during the budding process.

III. Hepatitis C virus entry

Hepatitis C virus entry into the hepatocytes is a multi-step process that requires a number of cell host factors enabling virus attachment to cells and internalization. Several entry factors have been described, however the precise mechanism of virus-receptor interactions is not well understood. The difficulty may lie in the amount of factors playing a role in HCV entry since not only host factors are implicated, but also viral glycoproteins and lipoproteins associated to the virus participate in the virus-host interactions. This paragraph aims to describe the entry process, focusing both on viral and cellular factors implied.

HCV entry proceeds in few stages; firstly virus interacts with non-specific attachment factors. Then following specific receptor interactions, the virus undergoes clathrin-dependent endocytosis. Finally the acidic pH induces conformational changes in the viral glycoproteins that leads to fusion with cellular membranes and liberation of RNA into the cytoplasm.

Figure 15 shows a putative model of the HCV entry process.

In addition to receptor-mediated HCV endocytosis, cell-to-cell spread has been also reported. Co-cultivation of infected B-cells or hepatoma cells with naïve target cells leads to direct virus transmission by cellular contact (Timpe et al., 2008; Valli et al., 2007; Valli et al., 2006).

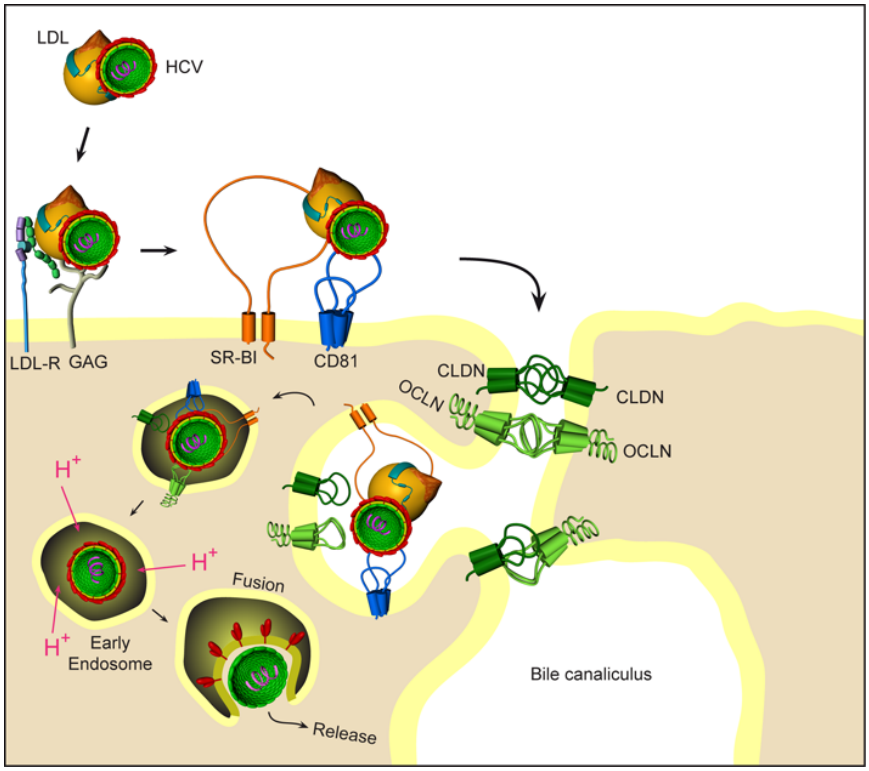

Figure 15. Putative model of the HCV entry process. The precise explanation is included in the text. Shortly, virus interacts with attachment factors LDL-R and GAG. Then specific interactions with scavenger receptor BI (SR-BI) and tetraspanin CD81 take place, enabling HCV internalization. Tight-junction proteins Claudin-1 (CLDN) and Occludin (OCLN) are also implicated in the internalization. In the endosomes, pH change mediates conformational changes in glycoproteins, which leads to fusion and release of viral genome into the cytoplasm. Adapted from (Tews and Dubuisson, 2009).

1. Biogenesis and functions of envelope glycoproteins

Envelope glycoproteins E1 and E2 play an essential role in the entry process. They interact not only with receptors on the cellular membrane but also mediate the fusion process. They are also suggested to participate in the virion assembly process (Wakita et al., 2005).

E1 and E2 are encoded in the structural part of the polyprotein. E1 consists of residues 192 to 384 and E2 residues 384 to 746. The cleavage of the polyprotein by a host signal peptidase gives rise to 31kDa E1 and 70kDa E2 proteins, which are glycosylated (Grakoui et al., 1993b). E1 and E2 form a non-covalent heterodimer that is essential for HCV infectivity (Bartosch et al., 2003b; Deleersnyder et al., 1997). Moreover, expression of E1 alone leads to E1 misfolding, highlighting that E2 is necessary for proper E1 folding (Michalak et al., 1997). The co-expression of E1 with E2 also helps in E2 folding (Cocquerel et al., 2003). E1 and E2 are type I membrane proteins as they contain a N-terminal ectodomain and a C-terminal transmembrane domain. During biogenesis of E1 and E2, the ectodomains are translocated into the ER lumen where post-translational modifications occur. ER stop-transfer signals are located in the C-termini of E1 and E2 (Cocquerel et al., 2002; Santolini et al., 1994). The transmembrane domains of the glycoproteins are responsible for the membrane anchoring, for ER retention and for the heterodimerisation process (Ciczora et al., 2007; Cocquerel et al., 2000; Op De Beeck et al., 2000). Additionally, a heptat repeat region at the C-terminus of E2 ectodomain has been also shown to participate in E1-E2 heterodimerisation (Drummer and Poumbourios, 2004).

Inside the ER lumen E1 and E2 undergo post-translational modifications. Importantly, they contain four to five and up to eleven potential glycosylation sites on E1 and E2, respectively. Most of these sites are N-glycosylated. The sites of glycosylation are often conserved among different HCV genotypes, which highlights their importance. Indeed they play a role in the glycoprotein folding, entry process and recognition by neutralizing antibodies (Falkowska et al., 2007; Goffard et al., 2005; Helle et al., 2007; Helle et al., 2010). The glycans associated with intracellular E1 and E2 glycoproteins were identified as oligomannosidase type (Duvet et al., 1998).

The glycoproteins produced in the context of HCVpp, thus in the absence of other viral proteins, assemble in a post-Golgi compartment (Sandrin et al., 2005). However, in the HCVcc system, E1 and E2 are supposed to assemble in an ER-derived compartment and this has been shown to lead to differences in protein-protein interactions as well as in glycan maturation as compared to HCVpp (Vieyres et al., 2010).

E1 and E2 mediate the interactions with cellular receptors (Op De Beeck et al., 2004). The role of E1 in this process is not specified. However, several regions in E2 have been described. Most importantly the E2 glycoprotein interacts with CD81 tetraspanin (Cocquerel et al., 2003; Pileri et al., 1998) and residues taking part in this interaction have been identified (Figure 16) (Drummer et

al., 2006; Owsianka et al., 2006; Rothwangl et al., 2008). Some reports suggest that E2 is also involved in SR-BI (scavenger receptor BI) interaction and that HVR1 may play a role in this process (Callens et al., 2005; Scarselli et al., 2002).

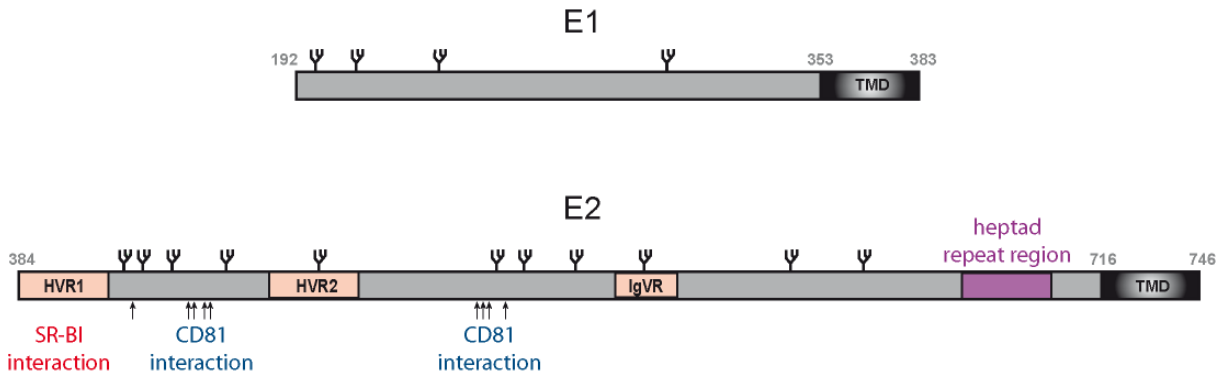

Figure 16. Schematic representation of E1 and E2 glycoproteins. Both glycoproteins contain transmembrane domain (TMD) at C-terminus of the protein. Glycosylation sites are marked by branched glycan symbols. E2 contains few distinguished regions. HVR1 (hypervaiable region 1) that was suggested to take part in SRBI interaction. Residues identified to interact with CD81 are indicated with arrows. Other regions indicated are HVR2 (hypervariable region 2), IgVR (intergenotypic variable region) and heptad repeat region.

In addition to their role in receptor binding, HCV glycoproteins are also responsible for the fusion between the viral envelope and a cellular membrane after the internalization process (Haid et al., 2009; Lavillette et al., 2006; Lavillette et al., 2007; Russell et al., 2009). Some previously published reports suggested that E1 contains a putative fusion peptide and plays a major role in the fusion process (Drummer et al., 2007; Russell et al., 2009). Others suggest that the determinants important for the fusion are located in both E1 and E2 (Lavillette et al., 2007). However, more recently, fusion peptide has been proposed to be located in E2 (Krey et al., 2010).

The exact structure of E1 and E2 has not yet been determined and no crystallography studies have been published. In consequence, the only data available come from similarity studies with other viral glycoproteins. However, a structural model for E2 has been proposed, based on the identification of disulfide bonds, which is supported by similarities among the viruses belonging to *Flaviviridae* family (Krey et al., 2010). This classifies E2 as a class II fusion protein. These observations led to a model of the E2 ectodomain, consisting of three separate domains. Domain I (DI) consists of eight β-strands and is extended on the N-terminus by HVR1. This domain contains determinants for CD81 interaction. DI is separated by Domain II (DII) that includes HVR2 and its most conserved part is suggested to act as a fusion loop (a.a. 502-520). DI is connected to domain III (DIII) by a linker region called inter-genotypic variable region (IgVR). Finally, DIII is connected to the TM domain by the flexible stem (ST) region (Krey et al., 2010). This model characterizes E2 as a complex structure in which intramolecular interactions as well as the

association with the E1 glycoprotein are required for receptor interactions and membrane fusion. The model of E2 is presented in Figure 17.

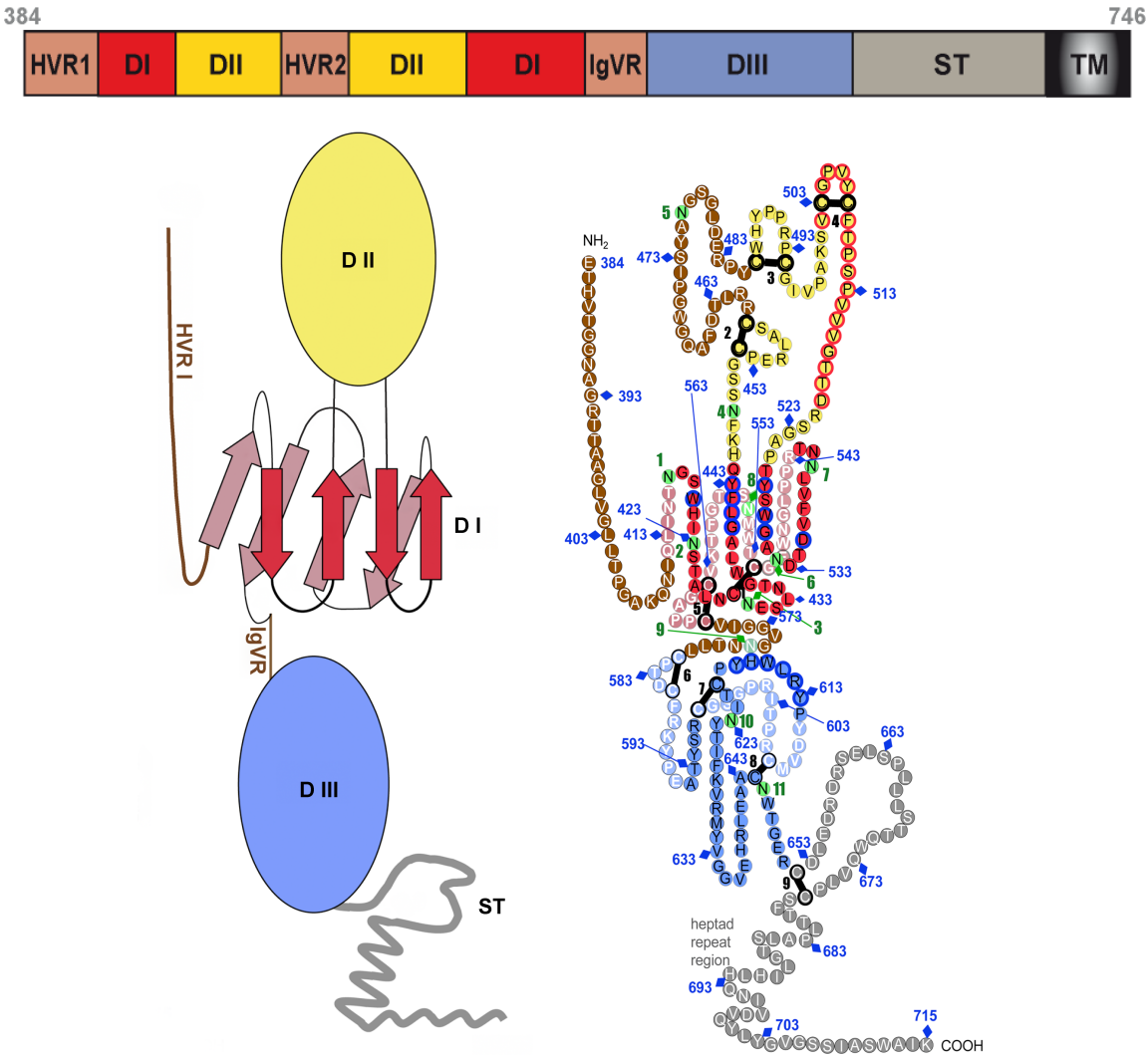

Figure 17. The E2 ectodomain model proposed by Krey et al., (Krey et al., 2010). Top panel shows domain organization on E2 glycoprotein. Left panel demonstrates the domain organization. Right panel describes more precisely the residues forming the domains and their role in the E2 protein function. Variable regions (HVR1, HVR2 and IgVR) are marked in brown, DI in red, DII in yellow, DIII in blue and Stem in grey. Aminoacids marked in green represent glycosylation sites. Aminoacids in blue circles were shown to play a role in CD81 interaction. Residues forming putative fusion peptide are marked in red circles. Disulfide bonds are shown in black. The numbering is based on reference H77 strain. Adapted from (Krey et al., 2010).

E2 contains several regions whose role in its assembly and function has been described. HVR1 on the N-terminus of E2 (a.a. 384-410) has its major role in the protection of the CD81 binding site from neutralizing antibodies. As indicated by its name, HVR1 is highly variable, and low conservation of this region is observed not only at the genotype level, but also in quasispecies produced in the chronic patients (Korenaga et al., 2001; Polyak et al., 1998). The inconstancy of this region provides not only the escape from humoral immunity but has also been associated with resistance to interferon treatment (Abbate et al., 2004; Grahovac et al., 2000; Pawlotsky et al.,

1999). Although substitutions in HVR1 are very frequent, their basic character remains stable between differing HCV genotypes, which makes the conformation of HVR1 rather conserved (Penin et al., 2001). This suggests that HVR1 may play a direct role in the entry process. As already mentioned, it may contribute to the attachment to glycosaminoglycans (GAG) (Penin et al., 2001) or interact with SR-BI receptor (Callens et al., 2005; Scarselli et al., 2002). Deletion of HVR1 attenuates infectivity of the virus (Forns et al., 2000); moreover it increases the sensibility to soluble CD81 neutralization and decreases sensibility to anti-SRBI antibodies (Bankwitz et al., 2010).

HVR2 (a.a. 471-482) is located in DII and its precise role is not known. It has been suggested that an interplay between HVR1 and HVR2 modulates CD81 binding (Roccasecca et al., 2003). Finally, a third variable region has been identified as intergenotypic variable region (IgVR) (McCaffrey et al., 2007). It may play a role during conformational changes preceeding the fusion process (Krey et al., 2010).

2. Attachment factors

The first step of HCV entry process is mediated by non-specific interactions between HCV glycoproteins and/or lipoproteins associated to the viral particle and the factors present on the cell surface. Two attachment factors have been identified for HCV: glycosaminoglycans (GAG) and low-density lipoproteins receptor (LDLR).

a. Glycosaminoglycans

Glycosaminoglycans represent the first binding site for many viruses, including those from the *Flaviviridae* family (Chen et al., 1997; Hulst et al., 2001; Iqbal et al., 2000; Kroschewski et al., 2003). Several types of GAG are described, however only heparan sulfate proteoglycans (HSPG) have been demonstrated to play a role during HCV attachment, mainly by showing that both HSPG and heparinase, an enzyme that degrades heparan sulfates, inhibit virus binding to cells (Barth et al., 2003; Barth et al., 2006; Basu et al., 2007; Germi et al., 2002; Koutsoudakis et al., 2006).

HVR1 in E2 glycoprotein has been suggested to mediate HSPG interaction (Barth et al., 2003; Penin et al., 2001). Interestingly, it has recently been shown that both intracellular and extracellular E2 can interact with heparin (HSPG homolog) (Vieyres et al., 2010), however the question whether heterodimers present on HCVpp or HCVcc interact directly with HSPG remains unanswered.

Interestingly, ApoE is a known ligand for GAG present on the cell surface, indeed uptake of lipoproteins by hepatocytes is mediated by the ApoE-lipoprotein lipase and GAG interplay (Arai et al., 1999; Cardin et al., 1989; McConathy and Wang, 1989; Saxena et al., 1993)(reviewed in (Mahley and Ji, 1999). With the present knowledge that ApoE is a part of HCV LVP (Chang et al.,

2007; Hishiki et al., 2010), one cannot exclude that in fact it is ApoE that mediates HCV attachment to GAG. Indeed, it has been shown that lipoprotein lipase enhances HCV binding to GAG (Andreo et al., 2007). Further studies using HCVcc system are required to solve this issue.

b. LDLR

The LDLR is localized on the hepatocyte surface and its major role is the clearance of cholesterol-transporting lipoproteins from the circulation. It binds ApoE and ApoB containing lipoproteins, which results is clathrin-dependent receptor-mediated endocytosis (Goldstein et al., 1985; Krul et al., 1985). LDLR consists of an extracellular domain, a transmembrane domain and a cytoplasmic tail. The extracellular domain is made up of ligand binding and EGF-precursor homology (epidermal growth factor-precursor) domains. Crystallography and NMR studies revealed the precise structure of LDLR (reviewed in (Rudenko and Deisenhofer, 2003)). The ligand-binding domain consists of seven cysteine-rich repeats responsible for the association to different ligands, whereas EGF-like domain contains three EGF-like repeats and a β propeller and plays a role in the ligand release in acidic endosomal pH. LDLR requires Ca^{2+} during ligand binding (reviewed in (Rudenko and Deisenhofer, 2003)). LDLR model is shown in Figure 18.

In the hepatocytes, the endocytosis of LDLR has been associated to the function of adaptor protein ARH (autosomal recessive hypercholesterolemia) that may promote LDLR clustering into clathrin-coated pits (Garuti et al., 2005; Mishra et al., 2002; Sirinian et al., 2005). The interactions occur via an internalization motif on the cytosolic tail of LDLR (Mishra et al., 2002). ARH participates in LDL but not IDL internalization (Jones et al., 2007a; Michaely et al., 2007). The mechanism of LDLR mediated internalization of lipoproteins is described in paragraph IV.3.

The first report associating LDLR to HCV entry has been published in 1999. The authors showed that anti-LDLR and anti-ApoE and ApoB antibodies block HCV internalization. Furthermore, chemical inhibition of LDLR activity also inhibited HCV uptake (Agnello et al., 1999). Other studies demonstrated that LDLR might participate in HCV binding to cells (Germi et al., 2002; Martin et al., 2008; Molina et al., 2007; Owen et al., 2009). However, no evidence exists to support the hypothesis that LDLR interacts with viral components of LVP. Most probably LVP binding to LDLR is mediated by ApoE or ApoB. Indeed, several reports show ApoE is important for HCV infectivity (Chang et al., 2007; Hishiki et al., 2010) and that anti-ApoE antibodies or intermediate-density lipoproteins (IDL or β-VLDL) inhibit infection (Owen et al., 2009). However, knowing that ApoE is also a ligand for GAG that are also engaged in HCV attachment, these results do not constitute the definite proof of a role for LDLR in HCV entry.

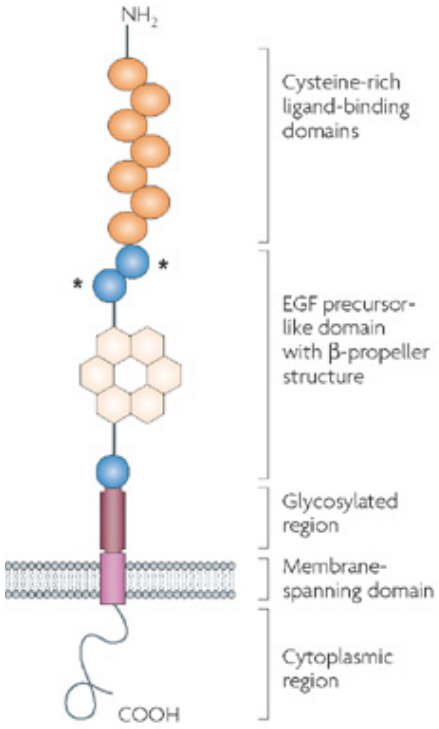

Figure 18. Schematic representation of LDLR. Stars are showning caldium-binding sites. Adapted from (Wasan et al., 2008)

The role of LDLR in HCV entry has also been supported by neutralizations with anti-LDLR antibodies (Agnello et al., 1999; Molina et al., 2007), siRNA down-regulation of LDLR (Owen et al., 2009) and chemical modification of LDLR expression (Molina et al., 2007; Owen et al., 2009). Although HCV particle binds to the LDLR, the interaction does not necessarily lead to a productive infection (Andreo et al., 2007). In their work, the authors showed that lipoprotein lipase (LPL) promotes HCV binding to cells, however it does not lead to virus replication. LPL may change LVP affinity to LDLR leading to increased internalization via LDLR and degradation (Andreo et al., 2007). LPL is an enzyme that modifies triglyceride-rich lipoproteins and targets them to the liver (Hide et al., 1992). LPL hydrolyses triglycerides in beta-lipoproteins and mediates interactions with heparan sulfates enabling lipoprotein clearance from the circulation (Mead et al., 2002). Furthermore, LPL has been shown to change LVP density, to reduce the level of HCV associated to ApoE and to inhibit infectivity (Shimizu et al., 2010).

The difficulty in the assessment of the role of LDLR in HCV entry may lie in the differences between models used in different studies. The lipoprotein component of LVP, which is a key factor in LDLR interaction, may depend on the way the virus is produced (e.g. *in vitro* versus *in vivo*).

3. Entry factors

As mentioned above, HCV entry is a very complicated process with many factors engaged. The specific receptors involved in HCV entry will be discussed here. At present, four cellular proteins

have been identified: scavenger receptor BI (SRBI), tetraspanin CD81, claudin-1 (CLDN1) and occludin (OCLN). The role of these factors in HCV entry is now clearly demonstrated, however we still lack knowledge about the interplay between these receptors and the virus during the entry process.

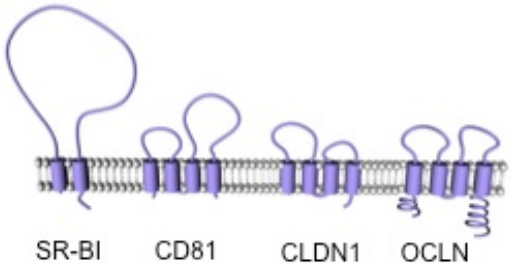

Figure 19. HCV entry factors.

a. CD81

CD81 belongs to superfamily of transmembrane proteins named tetraspanins. It consists of four transmembrane domains, short cytoplasmic domains and two extracellular domains: large extracellular loop (LEL) and small extracellular loop (SEL) (Seigneuret, 2006). Tetraspanins are multifunctional proteins implicated in many cellular processes. CD81 plays an important role in immune system regulations. It regulates membrane organization, acts as co-stimulatory molecule on T-cells, participates in B-cells activation and has many other functions as well (reviewed in (Levy and Shoham, 2005)).

CD81 was a first receptor identified in HCV entry. The authors found that soluble E2 bound to CD81-LEL, moreover antibodies neutralizing HCV *in vivo* block E2 interaction with CD81 (Pileri et al., 1998). At present, the role of CD81 in HCV entry is established, however the mechanism of action is still not known. We know that anti-CD81 antibodies and a soluble form of CD81-LEL inhibit HCVpp and HCVcc infection (Bartosch et al., 2003b; Hsu et al., 2003; Wakita et al., 2005; Zhang et al., 2004). Down-regulation of CD81 expression by siRNA significantly reduces infection (Bartosch et al., 2003c; Zhang et al., 2004) and the infectivity level has been correlated to CD81 expression (Akazawa et al., 2007; Koutsoudakis et al., 2007). Finally, cell lines that do not express CD81 are not permissive for HCV infection, however ectopic expression of CD81 promotes infection in cell lines like HepG2 (Bartosch et al., 2003c; Hsu et al., 2003; Lavillette et al., 2005b; Lindenbach et al., 2005; Zhang et al., 2004). Interestingly, the role of CD81 in HCV entry has also been confirmed in humanized mice (Meuleman et al., 2008).

CD81 may contribute to HCV species specificity as only human form of receptor efficiently promotes the entry process (Flint et al., 2006). However, virus can be adapted to murine CD81 by mutations in E2 glycoprotein (Bitzegeio et al., 2010).

The residues responsible for CD81-LEL binding have been mapped to amino acids 420, 437, 438, 441, 442, 527, 529, 530 and 535 in E2 Domain I (Drummer et al., 2006; Owsianka et al., 2006). Furthermore, residues in Domain III can modify CD81 interaction (Iacob et al., 2008; Rothwangl et al., 2008).

Tetraspanins interact with each other and with other membrane proteins forming so called tetraspanin-enriched microdomains (TEM). However, CD81 association with TEM does not seem to be essential during the HCV entry process (Rocha-Perugini et al., 2009). Recent studies suggest that CD81-CLDN1 complexes are necessary for the entry (Harris et al., 2010; Krieger et al., 2010).

CD81 can interact with many other proteins called partner proteins. At present only one partner has been shown to modify HCV entry. EWI-2wint (EWI-2 without its N-terminus) is a cleavage product of EWI-2 and its presence in different cell lines can potentially block infection. Cells permissive for HCV infection do not process EWI-2; thus EWI-2wint is not present in these cells (Rocha-Perugini et al., 2008). These findings are the first evidence that not only the presence of positive entry factors is essential for HCV entry, but also the absence of negative factors may play an important role.

CD81 might not participate in cell-to-cell spread, as anti-CD81 antibodies do not have an affect on virus transmission after infection *in vitro* and *in vivo* (Meuleman et al., 2008; Timpe et al., 2008; Witteveldt et al., 2009). However a recent raport suggests that CD81 is essential for cell-to-cell viral transmission (Brimacombe et al., 2010).

b. SRBI

SRBI is a class B scavenger receptor that mediates endocytosis of lipoproteins and plays an important role in lipid metabolism. It is highly glycosylated and consists of two short cytoplasmic tails, two transmembrane domains and one large extracellular loop (Acton et al., 1994; Calvo and Vega, 1993). SRBI is a receptor that binds HDL, which leads to selective cholesterol uptake (described in paragraph IV.5). It is mainly expressed in the liver and in nonplacental steroidogenic tissues (Acton et al., 1996).

The first evidence for the role of SRBI in HCV entry came from studies with soluble E2 glycoprotein. E2 was shown to selectively bind to human, but not murine SRBI and these interactions depend on the presence of HVR1 in E2 (Scarselli et al., 2002). Further studies revealed, that the natural SRBI ligand- HDL modifies HCV entry. Addition of HDL significantly enhanced HCVpp entry. This process seems to depend on the lipid transfer activity of SRBI (Bartosch et al., 2005; Voisset et al., 2005). Furthermore HDL protects HCVpp and HCVcc from neutralization with monoclonal and patient-derived polyclonal antibodies (Dreux et al., 2006; Voisset et al., 2006). One report showed that both HCVcc and plasma-derived virus interaction with SRBI could be

neutralized by anti-E2 antibodies (Grove et al., 2007). However, other study using patient delivered virus showed that the interaction between the virus and SRBI is not blocked by either anti-E2 or anti-HVR1 antibodies but by anti-β-lipoprotein antibodies. This suggests that LVP interact with SRBI via apolipoproteins and not via E2 (Maillard et al., 2006). Probably both viral and lipoprotein components take part in SRBI interaction.

Kinetics studies with anti-receptor antibodies showed that SRBI acted after virus binding to cells early in the entry process. This step was closely linked to CD81 action (Zeisel et al., 2007).

Finally a dual activity of SRBI in HCV entry has been established. The virus uses SRBI as a direct binding receptor but it also uses its lipid transfer activities in favor of infection (Dreux et al., 2009). This hypothesis may be supported by the finding that different regions of SRBI are implicated in HDL and HCV binding (Catanese et al., 2010).

Interestingly, SRBI is probably also involved in cell-to-cell transfer as anti-SRBI antibodies inhibit the infection both *in vitro* and *in vivo* when added after the inoculation. Therefore targeting SRBI could be potentially used to prevent graft re-infection after liver transplantation {Meuleman et al., 17th International Meeting on Hepatitis C Virus and Related Viruses, Yokohama, Japan, September 9-14, 2010).

c. Claudin-1

CLDN1 belongs to a family of 24 proteins that are responsible for tight junction formation. These proteins contain two extracellular loops, three intracellular domains and four transmembrane domains. Claudins contain characteristic and a highly conserved W-GLWC-C motif in the large extracellular loop (Van Itallie and Anderson, 2006).

CLDN1 is highly expressed in the liver and has been reported to contribute to the HCV tropism to hepatic cells. Ectopic expression of CLDN1 in non-hepatic cell lines enables infection (Evans et al., 2007). Residues in the first extracellular loop of CLDN1 are important for the HCV infectivity and the virus can be neutralized with antibodies against an epitope inserted in the extracellular loop of CLDN1. Finally, kinetics studies suggest that CLDN1 acts in the later stages of HCV entry, after attachment and CD81 binding (Evans et al., 2007).

Protein-protein interaction studies with FRET (fluorescence resonance energy transfer) technique showed that CLDN1 forms complexes with CD81 on permissive cells (Harris et al., 2008). CD81-CLDN1 complexes are present on the basolateral surface of polarized hepatocytes. Recently these complexes were shown to be important for HCV entry, while tight junction-associated CLDN1 does not seem to play a role in HCV infection (Harris et al., 2010). Moreover, anti-CLDN1 antibodies block CD81-CLDN1 interaction, which in turn prevents E2 binding (Krieger et al., 2010). Anti-

CLDN1 antibodies were also shown to inhibit the infection of primary human hepatocytes (Fofana et al., 2010).

CLDN1 may play a role in cell-to-cell transmission (Timpe et al., 2008). HCV infection significantly increases the CLDN1 expression level, which may be important for the viral spread in the liver (Reynolds et al., 2008).

Some reports suggest that CLDN6 and CLDN9 also participate in HCV entry (Meertens et al., 2008).

d. Occludin

OCLN is an integral tight junction protein that contains four transmembrane domains, two extracellular loops and three cytoplasmic regions including a long C-terminal tail (Furuse et al., 1993). Recently, OCLN has been shown to participate in HCV entry (Ploss et al., 2009). The authors showed that in addition to CD81, SRBI and CLDN1, OCLN is also necessary to infect mouse cells. Interestingly, human SRBI and CLDN1 can be replaced by the murine versions of these proteins; however only human CD81 and OCLN allow HCV entry. Overexpression of OCLN in non-permissive cells enhances HCVpp entry. In the contrary, the down-regulation of OCLN in permissive cells inhibits the entry process (Ploss et al., 2009). OCLN is suggested to play a role in a late stage of the entry process (Benedicto et al., 2009).

OCLN exists in different splicing forms and only variants containing the MARVEL motif enable HCV infection. This finding may lead to the conclusion that natural OCLN variants might contribute to HCV tissue tropism (Kohaar et al., 2010).

4. Clathrin-dependent endocytosis

Endocytosis is a process that regulates membrane composition and provides interactions between cell and environment. Several endocytic pathways are described now like caveolar-type endocytosis, Arf-6 dependent endocytosis, phagocytosis, macropinocytosis and many others. The best-described pathway is the clathrin-dependent endocytosis. It is based on formation of clathrin-coated vesicles and it dependents on many proteins like Rab5, Arf-6, clathrin and AP-2 (adaptor protein 2) (reviewed in (Doherty and McMahon, 2009).

HCV internalization is mediated by clathrin-dependent endocythosis. siRNA down-regulation of the clathrin heavy chain or chemical blockage of clathrin-coated pits formation by chloropromazine inhibits both HCVpp and HCVcc entry. Moreover, bafilomycin A and chloroquine that interfere with endosome acidification block HCV infection (Blanchard et al., 2006; Meertens et al., 2006). Clathrin-mediated endocytosis of HCV has also been confirmed in primary human hepatocytes (Codran et al., 2006). Finally, siRNA library screening demonstrated that

clathrin-dependent endocytosis associated proteins like clathrin, epsin and AP-2 are implicated in HCV entry (Coller et al., 2009).

5. Fusion process

After receptor-mediated clathrin-dependent endocytosis the virus needs to fuse with an endosomal membrane in order to release its RNA into the cytoplasm.

Studies in the HCVpp and HCVcc systems indicate that under pH change HCV glycoproteins undergo conformational changes into fusion active forms. Indeed, blocking endosomes acidification by bafilomycin A1 inhibits the fusion process (Bartosch et al., 2003c; Blanchard et al., 2006; Hsu et al., 2003; Koutsoudakis et al., 2006; Meertens et al., 2006). Conditions required for proper fusion process were characterized in an *in vitro* fusion assay using HCVpp and liposomes. Optimal pH was set as 5.5, moreover fusion was shown to be temperature dependent and not to require additional factors present on the liposomes surface. In addition, the presence of cholesterol in liposomes membrane facilitates the fusion process (Lavillette et al., 2006). Further studies with HCVcc also showed that the density of the particles is a modulating factor in a way that lower density virus has better fusogenic properties (Haid et al., 2009).

Interestingly extracellular HCV is not inactivated by low pH, like other pH dependent viruses. This suggests that an additional step is required during the entry process to render HCV glycoproteins sensitivity to low pH (Tscherne et al., 2006). Recently, it has been shown that E1 and E2 present on HCVcc form large covalent complexes bound together by disulfide bonds (Vieyres et al., 2010). This could explain virions resistance to low pH but again suggests that an additional step disrupting these covalent interactions is needed during the entry process.

The exact mechanism of E1E2 rearrangements during the fusion process is not known. However, as mentioned above, E2 belongs to class II fusion protein. In consequence, like other class II fusion proteins, under low pH conditions E1-E2 heterodimers are probably forming trimers that trigger membrane rearrangements (Krey et al., 2010). The model of flavivirus E glycoprotein fusion is shown in Figure 20.

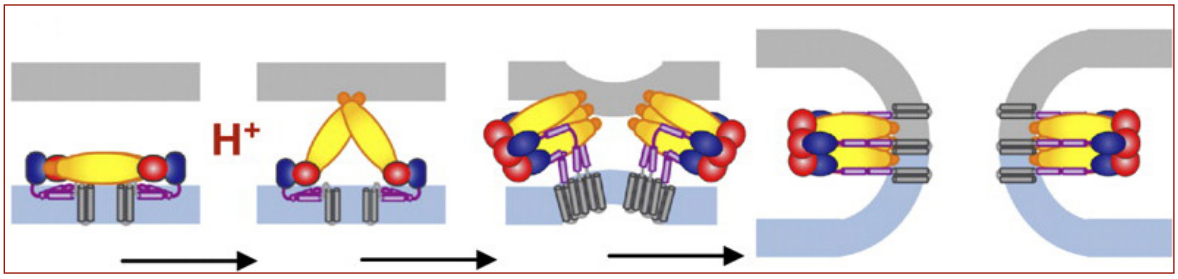

Figure 20. Schematic representation of flavivirus E glycoprotein fusion process. The color code of E domains is the same as for HCV E2 glycoprotein. As follows: DI in red, DII with fusion peptide in yellow, DIII in blue, stem in purple and TM in grey. From the left side: step 1 - E2 dimers in mature virions; step 2 – dimers dissociation at acidic pH, fusion peptide interaction with target membrane; step 3 – trimerisation, DIII and stem re-localization; step 4 – formation of post-fusion trimer and opening of the pore. Adapted from (Fritz et al., 2008).

IV. Lipoproteins and LDLR

The liver is an important organ implicated in uptake, storage, metabolism and secretion of different types of lipids. HCV, which targets hepatocytes, exploits this system in favor of its life cycle. This paragraph aims to explain the liver lipoprotein metabolism in order to better understand its interactions with HCV.

1. VLDL assembly pathway

VLDL (very low-density lipoproteins) are assembled in hepatocytes in two major steps. Firstly, lipids are transferred to ApoB by MTP. Later, the ApoB-containing precursor particle fuses with triglyceride droplets. ApoB is a highly lipophilic glycoprotein that plays a crucial role in triglyceride transport in vertebrates. Every VLDL particle contains a single molecule of ApoB that surrounds its surface like a belt. ApoB interacts with the neutral lipid core of VLDLs via amphipathic beta-like structures (Schumaker et al., 1994). Proper folding and stability of ApoB depends on polar and neutral lipids added cotranslationally to nascent ApoB. This process involves MTP–dependent transfer of lipids from the ER or other donor sites (Gordon and Jamil, 2000). The first step of VLDL assembly results in formation of small particles, approximately 25nm in diameter. Further increase in size is mediated by the fusion with protein-free triglyceride lipid droplets. MTP is not required during this fusion step, however, the formation of triglyceride droplets in the ER is MTP-dependent (Gordon and Jamil, 2000; Gordon et al., 1996). Maturation of VLDL also requires phospholipases, as for example phospholipase D that is implicated in metabolic channeling of phospholipids into VLDL triglyceride production (reviewed in (Shelness and Sellers, 2001)).

The secretion of VLDL also depends on the presence of CIDE-B (cell death-inducing DFF45-like effector B). CIDE-B interacts with lipid droplets and ApoB and promotes ApoB lipidation (Ye et al., 2009).

Synthesis of triglycerides for VLDL production involves acyl transfer to diacylglycerol by diacylglycerol acyltransferase (DGAT), an enzyme that belongs to the acyl coenzyme A family (Cases et al., 1998). Cholesterol esters for VLDL production are provided by two cholesterol esterifying enzymes, ACAT1 and ACAT2 (acyl coenzyme-A: cholesterol acyltransferase) (Shelness and Sellers, 2001)).

Lipid availability in hepatocytes dictates VLDL biosynthesis, as unlipidated or underlipidated ApoB is misfolded and degraded by the ubiquitin/proteasome pathway (Davidson and Shelness, 2000). Other processes can also regulate lipoprotein production. Interestingly, LDLR regulates VLDL production by a yet unknown mechanism. LDLR-knock-down hepatocytes secrete ApoB at

a 3.5-fold higher rate than wild-type hepatocytes (Twisk et al., 2000). In contrast, LDLR over-expression leads to degradation of 90% of newly synthesized ApoB. This is in agreement with observations in patients, as LDLR deficiency in familial hypercholesterolemia leads to overproduction of VLDL, whereas LDLR upregulation by statins reduces VLDL production (Twisk et al., 2000). It has also been shown that LDLR directs ApoB degradation in a post-ER compartment and that reuptake of ApoB through a constitutive endocytic pathway and of ApoE through a ligand-dependent pathway regulate this process (Blasiole et al., 2008).

An analogue assembly pathway of ApoB containing lipoproteins exists in the intestine. The lipoproteins secreted in this process are named chylomicrons and they contain an ApoB isoform, called ApoB48, whereas VLDL contain ApoB100 (Davidson and Shelness, 2000).

VLDL secreted from the liver contain other apolipoproteins, among them ApoE that functions as a ligand that mediates binding and uptake of lipoproteins by the LDL family receptors. ApoE also interacts with heparan sulfate proteoglycans (HSPG) facilitating lipoprotein clearance from the circulation. The human APOE gene is polymorphic with three possible alleles: E2, E3 and E4. Different forms of ApoE have different ligand-binding properties that manifests in differences in lipoproteins association with HSPG and LDLR (Mensenkamp et al., 2001).

VLDL particles secreted from the liver contain ApoB, ApoE and different ApoC particles. A core containing triglycerides and cholesterol esters is surrounded by a single phospholipid layer that contains free cholesterol molecules. A schematic model of a VLDL particle is shown in Figure 21. Modification of VLDL in the circulation changes its lipid profile and its protein content. This subject is discussed in the next paragraph.

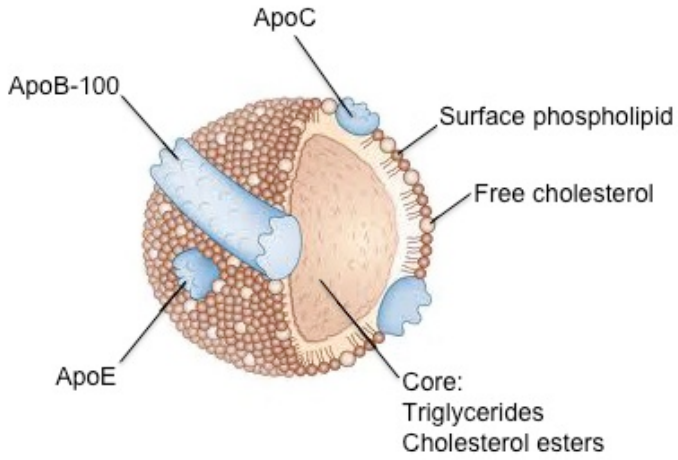

Figure 21. Schematic representation of VLDL particle. Adapted from (Mahley et al., 2008).

2. Lipoproteins in the circulation

Both VLDL and chylomicrons are rapidly cleared from the circulation by the liver with a half-time of 15 to 30 minutes. This clearance requires the enzymatic activity of lipoprotein lipase (LPL) and hepatic lipase (HL) (Chappell and Medh, 1998).

LPL plays a crucial role in VLDL metabolism. LPL simultaneously binds both lipoproteins and cell surface receptors/proteoglycans, which leads to the accumulation and uptake of lipoproteins. Its bridging function is not dependent on the catalytic activity of the enzyme (Merkel et al., 2002; Merkel et al., 1998). A dimeric form of LPL catalyses triglycerol hydrolysis in VLDL, enabling smaller remnants – IDL (intermediate density lipoproteins or β-VLDL) to enter the liver (Mead et al., 2002). IDL can be cleared from the circulation by uptake in the liver mediated by ApoE-LDLR interactions. Further hydrolysis of IDL decreases triglycerol content and ApoE moiety and leads to formation of more dense LDL particles, which are then internalized in the liver via ApoB-LDLR interactions. These two distinctive pathways of lipoproteins internalization will be discussed in the next paragraph.

The second enzyme that contributes to VLDL and VLDL remnants remodeling is HL. It is expressed in the liver and as LPL, it catalyzes triglycerol hydrolysis and acts as a bridge between lipoproteins and HSPG (reviewed in (Zambon et al., 2003)).

The mechanism of lipoproteins circulation is presented in Figure 22.

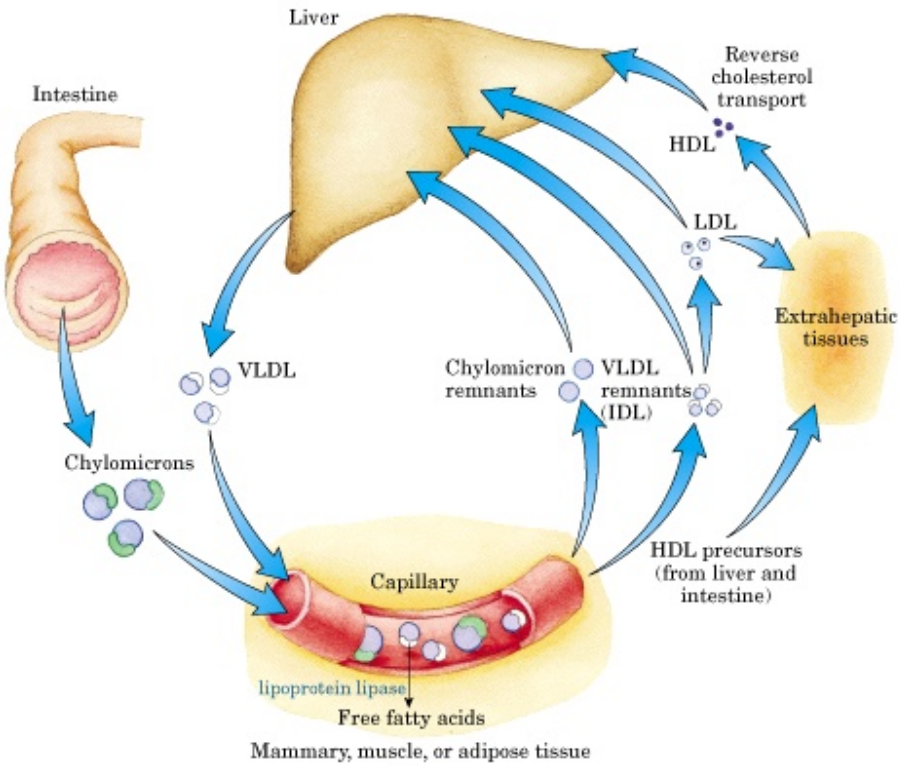

Figure 22. Circulation of lipoproteins. Chylomicrons and VLDL produced in intestine and liver, respectively, are modified by lipoprotein lipase in capillars. Chylomicron remnants, IDL and LDL are cleared from the circulation by liver uptake. HDL precursors and HDL take part in reverse cholesterol transport. Adapted from (Nelson and Cox, 2004)

The differences in lipid content and density of lipoproteins are shown in Table 2.

Table 2. The comparison of lipid content and density of lipoproteins. TG- triacyglycerol, C- free cholesterol, CE- cholesterol esters. Adapted from (Chappell and Medh, 1998).

	Chylomicron	VLDL	IDL	LDL
Lipids	TG > C + CE	TG > C + CE	C + CE > TG	C + CE > TG
Density [g/cm^3]	< 0.94	0.94 – 1.006	1.006 – 1.019	1.019 – 1.063

3. LDLR

As discussed above, LDLR is the major receptor responsible for lipoproteins uptake in the liver. The internalization of lipoproteins is mediated by the interplay between lipases, HSPG and LDLR. Some reports suggest that syndecan proteoglycan family can directly mediate ligand catabolism through a pathway independent of LDLR and coated-pits (Fuki et al., 1997; Williams and Fuki, 1997).

In spite of the existence of alternative uptake pathways, it is LDLR that plays a major role in lipoproteins clearance. The strongest evidence for this hypothesis is the homozygous form of familial hypercholesterolemia. In the absence of LDLR, LDL uptake is impaired and lipoproteins accumulate to very high levels in the serum, which induces a severe form of atherosclerosis (Goldstein and Brown, 1977).

The structure of LDLR has already been described before (Paragraph III.2.b). Here I would like to focus on the mechanism of lipoproteins uptake and the comparison of two existing endocytosis pathways.

Internalization of LDLR through clathrin-coated pits is dependent on ARH protein (Garuti et al., 2005; Mishra et al., 2002; Sirinian et al., 2005). ARH has an N-terminal phosphotyrosine-binding (PTB) domain that interacts with an FXNPXY–internalization motif in the LDLR cytosolic part. Moreover, ARH interacts directly with soluble clathrin trimers and with clathrin adaptors, which regulates clathrin-dependent LDLR internalization (Mishra et al., 2002).

LDLR can bind and internalize ApoB and ApoE containing lipoproteins. Internalization of VLDL remnants – IDL, requires ApoE but not ApoB. Further modification of IDL into LDL and loss of ApoE moiety changes LDLR-binding properties. In the case of LDL, it is ApoB that participates in LDLR binding (Krul et al., 1985). Recently it has been shown that inactivation of the FDNPVY motif by mutation prevented the uptake of LDL, but not IDL in human fibroblasts. This suggests that IDL are internalized via ARH-independent pathway (Michaely et al., 2007). Additionally, *in vivo* mouse studies showed that hepatocytes from ARH (-/-) mice (but not LDLR (-/-) mice)

internalized VLDL remnants, again suggesting that two distinct internalization pathways exist (Jones et al., 2007a).

Previously it has been shown that LDLR is a constantly internalized and recycled receptor, as it is present in coated pits without LDL and can be internalized in LDL absence (Anderson et al., 1982; Basu et al., 1981). However the IDL-internalization pathway seems to be a ligand-dependent because IDL can be still uptaken by the cells with LDLR mutant lacking the internalization FDNPVY motif (Michaely et al., 2007). In the ARH-dependent pathway, ARH mediates LDLR clustering into the coated pits by binding LDLR's FDNPVY motif and either clathrin or AP-2 (Garuti et al., 2005). In the ARH-independent pathway, LDLR clustering is probably mediated by LDLR dimerization induced by IDL binding. Additionally, IDL contain multiple copies of ApoE, thus can engage many copies of LDLR, spontaneously leading to LDLR concentration in coated pits (Michaely et al., 2007; Windler et al., 1980). The regulation of LDLR activity is probably mediated by the expression level of adaptors in different tissues and in different nutrient conditions.

LDLR-dependent internalization of lipoproteins through clathrin-dependent endocytosis is a very fast process. Lipoproteins are bound at the neutral pH of the extracellular environment. The acidification of endosomes releases lipoproteins from LDLR. After dissociation from their ligand, LDLR are recycled back to the cell surface in recycling endosomes. One round-trip of LDLR takes approximately 10 minutes (Brown et al., 1983).

4. LDLR role in lipid metabolism

Lipoproteins delivered by LDLR are an important source of lipids for the cells. HMG CoA reductase (3-hydroxy-3-methylglutaryl coenzyme A reductase) is an enzyme that catalyzes a rate-limiting step in cholesterol production. HMG CoA reductase activity is reduced in the presence of lipoproteins, whereas removing of cholesterol-containing lipoproteins from the medium strongly increases its activity. This is an evidence that cholesterol synthesis is a feedback-regulated process that depends on uptake of cholesterol via lipoproteins and LDLR (reviewed in (Goldstein and Brown, 2009)).

After the release of lipoproteins from the LDLR in endosomes, LDL protein components are rapidly digested into amino acids while cholesterol esters are hydrolyzed into free cholesterol. Degradation takes place in lysosomes and can be inhibited by chloroquine, which prevents lysosome acidification. Released free cholesterol inhibits a SREBPs (sterol regulatory element binding proteins) pathway that results in the suppression of transcription of the HMG CoA reductase and LDLR genes. It also regulates other cellular processes that control cellular cholesterol content. It activates ACAT enzymes that are responsible for cholesterol esterification so that excess

cholesterol can be stored in lipid droplets or can be used in VLDL assembly process (reviewed in (Goldstein and Brown, 2009)).

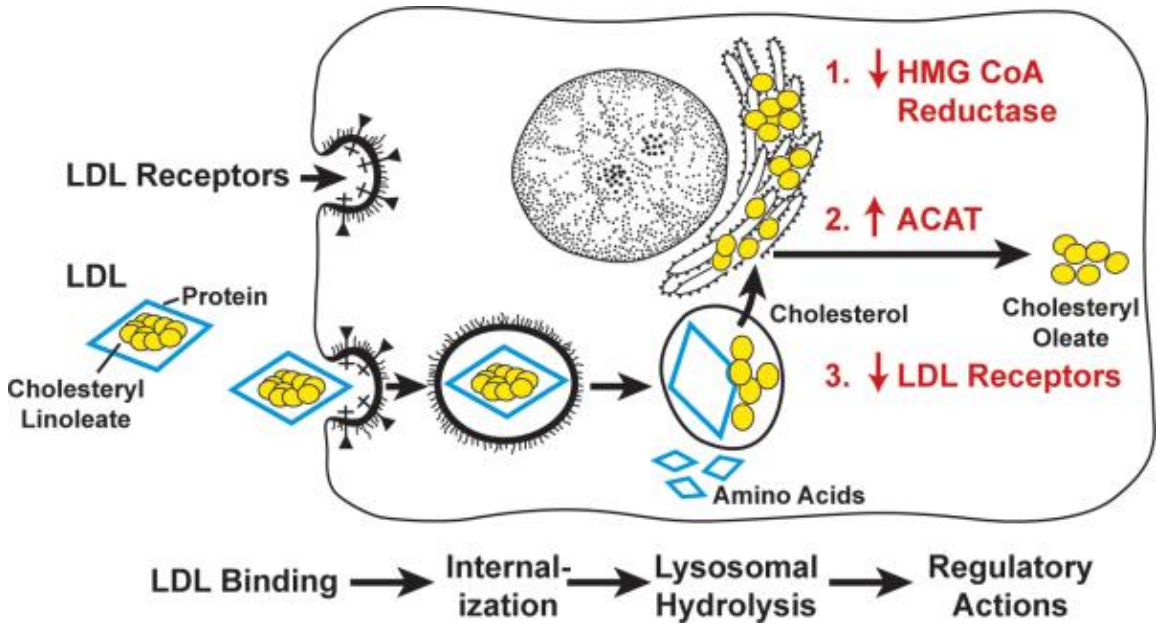

Figure 23. Sequential steps in LDLR pathway. LDL is internalized via LDLR. In lysosomes lipoproteins are digested into amino acids and free cholesterol. Cholesterol is directed into membranes of ER where it regulates cellular lipid metabolism. Precise description is placed in the text. Adapted from (Goldstein and Brown, 2009)

5. HDL, SRBI and reverse cholesterol transport

HDL (high density lipoproteins) are antiatherogenic as high HDL-cholesterol levels are associated with a lower risk of cardiovascular diseases. HDL takes part in the reverse cholesterol transport pathway. It removes excess cholesterol from the peripheral tissues and transports it back to the liver (von Eckardstein et al., 2001). HDL, by removing cholesterol and oxysterols from macrophages in the arterial walls have anti-inflammatory and immunosuppressive functions (Tall, 2008).

HDL are more dense that other lipoproteins. Indeed, their density ranges between 1.063 and 1.21 g/cm^3. They contain different ApoA isoforms, ApoC and ApoE. ApoAI is an important part of HDL as it is engaged in all stages of HDL metabolism: formation of nascent particles, remodeling and cholesterol delivery to the liver via SRBI (Rothblat and Phillips, 2010).

The HDL metabolism is a very complicated process that requires multiple enzymes and other factors. As the subject of this thesis is HCV, I will focus on the interplay between HDL and SRBI that has been shown to affect HCV entry (Bartosch et al., 2005; Dreux et al., 2006; Voisset et al., 2005).

The structure of SRBI has already been described in Paragraph III.3.b. SR-BI is responsible for the cellular selective uptake of cholesterol esters and other lipids from HDL (Rigotti et al., 2003). In

addition, SRBI mediates efflux of unesterified cholesterol from cells to lipoproteins or other acceptors (Yancey et al., 2003).

In normal conditions HDL is the major SRBI ligand. In the absence of LDLR, SRBI can also effectively deliver cholesterol to cells not only from HDL, but also from LDL (Stangl et al., 1998; Stangl et al., 1999). This shows that HDL are not the only SRBI ligands. The internalization of HDL through SRBI has been reported, however HDL was rapidly resecreted enabling cholesterol efflux (Pagler et al., 2006b). Only in specific lipoprotein-deficient conditions cells may internalize and degrade HDL and LDL in order to supply themselves in cholesterol (Pagler et al., 2006a). Recently, SRBI has been also shown to internalize IDL particles in the absence of LDLR (Rohrl et al., 2010).

As discussed above, the tissue-specific, posttranslational regulation of LDLR depends on ARH. A similar mechnism of regulation has been described for SRBI, which is regulated by the adaptor protein PDZK1 (postsynaptic density protein K1). PDZK1-deficient mice have elevated plasma cholesterol levels due to complete SRBI ablation (reviewed in (Yesilaltay et al., 2005)). Interestingly PDZK1 has been found to indirectly facilitate HCV entry through interactions with SRBI (Eyre et al., 2010).

Objectives

Entry is the initial step of the HCV life cycle. This process requires interactions between the viral particle and host entry factors.

The viral envelope glycoproteins, E1 and E2, are essential during attachment to host cells. They interact with specific receptors and play a crucial role in the fusion process. Moreover, these glycoproteins are also believed to participate in the virion assembly process. The function of E1E2 heterodimers is now established, however the role played by specific regions of these glycoproteins remains poorly understood. Crystal structures of E1 and E2 are not yet available thus all information about E1E2 structure is taken from comparative or bioinformatic studies. Research focused on identification of regions implied in inter or intramolecular interactions between glycoproteins and specific functions of different domains provides important information not only about E1E2 function, but it also helps understanding the structure of E1E2 heterodimers. Based on the hypothesis that HCV glycoproteins have co-evolved in different genotypes, we developed an approach using intergenotypic chimeras of HCV glycoproteins in order to identify less conserved but functionally important regions in E2 glycoprotein. The aim of the first part of my thesis was also to evaluate the function played by mapped regions using both HCVpp and HCVcc systems.

The second part of my thesis is focused on HCV host entry factors that allow virus attachment and internalization. LDLR has been proposed as a non-specific HCV entry factor that helps the virus bind to cells. Evidences confirming this hypothesis are however controversial. Indeed, HCV particles are associated with lipoproteins, therefore they contain ApoE molecule that is a ligand for LDLR. Nevertheless, the physiological function of LDLR – uptake and degradation of lipoproteins, does not seem to fit with HCV endocytosis. The aim of my project was therefore to evaluate the role of LDLR in HCV cell cycle and to understand the function played by ApoE. LDLR is an essential receptor that provides cells with lipids. HCV hijacks host lipid metabolism in favor of its infection. In this thesis we also address the question whether lipids provided by LDLR play a role in later stages of HCV cell cycle.

Results

I. Identification of new functional regions in Hepatitis C virus envelope glycoprotein E2

JOURNAL OF VIROLOGY, Feb. 2011, p. 1777–1792 Vol. 85, No. 4
0022-538X/11/$12.00 doi:10.1128/JVI.02170-10

Identification of New Functional Regions in Hepatitis C Virus Envelope Glycoprotein E2▽

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Received 15 October 2010/Accepted 29 November 2010

Little is known about the structure of the envelope glycoproteins of hepatitis C virus (HCV). To identify new regions essential for the function of these glycoproteins, we generated HCV pseudoparticles (HCVpp) containing HCV envelope glycoproteins, E1 and E2, from different genotypes in order to detect intergenotypic incompatibilities between these two proteins. Several genotype combinations were nonfunctional for HCV entry. Of interest, a combination of E1 from genotype 2a and E2 from genotype 1a was nonfunctional in the HCVpp system. We therefore used this nonfunctional complex and the recently described structural model of E2 to identify new functional regions in E2 by exchanging protein regions between these two genotypes. The functionality of these chimeric envelope proteins in the HCVpp system and/or the cell-cultured infectious virus (HCVcc) was analyzed. We showed that the intergenotypic variable region (IgVR), hypervariable region 2 (HVR2), and another segment in domain II play a role in E1E2 assembly. We also demonstrated intradomain interactions within domain I. Importantly, we also identified a segment (amino acids [aa] 705 to 715 [segment 705-715]) in the stem region of E2, which is essential for HCVcc entry. Circular dichroism and nuclear magnetic resonance structural analyses of the synthetic peptide E2-SC containing this segment revealed the presence of a central amphipathic helix, which likely folds upon membrane binding. Due to its location in the stem region, segment 705-715 is likely involved in the reorganization of the glycoprotein complexes taking place during the fusion process. In conclusion, our study highlights new functional and structural regions in HCV envelope glycoprotein E2.

Hepatitis C virus (HCV) infects approximately 3% of the world population (72) and is currently the major cause of chronic hepatitis, cirrhosis, and hepatocellular carcinoma (43). A vaccine is not yet available, and the treatment fails in around 50% of the cases, depending on the virus genotype (43). Although the cloning of the HCV genome more than 20 years ago (4) allowed for a rapid analysis of the genomic organization and a biochemical characterization of its proteins (reviewed in reference 57), the lack of a cell culture system to efficiently amplify this virus has long been a major obstacle for the study of the HCV life cycle. Fortunately, in 2005, the development of a cell culture system that allowed for a relatively efficient amplification of HCV (HCVcc) was finally reported (42, 71, 78).

HCV is an enveloped, positive-stranded RNA virus that belongs to the *Flaviviridae* family (41). Its genome encodes a single polyprotein of about 3,000 amino acids, which is cleaved co- and posttranslationally by cellular and viral proteases to yield at least 10 mature products (reviewed in reference 57). Cleavage of the viral polyprotein by a cellular signal peptidase gives rise to the envelope glycoproteins E1 and E2 (reviewed in reference 17). HCV envelope glycoproteins are type I transmembrane (TM) proteins containing a highly glycosylated N-terminal ectodomain (28) and a C-terminal TM domain (8). During their synthesis, E1 and E2 ectodomains are translocated inside the lumen of the endoplasmic reticulum (ER), and their TM domains are inserted in the membrane of this compartment (8). During their biogenesis, E1 and E2 assemble as noncovalent heterodimers, which are retained in the ER (11). Interestingly, the TM domains of HCV envelope glycoproteins have been shown to contain determinants of E1E2 interactions (53).

The development of retroviral pseudotypes containing HCV glycoproteins (HCVpp) has been the first tool available to study the role of HCV envelope proteins in virus entry (1, 14, 29). HCV glycoprotein heterodimers are involved in interaction(s) with a cellular receptor(s) (54) and mediate fusion with cellular membranes (27, 39, 40, 63). The tetraspanin CD81, the scavenger receptor BI (SR-BI), and the tight junction proteins claudin 1 and occludin have all been identified as essential for entry (reviewed in reference 61), but direct binding of the E1E2 heterodimer has been confirmed only for CD81 (7, 60).

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▽ Published ahead of print on 8 December 2010.

The secondary and tertiary structures of glycoproteins are supposed to be similar among the members of the *Flaviviridae* family, suggesting that HCV envelope glycoproteins should belong to class II fusion proteins (reviewed in reference 32). In this model, the fusion protein is located downstream on the polyprotein encoded by the virus, and the companion protein located immediately upstream is a chaperone involved in the folding of the fusion protein. These observations as well as the identification of E2 disulfide bonds led to a model of the E2 ectodomain, consisting of three separate domains (34). Domain I (DI) consists of eight β strands and is extended on the N terminus by hypervariable region 1 (HVR1). This domain contains determinants for CD81 interaction. Domain II (DII) includes hypervariable region 2 (HVR2), and its most conserved part is suggested to act as a fusion loop (amino acids [aa] 502 to 520). DI is connected to domain III (DIII) by a linker region called the intergenotypic variable region (IgVR). Finally, DIII is connected to the TM domain by the flexible stem (ST) region. This model characterizes E2 as a complex structure in which intramolecular interactions as well as the association with E1 glycoprotein are required for receptor interactions and membrane fusion.

HCV can be grouped into seven genotypes (24), but the overall structure and functions of the E1E2 heterodimer do not differ significantly between HCV genotypes. However, due to their cooperative interaction (38), HCV envelope glycoproteins have likely coevolved in the different genotypes, and this coevolution may lead to functional intergenotypic incompatibilities between E1 and E2. We therefore generated HCVpp containing HCV envelope glycoproteins from different genotypes to test the intergenotypic incompatibilities between these two proteins. We showed that several combinations of E1 and E2 from different genotypes were nonfunctional for HCV entry. We then used the HCVpp and HCVcc systems to map functional regions in HCV glycoprotein E2. This led to the identification of several regions of the E2 ectodomain that play a role in E1E2 assembly as well as a ST segment, which is involved in HCVcc entry. To better understand the role of the latter segment, an additional structural characterization of the C-terminal part of the ST was performed. Circular dichroism (CD) and nuclear magnetic resonance (NMR) analyses of a synthetic peptide denoted E2-SC revealed the presence of an amphipathic helix exhibiting lipid-binding properties. This helix is expected to fold upon membrane binding, but its limited stability suggests that it could easily switch from helical to random conformation, depending on its microenvironment and/or binding partners. Together, these data reveal new structure-function relationships for HCV envelope glycoprotein E2.

MATERIALS AND METHODS

Cell culture. HEK293T human embryo kidney cells and Huh-7 human hepatoma cells (52) were grown in Dulbecco's modified essential medium (Invitrogen) supplemented with 10% fetal bovine serum.

Antibodies. Monoclonal antibodies (MAbs) A4 (anti-E1) (16), 3/11 (anti-E2; kindly provided by J. A. McKeating, University of Birmingham, United Kingdom) (20), and anti-murine leukemia virus capsid (MAb R187; ATCC CRL1912) were produced *in vitro* using a MiniPerm apparatus (Heraeus) as recommended by the manufacturer.

Plasmids and mutagenesis. DNA sequences used in the studies were based on genotype 2a (JFH-1; GenBank accession number AB237837), 1a (UKN1A-14.42; accession number AY734972), 1b (UKN1B-5.23; accession number AY734976), 2b (UKN2B-1.1; accession number AY734982), and 1a (H77 strain; accession number AAB67037, with three amino acid changes at the following positions: R564C, V566A, and G650E). Sequences of E1 and E2 glycoproteins with their signal sequence were cloned together as a polyprotein or separately into the pcDNA3.1+ vector. To obtain chimeric constructs, full-length or fragments of glycoprotein E2 from isolate JFH-1 were replaced by the corresponding regions from isolate H77. These plasmids were constructed by two-step PCR using Native *Pfu* DNA polymerase (Stratagene). All of the constructs were sequenced and verified by CLUSTAL W software.

HCVpp assay. 293T cells were transfected with plasmids murine leukemia virus (MLV) Gag-Pol, MLV-luc, and pcDNA3.1+E1E2 as described previously (54). For some of the experiments, E1 and E2 were expressed from two separate vectors, pcDNA3.1+E1 and pcDNA3.1+E2. Plasmid pcDNA3.1+ containing no envelope protein gene was used as a negative control. After 48 h, supernatants containing pseudoparticles were filtered through a 0.45-μm-pore-size membrane and used to infect Huh-7 cells or pelleted by ultracentrifugation through a 20% sucrose cushion and analyzed by Western blotting. 293T cells were lysed with 1% Triton X-100 and analyzed by Western blotting. Infectivity of HCVpp on target Huh-7 cells was assessed after 48 h by using a firefly luciferase reporter gene activity kit (Promega), as recommended by the manufacturer. Results are presented as the means ± standard deviations of results of three independent experiments. Graphs were made using Prism software.

CD81 pull-down assay. Recombinant fusion proteins containing the large extracellular loop of CD81 fused to glutathione *S*-transferase (GST) were preadsorbed onto glutathione-Sepharose beads (Pharmacia Biotech) and then incubated with lysates of pseudoparticle-producing cells. After overnight incubation, beads were extensively washed with lysis buffer. Pull down was followed by Western blotting to detect E1 and E2.

Western blotting. After separation by sodium dodecyl sulfate (SDS)-PAGE, proteins were transferred to nitrocellulose membranes (Hybond-ECL; Amersham) by using a Trans-Blot apparatus (Bio-Rad) and revealed with specific antibodies (anti-E1 and anti-E2) followed by secondary immunoglobulin conjugated to peroxidase. The proteins of interest were revealed by enhanced chemiluminescence detection (ECL; Amersham) as recommended by the manufacturer.

HCVcc assay. Viral RNA of isolate JFH-1 containing the *Renilla* luciferase gene, A4 epitope, and cysteine and serine (CS) mutations in the capsid was prepared as described previously (12, 25). A replication-deficient clone containing a GND mutation in the NS5B active site and the assembly-deficient ΔE1E2 clone (71) were used as negative controls. Huh-7 cells (2×10^6) were mixed with 25 μg of RNA, placed in 0.2-cm cuvette (Bio-Rad), and electroporated with one pulse at 1,000 μF and 150 V using a GenePulser Xcell electroporator (Bio-Rad). After 10 min, cells were mixed with fresh medium and seeded into tissue culture dishes. After 4 h, a portion of the electroporated cells was lysed to verify the translation from the electroporated RNA for the different chimeras. Replication was tested after 24, 48, and 72 h posttransfection. Supernatants were collected after 72 h, centrifuged to remove cell debris, and used to infect Huh-7 cells. To assess intracellular infectivity, cells were washed with phosphate-buffered saline (PBS), trypsinized, and pelleted. Pellets were resuspended in medium and lysed by three freeze-thaw cycles. Cell lysates were clarified by centrifugation at $10{,}000 \times g$ for 5 min. Supernatants containing extracellular and intracellular virus were incubated with the cells for 2 h. Infectivity of the produced viral chimeras was verified after 48 h by using a *Renilla* luciferase activity kit (Promega), as indicated by the manufacturer. Results are presented as the means ± standard deviations of results of three independent experiments. Graphs were made using Prism software.

HCV core quantification assay. HCVcc supernatants were collected 72 h after electroporations. The core was quantified by an automated chemiluminescent microparticle immunoassay according to the instructions of the manufacturer (Architect HCVAg, Abbott, Germany) (47, 51).

Sequence analyses and predictions. Sequence analyses were performed using tools available at the Institut de Biologie et Chimie des Protéines (IBCP), i.e., by using the Network Protein Sequence Analysis (NPSA) website (http://npsa-pbil.ibcp.fr) (9). Provisional and confirmed genotyped HCV E2 sequences were retrieved from the European HCV database (http://euhcvdb.ibcp.fr/) (10). Multiple-sequence alignments were performed with CLUSTAL W (69), using the default options. The repertoire of residues at each amino acid position and their frequencies observed for natural sequence variants were computed by the use of a program developed at the IBCP (F. Dorkeld, C. Combet, F. Penin, and G.

Deleage, unpublished data). Protein secondary structures were deduced from a large set of prediction methods available at the NPSA website, including HNNC, SIMPA96, MLRC, SOPM, PHD, and Predator (see http://npsa-pbil.ibcp.fr/NPSA and the references therein). Interfacial hydrophobicity plots were generated with MPEx (http://blanco.biomol.uci.edu/mpex/) by using the scale developed by Wimley and White (74).

Peptide synthesis and purification. The E2-SC peptide, representing amino acids 684 to 719 of E2 from the HCV strain JFH-1 (accession number AB047639; SDLPALSTGLLHLHQNIVDVQYMYGLSPAITKYVVR), was synthesized in in-house facilities and purified by reverse-phase high-pressure liquid chromatography (HPLC) on a Nucleosil C_{18} column (120 Å, 5 μm) using a water/acetonitrile gradient containing 0.1% trifluoroacetic acid. The peptide was eluted as a single peak at 44% acetonitrile. The peak was identified by mass spectroscopy as the expected molecular mass peptide (observed molecular weight [MW + H^+], 4,013.35; calculated molecular mass, 4,013.68 Da).

CD. Far-UV circular dichroism (CD) spectra were recorded with a Chirascan spectrometer (Applied Photophysics, United Kingdom) calibrated with 1S-(+)-10-camphorsulfonic acid. Measurements were carried out at 25°C in a 0.1-cm-path-length quartz cuvette (Hellma), with a typical peptide concentration of 20 μM. Spectra were measured in a 180-nm to 260-nm wavelength range with a increment of 0.2 nm, band pass of 0.5 nm, and integration time of 1 s. Spectra were processed, baseline corrected, smoothed, and converted with Chirascan software. Spectral units were expressed as the mean molar ellipticity per residue by using the peptide concentration determined with UV light absorbance directly measured with a CD cell at 280 nm (ε = 3,900 M^{-1} cm^{-1}) of the peptide solubilized in a solution of 50% 2,2,2-trifluoroethanol (TFE) in water. Estimation of the secondary structure content was carried out using the DICHROWEB server facilities (http://dichroweb.cryst.bbk.ac.uk/) (73).

NMR spectroscopy. The purified peptide E2-SC was dissolved in either 100 mM deuterated SDS (SDS-d_{25}) or 50% deuterated TFE (TFE-d_2; 99%) in H_2O (vol/vol), and 2,2-dimethyl-2-silapentane-5-sulfonate was added to the NMR samples as an internal ^1H chemical shift reference. Multidimensional experiments were performed at 25°C with a Bruker Avance 500 MHz spectrometer using standard homonuclear pulse sequences, including nuclear Overhauser enhancement spectroscopy (NOESY) (mixing times, between 100 and 150 ms) and clean total correlation spectroscopy (TOCSY) (isotropic mixing time of 85 ms), as detailed previously (18, 58). Water suppression was achieved by presaturation. Bruker Topspin software was used to process all data, and Sparky was used for spectral analysis (http://www.cgl.ucsf.edu/home/sparky/). Intraresidue backbone resonances and aliphatic side chains were identified from homonuclear ^1H TOCSY experiments and confirmed with ^1H-^{13}C heteronuclear single-quantum correlation (HSQC) experiments in ^{13}C natural abundance. Sequential assignments were determined by correlating intraresidue assignments with interresidue cross peaks observed in bidimensional ^1H NOESY. NMR-derived $^1H\alpha$ and $^{13}C\alpha$ chemical shifts were reported relative to the random coil chemical shifts in SDS (65) and TFE (48), respectively.

NMR-derived constraints and structure calculation. Nuclear Overhauser enhancement (NOE) intensities used as input for structure calculations were obtained from the NOESY spectrum recorded with a 150-ms mixing time and checked for spin diffusion on spectra recorded at lower mixing times (100 ms). NOEs were partitioned into three categories of intensities that were converted into distances ranging from a common lower limit of 1.8 Å to upper limits of 2.8 Å, 3.9 Å, and 5.0 Å, respectively. Protons without stereospecific assignments were treated as pseudoatoms, and the correction factors were added to the upper distance constraints (76). Neither hydrogen bond nor dihedral angle constraints were introduced. Three-dimensional structures were generated from NOE distances with the standard torsion angle molecular dynamics protocol in the XPLOR-NIH 2.24 program (66) using the standard force fields and default parameter sets. A set of 50 structures was initially calculated to widely sample the conformational space, and the structures of low energy with neither distance nor dihedral angle restraint violations were retained. The selected structures were compared by pairwise root mean square deviation (RMSD) over the backbone atom coordinates (N, Cα, and C'). Statistical analyses, superimpositions of structures, and structural analyses were performed with MOLMOL (33), and the quality of the selected structures was checked with the Research Collaboratory for Structural Bioinformatics (RCSB) Protein Data Bank (PDB) validation server.

Accession numbers. The atomic coordinates for the NMR structure of peptide E2-SC and the NMR restraints in 50% TFE are available in the RCSB PDB under accession number 2KZQ (RCSB identification code 101755). The chemical shifts of all E2-SC residues have been deposited in BioMagResBank (BMRB) under accession number 17011.

RESULTS

HCVpp containing E1 and E2 from different genotypes identify genetic incompatibilities between some HCV genotypes. HCV is currently classified into seven genotypes and several subtypes that at the nucleotide level differ from each other by 31 to 33% and 20 to 25%, respectively (36, 67). To test the intergenotypic incompatibilities between E1 and E2, we selected a limited number of E1E2 sequences from different genotypes/subtypes (1a, 1b, 2a, and 2b). We constructed a series of plasmids expressing E1 alone or E2 alone, so we could easily coexpress E1 and E2 in *trans* from different genotypes/subtypes to produce HCVpp. However, since we did not have access to an anti-E1 antibody that recognizes all these genotypes, we mutated a few residues in E1 to generate the A4 epitope at the N terminus of the protein (16) to facilitate the detection of this protein in our experiments (Fig. 1C). This epitope is present in some HCV isolates of genotype 1a, and such a modification in the context of HCVcc does not alter infectivity (25). We confirmed that this mutation allows for the detection of E1 (Fig. 1E), and we showed that it had only a moderate effect on HCVpp infectivity. Indeed, the infectivity of A4 epitope-containing pseudoparticles was reduced to 24% and 33% of the wild-type HCVpp levels for JFH-1 and the genotype 2b isolate (data not shown), respectively, whereas an increase in HCVpp infectivity to 220% was observed for the genotype 1b isolate (data not shown). Since all the constructs tested remained infectious, the mutant E1 proteins could be used to test the intergenotypic incompatibilities between E1 and E2. For the detection of E2, we used the 3/11 MAb (20), which recognizes a conserved minimum epitope (29).

We then tested the intergenotypic incompatibilities between E1 and E2 by producing HCVpp containing different combinations of envelope glycoproteins and assessed their infectivity. The intergenotypic combinations were compared to HCVpp produced with E1E2 from the reference genotypes/subtypes. HCVpp infectivity was reduced to background levels for some combinations, whereas it was moderately affected for others (data not shown). Surprisingly, chimeras with glycoprotein E1 from genotype 1a always showed an increase in infectivity compared to the reference genotypes, which was not the case for E1 from other genotypes. To test whether this phenomenon is specific for E1 from the H77 strain or if it is a more general feature of genotype 1a isolates, we analyzed the phenotype induced by the presence of E1 from another genotype 1a isolate (the UKN1a.14.42 isolate). Only when coexpressed with E2 from genotype 2b did the UKN1a.14.42 isolate induce an increase in HCVpp infectivity, whereas the other combinations remained as infectious as the control HCVpp containing E1E2 proteins from the UKN1a.14.42 isolate (data not shown). These data indicate that E1 from genotype 1a is functionally compatible with E2 from different genotypes/subtypes. Furthermore, it can also potentially increase the entry efficiency of HCVpp in some combinations. Although this phenotype is potentially interesting to explore, it was beyond the scope of this work. We were indeed interested by combinations of E1 and E2 inducing a defect in infectivity. Importantly, our results showed that several combinations abolish HCVpp infectivity, indicating structural incompatibilities between some genotypes or subtypes.

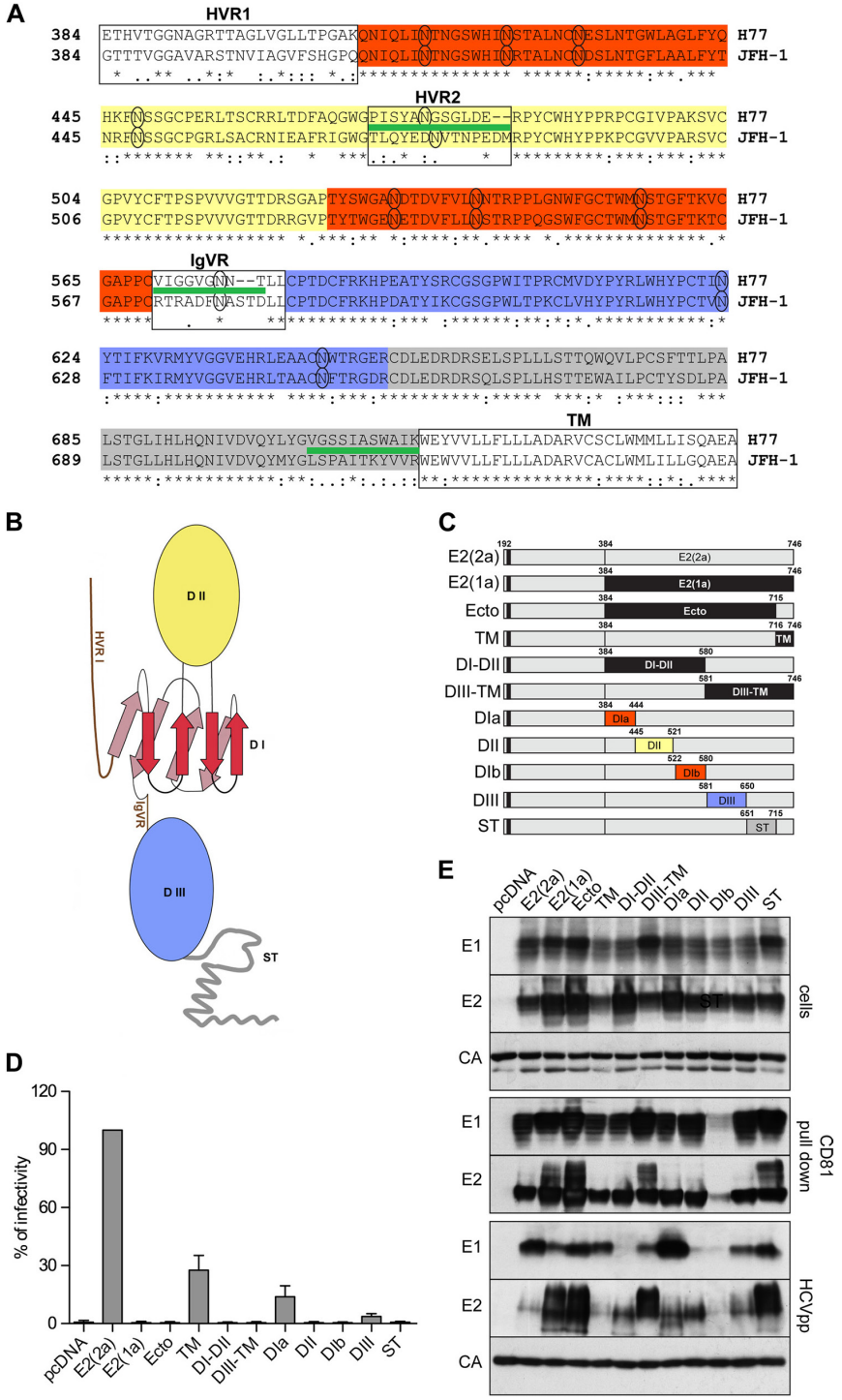

FIG. 1. Identification of regions within E2 responsible for intergenotypic incompatibilities. (A) Protein alignment of E2 glycoprotein from genotypes 1a (H77) and 2a (JFH-1). Previously characterized regions, such as HVR1, HVR2, IgVR, and TM, are marked with rectangles. Colors correspond to the E2 domains: DI in red, DII in yellow, DIII in blue, and ST in gray (34). Glycosylation sites are circled. Determinants of intergenotypic incompatibilities are underlined in green. Alignment was performed with CLUSTAL W software. (B) Hypothetical structural model of E2 glycoprotein (34). Colors of the domains correspond to the protein regions of the alignment presented in panel A. (C) Schematic

Several E2 regions are responsible for intergenotypic incompatibility. Although our first screening for nonfunctional chimeras indicated that several combinations lead to nonfunctional HCVpp, for a more in-depth analysis, we decided to focus on a single chimera for the identification of E2 determinants of intergenotypic incompatibility. We selected the combination containing E1 sequence from genotype 2a (JFH-1 isolate) and E2 from genotype 1a (H77 isolate). It should be noted that the A4 epitope was also reconstructed in this E1 sequence to facilitate its detection by Western blotting. In addition, derived constructs of this combination could easily be transferred into the HCVcc system (see below). Furthermore, we expressed E1 and E2 proteins from the same polyprotein to have both proteins expressed in *cis* instead of in *trans*. Under these conditions, we can be sure that the same amounts of E1 and E2 are coexpressed in cells producing HCVpp. Furthermore, this type of expression better mimics the polyprotein processing observed in the context of HCV infection.

To identify the E2 determinant(s) responsible for incompatibility between E1(2a) and E2(1a), we constructed a series of chimeras in the context of E1E2 of genotype 2a in which E2 regions were replaced by the corresponding sequence from genotype 1a (Fig. 1C), and their infectivity was analyzed in the context of the HCVpp system (Fig. 1D). These constructs were designed based on the recently proposed model of E2 (Fig. 1A and B) (34). HCVpp containing E1(2a)E2(2a) and E1(2a)E2(1a) was used as a positive and negative control, respectively. As shown in Fig. 1D, HCVpp was no longer infectious when E2(1a) ectodomain (Ecto) was introduced in the context of E1E2 of genotype 2a, whereas replacement of the TM domain only moderately reduced HCVpp infectivity, suggesting that the major determinant(s) leading to functional incompatibilities is located in the ectodomain of E2. Furthermore, when the DI-DII region or DIII-TM region of genotype 1a was introduced in the context of E1E2 of genotype 2a, HCVpp was also no longer infectious, indicating that at least two regions in the ectodomain of E2 contain determinants leading to intergenotypic incompatibility. Finally, analyses of chimeras DIa, DII, DIb, DIII, and ST indicated that regions responsible for intergenotypic incompatibilities are located in DII, DIb, and ST. The DIa construct was still moderately infectious, and DIII showed some residual infectivity. Even if they reduce HCVpp entry, these two regions do not contain genotype-specific determinants that are essential for HCV entry in the HCVpp system.

HCVpp generated with these chimeric proteins was also characterized for the presence of viral proteins and compared to cell lysates expressing these proteins. As shown in Fig. 1E, the levels of expression of MLV capsid, E1, and E2 were similar in cell lysates for all the constructs. However, the signals for E1 and E2 varied in some constructs in the context of HCVpp. Some of the variations in the intensity of E2 can be explained partly by differences in affinities of the MAb for its epitope in the context of the different genotypes as previously shown (46, 68). Indeed, when E2 or its ectodomain were from genotype 1a, the intensity of the signal was higher in HCVpp. A higher signal for E2 was also observed for the constructs containing the ST region of genotype 1a (Fig. 1E, DIII-TM and ST), suggesting that the ST region might modulate the recognition of the 3/11 epitope. Due to the distance between the 3/11 epitope (located in DI) and the ST region, this might be due to an indirect effect of the ST region. One possibility is that in the context of E1E2 of genotype 2a, the ST region of genotype 1a indirectly affects the processing of the glycans as suggested by a difference in the migration pattern similar to what has been observed by others when mutations are introduced in E1 (63). Glycan processing can in turn modulate the binding of MAb 3/11 as suggested for other anti-E2 MAbs (19). It is important to note that the level of incorporation of E2 into HCVpp does not reflect the level of infectivity. Indeed, mutant TM was the most infectious chimera; however, the level of incorporation of E2 appeared rather low for this virus. In the case of E1, the protein is from the same genotype, so the differences in the level of incorporation into HCVpp directly reflect the effect of the change induced by the chimeric E2 constructs. It is worth noting that the level of E1 was close to background for DI-DII, DII, and DIb constructs. The absence of infectivity of these constructs might therefore be due to the lack of incorporation of E1 into HCVpp. Surprisingly, in the case of DIa, a higher level of incorporation of E1 was observed. However, this was not correlated with a higher level of incorporation of E2 into HCVpp.

Finally, we also analyzed the recognition of intracellular E1E2 complexes by CD81. As shown in Fig. 1E, E1 and E2 obtained from cell lysates were precipitated with the CD81 large extracellular loop (CD81LEL) for all the constructs except DIb. These data indicate that, except for DIb, exchanging E2 regions between 1a and 2a genotypes does not dramatically affect E2 folding or its potential to interact with E1 or CD81.

representation of chimeric E1E2 constructs used in the study. All chimeras contain E1 from genotype 2a and E2 from genotype 2a in which corresponding regions from genotype 1a have been introduced. The numbers correspond to the first and the last residue of the exchanged regions based on the numbering of the reference strain H77. Fragments from genotype 2a are shown in light gray. Fragments from genotype 1a are marked in black or in the color corresponding to specific E2 domains. The DI construct includes DI and HVR1. DIa′ includes HVR1 and segment 412-444 from DI. The DIb construct includes segment 522-569 from DI and IgVR. Note that the N terminus of E1 has been modified to reconstruct the A4 epitope present in E1 of genotype 1a, as indicated by a black box at the N terminus of E1. For this purpose, amino acid sequence TSSSYMVTNDC at position 197 to 207 of E1 of genotype 2a has been modified to SSGLYHVTNDC (modified amino acids are underlined). (D) Infectivity of chimeric HCVpp. Huh-7 cells were infected with chimeric pseudoparticles. The infectivity levels were assessed by the activity of the reporter luciferase gene. The results are presented as the percentage of infectivity in comparison to wild-type 2a HCVpp. The noninfectious particles containing no envelope proteins (pcDNA) were used as a negative control. (E) Western blotting of chimeric HCVpp. Cells producing HCVpp were lysed and analyzed by Western blotting. HCVpp were concentrated on a 20% sucrose cushion and analyzed by Western blotting. E1E2 was pulled down from the cell lysates by a CD81LEL-GST assay. E1, E2, and capsid were detected using MAbs A4, 3/11, and R187, respectively.

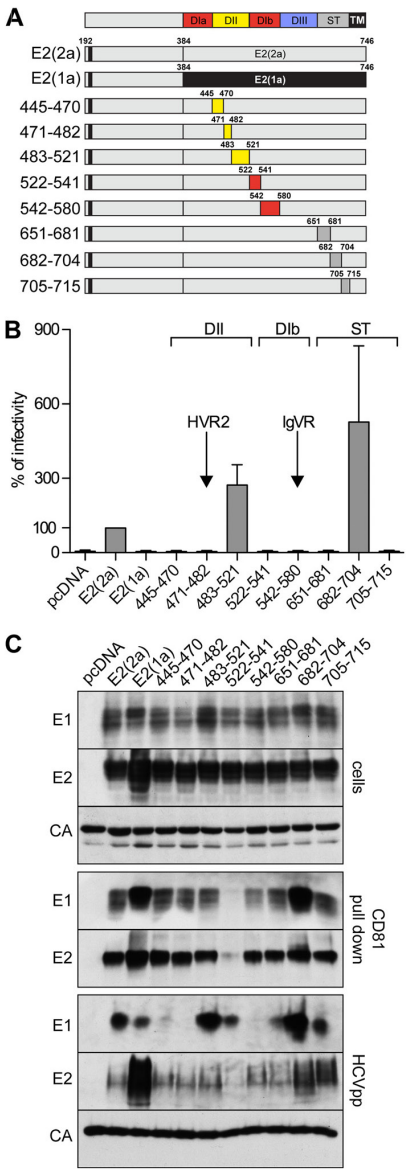

FIG. 2. Identification of E2 determinants of intergenotypic incompatibility. (A) Schematic representation of chimeric E1E2 constructs used in the study. All chimeras contain E1 from genotype 2a and E2 from genotype 2a in which corresponding regions from genotype 1a have been introduced. The numbers correspond to the first and last residues of the exchanged regions based on the numbering of the reference strain H77. Fragments from genotype 2a are shown in light gray. Fragments from genotype 1a are shown in black or in the color corresponding to specific E2 domains. As shown in Fig. 1C, the N terminus of E1 has been modified to reconstruct the A4 epitope present in E1 of genotype 1a as indicated by a black box at the N terminus of E1. (B) Infectivity of chimeric HCVpp with exchanged regions within DII (aa 445-470, 471-482, 483-521), DIb (aa 522-541, 542-580), and ST (aa 651-681, 682-704, 705-715). HCVpp infectivity was determined by measuring the activity of the luciferase reporter gene. Segment 471-482 corresponds to HVR2, and segment 542-580 includes part of DIb and IgVR. (C) Western blotting of chimeric HCVpp. Cells producing HCVpp were lysed and analyzed by Western blotting. HCVpp was concentrated on a 20% sucrose cushion and analyzed by Western blotting. E1E2 was pulled down from the cell lysates by a CD81LEL-GST assay. E1, E2, and capsid were detected using MAbs A4, 3/11, and R187, respectively.

Furthermore, the lack of infectivity of DIb is likely due to protein misfolding as shown in the CD81 pull-down.

Altogether, our data indicate that three regions (DII, DIb, and ST) contain determinants responsible for intergenotypic incompatibility. Furthermore, the lack of incorporation of E1 into HCVpp of DII and DIb chimeras suggests a defect in HCVpp assembly for these two constructs.

Identification of E2 determinants of intergenotypic incompatibility. To identify more precisely the E2 determinants of intergenotypic incompatibility, an additional series of chimeras was produced, which contained smaller segments of E2 of genotype 1a in the context of E1E2 of genotype 2a containing the A4 epitope (Fig. 2A). As shown in Fig. 2B and summarized in Table 1, changing the segment of aa 445 to 470 (segment 445-470) or 471-482 (HVR2) in DII, segment 522-541 or 542-580 (IgVR) in DIb, and segment 651-681 or 705-715 in the ST region abolished HCVpp infectivity. In contrast, changing segment 483-521 or 682-704 did not abolish HCVpp infectivity; inversely, it led to some increase in HCV entry.

HCVpp generated with these chimeric proteins were also characterized for the presence of viral proteins, and these results were compared to those for cell lysates expressing these proteins. As shown in Fig. 2C, the levels of expression of MLV capsid, E1, and E2 were similar in cell lysates for all the constructs. However, the signals for E1 and E2 varied in some constructs in the context of HCVpp. As discussed above, some variations were observed for E2, which might be due in part to differences in affinity of the 3/11 antibody for the E2 chimeras. In the case of E1, the level of incorporation into HCVpp directly reflects the effect of the change induced by the chimeric E2 constructs. It is worth noting that the level of E1 was close to background for 445-470, 471-482, and 542-580 constructs. The absence of infectivity of these constructs might therefore be due to the lack of incorporation of E1 into HCVpp. Furthermore, there was a lower level of incorporation of E1 and E2 for the 522-541 construct.

Finally, we also analyzed the recognition of intracellular E1E2 complexes by CD81. As shown in Fig. 2C, E1 and E2 obtained from cell lysates were precipitated with CD81LEL for all the constructs except 522-541. These data indicate that, except for the 522-541 segment, exchanging E2 regions between 1a and 2a genotypes does not dramatically affect E2 folding or its potential to interact with E1 or CD81. Furthermore, the lack of infectivity of the 522-541 construct is likely due to protein misfolding as shown in the CD81 pull-down.

Altogether, our data indicate that E2 contains six determinants responsible for intergenotypic incompatibility (445-470, 471-482, 522-541, 542-580, 651-681, and 705-715) (Fig. 2B). Furthermore, the lack of incorporation of E1 into HCVpp of 445-470, 471-482, and 542-580 chimeras as well as the reduced levels of E1E2 for the 522-541 chimera suggest a defect in HCVpp assembly for these four determinants (Fig. 2C). Finally, the lack of infectivity of chimeras 651-681 and 705-715

TABLE 1. Summary of the properties of E2 determinants tested in the HCVpp and/or HCVcc systems and their potential function in HCV assembly or entry

Domain	Region	HCVpp[a] infectivity	HCVcc[b]		Function
			Infectivity	Secretion	
Domain I	DIa 384-444	+	ND	ND	Both required for DI folding
	DIb 522-541	−	ND	ND	Both required for DI folding
Domain II	445-470	−	+	−	Assembly
	471-482 (HVR2)	−	−	−	Assembly
	483-521	+++	ND	ND	
IgVR	570-578	−	−	−	Assembly
Domain III	581-650	±	ND	ND	
Stem	651-681	−	+	+	
	682-704	+++	++	+	Entry[c]
	705-715	−	±	++	Entry
TM	716-746	+	ND	ND	

[a] For HCVpp infectivity data, +++, >90%; ++, between 30% and 90%; +, between 10% and 30%; ±, between 5% and 10%; −, <5%.
[b] ND, not defined. For HCVcc infectivity data, ++, <1 log lower than the wt; +, >1 log lower than the wt; ±, >2 logs lower than the wt; −, >3 logs lower than the wt. For HCVcc secretion data, ++, between 50% and 80%; +, between 20% and 50%; ±, between 10% and 20%; −, <10%.
[c] Suggested function based on our structural data.

despite a normal level of incorporation into HCVpp suggests a defect in virus entry.

Intradomain interactions within DI are crucial for E2 functions. Our data showed that the lack of infectivity of the DIb chimera is likely due to protein misfolding as shown in the CD81 pull-down (Fig. 1E). Since this construct contains only half of the predicted DI domain (34), we speculated that there might be an intradomain incompatibility between these two genotypes as reflected by the alteration of the CD81 binding region of the DI domain. We therefore made a new chimera containing DIa and DIb of genotype 1a in the context of E1E2 of genotype 2a to test this hypothesis. As shown in Fig. 3A, this chimera restored HCVpp infectivity by more than 50%. Furthermore, an increase in incorporation of E1E2 into HCVpp was observed, as was a better recognition of the intracellular form of E2 by CD81 (Fig. 3B).

Together, these data indicate that the two parts of the DI domain interact to form the CD81 binding region, which is in agreement with the recently proposed E2 model (34).

Role of the identified E2 regions in HCVcc assembly and infectivity. The HCVpp system is a well-established system to study the functions of HCV envelope glycoproteins. However, this system does not totally reflect the functions of HCV envelope proteins, since HCVpp is not assembled in the same compartment as HCVcc. Indeed, HCVcc assembles in an ER-derived compartment (49), whereas HCVpp is assembled in a post-Golgi compartment (64). Therefore, HCV envelope glycoproteins incorporated into HCVpp travel through the secretory pathway independently of the other viral components, whereas they are supposed to travel through the secretory pathway in association with nascent viral particles in the context of the HCVcc system. This can lead to differences in glycan maturation as well as in protein-protein interactions during assembly (70). We therefore analyzed some of our chimeric E2 proteins in the context of the HCVcc system.

We used the JFH-1 isolate containing the A4 epitope and the luciferase reporter gene in which we introduced separately the above-identified determinants of intergenotypic incompatibility. To further narrow down the determinants, we excluded some of the conserved fragments present in the HCVpp constructs. Moreover, we did not investigate the role of the 522-541 segment in the context of the HCVcc system, since we already showed (see above) that it is involved in intradomain interactions. However, we made an additional construct with the 682-704 segment, which induced a higher level of infectivity in the context of the HCVpp system. As controls, we used the wild-type JFH-1 isolate, a JFH-1 virus containing no envelope proteins (ΔE1E2), and the replication-defective mutant (GND) (71). None of the mutations affected genomic replication as analyzed by measuring luciferase activity at different times postelectroporation (data not shown). As shown in Fig. 4, chimeras 471-482 (HVR2) and 570-578 (IgVR) were noninfectious, and this lack of infectivity correlated with a defect in particle secretion as measured by a core release assay. Furthermore, we did not detect any intracellular infectivity for these two chimeras, indicating that the lack of infectivity of these mutants is not due to a defect in infectious particle secretion. It is worth noting that the lengths of HVR2 and IgVR differ by two amino acids between genotypes 1a and 2a. To determine whether the defect was not due to a change in the segment length, we extended HVR2 and IgVR of genotype 1a by inserting two corresponding amino acids from the 2a genotype or two alanine residues in the nonfunctional JFH-1 chimeras. However, none of the insertions was able to restore the infectivity of the chimeras (data not shown). This suggests that, instead of the length, it is the amino acid composition of the mutant viruses that affects E2 functions. Chimera 453-467 (within DII) was still infectious, but its extra- and intracellular infectivities were strongly reduced and this reduced infectivity also correlated with a low level of particle secretion (Fig. 4). Altogether, these data confirm some of the results obtained with the HCVpp system and they indicate that the 453-467

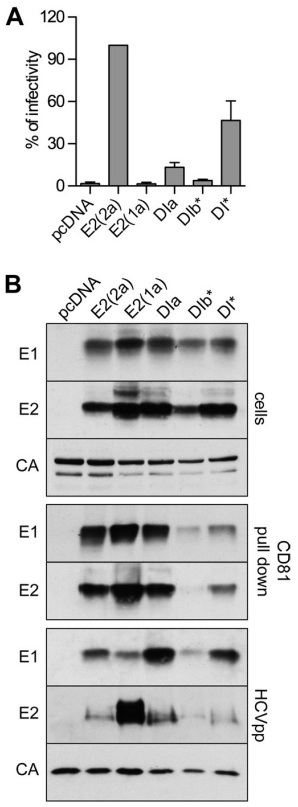

FIG. 3. Intergenotypic incompatibilities within CD81 binding domain DI. Wild-type 2a HCVpp [E2(2a)] and a noninfectious chimera [E2(1a)] were used as controls in the study. Chimeric constructs contained aa 384 to 444, corresponding to DIa and the nonconserved segment 522-541 within the second part of DI (labeled DIb*). Chimera DI* contained both regions. (A) Infectivity of chimeric particles assessed with the activity of reporter luciferase gene in infected Huh-7 cells. Results are presented as the percentage of infectivity in comparison to wild-type HCVpp [E2(2a)]. (B) 293T cells producing HCVpp were lysed and analyzed by Western blotting. HCVpp were pelleted from the supernatants on a 20% sucrose cushion and analyzed by Western blotting. E1E2 interaction with CD81 was verified by pull-down assay with CD81LEL fused to glutathione *S*-transferase. E1, E2, and capsid were detected using MAbs A4, 3/11, and R187, respectively.

region, HVR2, and IgVR of genotype 1a affect HCV infectivity by inducing a defect in particle assembly when introduced in the context of genotype 2a.

Chimeras with swapped segments within the ST region revealed some differences between the HCVpp and HCVcc systems. Chimera 667-681 was noninfectious in the HCVpp system (more precisely, segment 651-681 in Fig. 2B), whereas it showed less than 1 $\log_{10}$ decrease in infectivity in the HCVcc system (Fig. 4A). However, we cannot exclude the possibility that this difference is due to the Q-to-E change at position 661 (Fig. 1A), which is present in HCVpp chimera 651-681 but not in HCVcc chimera 667-681. Furthermore, chimera 682-704 was more infectious than the wild type in the HCVpp system (Fig. 2B), whereas its infectivity was reduced by 1 $\log_{10}$ in the HCVcc system (Fig. 4A). Core secretion of these chimeric viruses was lower than for the wild-type virus (Fig. 4B), and this likely contributes to their lower infectivity. Interestingly, the infectivity of chimera 705-715 was reduced by almost 3 $\log_{10}$ (Fig. 4A); however, this defect in infectivity was not due to a lack of particle secretion, since the level of secretion of core was close to that of the wild type (Fig. 4B). Importantly, the latter results correlate with those obtained with the HCVpp system and indicate that segment 705-715 plays a major role in HCV entry.

Characterization of the stem region determinant involved in the HCV entry process. Although the recently published E2 model provides some structural information for most of the regions identified in this work, the putative structure of the ST region remains limited to the identification of a highly conserved heptad repeat sequence necessary for heterodimerization with E1 (15, 59). Therefore, to better understand the role of the stem region, the structure and properties of this region were investigated. We first used bioinformatic tools to analyze this region. The degree of conservation among different genotypes was investigated. The amino acid repertoire derived from the alignment (Fig. 5A, panel b) revealed that amino acids are strictly conserved in 40% of the sequence positions (denoted by asterisks in Fig. 5A, panel a). In addition, the apparent variability is limited at most other positions, as indicated by both the similarity pattern (colons and dots) and the hydropathic pattern (Fig. 5A, panel c), where o, i, and n denote hydrophobic, hydrophilic, and neutral residues, respectively (see the legend to Fig. 5 for details). The conservation of the physicochemical properties at most positions indicates that the overall structure is certainly conserved among the different HCV genotypes. This is supported by secondary structure analyses that always predicted the presence of structured elements (α helix and/or β strand) for the same segments in the various genotypes as illustrated in Fig. 5A, panel d, for HCV clones H77 and JFH1 of genotypes 1a and 2a, respectively. Similar prediction patterns were observed for the other HCV genotypes (data not shown).

An examination of the hydrophobic plots in the E2 stem region indicates the presence of hydrophobic clusters, suggesting some potential binding to lipids. As illustrated in Fig. 5A, panel d, for E2 from HCV strain H77 and JFH1, the interfacial hydrophobicity plots calculated for sequences of various genotypes indicated that the segment of aa 663 to 703 or so, as well as segment 708-715, exhibits clear propensities to partition into the interface of a phospholipid bilayer (sequences highlighted in gray in Fig. 5A, panel d). To gain insight into the structure and lipotropic properties of the E2 aa 680-715 segment, the corresponding peptide of the JFH-1 strain (highlighted in bold in Fig. 5A, panel d; segment 684-719), designated E2-SC, was chemically synthesized, purified, and analyzed by CD and NMR. Note that the residues of this peptide were numbered according to the numbering of HCV reference strain H77, as recommended (35).

Conformation analyses of the E2-SC synthetic peptide by circular dichroism. Peptide E2-SC was soluble in water and gave a typical spectrum with a large negative band around 200 nm and a shoulder at 220 nm (Fig. 5B), indicating a mixture of random coil structures (~70%) with some poorly defined and/or residual secondary structures. The secondary structure of E2-SC was also examined in the presence of either lyso-

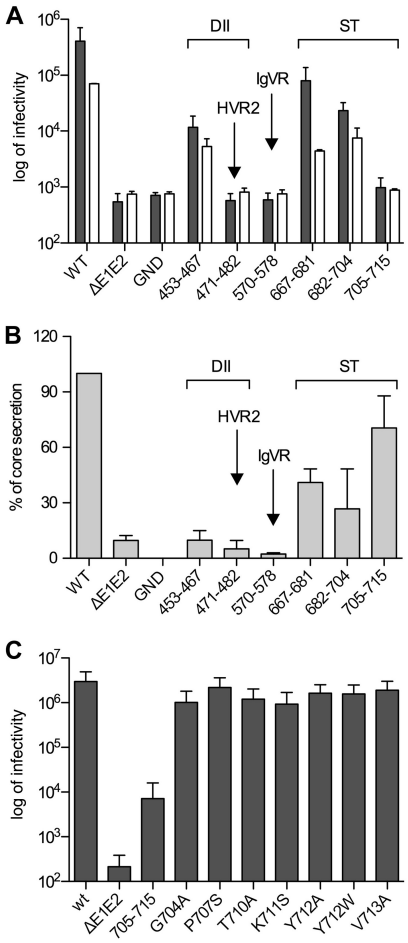

FIG. 4. Analysis of chimeric viruses produced in the HCVcc system. Wild type 2a virus (JFH-1) was used as a positive control. Viral RNA containing a deletion in the sequence encoding HCV envelope glycoproteins (ΔE1E2) and nonreplicative mutant containing a mutation in the polymerase sequence (GND) were used as negative controls. Chimeric viruses used in the study contained regions exchanged in DII (aa 453-467 and 471-482, corresponding to HVR2), aa 570-578, corresponding to IgVR, and aa 667-681, 682-704, and 705-715 within ST. (A) Extra- and intracellular infectivity of chimeric viruses. Viral RNA was electroporated into the Huh-7 cells. After 72 h, cells were lysed, and cleared supernatants were used to infect Huh-7 cells (intracellular infectivity is shown with white bars). Supernatants containing secreted virus were used to infect naïve Huh-7 cells (extracellular infectivity is shown with gray bars). Infectivity was assessed with the activity of the luciferase reporter gene in infected Huh-7 cells. The results are presented as the logarithm of infectivity. Note that in our JFH1 constructs, the N terminus of E1 has been modified to reconstruct the A4 epitope present in E1 of genotype 1a. (B) Secretion of chimeric viruses from electroporated cells. Supernatants collected from the electroporated cells were used to perform a core secretion assay. The presence of core in the samples is presented as the percentage of the core amount in comparison to wild-type JFH-1 virus. (C) Infectivity of HCVcc containing point mutations within aa 705-715 in the stem region. Wild-type 2a virus (JFH-1) was used as a positive control. Viral RNA containing a deletion in the sequence encoding HCV envelope glycoproteins (ΔE1E2) was used as a negative control. Chimera 705-715 was used to compare its infectivity with the infectivity of mutant viruses containing single amino acid changes. Viral RNA was electroporated into Huh-7 cells. After 72 h, the supernatants were collected and used to infect Huh-7 cells. Cells were lysed after 48 h, and the infectivity level was assessed by the activity of the luciferase reporter gene. The results are presented as the logarithm of the infectivity level.

phosphatidyl choline (LPC) or various detergents (SDS, *N*-dodecyl-β-D-maltoside [DM], dodecyl phosphocholine [DPC]) or cosolvents (TFE-water mixture) that mimic the membrane environment (Fig. 5B). These membrane mimetics were selected to reflect the various conditions in a true membrane, in order to gain a more comprehensive picture of the peptide conformational preferences. In the presence of the various detergents, CD spectra of the peptide exhibit the typical spectrum of α-helical folding, with a maximum at 190 nm and two minima, at 208 and 222 nm. The various CD deconvolution methods used indeed indicate predominant α-helix content (~40%), whatever the detergent used. The potential conformational preferences of E2-SC peptide were also probed in the presence of TFE, which is known to stabilize the folding of peptidic sequences, especially those exhibiting an intrinsic propensity to adopt an α-helical structure (3, 50). The peptide folding titration with increasing proportions of TFE gave spectra that were characteristic of α-helical folding, as illustrated in Fig. 5B (TFE 50%). Maximal amplitude was reached at 40% TFE and corresponds to an α-helical content of ~65% as measured. An isodichroic point was observed at 204 nm (data not shown), indicating that the peptide undergoes a simple transition from random coil to α helix, and according to the two-state model, equilibrium exists between the two conformers. This equilibrium together with the high α-helix content compared to that observed with detergents are consistent with an improved stabilization of the helical region in TFE, which is generally observed with these media. In summary, CD spectral analyses indicated the high propensity of E2-SC to interact with lipids and to adopt an α-helical structure upon lipid binding.

The E2-SC segment comprises an amphipathic α helix. Deuterated micellar SDS and DPC are popular membrane mimetic media for structure analyses of membrane peptides by liquid NMR (55). Unfortunately, samples of E2-SC peptide prepared in SDS and DPC displayed broad, poorly resolved NMR spectra. Nevertheless, an almost complete amino acid sequential attribution and ^{1}Hα chemical shift variation analysis were possible from spectra recorded with SDS, as were the identification of many sequential and medium-range NOE connectivities, as illustrated in Fig. 6A. However, this limited set of data did not permit the accurate modeling of the E2-SC peptide structure. We thus studied the three-dimensional structure of E2-SC dissolved in 50% TFE-d_2, which yielded well-resolved NMR spectra (data not shown). Sequential attribution of all spin systems was complete, and an overview of the sequential and medium-range NOE connectivities is shown in Fig. 6B. The NOE connectivity patterns demonstrate that the central part of the peptide, including residues 687 to 703, displays most characteristics of an α helix, including strong dNN(i, i + 1) and medium dαN(i, i + 1) sequential connectivities as well as weak dαN(i, i + 2), medium or strong dαN(i, i + 3) and dαβ(i, i + 3), and weak dαN(i, i + 4) medium-range

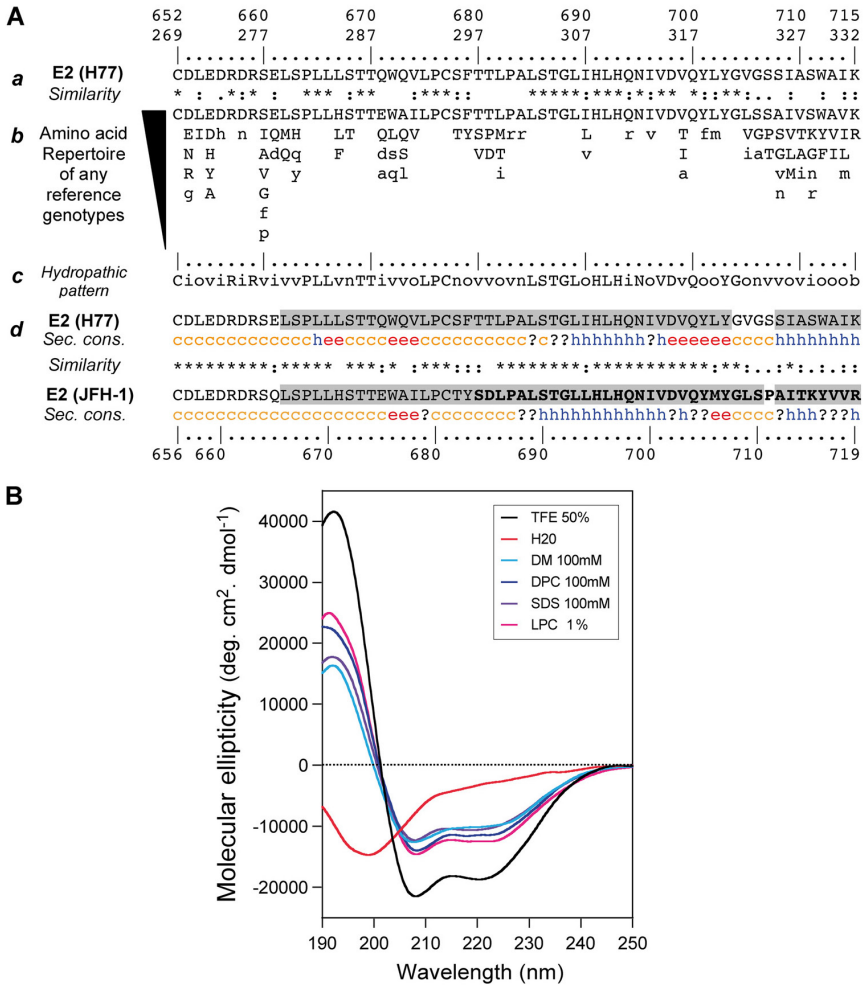

FIG. 5. Sequence analyses, predictions of secondary-structure, and membrane binding properties of the glycoprotein E2 stem region. (A) Amino acid repertoires. Amino acids are numbered with respect to the HCV polyprotein and E2 protein (top row) of the HCV H77 infectious clone (GenBank accession number AF009606). The amino acid repertoire of the 27 representative E2 sequences from confirmed HCV genotypes and subtypes (listed with accession numbers in Table 1 in reference 67), including the recently described genotype 7a (accession number EF108306; see the European HCV database for details [http://euhcvdb.ibcp.fr/; 10]), is given in panel b. The degree of amino acid conservation at each position can be inferred from the extent of variability (with the observed amino acids listed in decreasing order of frequency from top to bottom) together with the similarity index (a) according to the CLUSTAL W convention (asterisk, invariant; colon, highly similar; dot, similar) (69). Amino acids observed only one time at a given position among the 27 sequences are indicated by lowercase letters. (c) The physicochemical conservation of residues at each position is outlined by the consensus hydropathic pattern deduced from the consensus amino acid repertoire: o, hydrophobic position (F, I, W, Y, L, V, M, P, and C); n, neutral position (G, A, T, and S); i, hydrophilic position (K, Q, N, H, E, D, and R); v, variable position (i.e., when both hydrophobic and hydrophilic residues are observed at a given position). Fully conserved residues in any genotype are indicated by their one-letter code. (d) Consensus secondary-structure predictions of E2 from H77, and JFH1 clones (genotypes 1a and 2a, GenBank accession numbers AF009606 and AB047639, respectively), are indicated as helical (h, blue), extended (e, red), turn (t, green), or undetermined (coil [c], yellow). Predictions were made by using the Web-based algorithms DSC, HNNC, MLRC, PHD, Predator, SOPM, and SIMPA96 available at the NPSA website (http://npsa-pbil.ibcp.fr; reference 9 and the references therein). Amino acid sequence segments predicted to exhibit a propensity to partition into the interface of a phospholipid membrane are shaded in gray. Interfacial hydrophobicity plots were calculated with MPEx (http://blanco.biomol.uci.edu/mpex/) using the Wimley and White scale of interfacial hydrophobicity (74) and a window of 19 residues. The sequence comprising E2 aa 684 to 719 from the HCV JFH-1 infectious clone, which was used to prepare the peptide E2-SC described in this study, is indicated in bold. (B) Far-UV circular dichroism (CD) analyses of synthetic peptide E2-SC in various environments. CD spectra were recorded in water (H2O), complemented with either 50% 2,2,2-trifluoroethanol (TFE) or 1% L-α-lysophosphatidyl choline (LPC) or the following detergents: 100 mM sodium dodecyl sulfate (SDS), 100 mM *N*-dodecyl-β-D-maltoside (DM), or 100 mM dodecyl phosphocholine (DPC). sec. cons., secondary-structure consensus; deg., degree.

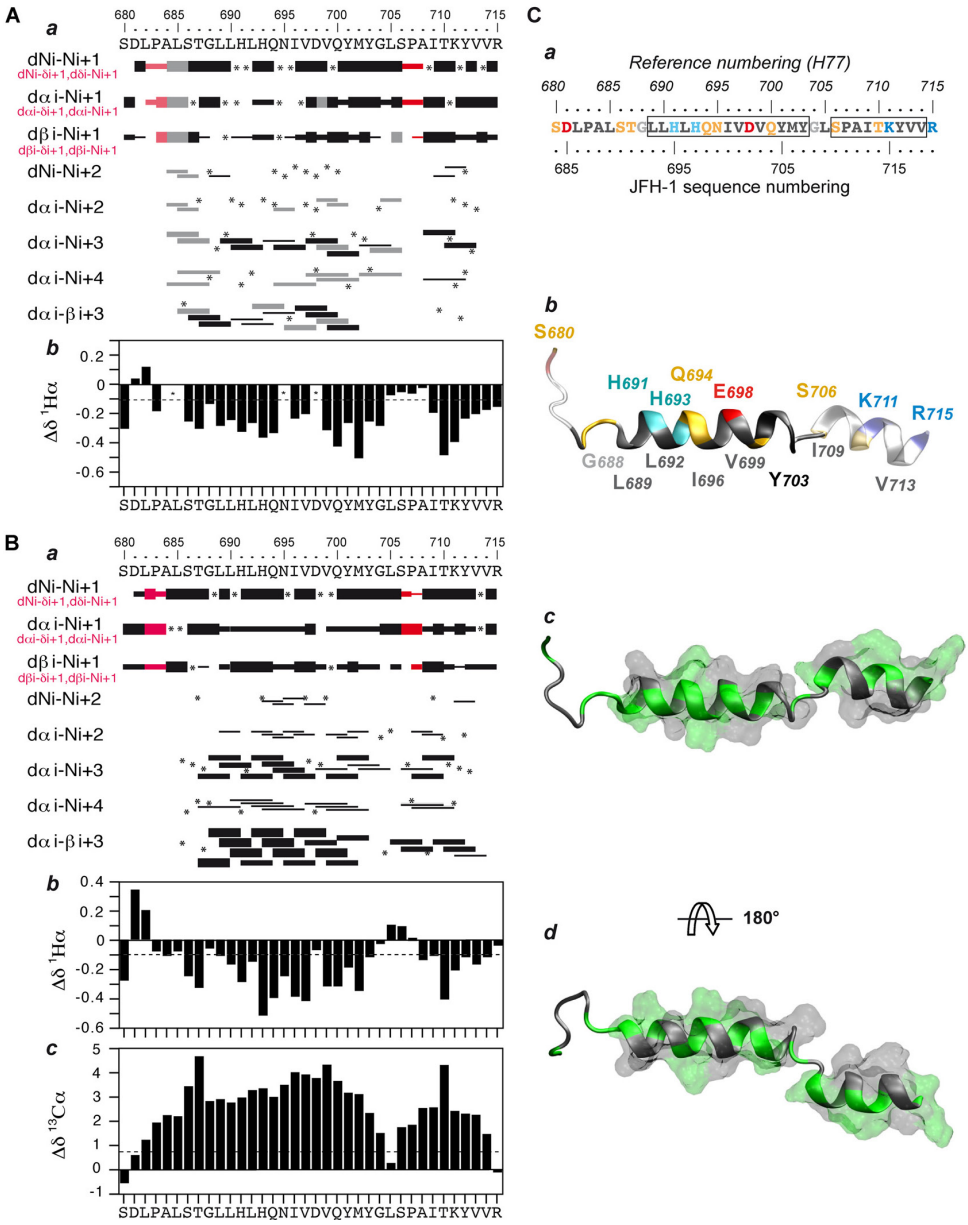

FIG. 6. NMR analysis and structure calculation of the E2-SC peptide. (A) Summary of sequential ($i, i + 1$) and medium-range ($i, i + 2$ to $i, i + 4$) NOEs (panel a) and ^{1}Hα chemical shift differences (in parts per million) (b) in 100 mM SDS. (B) Summary of sequential ($i, i + 1$) and medium-range ($i, i + 2$ to $i, i + 4$) NOEs (panel a) and ^{1}Hα and ^{13}Cα chemical shift differences (b and c) in 50% TFE. Sequential NOEs allowing the assignment of proline residues are indicated in red. Asterisks indicate that the presence of a NOE cross peak was not confirmed because of overlapping resonances (a) or the lack of Hα assignment (b). Intensities of NOEs are indicated by the height of the bars. Bars in gray indicate the NOEs that could not be unambiguously defined because of the incomplete assignment of residues. NMR-derived ^{1}Hα and ^{13}Cα chemical shift differences were calculated by subtraction of the experimental values from the reported random coil conformation values in either SDS (65) or TFE (48), respectively. The dashed lines indicate the standard threshold value of ΔHα (−0.1 ppm) or ΔCα (0.7 ppm [c]) for an α helix. (C). Amino acid sequence and NMR representative structure of E2-SC. (a) Sequence numbering refers to the H77 strain for reference numbering (35) and JFH-1 sequence numbering. Boxes indicate the α-helical segments. Residues are color-coded according to their physicochemical properties. Hydrophobic residues (A, V, L, F, M, I, W, and Y) are dark gray, and glycine residues are light gray. Polar residues (S, N, Q, and T) are yellow, and positively and negatively charged groups of basic (K, R) and acidic (E) residues are blue and red, respectively. Histidine residues are cyan. (b to d) Representative structure model of E2-SC showing the amphipathic character of α-helix 688-702 (aa 694 to 706 in the JFH-1 strain). (b) Side view with backbone residues (ribbon representation) colored as in panel a. (c and d) Hydrophilic side and hydrophobic side views of backbone and surface of amphipathic α-helix 688-702. Hydrophobic and hydrophilic residues are colored gray and green, respectively. Figures were generated from structure coordinates (PDB entry 2KZQ) using VMD (http://www.ks.uiuc.edu/Research/vmd/; [30]) and rendered with POV-Ray (http://www.povray.org).

TABLE 2. Statistics of final simulated annealing structures of the E2-SC peptide

Parameter	Value ± SD
Constraints used	
No. of distance restraints	
Intraresidue	0
Sequential	129
Medium range	87
Total	216
Statistics for the final X-PLOR structures	
No. of structures in the final set	36
X-PLOR energy (kcal · mol^{-1})	−91.9 ± 13.2
NOE violations	
No. > 0.5 Å	None
RMSD (Å)	0.081 ± 0.007
Deviation from idealized covalent geometry	
Angles (°)	0.54 ± 0.01
Impropers (°)	0.345 ± 0.008
Bonds (Å)	0.0037 ± 0.0001
RMSD (Å)	
Backbone (C′, Cα, N)	
Helix segment 689-700	0.44 ± 0.13
All residues	5.45 ± 1.34
All heavy atoms	
Helix segment 689-700	1.14 ± 0.19
All residues	6.78 ± 1.40
Ramachandran data (for 1,080 residues)[a]	
Residues in most-favored regions (%)	71.4
Residues in allowed regions (%)	27.0
Residues in generously allowed regions (%)	1.6
Residues in disallowed regions (%)	0

[a] Ramachandran data are from PROCHECK (37).

connectivities. Apart from this central helix, typical connectivities of the α-helical fold but of weaker intensities are also present in the C terminus of the peptide (aa 706 to 712 or so), indicating the presence of a fraying helix. The NOE-based indications of α-helical conformation were supported by the deviation of the ^{1}Hα and ^{13}Cα chemical shifts from random coil values (75), as shown in Fig. 6B, panels b and c. The long series of negative variation of ^{1}Hα chemical shifts ($\Delta\delta^{1}$Hα, <-0.1 ppm) as well as the positive variation of ^{13}Cα chemical shifts ($\Delta\delta^{13}$Cα, >0.7 ppm) are indeed typical of α-helical conformation.

The comparison of NOE connectivities and variations of Δd^{1}Hα chemical shifts observed with 50% TFE and 100 mM SDS along the peptide sequence reveals that the same segments exhibit α-helical folding (Fig. 6A and B). Interestingly, the N terminus of the central helix seems to extend up to residue 684 in 100 mM SDS. However, the weaker NOE intensities indicate that both helices are flexible and/or lack stability in SDS compared to results observed for 50% TFE. This is consistent with the higher content of α helix as measured by CD in 50% TFE and corresponds to the well-known stabilization of α-helical folding in this medium (3).

Based on the NOE-derived interproton distance constraints obtained with 50% TFE, a set of 50 structures was calculated with X-PLOR, and a final set of 36 low-energy structures that fully satisfied the experimental NMR data was retained. The number and types of NOE constraints used for the structure calculations as well as the statistics for this final set of 36 structures are given in Table 2. Superimposition of the 36 structures (Fig. 7) shows two quite well defined helices connected by a small flexible segment around Gly 704, due to presence of a proline residue in position $i + 3$ (Pro 707). The main part of the central helix (residues 689-700) is well de-

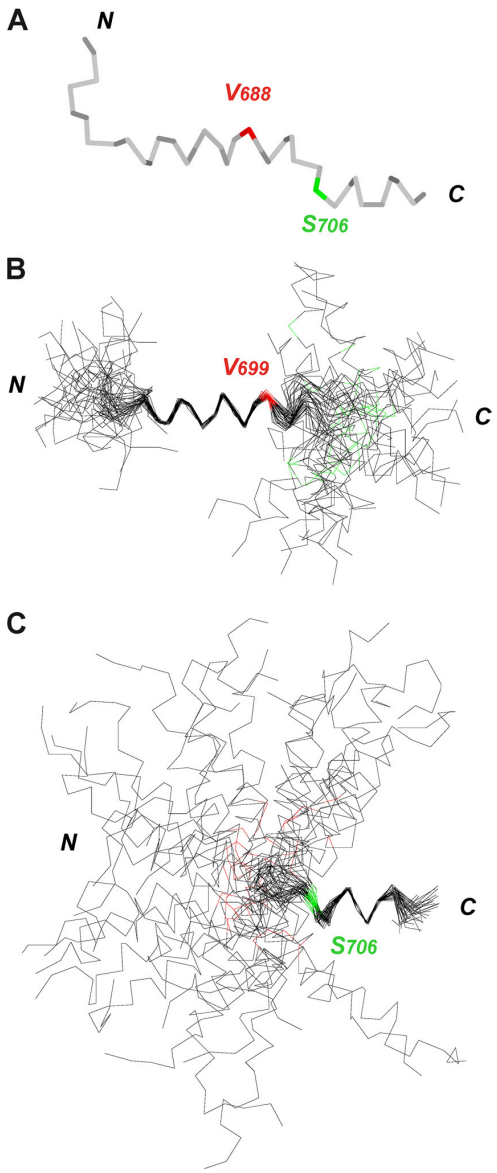

FIG. 7. Structural characterization of the E2-SC peptide in TFE 50%. (A) Representative structure model. (B and C) Superimposition of the backbone heavy atoms (N, Cα, and C′) of the 36 final structures (PDB entry 2KZQ) for the best overlap of residues 688-699 and 709-712, respectively, which correspond to canonical α-helical residues.

fined, with a backbone RMSD of 0.44 Å (Table 2). This helix extended also on some structures up to residue 703. The C-terminal helix is less well defined and behaves as a fraying helix, including residues 706 to 714 or so. The apparent instability of this helix in the E2-SC peptide context is likely due to the lack of the downstream sequence corresponding to the predicted transmembrane helical domain of E2.

As illustrated in Fig. 6C, the asymmetric distribution of polar and hydrophobic residues on each side of the central helix clearly reveals the mainly amphipathic character of this α helix. The hydrophobic residues, including two tyrosine residues, are quite well positioned within or on the edges of the hydrophobic side, suggesting their essential role in a putative interaction with the membrane interface. Indeed, tyrosines are frequently found at the membrane interface (26). These structural features, together with the clear propensity of the E2-SC peptide to adopt an α-helical structure upon binding to lipid-like molecules, suggest that the central amphipathic α helix associates with the membrane interface, at least transiently, in an in-plane topology. The C-terminal helix also appeared to be relatively amphiphilic, with a hydrophobic face formed by amino acids 709-710 and 712-714 and a hydrophilic side of amino acids 711 and 715. Although variability between genotypes 2a and 1a was observed, the conformational properties of this region are conserved. The amphiphilicity of this helix together with the presence of aromatic residues and its location upstream of the TM domain of E2 suggest that it is located at the membrane interface, maybe in an in-plane topology.

The structural data obtained for E2-SC provided us with a framework for additional mutations in the 705-715 region identified in our biological experiments. To further characterize the role of segment 705-715 in HCV entry, we therefore introduced point mutations in this region in the context of the HCVcc system. Based on sequence comparison between genotypes 1a and 2a, we designed a panel of mutants: G704A, P707S, T710A, K711S, Y712A, Y712W, and V713A. Alanine substitutions were introduced to identify the potential role of specific amino acids, whereas the other substitutions were based on the differences between 2a and 1a genotypes. Although a slight decrease in HCVcc infectivity was observed for the G704A, T710A, and K711S mutants, none of the individual mutations can explain the defect in infectivity of chimera 705-715 (Fig. 4C). As these single mutations are not supposed to affect the α-helix structure, these data suggest that several residues within segment 705-715 cooperate to play a role in HCV entry, maybe thanks to multiple binding points with an interacting partner.

DISCUSSION

Viral envelope glycoproteins play an important role at different steps of the viral life cycle. During virion morphogenesis, they take part in the assembly process, whereas in the early steps of the viral life cycle, they are involved in receptor binding and in fusion between the viral envelope and a cellular membrane. To fulfill these functions, viral envelope glycoproteins have to adopt dramatically different conformations at these different steps of the infectious cycle. Importantly, these conformational changes have to occur at a precise time and thus have to be tightly controlled. Despite extensive research on HCV envelope glycoproteins, the structures and functions of these proteins remain poorly understood. Here, we used a genetic approach to identify functional determinants in HCV glycoprotein E2. By generating HCVpp containing E1 and E2 from different genotypes, we identified intergenotypic incompatibilities between these two proteins. By using a nonfunctional E1E2 complex, we identified new functional regions in E2 by exchanging protein regions between two incompatible genotypes. This led to the identification of several determinants of the E2 ectodomain that play a role in E1E2 assembly (Table 1). Furthermore, we also characterized the structural and lipid binding features of the C-terminal part of the ST segment by CD and NMR using a synthetic peptide denoted E2-SC. This segment, which is involved in HCV entry and located close to the TM domain, includes a central amphipathic helix, which folds upon binding to lipid mimetics. Its features suggest that this helix could easily switch from helical to random conformation, depending on its microenvironment and/or binding partners. Together, these data highlight new functional regions in HCV envelope glycoprotein E2.

Intergenotypic incompatibilities exist between HCV glycoproteins E1 and E2 from different genotypes. Although the overall structure of HCV proteins is not expected to differ significantly between HCV genotypes, coevolution within a genotype or subtype can potentially lead to functional incompatibilities between partner proteins. Such genetic incompatibilities have indeed already been reported for HCV, highlighting potential protein-protein interactions between viral polypeptides (2, 77). In the case of HCV envelope glycoproteins, biochemical analyses have shown that they assemble in the cell as noncovalent heterodimers (11). Furthermore, these interactions are important for the cooperative folding of these two proteins (reviewed in reference 38). It is therefore not surprising that HCV envelope glycoproteins have coevolved in the different genotypes and that this coevolution can lead to functional intergenotypic incompatibilities between E1 and E2. It is however more puzzling that in the case of genotype 1a, E1 was compatible with the E2 of any genotype tested. A potential explanation for this phenotype is that the E1 protein of genotype 1a has more flexibility to accommodate changes in conformation or oligomerization during the fusion process. However, this needs further investigation.

Residues of the ST region close to the TM domain play a major role in HCV entry. Indeed, HCVcc infectivity was reduced by almost 3 logs when segment 705-715 of genotype 1a was introduced in the context of E1E2 of genotype 2a. We have previously shown that the interactions between TM domains play a major role in E1E2 heterodimerization (8) as well as in virus entry (5, 6). Since segment 705-715 corresponds to the upstream sequence of the E2 transmembrane domain, these two regions might be functionally connected for the assembly of the E1E2 heterodimer. However, heterodimerization was not affected for the 705-715 chimera, as shown by coprecipitation in a CD81 pull-down assay. Furthermore, particle assembly and release was not affected by this mutation. The incompatibility between genotypes 1a and 2a in the ST region therefore highlights a role for the 705-715 segment in the entry process. The ST region is relatively flexible and is supposed to play a major role in the reorganization of the envelope glycoproteins during the fusion process (34). Amino acid residues

705 to 715 are therefore likely involved in interactions with E1 region at some stage of the fusion process.

The aa sequence analyses and the structural investigations of the corresponding synthetic peptide E2-SC by CD in various media clearly show that this region exhibits potential lipid binding properties and could fold into α helices upon binding. The three-dimensional structure analysis of this peptide in SDS or 50% TFE used to probe the peptide conformational preferences as well as to mimic the membrane environment revealed that the major structural elements consist of a central amphipathic helix (689 to 703, but it could extend to aa 684 as revealed by SDS analysis) and a C-terminal fraying helix (706 to 714 or so) connected by a short flexible segment, including a glycine residue (704). Both helices require a hydrophobic environment for folding, indicating that lipid interactions and/or protein interactions could contribute to their structural stability. In addition, the fraying of the C-terminal helix could be due to the absence of the downstream transmembrane sequence, which would likely stabilize this helix. Its relative amphiphilicity and its connection with the TM of E2 suggest that this helix should be located at the membrane interface, maybe with an in-plane topology. The amphipathic nature of the highly conserved central helix 689-703 together with the helix folding upon binding to lipid mimetics suggest that this helix could bind in-plane to a membrane interface. However, the relatively low free energy of membrane association, as calculated using the MPEX program (74), suggests that this helix could be easily released from the membrane interface. This is consistent with the limited stability of this amphipathic helix in SDS. One can thus hypothesize that this helix is able to ensure a conformational transition between helix folding when bound to the membrane interface and a coil state upon membrane release. According to the conformational change of the stem region proposed in the model of fusion events for class II glycoproteins (22, 23), it is tempting to speculate that this amphipathic helix could ultimately fold again upon binding to the trimeric postfusion complex of glycoproteins.

HVR2 and IgVR are essential determinants for HCV particle assembly. Based on results with HVR1 mutants, it was thought that the most variable regions on of E2 would be dispensable for HCV infectivity. Indeed, although it reduces infectivity, the deletion of HVR1 in the context of an infectious virus is not lethal (21). Furthermore, deletion of the three variable regions HVR1, HVR2, and IgVR in a truncated form of E2 does not seem to affect its folding as measured by binding of conformation-dependent MAbs and CD81 pull-down (44). However, very recent data indicate that deletion of HVR2 or IgVR is lethal in the HCVcc system, suggesting that the presence of these regions in E2 can play a functional role in virus assembly (45). In our case, we used a less drastic approach consisting of replacing these regions with the corresponding segment from another genotype. In such chimeric viruses, introducing HVR2 or IgVR from genotype 1a in the context of an infectious clone of genotype 2a was also lethal for the production of infectious virus, which was due to an alteration in the production of viral particles as measured by a core release assay.

Exchanging HVR2 or IgVR affects the incorporation of E1 into HCVpp. In the context of the HCVpp system, the recognition of the chimeric E2 by CD81 suggests that at least domain DI of this protein is properly folded after replacing HVR2 or IgVR by the corresponding region from another genotype. However, the level of incorporation of the E1 into HCVpp was barely detectable for these chimeras. This lack of E1 incorporation into HCVpp is in contrast with the interactions between E1 and E2 as observed in the CD81 pull-down assay. However, further analyses of the intracellular E1E2 complexes recognized by CD81 indicated that E1 formed disulfide bond-linked high-molecular-weight complexes with these chimeric E2 proteins (data not shown). Interestingly, we have recently shown that in the context of the HCVcc system, virion-associated E1 and E2 envelope glycoproteins form large covalent complexes stabilized by disulfide bridges, whereas the intracellular forms of these proteins assemble as noncovalent heterodimers (70). The presence of disulfide bridges between HCV envelope glycoproteins suggests that lateral protein-protein interactions assisted by disulfide-bond formation might play an active role in the budding process of HCV particles. Therefore, we cannot exclude the possibility that in the context of our chimeric viruses, the introduction of HVR2 or IgVR from another genotype leads to the formation of premature intermolecular disulfide bonds within infected cells, which is no longer coordinated with the other steps of the assembly process. It is worth noting that, when the whole ectodomain of E2 was replaced by the corresponding sequence from genotype 1a, E1 was correctly incorporated into HCVpp, suggesting that chimeras containing HVR2 or IgVR induce an additional defect, which is likely due to interdomain incompatibilities within E2. There might indeed be molecular cross-talk between HVR2 or IgVR and domain DIII and/or the ST region, since a defect in assembly was also observed when we dissociated the DI-DII region from the DIII-TM region (Fig. 1).

In addition to interdomain incompatibilities within E2, we also identified a genetic incompatibility within a single E2 domain. Indeed, exchanging sequences between genotypes 1a and 2a within domain DI can lead to protein misfolding. as shown by replacing DIb of genotype 2a with the equivalent sequence from genotype 1a, which led to the absence of recognition of E2 by CD81. Importantly, infectivity and CD81 binding were restored when both DIa and DIb of genotype 1a were introduced in the context of E1E2 of genotype 2a. Therefore, our data indicate that the two parts of the DI domain interact together to form the major CD81 binding determinant, which is in agreement with the recently proposed E2 model (34). It is worth noting that the chimeric E2 protein containing DI domain from genotype 1a was still less efficiently pulled down by CD81, suggesting that in addition to the major CD81 determinants identified in DI (13, 56), other residues within DIII might also modulate CD81 binding, as suggested previously (34, 62). However, we cannot exclude the possibility that the involvement of domain DIII is indirect since mutations in this domain, which affect CD81 binding, can affect DIII folding (31).

In this study, we cannot exclude the possibility of some effect of the changes introduced in E1 in reconstructing the A4 epitope. Indeed, we observed some decrease in HCVpp infectivity when the A4 epitope was engineered with E1 of genotype 2a, suggesting some modulation of E1E2 interaction mediated by this region. However, JFH1 infectivity was very similar in the presence or absence of the modified epitope, suggesting

that the change in the A4 epitope has only minor effects in the HCVcc system.

In conclusion, we identified several important determinants of E2 ectodomain that are involved in virion assembly or in HCV entry. We also revealed the presence of a well conserved amphipathic helix in the stem region, which likely undergoes conformational changes during the fusion process. Together, these data highlight the complexity of the intermolecular interplay between E1 and E2.

ACKNOWLEDGMENTS

We are grateful to Czeslaw Wychowski, Nathalie Callens, François Helle, and Costin-Ioan Popescu for their scientific input. We thank Sumedha Roy and Bhavna Tandon for their technical assistance. We also thank Sophana Ung for advice on preparing the figures. We are also grateful to T. Wakita, F. L. Cosset, R. Bartenschlager, and J. McKeating for providing reagents. Peptide synthesis and CD experiments were performed with the platforms Centre Commun de Microanalyse des Proteines and Production et Analyze de Proteines from the IFR 128 BioSciences Gerland—Lyon Sud.

This work was supported by the French Agence Nationale de Recherche sur le sida et les hépatites virales (ANRS), a Marie Curie Research Training Network grant (MRTN-CT-2006-035599), and the European Commission through the EMBRACE project (LHSG-CT-2004-512092). A.A. was successively supported by a Marie Curie Research Training Network grant (MRTN-CT-2006-035599) and a fellowship from the ANRS. J.D. is an international scholar of the Howard Hughes Medical Institute.

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II. Role of LDL receptor in the Hepatitis C virus life cycle

ROLE OF LDL RECEPTOR IN THE HEPATITIS C VIRUS LIFE CYCLE

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Running title: LDL receptor and HCV life cycle

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ABSTRACT

Hepatitis C virus (HCV) particles are known to be in complex with lipoproteins. As a result of this interaction, the LDL receptor (LDLR) has been proposed as a potential entry factor for HCV. However, the role of this receptor in lipoprotein degradation is in contradiction with an implication of the LDLR in a productive HCV entry. Here, we re-investigated the role of the LDLR in the HCV life cycle by comparing virus entry to the mechanism of lipoprotein uptake. A soluble form of the LDLR inhibited HCV entry, suggesting a direct interaction between HCV particle and the LDLR. Furthermore, a siRNA targeting the LDLR in Huh7 cells reduced HCV infectivity, confirming that this receptor plays a role in the life cycle of infectious virus generated in cell culture. However, the knock down the LDLR-specific adaptor protein autosomal recessive hypercholesterolemia (ARH) did not affect HCV infectivity, and the half-time of HCV internalization was different from the kinetics of internalization of lipoproteins. Furthermore, a decrease in HCV RNA replication was observed by blocking the LDLR with a specific antibody, and this was associated with an increase in the ratio of phosphatidylethanolamine to phosphatidylcholine in host cells. Finally, we confirmed that modification of HCV particles by the lipoprotein lipase reduced HCV infectivity but increased HCV binding. Altogether, these data suggest that LDLR internalization might not be essential in the HCV life cycle, whereas the physiological function of this receptor is important for optimal replication of HCV genome.

INTRODUCTION

With 120 to 180 million chronically infected individuals worldwide, hepatitis C virus (HCV) infection represents a major cause of severe liver disease (41). This virus primarily infects human hepatocytes, which over time leads to chronic inflammation, progressive fibrosis and development of hepatocellular carcinoma. HCV is a positive-stranded RNA virus that belongs to the *Flaviviridae* family (39). For a long time, it remained difficult to study the HCV life cycle due to problems in propagating this virus in cell culture. Fortunately, the development of a cell culture system that allows for a relatively efficient amplification of HCV (HCVcc) (37, 67, 70) has brought to an end the difficulties in working on this virus at the cellular level.

To initiate its life cycle, HCV has to cross the plasma membrane of hepatocytes and gain access to the cytosol of these cells. Increasing evidences indicate that HCV entry is a complex and tightly regulated process (reviewed in (18)). Several studies suggest that heparan sulfate proteoglycans may serve as an initial docking site for HCV attachment (6, 7, 33). After the initial attachment to the host cell, a virus generally binds to specific entry factors, which are responsible for initiating a series of events eventually leading to the release of the viral genome into the cytosol. Several cell surface proteins have been described as specific entry factors for HCV. They include the tetraspanin CD81 (56), the scavenger receptor BI (SRBI)(63) and the tight junction proteins Claudin-1 (20) and Occludin (58). However, the precise role of these specific entry factors in HCV entry remains to be determined. Furthermore, the identification of EWI-2wint as a new partner of CD81 that blocks E2-CD81 interaction provides additional evidence of the complexity of the HCV entry process (60). Finally, after binding to the plasma membrane and interaction with specific entry factors, HCV enters target cells by clathrin-mediated endocytosis (10) and fusion has been proposed to take place in the early endosomes (43).

The HCV particle is composed of a nucleocapsid surrounded by a host cell-derived membrane envelope that contains the viral glycoproteins E1 and E2 (reviewed in (34)). Importantly, plasma-derived HCV particles have been reported to be in complex with very low-density lipoproteins (VLDL) (reviewed in (3)). It has also been shown that HCV production in hepatoma cells is dependent on assembly and secretion of VLDL lipoproteins (23, 30). Furthermore, the composition or maturation of the lipoproteins associated with HCV particles has been suggested to modulate their infectivity (24, 38). Finally, the participation of different apolipoproteins in the make up of cell culture derived virus has been confirmed by the neutralization of HCV infectivity with antibodies against ApoB, ApoE or ApoCI (4, 14, 29, 44). Thus, it appears that HCV particles interact with VLDL during the assembly of the lipoprotein particles and are secreted together with VLDL in the form of what has been called lipoviroparticle (3). However, the nature of the

association between HCV and VLDL remains unclear. Whatever the nature of this interaction, it gives the virus the opportunity of using lipoprotein receptors to bind to target cells (reviewed in (13)).

As a result of the association between HCV and lipoproteins, the LDL receptor (LDLR) has been proposed as a potential entry factor for HCV (1, 48). Cell surface adsorption of HCV particles isolated from patients and accumulation of intracellular viral RNA can be inhibited by antibodies directed against the LDLR as well as by purified LDL or VLDL. Furthermore, a correlation has been shown between the accumulation of HCV RNA into primary hepatocytes, expression of LDLR mRNA and LDL entry (47). The potential involvement of the LDLR in HCV entry has also been recently reported in the HCVcc system (55).

Lipoproteins are released from the liver in the VLDL form. Nascent VLDL particles released into plasma are not ligands for the LDLR. However, upon processing by lipoprotein lipase (LPL), which hydrolyzes the triglycerides in the core of the lipoprotein particles, a large proportion (70%) of the resulting intermediate density lipoproteins (IDL) is efficiently removed from plasma by the hepatocytes. This process is supposed to depend on the interaction between LDLR and apoE located on IDL. The remaining IDL in the circulation is converted to LDL by a reaction catalyzed by hepatic lipase, which further reduces the amount of triglycerides in the lipoprotein particles (15). More recently, it has also been proposed that SRBI could also be used as an alternative uptake pathway for processed VLDL (61).

Although several data are in favor for a role of the LDLR in HCV entry, some discrepancies remain. Indeed, lipoproteins are released from the LDLR at low pH in late endosomes (62), which might potentially lead to viral particle degradation. Furthermore, the kinetics of internalization of the LDLR (12) does not fit with the slow internalization suggested for HCV (43). Finally, other steps of the HCV life cycle are dependent on lipid metabolism (reviewed in (69)), and interfering with the LDLR might also potentially affect a post-entry step of the HCV life cycle. Here, we re-investigated the role of the LDLR in the HCV life cycle by comparing virus entry to the mechanism of LDL and IDL lipoproteins uptake. We show that HCV particle can interact with the LDLR. However, this interaction does not seem to lead to a productive infection. Furthermore, our data are in favor for a role of the LDLR as lipid providing receptor, which modulates viral RNA replication.

MATERIALS AND METHODS

Cell culture

Huh-7 human hepatoma cells (50), HEK293T cells were grown in Dulbecco's modified essential medium (Invitrogen) supplemented with 10% fetal bovine serum. BHK-21 cells were grown in Minimum essential medium (Invitrogen) supplemented with 10% fetal bovine serum.

Antibodies and reagents

Monoclonal antibodies C7 anti-LDLR and Q-13 anti-ARH were from Santa Cruz Biotechnology. Polyclonal anti-LDLR antibody was from Progen Biotechnik. Polyclonal anti-SRBI antibody was from Novus Bio. Polyclonal anti-apoE antibody was from Millipore. Monoclonal antibody (Mab) 5A6 anti-CD81 was kindly provided by S. Levy (Stanford University). Recombinant soluble form of human LDLR (sLDLR) was provided by R&D Systems. LPL from bovine milk and LPL inhibitor tetrahydrolipstatin (THL) were purchased from Sigma-Aldrich. Low-density lipoproteins conjugated to DiI (DiI-LDL) and DiI lipophilic tracer were from Molecular Probes, Invitrogen. IDL and LDL from human plasma were purchased from Athens Research & Technology.

Production of HCV and Sindbis virus

In this work, we used a modified version of the plasmid encoding the full-length JFH1 genome (genotype 2a; GenBank access number AB237837), kindly provided by T. Wakita (National Institute of Infectious Diseases, Tokyo, Japan) (67). This modified virus contains mutations at the C-terminus of the core protein leading to amino acid changes F172C and P173S, which have been shown to increase the viral titers (16). Furthermore, the N-terminal E1 sequence encoding residues ^{196}TSSSYMVTNDC has been modified to reconstitute the A4 epitope (SSGLYHVTNDC) (19) as described (26). In some experiments, we also used a JFH-1 virus containing a *Renilla* luciferase reporter gene. In this construct, the *Renilla* luciferase gene is fused with the viral open reading frame in a monocistronic configuration. Stocks of JFH1 and JFH1-Luc were generated by transfection of in vitro-transcribed RNA into Huh-7 cells. HCV RNA was prepared as described previously (16, 26) with mMESSAGE mMACHINE T7 *in vitro* transcription kit (Ambion). A GND replication-deficient clone with a mutation in NS5B active site was used as a negative control (67). Stocks of Toto1101/Luc (9), a Sindbis virus (SINV) expressing the *Firefly* luciferase (kindly provided by M. MacDonald from the Rockefeller University), were generated by electroporation of in vitro-transcribed RNA into BHK-21 cells. Briefly, 15μg of RNA was mixed with 4 x 10^6 BHK-21 cells and cells were electroporated with one square wave at 25μF and 140V. Supernatant was collected after 48h.

HCVpseudoparticle (HCVpp) assay

HCVpp containing the *Firefly* luciferase reporter gene were produced as described previously (8, 53, 54). pcDNA3.1 plasmids expressing HCV envelope glycoproteins from JFH1 isolate or VSV G protein were used to generate HCVpp. Supernatants containing the pseudotyped particles were harvested 48h after transfection and filtered through 0.45 μm pore-sized membranes. HCVpp were added to Huh-7 cells seeded the day before in 24-well plates and incubated for 2h at 37°C. The supernatants were then removed, and the cells were incubated in Dulbecco's modified essential medium, 10% fetal bovine serum at 37 °C. At 48h post-infection, luciferase activities were measured as indicated by the manufacturer (Promega).

siRNA transfections

ON-TARGETplus SMARTpools containing siRNA targeting SRBI (SCARB1), CD81, ARH and non-targeting siRNA were provided by Dharmacon. siRNA duplex of GGACAGAUAUCAUCAACGA and UCGUUGAUGAUAUCUGUCC targeting LDLR (55) was provided by Sigma. Transfections using Oligofectamine (Invitrogen) were performed as described previously (26). Subconfluent cultures of Huh-7 cells in 6-well plates were transfected twice with synthetic double-stranded siRNA. The interval between both siRNA transfections was 48h. Cells were trypsinized 24h after the second siRNA transfection and plated in 24-well and 12-well plates. The following day, cells from the 12-well plates were lyzed with 1% Triton-X 100 and used for Western blotting analysis to verify protein down-regulation. Cells in the 24-well plates were infected with HCVcc, HCVpp, VSVpp or SINV and lyzed at 16h (SINV) or 48h (HCVcc, HCVpp and VSVpp) post-infection to measure the luciferase activity. *Firefly*/*Renilla* luciferase activity was measured with a kit from Promega, as recommended by the manufacturer.

Neutralization and inhibition assays

Supernatants containing viruses or pseudotypes were preincubated with different concentrations of antibodies or sLDLR for 1h or 2h at 37°C. Preincubation was followed by 2h infection of Huh-7 cells at 37°C. After removing the antibodies or sLDLR, cells were washed and fresh medium was added. At 48h post-infection, luciferase activities were measured. For the experiments of the kinetics of neutralization, antibodies were added at different time points after infection. To analyze the effect of LPL on virus infectivity, viruses were preincubated with different concentrations of LPL and/or THL for 1h at 4°C or 37°C followed by 1h infection of naïve cells at 4°C or 37°C. The virus was then removed and fresh medium added. At 48h post-infection, luciferase activities were measured.

Replication analysis

Huh-7 cells were electroporated with 15μg of *in vitro* transcribed viral RNA as described above. Ten min after electroporation, cells were transferred into culture medium containing Mab C7 or a non-specific Mab. The final antibody concentration was set to 5μg/ml. Cells were seeded into 24-well plates and replication was assessed after 4, 24, 48 and 72 h by measuring *Renilla* luciferase activities in electroporated cells with a Berthold CentroXS3 LB 960 Luminometer as indicated by the manufacturer (Promega).

Lipid analysis

Cells treated with Mab C7 or a non-specific Mab, as described above, were processed for lipid analysis. Cells were homogenized with 0.5ml of methanol/ 5mM EGTA (2:1 v/v). 50μl aliquots were evaporated, the dry pellets were dissolved in 0.25 ml of NaOH (0.1M) overnight and proteins were measured with the Bio-Rad assay. Neutral lipid analysis was performed as follows. Lipids corresponding to 0.1 ml of the homogenat were extracted according to Bligh and Dyer (11) in chloroform/methanol/water (2.5 :2.5 :2.1, v/v/v), in the presence of the internal standards : 6 μg of stigmasterol, 3 μg of cholesteryl heptadecanoate, 6μg of glyceryl trinonadecanoate. Chloroform phase was evaporated to dryness, and dissolved in 20μl of ethyl acetate. 1μl of the lipid extract was analyzed by gas-liquid chromatography on a FOCUS Thermo Electron system using a Zebron-1 Phenomenex fused silica capillary columns (5m X 0,32mm i.d, 0.50 μm film thickness)(5). Oven temperature was programmed from 200°C to 350°C at a rate of 5°C per min and the carrier gas was hydrogen (0.5 bar). Injector and detector were at 315°C and 345°C, respectively. Phospholipids were analyzed as follows. Lipids corresponding to 0.35 ml of the homogenat were extracted according to Bligh and Dyer (11) in chloroform/methanol/water (2.5 :2.5 :2.1, v/v/v). Chloroform phase was evaporated to dryness. Lipid extract was analysed by HPLC (DIONEX Summit) on a Uptisphere6OH analytical column (5 μm particle size, 250 x 2.1 mm) fitted with a DIOL guard column cartridge (10X2.1mm mm; INTERCHIM) and coupled to a light scattering detector (Polymer Laboratory ELS 2100, nitrogen flow 1.8 ml/min, evaporating temperature 50°C, and nebulizer temperature 80°C). Separation was achieved at a flow rate of 0.25 ml/min using a gradient of B (isopropanol:water:triethylamin:acetic acid (v/v/v/v ; 85/15/0.014/0.5)) in A (Hexane:isopropanol : Triethylamin:acetic acid (v/v/v/v ; 82/18/0.014/0.5) from 5 to 35% of B in 35min.

Production of DiI-conjugated lipoproteins, internalization assay and flow cytometry analyses

IDL (0.2 mg) were mixed with 10 μg of DiI in 0.5 ml total volume of PBS with 0.5% BSA. After

overnight incubation at 37°C, the suspension was adjusted to a density of 1.019 g/mL with NaBr, followed by ultracentrifugation for 4h at 435,000 x g in TLA110 Beckman rotor. Lipoproteins were collected from the top of the gradient and dialyzed 5 times against PBS. Protein content in the lipoprotein suspension was determined by BCA protein assay (Pierce). Ten μg/ml of DiI-conjugated lipoproteins was incubated with Huh-7 cells for 2h at 37°C in serum free medium in the presence or absence of antibody. Cells were then extensively washed with cold PBS containing 2% BSA and then incubated for 1h at 4°C with 2mM EDTA in PBS. Detached cells were rinsed once with PBS 2% bovine serum albumin and then fixed with 10% paraformaldehyde. The fluorescence was measured using FL2 channel by FACS Beckman EPICS-XL MCL.

Kinetics of internalization

HCVcc or HCVpp were incubated with Huh-7 cells for 1h at 4°C. Cells were then rinsed with culture medium and the temperature was shifted to 37°C. At different time points, internalization was stopped by washing cells once with PBS, followed by trypsynization for 1h at 4°C to remove virus attached to the cell surface. Trypsinized cells were then seeded into new wells and incubated for an additional 48h. The kinetics of internalization was determined by measuring the luciferase activity. For the kinetics of internalization of lipoproteins, 10 μg/ml of DiI-LDL or DiI-IDL were incubated with Huh-7 cells for 1h at 4°C in DMEM without bicarbonate containing 25mM HEPES buffer. Cells were rinsed once with cold PBS and the temperature was shifted to 37°C by adding warm DMEM to allow internalization. The reaction was stopped at different time points by 1h incubation with 10 mg/ml of heparin at 4°C, which allowed the release of cell bound non-internalized lipoproteins (25). Internalization was then analyzed by flow cytometry.

RESULTS

Soluble LDLR inhibits HCV infection

As a result of the association between HCV and lipoproteins, the LDLR has been proposed as a potential entry factor for HCV (1, 48). Furthermore, the potential involvement of the LDLR in HCV entry has recently been reported in the HCVcc system (55). However, due to the role of the LDLR in the lipid delivery and the dependence of HCV on lipid metabolism (59, 69), it remains difficult to draw clear conclusions on how this receptor is involved in the HCV life cycle. To investigate the role of the LDLR in HCV entry we first used a soluble form of the LDLR (sLDLR) to determine whether it would inhibit HCV infectivity. As shown in Figure 1, sLDLR inhibited HCVcc infectivity in a dose dependent manner with a residual infectivity of approximately 20% at a

concentration of 5 μg/ml. In the same conditions, 10 μg/ml of CD81-LEL decreased HCV entry only by 40% (data not shown), suggesting that on HCVcc particle, the lipoprotein component is more exposed than E1E2 complexes. As expected, there was no effect of sLDLR on SINV infectivity, a virus that is not dependent on LDLR for its entry. In contrast to the HCVcc system, sLDLR had barely any effect on HCVpp infectivity (Figure 1). This is likely due to differences in the composition of HCVpp as compared to HCVcc particles. Indeed, due to their production in 293T cells and their difference in the assembly process, HCVpp are not supposed to be associated with VLDL. Together, these results suggest that the LDLR can potentially interact with infectious HCV particles generated in cell culture.

The LDLR plays a role in the HCV life cycle

To further investigate the role of the LDLR in the HCV life cycle, we used RNA interference to knock down LDLR expression. In addition, siRNA targeting other entry factors (CD81 and SRBI) were used as positive controls. As previously shown, the knock down of CD81 and SRBI strongly reduced HCVcc infectivity, with approximately 20% and 30% of residual infectivity for CD81 and SRBI, respectively (Figure 2A). Furthermore, HCVcc infectivity was reduced to approximately 50% in cells treated with the LDLR siRNA. Although the LDLR siRNA was less drastic in reducing HCV infectivity as compared to CD81 and SRBI siRNAs (Figure 2A), the efficacy of knock down on the LDLR was also less pronounced (Figure 2B), suggesting that our analysis might underestimate the effect of the LDLR knock-down on HCV infectivity. Similar results on the effect of LDLR knock-down in HCV infection have indeed been recently reported (55).

We also analyzed the effects of knocking down the LDLR in the HCVpp system. As shown in Figure 2A, HCVpp infectivity was reduced to a similar level as for HCVcc in cells treated with CD81 or SRBI siRNA, which is in agreement with the role of these molecules in HCV entry. Retroviral pseudoparticles containing VSV G protein were only slightly affected by CD81 and SRBI knock down. In contrast to HCVcc, HCVpp entry was barely affected by the knock down of the LDLR. As discussed above, this is likely due to differences in the composition of the HCVpp as compared to HCVcc particles.

Although the effects of LDLR knock down supports a role for this receptor in the HCV life cycle, it does not prove that the LDLR is used by the virus to enter the hepatocyte. Since LDLR is known to internalize lipoproteins, viral particles associated to such lipoproteins could be internalized by this receptor. Another approach to investigate the role of the LDLR in the HCV life cycle is therefore to block its uptake without affecting its expression. We therefore also knocked down the LDLR-specific adaptor protein autosomal recessive hypercholesterolemia (ARH) (21, 46). Indeed, clathrin-mediated endocytosis of the LDLR in cultured hepatocytes is strictly

dependent on ARH adaptor (22, 45, 65). Despite a strong reduction in ARH expression in siRNA treated cells, HCVcc infectivity remained close to 80% when this adaptor molecule was knocked down (Figure 2A), suggesting that LDLR uptake might not be essential in the HCV life cycle.

Together, these results suggest that the LDLR can play a role in the life cycle of infectious virus generated in cell culture. However, LDLR internalization might not be essential in the HCV life cycle.

Comparison of the internalization kinetics between HCVcc and lipoproteins

It is thought that HCV is slowly internalized by the hepatocytes. Indeed half-maximal HCVpp internalization has been shown to occur approximately 50 min after initiation of entry, as determined by proteinase K sensitivity (43). In contrast, the kinetics of internalization of the LDLR is much more rapid. Indeed, this receptor is known to undergo a continuous process of constitutive recycling via clathrin-coated pits on the cell surface at the rate of one cycle every 3–12 min (12, 27). Besides the fact that the knock down of ARH barely affects HCVcc infectivity, the differences in the kinetics of internalization are not in favor for a role of the LDL receptor in HCV internalization. To further investigate this discrepancy, we analyzed the kinetics of internalization of the viral particle in the context of the HCVcc system, which might potentially be different from HCVpp uptake due to the presence of lipoproteins. For this experiment, infections were synchronized by performing the virus attachment step at 4°C and then shifting the temperature to 37°C to enable virus internalization. Internalization was analyzed at different time points by removing surface-bound virus with trypsin and determining viral infectivity at 48h post-infection. As shown in Figure 3A, similarly to what was observed for the HCVpp particles, HCVcc was also slowly internalized in Huh-7 cells with a half-maximal rate of internalization of approximately 50 min for both types of particles. These observations indicate that the lipoproteins associated with the viral particles in the HCVcc system do not affect the rate of internalization of this virus.

Internalization of DiI-conjugated lipoproteins was tested by flow cytometry. Kinetics of internalization was performed similarly to virus experiments, however, surface-bound lipoproteins were removed by heparin treatment instead of trypsin (25). In contrast to what was observed for HCV particles, internalization of lipoproteins was very rapid as reported before (12). As shown in Figure 3B, all IDL and LDL used in the experiment were inside the cells after 30 min. At later time points, fluorescence decreased, likely due to lipoprotein degradation. These results demonstrate that HCV is not internalized with the same kinetics as lipoproteins, suggesting two distinct uptake pathways.

LDLR function is important for HCV replication

To further analyze the role of the LDLR in the HCV cell cycle we analyzed the effect of blocking this receptor with a specific antibody. For this approach, we used Mab C7, a well characterized antibody which binds the first repeat of the ligand-binding domain of the LDLR and partly blocks lipoprotein binding (51). We first verified whether Mab C7 could reduce lipoprotein internalization in our experimental conditions. Huh-7 cells were incubated with DiI conjugated lipoproteins for 2h in the presence or absence of Mab C7 and lipoprotein internalization was determined by flow cytometry. As shown in Figure 4A, the antibody partially reduced LDL and IDL internalization (87% of IDL and 78% of LDL). We then analyzed the effect of this antibody on HCVcc infectivity. As shown in Figure 4B, when the antibody was present only during the 2h of virus infection, no decrease in HCVcc infectivity was observed. In contrast, we observed a strong decrease in HCVcc infectivity when the antibody was present in the cell culture supernatant for about 24h. In these conditions, HCVcc infectivity was reduced to approximately 40% (Figure 4B). Furthermore, when the antibody was added overnight at 8h post-infection, we also observed a drop in HCVcc infectivity to 56%.

These observations suggest that instead of playing an active role in HCV entry, the LDLR might rather be involved in a post-entry step. To further investigate this hypothesis, Huh-7 cells were electroporated with HCV RNA and incubated in the presence or absence of Mab C7. As shown in Figure 5A, a decrease in HCV replication was observed as measured by the luciferase activity of the reporter gene. Indeed, the luciferase activity was reduced by 1 $\log_{10}$ at 24h post-electroporation. However, no further decrease was observed over time since the replication curves remained parallel at 48h and 72h. However, we cannot exclude that, at later time points, replication in the presence Mab C7 was less affected due to constant antibody internalization leading to a decrease in their concentration. Furthermore, upon HCV infection intracellular lipid biosynthesis pathways can be upregulated, potentially decreasing the role of lipids taken up by the LDLR. Finally, we cannot exclude that the initiation of HCV replication may require different lipid conditions than later during established infection. It is worth noting that the drop in luciferase expression does not seem to be due to an effect on translation since the luciferase values did not differ at 4h after electroporation (Figure 5A). As a control experiment, we also incubated Huh-7 cells electroporated with SINV to determine whether the effect would be specific for HCV. As shown in Figure 5A, only a slight decrease in SINV replication was observed at 24h post-electroporation. Furthermore, the level of replication was the same as for the controls at 48h, whereas at 72h a slight increase in replication was observed. It is worth noting that due to differences in the kinetics of replication between HCV and SINV, the curves had different shapes for these two viruses.

The effect of Mab C7 on HCV replication can potentially be due to a decrease in lipoprotein uptake, which might results in intracellular decrease of some lipids essential for HCV replication. Therefore, we analyzed the lipid content of Huh-7 cells after 24h of Mab C7 treatment and we found indeed that both neutral lipid and phospholipid content is modified. As shown in Figure 5B, in cells treated with C7 Mab, the ratio between free cholesterol and cholesterol esters (CE) is shifted in favor of CE. Moreover, changes are also observed in phospolipid content. Phosphatidylethalonamine (PE) and phosphatidylcholine (PC) are the major phospholipids in cell membranes. In C7 Mab treated cells, PE content was higher than in control cells. In contrast, PC content was lower, indicating that the proportion between these two phospolipids is affected. Together, these results indicate that blocking the LDLR with C7 Mab affects the ratio of several host cell lipids.

Effect of LPL on HCV infectivity

Changes in the composition of the lipoproteins are known to affect their interaction with the LDLR. In the blood circulation, lipoproteins are known to be altered by LPL, an enzyme that modifies triglyceride-rich lipoproteins and targets them to the liver (28). LPL hydrolyses triglycerides in VLDL and mediates interactions with heparan sulfate enabling lipoprotein clearance from the circulation (42). Due to the potential role of lipoproteins in HCV entry, by modifying their composition, LPL might potentially affect HCV entry. Indeed LPL has already been shown to increase HCV binding to target cells and to inhibit virus infection (4), suggesting that LPL might promote HCV internalization by the same mechanism as lipoprotein uptake, leading to non-productive virus uptake. Furthermore, the modification of HCV-associated lipoproteins by LPL seems to be the reason for the loss of infectivity (64).

To further investigate the effect of LPL on HCV entry, HCVcc were pre-incubated with 5μg/ml of LPL at 4°C or 37°C in the presence or absence of the THL inhibitor, followed by viral infection at 4°C or 37°C, respectively. When HCVcc were incubated with LPL at 4°C, infectivity did not change unless the THL inhibitor was present. In this latter condition, an increase of infectivity of approximately 2 $\log_{10}$ was observed (Figure 6A). In contrast, when HCVcc were incubated with LPL at 37°C, infectivity was reduced by 1 $\log_{10}$ in the absence of THL, whereas the level of infectivity was similar as the treatment at 4°C when the THL was added (Figure 6A). Similar experiments were performed in the HCVpp system. In contrast to the effects observed on HCVcc, treatment with LPL in the absence of THL at 37°C did not reduce HCVpp infectivity (Figure 6B). Rather, a slight increase in virus entry was observed, suggesting that the enzymatic activity of LPL does not affect HCVpp infectivity. However, a stronger increase in HCVpp infectivity was observed in the presence of THL. This effect seems to be specific of the HCVpp since a similar

treatment of pseudoparticles containing VSV G envelope protein had no effect (Figure 6C). This observation is more difficult to interpret. One possibility could be that THL stabilizes LPL in a conformation that is more appropriate to facilitate HCVpp entry.

Together, our data suggest that LPL plays two antagonist effects on HCVcc. In the absence of enzymatic activity, it enhances HCVcc infectivity, probably by increasing virus binding to cellular heparan sulfate proteoglycans as proposed (4). In contrast, if the enzymatic activity is functional, LPL affects the apolipoprotein and lipid composition of the lipoproteins associated with the viral particle as reported (64), leading to a decrease in infectivity probably through LDLR-mediated endocytosis.

To investigate a potential change in lipoprotein composition of the HCVcc, we analyzed the sensitivity of the virus to neutralization by an anti-apoE antibody with or without LPL treatment at 37°C. In the absence of LPL, the anti-apoE antibody was able to neutralize up 90% of virus infectivity at the highest concentration used, whereas only 55% of the virus was neutralized by the anti-ApoE antibody in the presence of LPL (Figure 6D). This observation is in agreement with a decrease in apoE content of the viral particle, which is in line with a recently reported paper indicating that LPL treatment increases virus density and reduces the amount of HCV-associated ApoE (64).

SR-BI takes part in IDL internalization

Lipoproteins associated with HCV particles have been suggested to be in the form of processed VLDL or IDL (52, 55). In contrast to IDL, LDL do not contain apoE. LDL are therefore less likely to be involved in HCV entry since apoE has been shown to play an active role in HCV entry (29). Both LDL and IDL can potentially bind the LDLR. However, it has also been proposed that SRBI could be used as an alternative uptake pathway for processed VLDL (61). Since SRBI is also involved in HCV entry, one cannot exclude that the apoE-containing lipoproteins associated with HCV would facilitate HCV entry through SRBI interaction. We therefore analyzed the internalization of fluorescently labeled LDL and IDL in Huh-7 cells treated with siRNA targeting the LDLR or SRBI. Furthermore, siRNA targeting CD81 were used in a control experiment. Down-regulation of CD81 had no effect on IDL and LDL internalization. As expected, down-regulation of the LDLR reduced both IDL and LDL uptake, to 76 and 62%, respectively (Figure 7). Interestingly, SRBI knock-down also reduced both IDL and LDL uptake, but it had a much stronger effect on IDL uptake. Indeed, this reduced IDL and LDL uptake to 47% and 70%, respectively. These results suggest that the lipoprotein moiety of the HCV particle might preferentially target the viral particle to SRBI instead of the LDLR.

DISCUSSION

HCV production in hepatoma cells is dependent on assembly and secretion of VLDL lipoproteins (23, 30), and HCV particle is secreted in association with these lipoproteins in the form of lipoviroparticle (3). Although such a combination provides the virus with the opportunity of using the LDLR internalization pathway to enter the hepatocyte, the role of this receptor in lipoprotein degradation is in contradiction with an implication of the LDLR in productive entry of this virus. Our data indicate that HCVcc is able to interact with the LDLR and that this receptor plays a role in the HCV life cycle. However, internalization of the LDLR does not seem to be essential for HCV infectivity and its physiological function is rather important for optimal replication of HCV genome.

A functional LDLR is important for HCV replication. By internalizing lipoproteins, the LDLR contributes to the hepatocyte content in cholesterol and potentially in other lipids, which could affect HCV replication since genomic RNA replication is tightly linked to the lipid metabolism. Indeed, during the early stages of HCV infection in chimpanzee, host genes involved in lipid metabolism are differentially regulated (66). Furthermore, lipidomic analyses have identified numerous temporal perturbations in select lipid species that can play important roles in viral replication (17). It has also been shown that inhibitors of cholesterol synthesis and sphingomyelin synthesis inhibit HCV replication (2, 32, 68). In addition, it has also been shown that poly-unsaturated fatty acids inhibit HCV replication (32, 35). Interestingly, Huh-7 cells treated with the anti-LDLR antibody showed some changes in lipid composition. In this context, the increase in the ratio of PE to PC is particularly interesting to note. PE and PC are indeed major phospholipids in mammalian membranes and they are the major components of the ER membrane. Since HCV replicates its genome in association with ER-derived membranes (49) and the ratio of PC to PE has been shown to influence membrane integrity (36), we can speculate that this lipid composition change, induced by Mab C7, may affect HCV replication.

HCV particles are internalized more slowly than lipoproteins. It has previously been shown that the half-maximal HCVpp internalization is approximately 50 min after initiation of entry (43). However, in contrast to HCVcc, HCVpp do not associate with lipoproteins. It was therefore important to revisit the kinetics of internalization of the viral particle in the context of the HCVcc system. Our data indicate that HCVcc are internalized at the same pace as HCVpp, indicating that the association of the particle with lipoproteins has no effect on the rate of HCV entry within target cells. This contrasts with the continuous process of LDLR recycling via clathrin-coated pits on the cell surface at the rate of one cycle every 3-12 min (12, 27). It is also worth noting that clathrin-mediated endocytosis of the LDLR in cultured hepatocytes is strictly dependent on ARH adaptor

(45) (22, 65); however, the ARH knock down did not really affect HCVcc infectivity. This observation is also in favor for the absence of a direct contribution of LDLR internalization in the HCV life cycle. It is worth noting that even if ARH is essential for LDL internalization in hepatocytes, it does not seem to be required for the uptake of VLDL by the liver (31), suggesting another uptake pathway for VLDL.

The lack of effect of ARH knock-down on HCV infectivity contrasts with the effect of LDLR down regulation on HCV infectivity. However, it is known that plasma LDL levels are substantially lower in ARH patients than in familial hypercholesterolemia due to mutations in the LDLR (57). Furthermore, the rate of VLDL clearance has been shown to be significantly higher in ARH knock-out mice than in LDLR knock-out mice, suggesting an alternative pathway for VLDL uptake in the absence of ARH (31).

The state of maturation of the lipoproteins associated with HCV particles needs to be taken into account in virus entry studies. VLDL are triglyceride-rich lipoprotein particles assembled by the liver and they are found in association with HCV particles. Upon entry into the plasma, the triglyceride component of VLDL is rapidly hydrolyzed by LPL and they are converted to cholesterol-rich IDL, which are cleared by the liver (15). IDL are rich in apoE, whereas in LDL, the vast majority of apoE and apoC have been removed. The processing of the lipoprotein moiety associated with HCV particle should therefore be considered when studying HCV entry. Indeed, LPL treatment of the viral particles affects HCV infectivity as previously shown (4, 64) and confirmed in this study. The decrease in HCV infectivity after LPL treatment seems to be due to the loss of apoE associated with the viral particle. It is therefore likely that, in infected individuals, HCV entry functions can be affected by the state of maturation of the lipoprotein associated with HCV particle, with apoE-rich lipoviroparticles being involved in a productive entry process and apoE-depleted particles potentially targeted to non-productive entry. Furthermore, since LPL facilitates the interaction between lipoproteins and the LDLR (15), it is very likely that this receptor targets LPL-processed HCV particles in a degradation pathway.

Lipoprotein interaction with SRBI might play a role in HCV entry. Recent evidence suggests that SRBI plays a physiological role in VLDL metabolism (61). SRBI has indeed been reported to mediate processed-VLDL uptake in CHO cells. Furthermore, we show in our work that IDL internalization is also very dependent on SRBI in Huh-7 cells, confirming the role of this receptor in VLDL metabolism. Although a direct interaction between HCV glycoprotein E2 and SRBI has been shown (63), the lipoproteins associated with the viral particle have also the potential to bind this receptor (40). Moreover, this interaction can mediate direct internalization of HCV into the cell (40).

Inhibition of HCV infectivity by preincubation of the virus with sLDLR suggests that this receptor can potentially interact with infectious viral particles generated in cell culture. This observation contrasts with the lack of effect of Mab C7 on virus entry. Furthermore, our other data are not in favor for the involvement of the LDLR in productive HCV entry. ApoE is present on HCV lipoviroparticles and it plays an essential role in HCV entry (14, 29). It is therefore not surprising that sLDLR inhibits HCVcc entry since apoE associated with processed VLDL is a ligand for this receptor (15). In the context of a viral infection, HCV lipoviroparticle will encounter several lipoprotein receptors, including the LDLR and SRBI, and we cannot exclude that the lipoprotein associated with the viral particle could have a higher affinity for SRBI than for the LDLR. Indeed, as shown in Figure 7, the SRBI knock down has a dramatic effect on IDL internalization, suggesting that this receptor could be preferentially used by this type of lipoprotein.

In conclusion, our data suggest that LDLR could take part in a non-productive entry of HCV particles, whereas the physiological function of this receptor is important for optimal replication of HCV genome. Based on what is known on lipoprotein metabolism, we can expect that in infected people, some of the lipoproteins associated with HCV particles will be further processed into LDL which is a ligand preferentially used by the LDLR. This would likely lead to LDLR mediated uptake and potentially to degradation of such particles. This dead-end pathway might just be a fortuitous consequence of the exploitation of the VLDL assembly process by HCV. However, we cannot exclude that it also provides an as yet undetermined selective advantage for the virus.

ACKNOWLEDGMENTS

We are grateful to Birke Andrea Tews, Ngoc Vu-Dac, Laurence Cocquerel, Lucie Goueslain and Czeslaw Wychowski for their scientific input. We are also grateful to T. Wakita, F.L. Cosset, R. Bartenschlager and M. MacDonald for providing reagents. This work was supported by the French “Agence Nationale de Recherche sur le Sida et les hépatites virales” (ANRS) and by a Marie Curie Research Training Network (MRTN-CT-2006-035599). A.A. was successively supported by a Marie Curie Research Training Network (MRTN-CT-2006-035599) and by a fellowship from the ANRS. J.D. is an international scholar of the Howard Hughes Medical Institute.

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FIGURE LEGENDS

Figure 1: Soluble LDLR neutralizes HCVcc infection in a dose dependent manner. Supernatants containing luciferase reporter viruses HCVcc, HCVpp or SINV were preincubated with different concentrations of sLDLR at 4°C for 2h. These viruses were then used to infect Huh7 cells for 2h at 37°C. Infected cells were rinsed and further incubated for 48h (HCVcc and HCVpp) or 16h (SINV) in culture medium. The levels of infection were then determined by measuring the luciferase activity. Results from three independent experiments are presented as the percentage of infectivity in comparison to non-treated cells ± SD.

Figure 2: LDLR down-regulation decreases HCV infection. Huh-7 cells were transfected with siRNAs as indicated in Materials and Methods. (A) siRNA treated cells were seeded into 24-well plates 24h before infection and then infected with luciferase reporter viruses HCVcc, HCVpp or VSVpp for 2h at 37°C. After 48h of incubation, cells were lyzed and the levels of infection were determined by measuring the luciferase activity. Results from three independent experiments are presented as the percentage of the infectivity in comparison to cells transfected with non-specific siRNA, ± SD. Down-regulated proteins are indicated on the X axis. (B) siRNA transfected cells were lyzed with 1% Triton X-100 and analyzed by Western Blotting under reducing (SRBI, LDLR, ARH, Actin) or non-reducing conditions (CD81) with specific antibodies. The actin content was also analyzed to verify that equal amounts of cell lysates have been loaded.

Figure 3: Kinetics of virus internalization in comparison to lipoproteins. (A) HCVcc and HCVpp infections were synchronized by binding for 1h at 4°C. Internalization was then allowed by shifting the temperature to 37°C. At different time points, cell-bound particles were removed by trypsin treatment. Infectivity was determined after 48h by measuring the luciferase activity. Results are presented as means of three independent experiments ± SD. The 100% value is set as the maximal luciferase activity value after 240 min of internalization. HCVcc and HCVpp internalizations are shown as a solid and dashed lines, respectively. (B) DiI-LDL and DiI-IDL (10 μg/ml) were bound to cells for 1h at 4°C. Internalization was then allowed by shifting the

temperature to 37°C. At different time points the reaction was stopped and non-internalized lipoproteins were removed from cell surface by heparin treatment and cells were analyzed by flow cytometry. The data from three independent experiments are shown as the mean of percentage of the maximum value, ± SD. DiI-LDL and DiI-IDL internalizations are shown as a solid and dashed lines, respectively.

Figure 4: LDLR plays a role in later stages of HCV cell cycle. (A) Huh-7 cells were incubated for 2h with DiI-LDL or DiI-IDL (10 μg/ml) for 2h at 37°C in the presence (green lines) or absence (black lines) of Mab C7 (10 μg/ml). Cells were then processed for flow cytometry analysis. Grey lines show cells processed in the absence of lipoproteins. (B) Huh-7 cells were infected with HCVcc in the presence or absence of Mab C7 (5 μg/ml) and further incubated in the presence or absence of Mab C7 as indicated in the table. Infectivity was determined after 48h by measuring the luciferase activity. ON is for overnight incubation.

Figure 5: LDLR function is important for HCV replication. (A) Huh-7 cells were electroporated with viral RNA (HCV or SINV) and then incubated with Mab C7 or a non-specific antibody at a concentration of 5 μg/ml. At different times post-electroporation, replication was determined by measuring the luciferase activity. A non-replicating HCV GND mutant was used as a negative control of replication. Results are shown as the mean for three independent experiments, ± SD (B) Analysis of Huh-7 lipid composition in the presence of Mab C7. Neutral and phospholipid cell content was tested as described in Materials and Methods. The total amount of neutral lipid/phospholipid was set to 100%. Content of different lipids was caluculated as the percentage of the total lipid amount. Abbreviations correspond to: CE – cholesterol esters, TG – triglycerides, PE – phosphatidylethanolamine, PC – phosphalidylcholine, SM – sphingomyelin, PS – phosphatidylserine, PI – phosphatidylinositol.

Figure 6: Effect of LPL on HCV infectivity and sensibility to anti-ApoE antibody. HCVcc (A), HCVpp (B) or VSVpp (C) were preincubated for 1h at 4°C or 37°C with LPL (5 μg/ml) in presence or absence of THL (LPL inhibitor, 12.5 μg/ml). These viruses were then used to infect Huh-7 cells for 1h at 37°C. Infected cells were rinsed and further incubated for 48h. The levels of infection were then determined by measuring the luciferase activity. Results from three independent experiments are presented as mean, ± SD. (D) HCVcc were preincubated in the presence (●) or absence (□) of LPL (5 μg/ml) for 30min at 37°C. Then, different concentrations of anti-apoE antibody were added to the mixture and incubated for another 30 min. Finally, these viruses were

used to infect Huh-7 cells for 1h at 37°C. Infected cells were rinsed and further incubated for 48h. The levels of infection were then determined by measuring the luciferase activity. Results fro three independent experiments are presented as the mean of percentage of infectivity in comparison to a non-treated control, ± SD.

Figure 7: Role of SRBI in IDL internalization. (A) Cells transfected with siRNA as described in Figure 2 were seeded into 24-well plates. 24h later, lipoproteins internalization was performed for 2h at 37°C with 10 μg/ml of DiI-LDL and DiI-IDL. Fluorescence was assessed by flow cytometry. Quantification was performed using median of total fluorescence. Results from three independent experiments are presented as the mean of percentage of the fluorescence in comparison to control cells, transfected with non-targeting siRNA, ± SD. Down-regulated proteins are indicated on the X axis

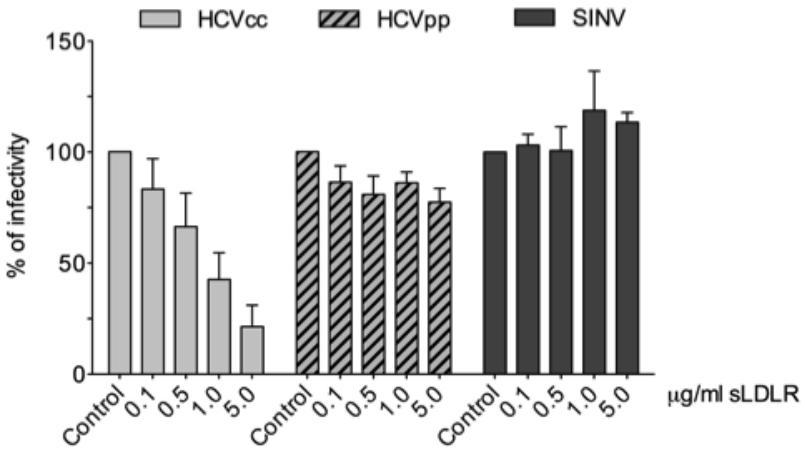

Fig. 1

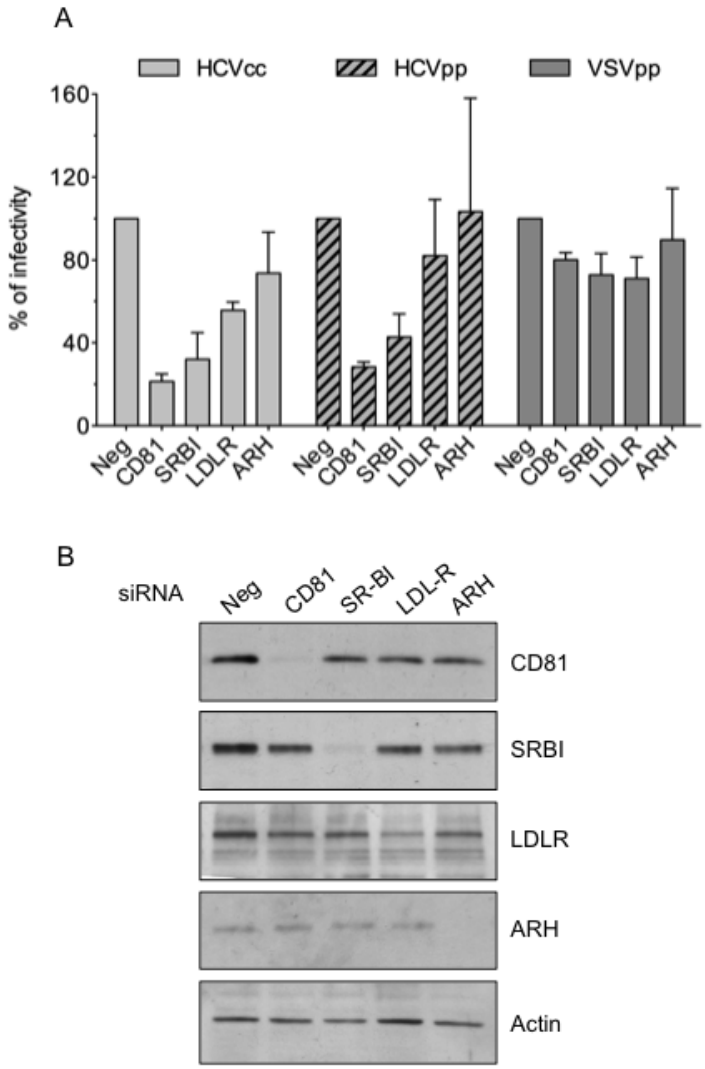

Fig. 2

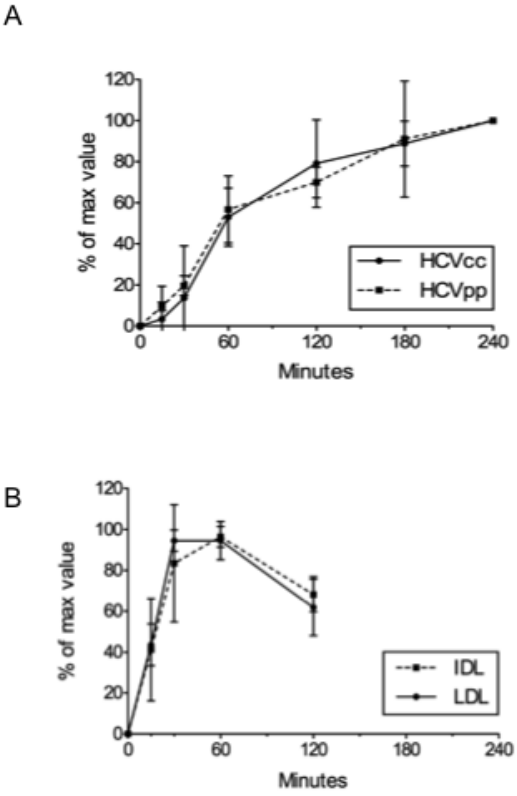

Fig. 3

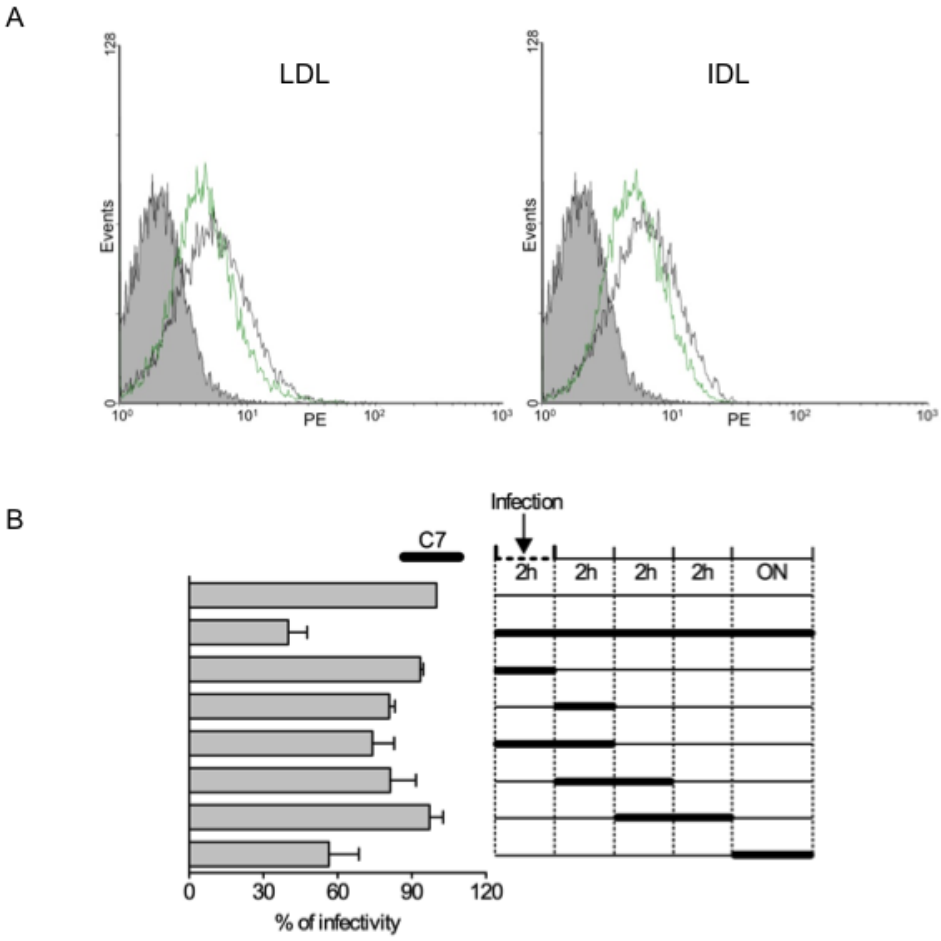

Fig. 4

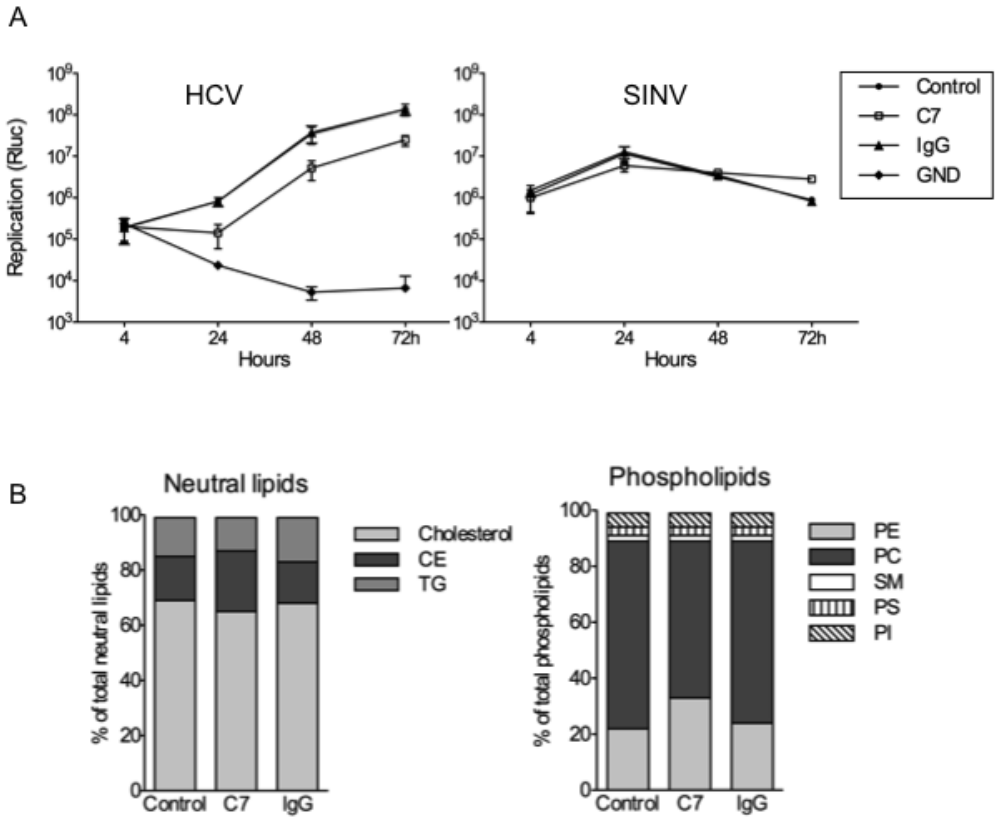

Fig. 5

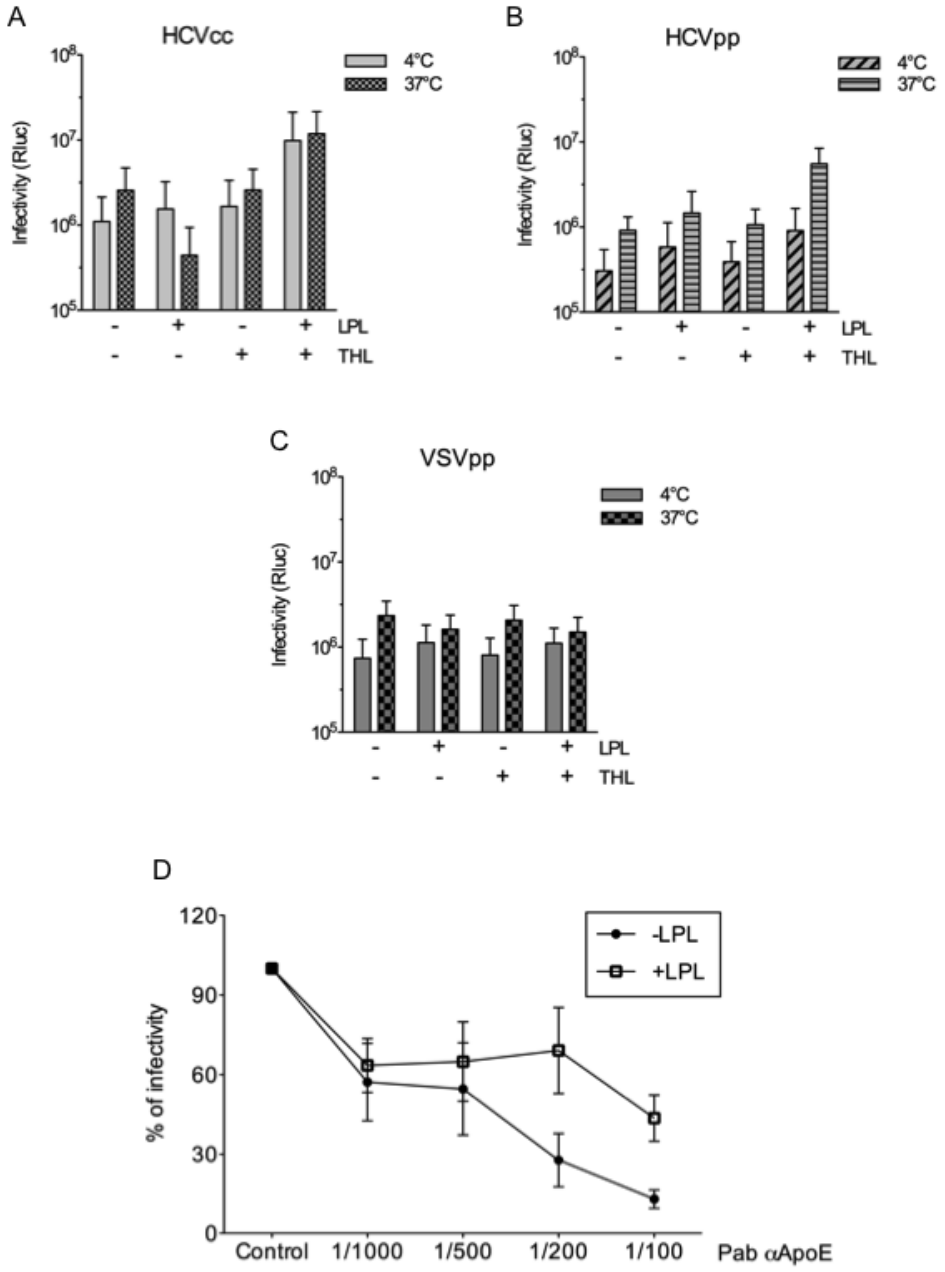

Fig. 6

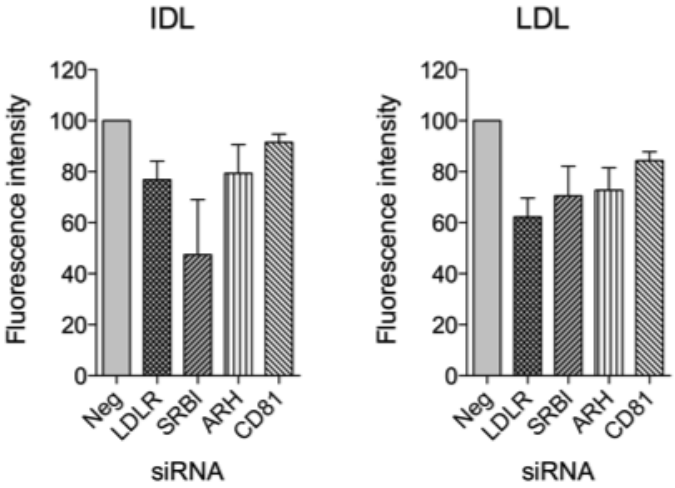

Fig. 7

Discussion

I. Functional regions within E2 glycoprotein

HCV envelope glycoproteins, E1 and E2, play an important role during virus assembly and entry. It has been previously shown in our laboratory that the interactions between TM domains of E1 and E2 are essential for the formation of a functional heterodimer. Furthermore, E2 TM domain is also important during the entry process (Ciczora et al., 2005; Ciczora et al., 2007). In my work, we focused on determining whether interactions occur also between the ectodomains of the glycoproteins. Using HCVpp chimeras containing E1 and E2 from differing genotypes we confirmed our hypothesis that co-evolution of glycoproteins led to development of intergenotypic incompatibilities between E1 and E2. Regions that take part in these interactions are not conserved among the genotypes; therefore, chimeric HCVpp containing glycoproteins from two differing genotypes are often non-functional.

Interestingly, we found that combining E1 from H77 strain (genotype 1a) with E2 from other genotypes led to generation of HCVpp that are not only functional but also more infectious than wild type combinations. This effect was observed when E1(H77) was produced with E2 from genotypes 1b, 2a, 2b and 4. To verify whether this effect is specific for H77 isolate we repeated the experiments using another 1a isolate - UKN1a.14.42. In this case, a high increase in infectivity was only observed in the combination of E1(1a) with E2(2b). However, in other combinations the infectivity of chimeras was also preserved, meaning that E1 from genotype 1a has the ability to efficiently interact with E2 glycoprotein from other genotypes. This feature is unusual and only observed with E1 from genotype 1a. Interestingly, the opposite combinations, for example E2(1a) with E1(2a), are usually not infectious. These results suggest that E1 from genotype 1a is functionally compatible with E2 from other genotypes. This phenomenon is difficult to interpret. Comparison of E1-1a structure with other E1 glycoproteins could help in understanding the unique character of this protein. However, a structure of E1 has not been solved yet. Potentially, E1(1a) could be more flexible than other E1 proteins and therefore could be able to adjust to different E2 proteins. Structure prediction in comparison to other genotypes could clarify this hypothesis.

Part of the experiments concerning chimeric E1-E2 constructs was performed in trans, meaning that E1 and E2 were expressed from separate plasmids. These conditions allowed us to test many combinations, however they do not reflect natural conditions of the HCV translation when a single polyprotein molecule gives rise to all HCV proteins. Incorporation of E1 into HCVpp depends on the presence of E2 (Sandrin et al., 2005). During assembly of HCVpp produced in trans, the interactions between E1 and E2 necessary for proper folding of heterodimer may be limited. This

may be a reason for a slightly lower infectivity of HCVpp generated in trans in comparison to HCVpp generated in cis (Figure 24). However switching E1(2a) with E1(1a) led to a significant increase in infectivity mostly when the proteins were expressed in trans (Figure 24). E1(1a) may be less prone to aggregation than other E1 proteins, thus even in suboptimal conditions, it may form functional heterodimers with E2 leading to increased HCVpp infectivity.

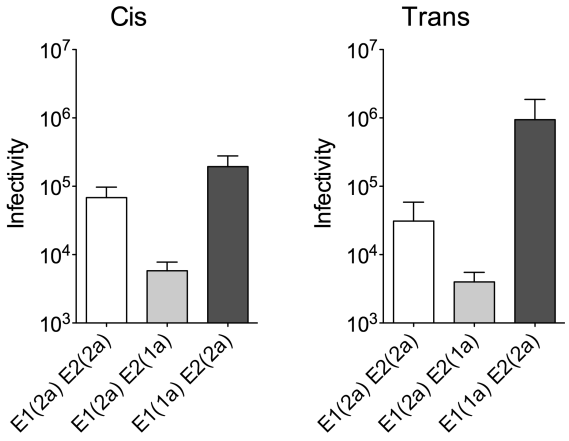

Figure 24. Comparison of infectivity of HCVpp from genotype 2a combined with 1a produced in cis and in trans. HCVpp were generated in 293T cells by transfection with plasmids Gag-Pol MLV, MLV-luc and pcDNA3.1+ containing both (cis) or single (trans) E1E2 proteins. Supernatants containing pseudoparticles were used to infect Huh-7 cells. Infectivity was verified using luciferase activity assay.

Because of these reasons, later experiments were performed by expressing E1E2 from the same plasmid. Based on the E2 model published recently (Krey et al., 2010), we generated a panel of constructs containing E1(2a) and chimeric E2(1a-2a). Chimeric HCVpp allowed us to confirm the hypothesis that interactions within ectodomains are necessary for the functionality of E1E2 heterodimer. Indeed, we identified several regions important for HCVpp infectivity. We assumed that in case of non-functional chimeras, the lack of proper interactions resulted in intergenotypic incompatibilities.

Using HCVpp system, we identified six regions: a.a. 522-541 (within DI), a.a. 445-470 and 471-482 corresponding to HVR2 (within DII), a.a. 570-578 corresponding to IgVR and two regions within ST: a.a. 651-678 and a.a. 705-715 (shown in Figure 25).

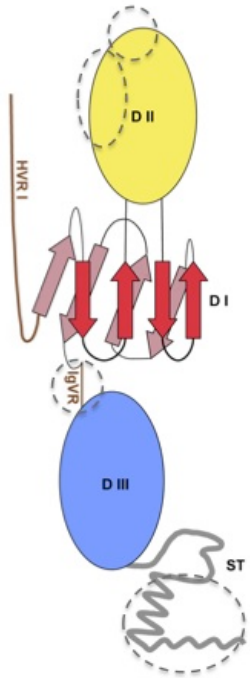

Figure 25. Putative model of E2 glycoprotein with functional regions identified in our studies.

DI is predicted to contain eight β-strands and is responsible for CD81 interaction. The residues responsible for the CD81 binding have been mapped to: 420, 437, 438, 441, 442, 527, 529, 530 and 535 (Drummer et al., 2006; Owsianka et al., 2006). When we swapped the first part of DI (DIa) containing residues 420, 437, 438, 441 and 442 the infectivity of HCVpp was decreased but not lost. Comparison of protein sequences of genotypes 1a and 2a revealed that these aminoacids are conserved and only a minor substitution (W437F) that does not change amino acid character is observed. However, when we swapped the second part of DI (DIb), the infectivity was lost. Moreover, Western blotting analysis showed that the lack of infectivity is probably due to a defect in CD81 interaction. Since the residues involved in interaction with CD81 are conserved between the genotypes, the lack of interaction with CD81 is likely due to a defect in DI folding. To verify this hypothesis we made an additional construct containing the complete DI from 1a genotype in the 2a background. This chimera restored CD81 interaction and infectivity confirming the hypothesis that not only identified residues play a role in CD81 recognition but also genotype specific interactions within DI. Indeed, the structural model of E2 suggests interactions between β-strands within DI involving disulfide bonds (Krey et al., 2010). Therefore, even small changes in aminoacids between genotypes may change the structure of the region and prevent the interaction. Our results are a first biological proof for the reliability of the model, at least in the DI region.

DII forms an insertion in DI and contains HVR2 and the putative fusion peptide. This part of E2 seems to be unstructured and the stability of the domain could be provided by E1 (Krey et al., 2010). Therefore DII may be a potential site of E1E2 interactions. Our HCVpp studies revealed that

swapping two regions within this domain led to intergenotypic incompatibilities. Similar results were observed for the linker region – IgVR. Studies in HCVcc system showed that the lack of infectivity was due to poor secretion of the viruses. Interestingly, region 445-470, which was not infectious in the HCVpp system, remained infectious in the HCVcc system in spite of poor secretion. This can indicate that poorly secreted particles are still infectious. In fact these results can suggest that this virus is even more infectious than the wild type. Discrepancies between the HCVpp and HCVcc systems are observed in this case. We did not detect a defect in HCVpp assembly, however the infectivity was lost. In contrast, in HCVcc, the assembly process was affected but still, the virus remained infectious. Nevertheless, HCVcc system, which reflects the complete HCV life cycle is a more reliable model to study the functions of HCV glycoproteins. Therefore, we can conclude that region 445-470 provides important interactions during the assembly process.

In the case of HVR2 and IgVR, the results obtained in HCVpp and HCVcc led to the same conclusions. In both systems, the lack of infectivity seems to be due to a defect in assembly. In HCVpp system, we observed poor incorporation of E1 into the particles. To better understand the lack of incorporation of E1, CD81-LEL-GST pull-down assay experiments using these chimeras were also performed under non-reducing conditions. As shown in Figure 26, under reducing conditions, the monomeric form of E1 is present although at a lower intensity as compared to the wild type. Under non-reducing conditions, the monomeric E1 band is absent, suggesting that E1 is forming aggregates. Since E2 plays a role in E1 folding (Michalak et al., 1997), our results with HVR2 and IgVR chimeras suggest that these regions affect the chaperone role of E2, likely by altering intermolecular interactions within E2.

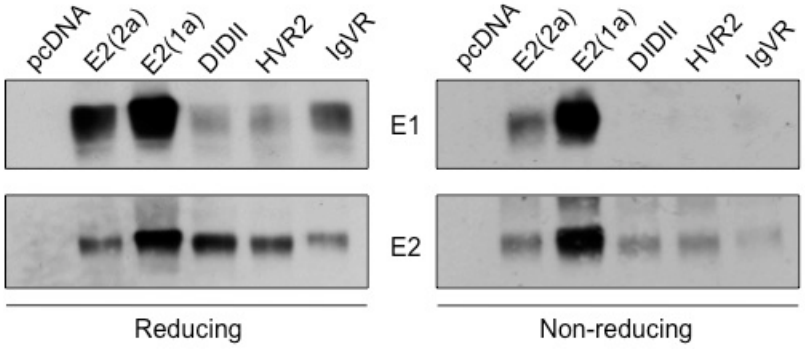

Figure 26. E1 aggregation during the assembly of chimeric HCVpp containing swapped HVR2 and IgVR regions. Lysates of HCVpp producing 293T cells were used in CD81-LEL-GST pull down assay. The presence of precipitated proteins was analyzed by SDS-Page in reducing or non-reducing conditions and followed by Western blotting to detect E1 and E2.

HCVpp results were confirmed in HCVcc system since both chimeric viruses lost their infectivity due to assembly defect. We hypothesized that maybe a difference in segment length between 1a and 2a genotypes could be the reason for the functionality defect. Therefore, we inserted two alanines or

the two corresponding aminoacids from 2a genotype into the non-functional chimeras to restore the length of the segment. Interestingly, these insertions were not able to rescue virus infectivity suggesting that not the length but the amino acid composition of these regions play a functional role during the assembly process.

Our study also focused on ST region. Again, we observed some discrepancies between HCVpp and HCVcc systems. Swapping the first fragment of ST, a.a. 651-681, led to production of non-infectious pseudotypes. However, the equivalent HCVcc chimera was well secreted and the infectivity maintained. The second segment of ST, a.a. 682-704, is well conserved between 1a and 2a genotypes. Two residue changes (I690L and L702M) led to significant increase in HCVpp infectivity, though infectivity of HCVcc was reduced showing again differences between HCVpp and HCVcc systems. Our structure-based studies showed that this region contains an amphipathic helix within region 684-703 (Figure 27). Interestingly, the α-helical structure of this region depends on association with lipids, meaning that only in the presence of lipid-like molecules the α-helix is formed. Moreover, the amphipathic character of this region and the TM domain proximity can indicate that the α-helix associates with a membrane interface, in an in-plane topology. These results are in agreement with the model of the fusion process suggested for class II glycoproteins (Gibbons et al., 2003; Gibbons et al., 2004). In pre-fusion state, the α-helix would be bound to a membrane interface. However, pH change in endosomes could lead to membrane release and formation of coil, mediating translocation of the fusion complex. The α-helix could be again formed upon binding to the trimeric post-fusion complex of glycoproteins.

As shown in Figure 27, a small α-helix is also predicted within region 706-712. Chimeric HCVpp containing swapped 705-715 segment were no longer infectious. Moreover, in the HCVcc system, a strong decrease in infectivity was observed in spite of good secretion levels.

This indicates that this region indeed plays a role during the entry process. Structural analysis revealed that region 706-712 comprises an α-helical fold but of weaker strength than 684-703 helix. This region is also amphipathic with aminoacids 709-710 and 712-714 forming a hydrophobic face and aminoacids 711 and 715 at hydrophilic face. Proximity of the TM and aromatic residues suggests that this segment is also located at the membrane interface. We propose that this region also participates in the pH-mediated reorganization of glycoproteins during fusion. Our biological data demonstrate the importance of this region and its genotype-specific properties. Although aminoacid residues of this region are not well conserved, the character of the segment is preserved in both 1a and 2a genotypes. As predicted, single substitutions within this region do not disturb the general structure and therefore do not lead to infectivity loss as shown in Article I. However swapping bigger 705-715 fragment, blocked entry both in HCVpp and HCVcc systems. To verify

which aminoacids are responsible for intergenotypic interactions within this region, 2 or 3 residues substitutions could be designed.

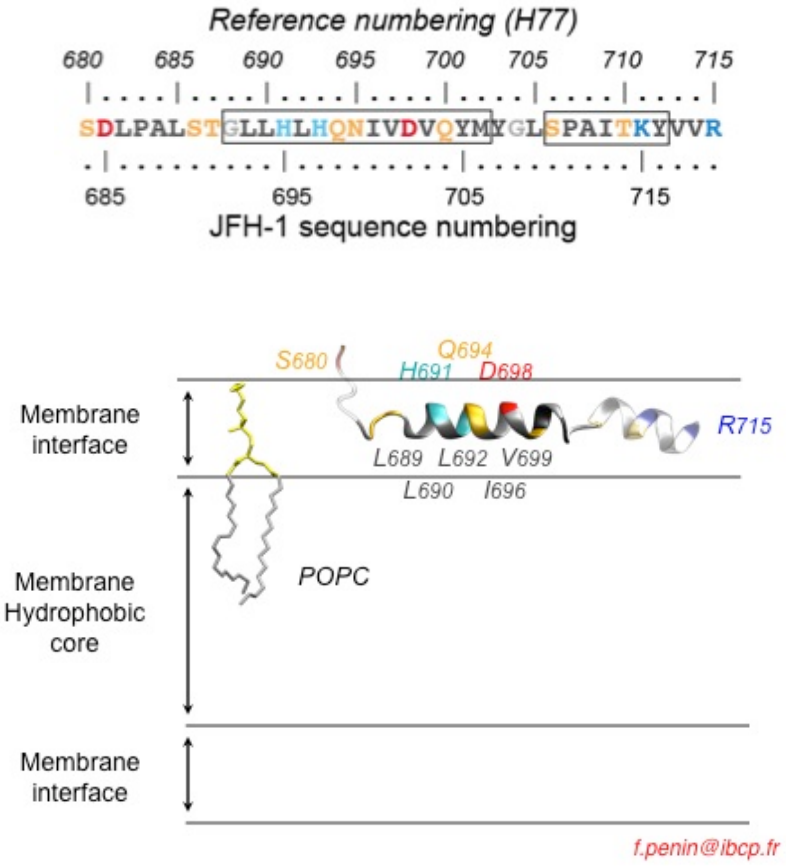

Figure 27. α-helixes in ST region. Top panel: NMR predicted structure of E2-SC JFH-1 peptide (680-715) in SDS or TFE 50%. Studies revealed two α-helices: 688-702 and 706-712 (in boxes). Residues numbering is shown in both H77 reference strain and in JFH-1. Bottom panel: suggested position of the helixes at the membrane interface, in-plane to the membrane bilayer. The proportions between peptide and membrane are preserved. POPC stands for palmitoyl-oleoyl-sn-glycero-phosphocholine. Colors of aminoacids show the character of the residue: blue – polar positive (H, R, K), red – polar negative (D), yellow – polar neutral (S, Q, N, T), grey – non-polar (L, V, I). The model was designed by François Penin (f.penin@ibcp.fr).

Our results identified the presence of the conserved α-helix structure in region of ST. This region is likely to play a role in the fusion process, therefore it possibly interacts with other region within E2 or with E1. Following this hypothesis we tested whether E2-SC peptide synthesized for structural studies, can interfere with the fusion process and block virus entry. We hypothesized that the peptide added during infection would bind to its partner region and therefore would prevent interaction between this region and ST during fusion. Unfortunately, no inhibitory effect was observed. In contrast, high concentration of peptide (50-100μg/ml) increased virus infectivity. This interesting feature of E2-SC peptide remains however to be explained. *In vitro* fusion experiments

using artificial membranes like liposomes and different chimeric viruses in the presence or absence of the peptide could bring more information of the effect of this peptide.

Searching for partner regions interacting with identified E2 segments, we tried to adapt our chimeric viruses to cell culture in order to identify second-site mutations and further characterize E1E2 interactions. We focused on chimeras that preserved residual infectivity: a.a. 445-470 within DII and a.a. 705-715 within ST. Electroporated cells were cultured for two months. Supernatants were collected every second passage and verified for infectivity. Unfortunately, we did not observe any significant increase of infectivity and no adaptive mutations appeared. Our protocol for selection of mutants likely needs to be optimized to increase our chance of identifying mutations.

In our project, we identified regions that play a functional role during HCV assembly and entry. These regions are non-conserved between the genotypes and their swapping abolishes HCV functionality. Our results provide an important knowledge on E2 glycoprotein, highlighting that interactions within this glycoprotein play a role in both assembly and entry processes. Moreover, some of our biological data constitute a functional prove in support of the recently published E2 structural model (Krey et al., 2010).

To complete our story, partner regions interacting with the identified segments should be search for. It is possible that regions responsible for intergenotypic incompatibilities interact with other non-conserver parts of E2. In this case, it is likely that we have already identified all players and now it remains to verify which regions are partners. For example, both regions in DII (445-470 and HVR2) that are in close proximity, may interact with IgVR, since all these segment seem to play a role in assembly. However, since HCVpp chimera DI-DII also demonstrated assembly defect (Figure 26), it is likely that a region within the second part of E2 (DIII-ST-TM) and/or E1 glycoprotein also takes part in these interactions. Additional chimeric viruses should be generated to address this issue.

Importantly, E1 structural model is not yet available; therefore, similar studies focused on E1 could bring important information and help designing a structural model for E1. As for E2, a first description of the intramolecular disulfide bonds within E1 would help in the design of chimeric proteins used for structure-function studies.

II. LDLR – necessary entry factor or suicidal pathway?

LDLR has been previously suggested as an entry factor for HCV (Agnello et al., 1999; Monazahian et al., 1999). Later studies indeed confirmed that LDLR might potentially participate in HCV attachment (Martin et al., 2008; Molina et al., 2007; Owen et al., 2009). The goal of our project was to verify this hypothesis and to understand whether LDLR function is essential during HCV cell cycle.

An important feature of HCV in its LVP form is that it contains apolipoproteins. These are ligands for several molecules present in the cellular membrane, thus can play a role in HCV binding and entry. Among the apolipoproteins, ApoE plays a crucial role and affects the HCV infectivity (Hishiki et al., 2010; Owen et al., 2009). ApoE mediates the interaction between IDL (βVLDL) and LDLR. Therefore, to verify whether LVP-associated ApoE, can interact with LDLR we used the sLDLR form in HCVcc infection assay. Pretreatment of virus with sLDLR inhibited HCV entry in a dose-dependent manner. In contrast, no effect was observed for control viruses. These experiments confirm that HCV can interact with LDLR, but they do not constitute the proof that LDLR is used in HCV infection. Importantly, ApoE can also interact with HSPG (Cardin et al., 1989) and probably with SRBI (Rohrl et al., 2010). Therefore, virus binding to sLDLR can inhibit ApoE interactions with other molecules that play a role in HCV entry. HCV binds to HSPG and to verify whether sLDLR can block these interactions heparin pull-down could be performed. In this case precipitation of virus with heparin could be prevented in the presence of sLDLR. We will try to solve this issue in upcoming weeks. In addition, the second hypothesis concerning the interaction with SRBI is also an interesting question to answer. However, this point will be discussed later.

Using siRNA transfections we confirmed that LDLR somehow participates in the HCV life cycle. Here I would like to discuss the role of ARH and how it can affect the virus. ARH is an adaptor protein that plays a role in LDLR internalization (Garuti et al., 2005; Mishra et al., 2002; Sirinian et al., 2005). However, some studies suggest that ARH-independent pathway responsible for uptake of VLDL remnants exists (Jones et al., 2007a; Michaely et al., 2007). This can be tissue-specific and has not been confirmed yet in human hepatocytes. Our siRNA experiments do not show a clear role for ARH. If an ARH-independent uptake pathway exists in Huh-7 cells then we can suggest that HCV uses this route of LDLR internalization, therefore ARH would not be necessary. In contrast, HCV uptake through LDLR could lead to virus degradation in lysosomes. In this case, blocking ARH could prevent this process and should increase the productive uptake of HCV. However, our results show that LDLR is important during the replication, therefore the presence of two opposing effects can lead to intermediate phenotype. Finally, our lipoprotein internalization studies show that the effect of LDLR downregulation is similar to ARH effect on both LDL (~30% inhibition) and

IDL (~22% inhibition). This indicates that in Huh-7 cells ARH is used during the uptake of both LDL and IDL, however the inhibition is not so strong. Therefore, the effect on the virus may be not so visible.

Our results clearly demonstrate that HCV is not internalized through the same pathway as the lipoproteins. As already shown many years ago, lipoprotein uptake is a very fast process (Brown et al., 1983). In our experiments we observe that all lipoproteins used in the experiments are inside the cells after 30 minutes. In contrast, after the same time approximately 15% of HCVcc or HCVpp is protected from trypsin. This significant difference suggests that the virus is not using the LDLR for its internalization. Interestingly, the lack of distinction between HCVcc and HCVpp internalization kinetics indicates that the lipoprotein components of the virus do not affect the uptake mechanism. These results and the fact that HCVpp do not interact with sLDLR as shown in Article II, again show that LDLR is unlikely to be used during productive HCV entry.

Another argument against the role of the LDLR in HCV binding comes from our antibodies experiments. We used the well-described C7 Mab that partially inhibits lipoproteins uptake. We confirmed that this Mab indeed reduces LDL and IDL internalization. We first tried to determine whether C7 Mab inhibits virus binding to cells. Cell treatment with Mab C7 at 4°C was followed by virus binding also at 4°C. No inhibition was observed in these conditions suggesting the lack of role of LDLR in virus attachment. Then, we performed the experiments at 37°C but in parallel with the Mab C167 that blocks virus interaction with SRBI (Catanese et al., 2007). As shown in Figure 28, in contrast to C7, C167 inhibited HCV entry.

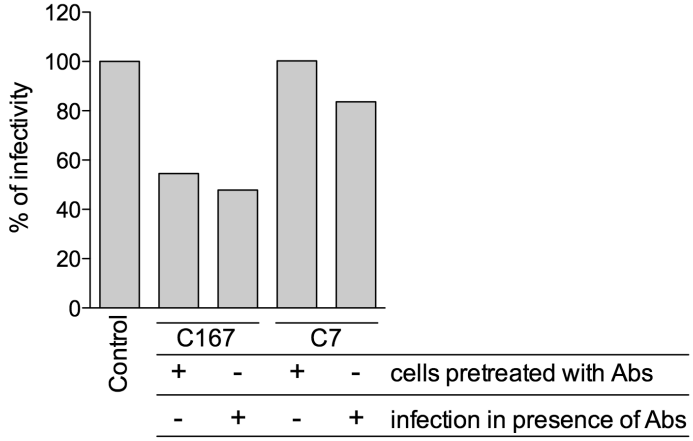

Figure 28. Comparison of the effect of C167 and C7 Mab on HCV entry. Cells were pretreated with Abs before infection or infected with HCVcc in the presence of Abs. Cells were lyzed after 48h and infection levels were quantified by the luciferase reporter gene activity assay.

To verify whether reduction of lipoprotein uptake by LDLR can affect later stages of entry we performed the experiments in which the C7 Mab was present during different periods of the infection. As shown in Article II, it occurred that C7 treatment affected a late stage of the virus life cycle. Electroporation experiments in the presence of C7 confirmed our hypothesis that indeed

LDLR function is important for HCV replication. LDLR plays a significant role in lipid metabolism in the liver as discussed in Paragraph IV of the Introduction. Cholesterol delivered by LDLR pathway regulates cellular lipid homeostasis by controlling the behavior of different transcription factors responsible for the expression of proteins engaged in lipid metabolism. Interestingly, these regulation events take place in ER membranes. It is now thought that the membranous webs, which are the sites of HCV replication originate from ER membranes (Romero-Brey et al., 17th International Meeting on Hepatitis C Virus and related Viruses, Yokohama, Japan, September 9-14, 2010). Moreover, the lipid composition of the membranous webs affects the replication process (Gosert et al., 2003; Kapadia and Chisari, 2005). Finally, the HCV life cycle induces changes in cellular lipid metabolism; for example it elevates the expression of genes responsible for cholesterol biosynthesis (Kapadia and Chisari, 2005; Su et al., 2002). All these data can suggest that disregulation of lipid metabolism by LDLR inhibition can affect HCV replication. Indeed, the lipid profile analysis of C7 treated cells revealed some differences in ratios between lipids. The analysis of neutral lipid composition showed an increased level of cholesterol esters in comparison to free cholesterol and triglycerides. This can have an impact on SBEBPs transcription control, which is regulated by cholesterol (Brown and Goldstein, 1997). More importantly, differences were also observed in phospholipids composition. Mainly, the ratio between phosphatidylethanolamine (PE) and phosphatidylcholine (PC) was affected in favor of PE.

PC and PE are the most abundand phospholipids in membranes. Changes in lipid composition of ER activate phosphocholine cytidylyltransferase, an enzyme that plays a crucial role in the biosynthesis of PC. Both PE and PC are produced in the ER in Kennedy pathway by different isoforms of diacylglycerol choline /ethanolaminephosphotransferase. Conversion of PE into PC is mediated by another enzyme, however this process is believed to be restricted to mitochondria-associated membranes (Fagone and Jackowski, 2009). The regulation of phospholipids biosynthesis and its influence on membrane properties are highly complicated processes. Interestingly, the ration of PC to PE influences ER membrane integrity (Li et al., 2006). Since HCV replicates on ER derived membranes, these alterations likely affect viral replication. However, more studies are required to understand this mechanism. Furthermore, we cannot exclude a link between phospholipids content and SREBPs lipid metabolism regulation.

Our results show that LDLR does not participate in productive HCV entry, however its function is important in regulation of membrane lipid composition, which dictates the efficacy of the replication process. Furthermore, we wanted to investigate the role of ApoE in the entry process. It is described that LPL enzymatic activity modifies VLDL lipid and protein composition, decreasing ApoE moiety in the particles (Mead et al., 2002). Independently of its catalytic activity, LPL also helps lipoproteins bind to cells (Merkel et al., 2002; Merkel et al., 1998). It has been shown on

HCV that LPL indeed increases virus binding to cells, however this leads to a decrease in infectivity (Andreo et al., 2007). The infectivity reduction has been correlated to a decrease in ApoE moiety after LPL treatment (Shimizu et al., 2010). We also observed that inactivated LPL increased HCV infectivity probably by increasing its binding to cells. The same effect was clearly visible with IDL treated with LPL. LPL treatment strongly increases IDL binding as observed in confocal microscopy studies (Figure 29). In contrast, the catalytically active enzyme reduced HCV infectivity, suggesting that LPL-mediated change in LVP composition somehow inhibits the entry process. As suggested by Shimizu et al., LPL treatment shifts HCV particles into higher densities and lowers the amount of ApoE associated to the viruses (Shimizu et al., 2010). Indeed, our observations are in agreement with these results, since LPL-modified virus is less sensible to neutralization with anti-ApoE Abs.

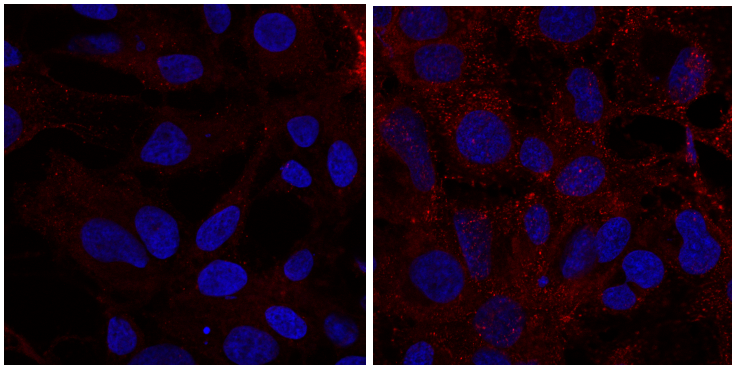

Figure 29. LPL increases IDL internalization. On the left panel, internalization of IDL during 15 minutes at 37°C. On the right panel, internalization of IDL pretreated with LPL. Nuclei are labeled with DAPI (blue), lipoproteins are conjugated to DiI (red).

As suggested before, HCV-associated ApoE could mediate HSPG or SRBI interactions. Experiments of lipoprotein internalization using cells transfected with siRNA allowed us to propose a hypothesis that ApoE particles mediate HCV-SRBI interactions. It occurred that, in SRBI downregulated cells, IDL internalization is strongly impaired (~50% inhibition). In contrast, LDL uptake was less affected in these cells, suggesting a role for SRBI in IDL binding. Connecting these results to our previous experiments led us propose, that IDL dependence on SRBI could be reduced by LPL treatment. We think that ApoE molecules interact with SRBI, therefore decrease in ApoE content would reduce SRBI interaction. This hypothesis could be verified by internalization of LPL-treated lipoproteins again into the SRBI-downregulated cells.

Since it is associated with lipoproteins, LVP likely behaves as IDL during some steps of the entry process. Virus-associated ApoE could indeed interact with SRBI. It has been previously suggested that the lipid transfer function of SRBI and LVP-associated apolipoproteins affect HCV entry (Dreux et al., 2009; Maillard et al., 2006). In this case, ApoE binding to SRBI could modify LVP

composition and possibly expose previously hidden regions of glycoproteins allowing SRBI-E2 interactions, as suggested (Catanese et al., 2010; Dreux et al., 2009; Scarselli et al., 2002). In contrast, LPL-induced ApoE loss could inhibit this process, therefore blocking HCV entry.

To further explore this hypothesis, we need to develop a binding assay to describe which entry factors are indeed used by the viral particle. This will be done by expressing these entry factors in CHO cells lacking LDLR. We are currently producing CHO cell lines stably expressing SRBI or LDLR. These cells should allow us to study virus binding to SRBI or LDLR in different conditions. Our major goal is to verify whether purified infectious virus binds preferably to SRBI and to determine whether LPL treatment would reduce SRBI binding. It will also be interesting to check how HCV binding to SRBI and LDLR is modified not only by LPL, but also by sLDLR, IDL, LDL and anti-ApoE Abs. In these experiments we will use concentrated and sucrose-gradient purified virus. This kind of preparation should decrease the background level associated to binding of non-infectious viral particles. The binding will be assessed by quantitative real time RT-PCR. We hope that these experiments will allow us to fully understand the mechanism of LPL-induced infectivity reduction. We also hope that the binding studies will confirm our results and bring more definite proofs for our hypotheses.

Finally, we predict that LDLR can induce HCV internalization. Indeed, LVP-associated ApoE binding to LDLR should lead to virus entry following the lipoprotein uptake process. However, the lack of interaction with other HCV entry factors like CD81, would prevent proper entry process resulting in fusion with endosomal membrane and release of viral RNA. In this case, virus release from LDLR, mediated by acidification of lysosomes would lead to virus degradation. This hypothesis has not been confirmed yet. However, older reports suggesting LDLR role in HCV entry, where internalization, not infection assays were used, could stand in favor of this hypothesis. A potential experiment to verify this hypothesis could be the comparison of infectivity level with the amount of internalized RNA after LPL treatment. Although LPL reduces infectivity, it might not affect LDLR binding. Viral particles that lose ApoE could still interact with LDLR through ApoB particles that are normally exposed on the LDL after LPL modification. In this case, in spite of infectivity reduction, the RNA uptake might not be affected, indicating that particles that lose the ability to interact with SRBI are still internalized by LDLR. Of course these kinds of experiments will not show the direct role of LDLR, however they could bring us closer to understanding this mechanism. More reliable studies should be performed to definitely establish LDLR role in HCV internalization. Functional, fluorescent HCV particles would be a perfect tool enabling direct observation of HCV entry process in co-localization with different entry factors. This strategy is now being designed in some laboratories (Coller et al., 2009).

In conclusions, I would like to highlight that our studies are the first to show the role of LDLR in HCV replication. We were also able to show that LDLR inhibition-mediated changes in replication might be caused by changes in cellular lipid composition. Furthermore our data are not in favor for a role of the LDLR in productive HCV entry. We now hypothesize that virus-associated ApoE might play a role in HCV binding to SRBI. We hope that binding experiments that are now under development will confirm our hypothesis. In Figure 30 we present a model of HCV entry that summarizes our work. Shortly, HCV interacts with SRBI through ApoE molecules. This leads to further interactions with other HCV entry factors enabling virus endocytosis followed by RNA release and replication. LPL prevents ApoE-SRBI interaction, however virus can still interact with LDLR that results in HCV uptake and degradation.

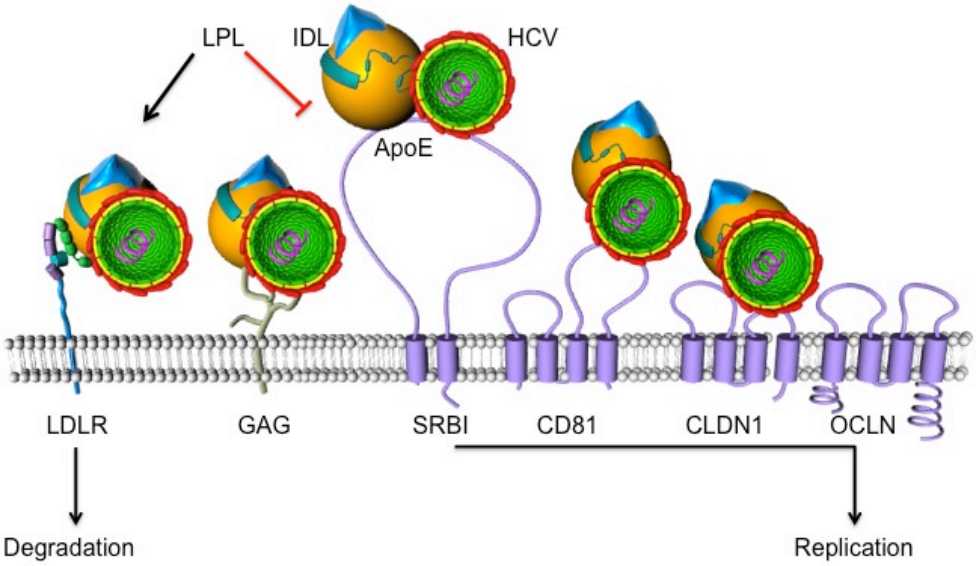

Figure 30. Model of two pathways of HCV endocytosis. Description is placed in the text.

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