UNIVERSITA' DEGLI STUDI DI PADOVA

Sede Amministrativa: Università degli Studi di Padova
Dipartimento di Scienze Biomediche Sperimentali

SCUOLA DI DOTTORATO DI RICERCA IN : BIOSCIENZE
INDIRIZZO: BIOLOGIA CELLULARE
CICLO: XX°

Production of HCV infectious viral particles through trans-complementation of gpE1/gpE2 and characterization of early events that follow HCV binding to target cells

Direttore della Scuola : Ch.mo Prof. Tullio Pozzan
Supervisore : Ch.mo Prof. Cesare Montecucco
Supervisore esterno: Dott.ssa Stefania Crotta

Dottoranda : Dott.ssa Sara Filippini

31 gennaio 2008

RINGRAZIAMENTI

Vorrei ringraziare innanzitutto la Dott.ssa Michela Brazzoli, che oltre a seguirmi scrupolosamente ed attivamente nell'attività di ricerca svolta in questi tre anni di dottorato, ha saputo trasmettermi parte dell'entusiasmo e della fiducia che caratterizzano il suo carattere solare e disponibile. Desidero inoltre ringraziare la Dott.ssa Stefania Crotta e il Dott. Konrad Stadler per l'esperta supervisione dell'attività di ricerca svolta nel corso del Dottorato e per i materiali messi a disposizione, ma soprattutto per i validi e utili consigli relativi ai temi di Biologia Molecolare e di Virologia trattati in questa tesi. Un grazie sentito anche al Prof. Cesare Montecucco dell'Università di Padova e a tutte le persone con cui ho avuto il piacere di collaborare durante il mio periodo di permanenza alla Novartis Vaccines & Diagnostics di Siena.

Un immancabile ringraziamento va, come sempre, alla mia famiglia che non manca mai di sostenermi e di incoraggiarmi.

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RIASSUNTO

La trans-complementazione di gpE1/E2 consente il recupero di paricelle infettive di HCV

Sebbene esperimenti di trans-complementazione siano già stati attuati in alcuni virus appartenenti alla famiglia dei Flaviviridae come quello della diarrea virale bovina o il virus di Kunjin, essa non ha mai avuto notevoli risultati nel caso del virus dell'HCV, soprattutto quando questo approccio è stato utilizzato per ripristinare la funzionalità di repliconi difettivi. La scarsa efficienza del sistema in HCV fa pensare ad una natura indipendente ed isolata dei complessi di replicazione virale, che hanno poco o nessuno scambio tra loro.

Con il presente lavoro siamo state in grado di dimostrare per la prima volta in HCV che è possibile avere la produzione di paricelle virali infettive anche quando le proteine strutturali dell'envelope virale E1 ed E2 vengono fornite *in trans*.

Attraverso un sistema lentivirale abbiamo utilizzato delle cellule di epatoma umano (S6.1) per creare tre linee cellulari geneticamente modificate in grado di sintetizzare in modo costitutivo le proteine virali E1E2 appartenenti a tre genotipi diversi di HCV: GT 2a, GT1a, e GT2b.

Quando la linea cellulare S6.1/E1E2:2a è stata trasfettata con l'RNA dell'isolato JFH-1 (GT 2a) privato delle proteine dell'envelope virale (JFHΔE1E2), ciò a portato al rilascio di particelle virali infettive, dando prova che per una corretta formazione e rilascio di virioni le glicoproteine dell'envelope virale possono essere fornite *in trans*.

Questi risultati, confermati da cellule S6.1 naïves trasfettate con l'RNA completo dell'isolato JFH-1, usato come controllo positivo, non sono stati invece riscontrati nel caso delle altre due linee cellulare trasfettate con JFHΔE1E2.

Probabilmente, nel caso di trans-complementazione eterologa tra genotipi diversi (1a/2a; 1b/2a) un'incompatibilità genetica esistente tra lo scheletro JFH-1 e le proteine strutturali E1E2 ha impedito la formazione o il rilascio di particelle virali nei fluidi extracellulari, ma questa ipotesi necessiterebbe comunque di verifiche attraverso nuovi esperimenti di trans-complementazione.

Caratterizzazione dei primi eventi che seguono l'attacco del virus dell'HCV alla cellula bersaglio

Come abbiamo visto nello studio precedente, è possibile produrre particelle virali infettive di HCV trasfettando delle cellule di epatoma umano con l'RNA derivante dall'isolato JFH-1. Queste particelle poi possono essere isolate ed utilizzate in esperimenti riguardanti il ciclo vitale del virus, e per investigare i processi legati all'attacco del virus ai recettori di membrana, piuttosto che i meccanismi di entrata.

Oltre ai due recettori principali identificati per HCV, cioè CD81 e SR-BI, sembrano esistere molti altri fattori che mediano l'attacco e l'ingresso del virus nella cellula. Recentemente Evans *et al*. (2007) hanno identificato una proteina facente parte delle giunzioni occludenti di membrana (tight junctions), la Claudina-1 (CLDN-1), che sembra essere coinvolta nell'entrata di HCV nella cellula più tardivamente rispetto a CD81.

Nel nostro studio abbiamo dimostrato che esiste effettivamente una stretta correlazione funzionale tra CD81 e CLND-1 durante le prime fasi dell'entry virale. Infatti la stimolazione di CD81 attraverso l'utilizzo di proteine virali ricombinanti o di specifici anticorpi anti-CD81 ha portato ad una traslocazione di questo recettore dalle varie zone della membrana cellulare, dove è normalmente diffuso, alle sole aree di connessione fra le cellule, dove risiedono le giunzioni occludenti e la CLDN-1.

L'utilizzo di inibitori chimici per la polimerizzazione dello scheletro di actina, come la Latrunculina A e il Jasplakinolide e lo studio della cinetica di azione hanno evidenziato l'effettivo coinvolgimento dello scheletro di actina in questo processo di rilocalizzazione del recettore,oltre a dimostrare quanto un'actina funzionale sia importante anche per l'internalizzazione del virus stesso. Un notevole calo dell'infettività virale si è rilevato anche in cellule trattate con inibitori o silenziate per l'espressione delle proteine che sono maggiormente coinvolte nella riorganizzazione dello scheletro di actina cellulare, ovvero le Rho GTPasi Rho, Rac e Cdc42.

ABSTRACT

Trans-complementation of gpE1/gpE2 allows recovery of infectious Hepatitis C virus

Although observed for other members of the Flaviviridae including bovine diarrea virus and Kunjin virus, trans-complementation of replication-defective replicons was never observed for hepatitis C virus (HCV). This suggested an isolated and independent nature of the HCV replication complexes, with little or not exchange of factors between them. In particular, HCV non structural proteins seem to remain associated to their respective complexes and may not be able to access other complexes.

In the present work we demonstrated for the first time for HCV that when the structural proteins E1 and E2 are provided *in trans* by the use of complementing cell lines constitutively expressing them it is possible to achieve production of infectious viral particles.

Using a 293T cells retroviral system we created three packaging cell lines constitutively expressing the two surface proteins E1 and E2 from genotype 2a (S6.1/E1E2:2a), GT 1a (S6.1/E1E2:1a), and GT 1b (S6.1/E1E2:1b).

When viral backbone RNA lacking the E1E2 genes (JFHΔE1E2) deriving from the JFH-1 isolate (GT 2a) was transfected into S6.1/E1E2:2a cells, HCV infectious particles were released, indicating that for successful virus assembly, budding and release the structural glycoproteins E1 and E2 can be provided in *trans*. These results were consistent with what we observed in S6.1 naïve cells transfected with the full length RNA genome of JFH-1, use as positive control. The release of infectious viral particles, though, could not be detected in S6.1/E1E2:1a and S6.1/E1E2:1b transfected with JFHΔE1E2.

We can hypotize that in the case of heterologous trans-complementation, genetic incompatibility between JFH-1 backbone and the structural proteins of GT 1a and GT 1b could have totally or partially affected the production of viral particles in our system, although these is only a speculative hypothesis that need to be verified in other experiments of homologous and heterologous trans-complementation, characterizing the relationships among HCV proteins and other viral factors

Characterization of early events that follow HCV binding to target cells

As we have previously seen, by transfecting permissive cells with the mRNA of the HCV isolate JFH-1 it is possible to produce infectious viral particles that might be used for experiments on the viral life cycle, as binding, attachment and entry. One of the main advantages of the cell culture-derived HCV (HCVcc) in fact is probably the mechanisms of interaction of these viral particles with the host cell receptors that closely mimic what happens in a natural infection event.

Besides the two main HCV receptors identified so far, CD81 and SR-BI, many cellular factors act in concert to mediate HCV binding and entry into hepatocytes. Recently Evans *et al*. (2007) identified a tight junction protein that is highly expressed in liver cells, Claudin-1 (CLDN-1), which seems to act late in the entry process, after virus binding and interaction with the HCV co-receptor CD81.

In the present study we demonstrated that there is a strict connection among CD81 and CLND-1 during the early events of viral attachment and entry. In fact engagement of CD81 by the use of the recombinant protein E2, the E1E2 heterodimer complex or anti-CD81 antibody led to a translocation of this receptor, normally present on the whole cellular surface, to the areas of cell-cell junctions, where CLDN-1 dwells.

The use of chemical inhibitors of actin polymerization, such as Latrunculin A and Jasplakinolide, proved the involvement of the actin cytoskeleton in CD81 translocation. Time course experiments with LatA demonstrated that an intact and functional cytoskeleton is required at a very early stage in the relocalization process, beside having also a relevant physiological importance on viral internalization. In fact, pretreatment of cells with these specific actin inhibitors as well as silencing the expression of some proteins of the Rho GTPases family, such as Rho, Rac ad Cdc42, which normally modulate the actin rearrangement, greatly reduced HCVcc infectivity.

§ 1. INTRODUCTION

1.1 Impact of HCV in the World. Different GTs.

Hepatitis C virus (HCV) is a major cause of acute and chronic hepatitis world-wide. The liver, and the hepatocytes in particular, are the HCV virus primary targets, although it has been shown that the viral RNA is able to replicate also in some hematopoietic cells, as dendritic cells and B lymphocytes (Sung *et al*., 2003; Goutagny *et al*., 2003). To date, the World Health Organization (WHO) reports that about 170 million people are currently infected with HCV, but the acute infection is usually asymptomatic, making the early diagnosis difficult to achieve. In general, it has been estimated that in 20-30% of acutely infected individuals there is a spontaneous viral clearance that results in a resolution of the infection without any health complications, but the rest of the acute infections become persistent. When individuals become chronically infected by HCV they have a very high risk of developing serious liver diseases that may begin with liver cirrhosis and progress to hepatocellular carcinoma (Zeisel *et al.*, 2007a; Chisari, 2005).

In common with hepatitis B (HBV) and human immunodeficiency virus (HIV), HCV may be transmitted through the use of unscreened blood transfusions and related products, and by organ transplantation, but today the category primarily hit by HCV is the one related to injecting drug users, for the re-use of needles and syringes that have not been adequately sterilized, and their sexual partners.

Although many efforts have been made to develop an efficacious vaccine against HCV with therapeutic or prophylactic activity, the target of these studies has still to be achieved. The only way to resist the infection to date is based on the combined use the antiviral drugs interferon-α and ribavirin, which unfortunately are effective only in 50% of the infected population, depending on individuals and, mostly, on the HCV genotype that is present in patients' blood. Hepatitis C virus in fact is present around the world in six main different genotypes and a large number of subtypes, and some are mainly distributed in certain areas compared to the others. Genotype 1, for example, is prevalent in Northern Europe and USA (subtype 1a), as well as in Corea, Japan, Southern and East Europe (subtype 1b) whilst genotype 5 is only present in South Africa and genotypes 4 and 6 are more frequent in Asia and in Australia. (Simmonds *et al*., 2005; Chevaliez and Pawlotsky,

2007). Genotypes 2 and 3 are widely distributed throughout the world, from the Mediterranean Countries to the Far East, and it is against them that treatments with antiviral drug cocktails achieved the best results, with viral eradication or diminishing of viral load in 50-60% of patient treated (Dago, 2005; Yoon *et al*., 2007).

1.2 Hepatitis C virus life cycle

HCV has been classified as the sole member of a distinct genus called *hepacivirus* in the family Flaviviridae, which includes the flavivirus and the animal pathogenic pestiviruses. (Reed and Rice, 2000; Lindenbach and Rice, 2001). These viruses have in common an envelope particle harboring a plus-strand RNA that, in the case of HCV has a length of about 9600 nucleotides. The HCV virus is able to infect only humans and chimpanzees and the lack of small animal models has been for a long time a strong obstacle for studies on viral life cycle. It's been only two years since researchers have been able to produce HCV infectious viral particles *in vitro* (Lindenbach *et al.*, 2005; Wakita *et al*., 2005; Zhong *et al*., 2005), and although this result represents a success and a great step forward in the study of HCV life cycle, the particle production of the HCV cell-culture system (HCVcc) is still too low to be applied in all HCV research fields. These are the reasons why nowadays little is known about the mechanisms and the host functions involved in viral entry, uncoating, trafficking, assembly and egress, but all around the world researcher are investigating on many of these topics and some details begin to emerge due to the availability of efficient *in vitro* systems, that can be used as surrogates of the HCVcc when this can not satisfy experimental requirements. These alternative methods comprise the replicon system (Lohmann *et al*., 1999; Blight *et al*., 2000), HCV pseudoparticles, HCVpp, (Bartosch *et al*., 2003b; Hsu *et al*., 2003), and the recombinant proteins E2 or the E1E2 complex (Spaete *et al*., 1992; Choo *et al*.,1994).

To initiate its life cycle, HCV has to cross the plasma membrane of hepatocytes and gain access to the cytosol, where everything takes place. The HCV particles consist in a nucleocapsid surrounded by a lipid bilayer where the two envelope glycoprotein E1 and E2 are anchored (Deleersnyder *et al*., 1997; Op De Beek *et al*., 2004). These two proteins form noncovalent heterodimers, which interact with HCV receptors on the surface of the

host cells, playing a major role in HCV fusion process and entry (Hsu *et al*., 2003; Ciczora *et al*., 2007).

The detailed mechanism of interaction between the virus and the host cell is complex and still under close investigation. A number of receptors on the cellular surface interact with the HCV. Among them, for example, the low density lipoprotein receptor LDLr might be utilized by the HCV virus to enter cells exploiting its function of carrier of cholesterol-containing lipoprotein particles from the extracellular medium into cells, and the glycosaminoglycan heparan sulfate HS, might allow viruses to adhere to target cells before high affinity receptors induce specific adhesion and entry. Nevertheless, three molecules seem to be mainly involved in viral binding and entry: the scavenger receptor group B type I (SR-BI), the tetraspanin CD81, and the transmembrane protein Claudin-1 (CLDN-1). The ubiquitously expressed CD81 was the first molecule to be identified as putative receptor for HCV by Pileri and coworkers (1998), and, as a member of the tetraspanins family, it is probably involved in the formation of an extended network on the cell surface acted to organize the membrane by protein interactions. Regarding the mechanisms of viral interaction in which the main receptor molecules might be involved, it has been postulated that SR-BI and CD81 act in concert to allow viral entry, and probably a first contact with SR-BI might be necessary before the particle interacts with CD81 (Zeisel *et al*., 2007b; Evans *et al*, 2007). The recent discovery by Evans and coworkers (2007) that identify the Claudin-1 as a new potential HCV receptor involved in viral entry added a new step in this process. This protein is predominantly expressed in the liver but it is also found in other epithelial tissues, and belongs to a family responsible for the formation of specialized structures in areas of cell-cell contact: the tight junctions. It is interesting to highlight the different cells distribution of these receptors, with CLDN-1 strictly localized to the tight junctions in polarized hepatocytes, and SR-BI and CD81 uniformly distributed on the whole cellular surface, because it might hide peculiar mechanisms of interaction among the HCV virions and these molecules.

After binding to the cellular receptors HCV virus has to cross the plasma membrane to be internalized (Fig. 1). Viruses that require internalization for entry mostly take advantage of clathrin-mediated endocytosis, which is the main route of receptor internalization into cells (Brodsky *et al.*, 2001). Clathrin and associated proteins assemble on the intracellular face

of the plasma membrane to form invaginations that vesiculate into the cell (Conner and Schmid, 2003; Mousavi *et al.*, 2004). As they mature into early endosomes, clathrin-coated vesicles shed their protein coat and become acidified. Viruses entering through this pathway generally follow the course of their attachment receptors until the low pH in early or late endosomes trigger fusion and delivery of the viral capsid to the cytoplasmic site of replication. Recent studies support the hypothesis that also the HCV virus exploits this mechanism of internalization by clathrin-coated vesicles (Blanchard *et al*., 2006; Meertens *et al*., 2006) followed by delivery to early endosomes. In sustain of this latter statement, using retroviral particles pseudotyped with E1E2 (HCVpp), and cell-culture derived HCV (HCVcc), different laboratories established that inhibitors of endosomal pH acidification markedly reduced the infectivity of these particles, to delineate a pH-dependent route of entry for the hepatitis C virus (Bartosch *et al.*, 2003c; Hsu *et al*., 2003; Tscherne *et al*., 2006), in common with other viruses of the Flaviviridae family. In particular, Bartosch *et al*. (2003c) proposed that once the HCV virions bind to their receptors, one or maybe more of these proteins should traffic virions to endosomal compartments. SR-BI was indicated as the strongest candidate in this respect, because it normally mediates the selective uptake of macro-molecules such as high-density lipoproteins and lipopolysaccharides (LPS), carrying them in intracellular compartments. This indicated that SR-BI may have the capacity to traffic receptor-bound virions in clathrin-coated vesicles to endosomal compartments where the low pH could activate the fusion between the viral and the endosomal membranes(Meertens *et al*., 2006; Tscherne *et al*., 2006), with the released of the viral nucleocapsid in the cellular cytoplasm. Nevertheless, the exposure of the host cells to low pH could not induce fusion of the HCVcc with the cell membrane, suggesting that a post-binding maturation step is required to render the virus competent for low-pH-triggered entry (Tscherne *et al*., 2006). It may be that the HCV attached to the cellular surface via a primary bind determinant such as CD81 may require interaction with a co-receptor to stabilize binding and activate the virus for the entry step induced by low pH.

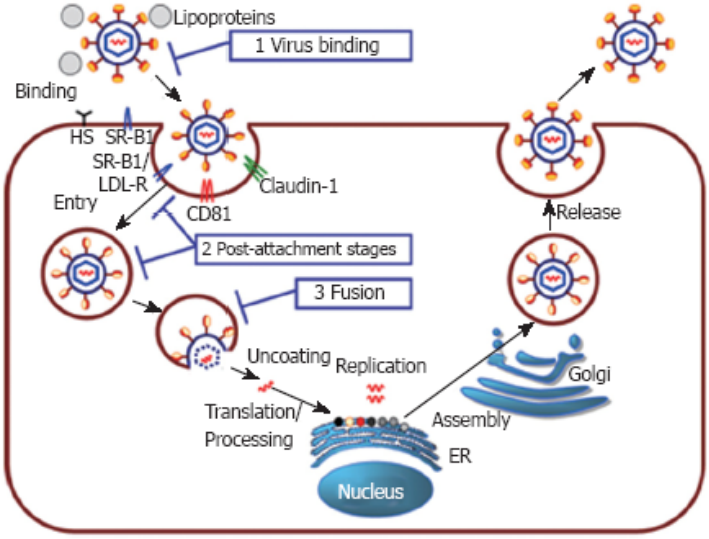

Fig. 1. HCV life cycle. When the HCV virus comes in contact with the cellular surface it interacts with a number of receptor proteins. The precise role and function of some of these has still to be defined, as for the Heparan Sulphate (HS) or for the Low Density Lipoprotein receptor, but the importance of others is much more clear. HCV certainly interacts with SR-BI, CD81 and, in a later step, with Claudin-1 in its process of binding and entry. Probably bound to SR-BI, HCV is trafficked in clathrin-coated vesicles to early endosomes where the low pH triggers the membrane fusion and nucleocapsid release into the cytoplasm. After the uncoating the plus-strand RNA genome is translated to initiate virus replication. Virus assembly occurs onto intracellular membranes derived from the endoplasmic reticulum and leads to the formation of naïve viral particles that are released by exocytosis.

Once inside the early endosomes the uncoating of the viral genome will soon take place, leading to the translation of the plus-strand RNA into viral proteins. The HCV genome is a linear molecule with a length of about 9600 nucleotides, coding for a single polyprotein of about 3000 amino acids. The RNA sequence carries a long open reading frame that is flanked at the 5' and at the 3' ends by two non-translated region (NTR); it is inside the NTR at the 5' end that resides the internal ribosome entry site (IRES). This short sequence is required for the translation of the HCV genome since it binds directly to the 40S ribosomal subunits of the host cell, without the need of any pre-initial factors, enabling the start of the translation process as soon as the entire 80S ribosome has formed. Directed by the IRES sequence the large HCV polyprotein is translated at the rough endoplasmic reticulum and cleaved co- and post-translationally by host cell signalases and two viral proteinases. The translation process leads to the consequential expression of viral structural proteins, that are core and the envelope proteins E1 and E2, of a small integral membrane protein p7, that seems to function as an ion channel, and of the non-structural

proteins (NS) NS2, NS3, NS4A, NS4B, NS5A, NS5B, which coordinates the intracellular processes of the virus life cycle (Bartenschlager and Lohmann., 2000; Lindenbach and Rice, 2005; Appel *et al.*, 2006) (Fig. 2).

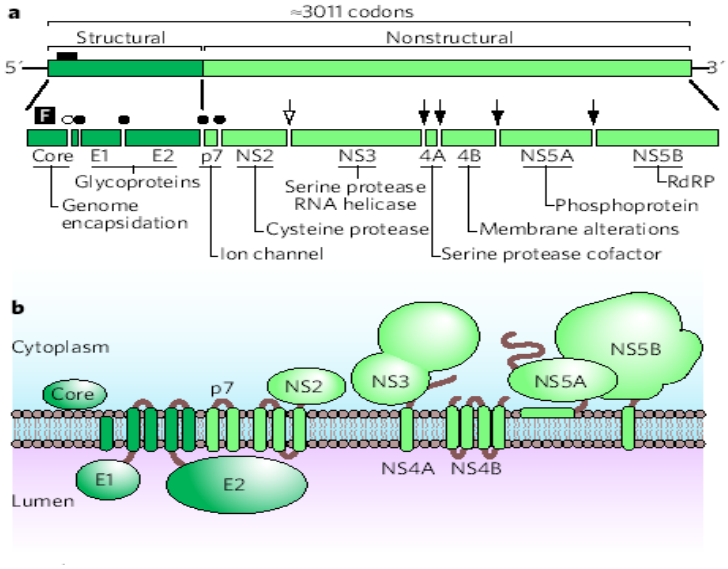

Fig.2. HCV genome and gene products. (a).The structure of the viral genome is schematically represented, including the 5' and 3' non-coding regions, the structural and non-structural genes. The polyprotein processing scheme is shown below: closed circles indicate cleavages by the ER signal peptidase, the white arrow indicates the self-cleavage by HCV NS2-NS3 protease, whilst the black arrows indicate the cleavage sites of the NS3 protease. (b). The topology of HCV proteins with respect to the membrane of the endoplasmic reticulum.

The first protein to be translated from the polyprotein sequence is Core. During translation, the nascent polypeptide is targeted to the endoplasmic reticulum for the translocation of the E1 protein ectodomain into the lumen of this intracellular compartment. This occurs thanks to the presence of an internal signal sequence between core and E1, that is than cleaved by host signal peptidases, leading to the formation of an immature core protein, which still contains the E1 signal sequence at its C-terminus. The mature core protein is then produced by the processing of this immature form by an ER signal peptide peptidase, which cleaves off the E1 signal peptide. The hydrophobic nature of the C-terminus of the mature core protein is responsible for its association with ER membranes or with lipid droplets in mammalian cells (Hope and McLauchlan, 2000) and determines its perinuclear localization inside the cell. The capability of core to interact both with viral RNA and the envelope protein E1 suggest its function in determining the formation of the virions nucleocapsid, although the precise mechanisms of capsid assembly are still to be defined (Kato, 2001; Penin *et al.*, 2004).

Other two very important proteins for the HVC life cycle are the envelope proteins E1 and E2, which play a pivotal role in virus-host cell interaction, in particular in binding and entry processes. E1 and E2 are type-I- transmembrane glycoproteins of about 30 and 60 kD respectively, with an N-terminal ectodomain and a short C-terminal transmembrane domain (TM), and they assembly in the virions as non-covalent heterodimers. It has been shown that the N-terminal sequences of E2 (aa 415-500 in the polyprotein) and also in E1 are important for the glycoprotein interaction (Patel *et al*., 1999), and the assembly of E1E2 complexes is not affected in mutated forms of E1 and E2 in which the TM domains have been removed, suggesting again that it is the ectodomains of E1 and E2 that contain the determinants for heterodimerization (Michalak *et al*., 1997; Takikawa *et al*., 2000). The transmembrane domains of E1 and E2 are composed by two stretches of hydrophobic residues separated by a short segment containing conserved charged aminoacids (Cocquerel *et al*., 2000), and the second one functions as a signal sequence for the ER localization of these proteins, beside being important for anchoring them to the ER membranes (Op De Beek *et al*., 2001; Cocquerel *et al*., 2002). Envelope proteins are targeted to the endoplasmic reticulum by these signal sequences in the polypeptide form, and then are co- and post-translationally separated from each other by host signal peptidases, remaining anchored to the ER membranes by the hydrophobic sequence located at their C-terminus, with the ectodomain in the ER lumen. The processing and the complex folding pathway of the viral glycoproteins is assisted by several ER-resident chaperones (Bartenschlager and Pietschmann, 2004), but recent studies affirm that probably the two proteins may influence each other's folding, and in particular it seems that E1 can not reach the native structure in absence of E2. Beside this, the conformational maturation of E2 can be the limiting step in the conformation of native heterodimers E1E2, as it is sustained by studies that shown that E2 expressed alone in mammalian cells are not recognized by human conformational antibodies and showed a weaker binding to the putative receptor CD81 (Deleersnyder *et al*., 1997; Cocquerel *et al*., 2003a; 2003b; Brazzoli *et al*., 2005). In the ectodomain of the E2 protein it has been identified an hypervariable region (HVR1) constituted of 27 aminoacids that differ by up to 80% among HCV different genotypes; this short region represents the only neutralization epitope so far identified for the major envelop protein E2 (Weiner *et al.*, 1991; Kato, 2001; Farci *et al*., 1996). Another hypervariable region, HVR2, has been described in the E2 glycoprotein of the HCV genotype 1 stains; and it is represented by only 7 aminoacids that have a 100%

difference among genotypes. Together, HVR1 and HVR2 seem to modulate the binding of the E2 glycoprotein with the putative receptor CD81 on the host cell surface (Roccasecca *et al*., 2003), highlighting the great importance that the envelope proteins, and in particular E2, have in virus-host cell interactions.

Following the translation of E1 and E2, the little peptide p7 is expressed in the ER. This is a small protein of 63 aminoacids long with a double membrane spanning topology in which the N- and C-termini, released from the polyprotein by host signal peptidases, face the ER lumen. This protein belongs to the viroporin family and recent data sustained that it could have important roles in viral particle release and maturation, being essential for the production of infectious progeny virus (Carrasco, 1995; Harada *et al*., 2000; Jones *et al*., 2007).

The 3' half of the polyprotein is involved in the expression of the Non Structural proteins, which are indispensable for RNA replication.

The NS proteins include two important proteinases: the NS2-NS3 zinc-dependent metalloproteinase, and the NS3 serine protease. The former is responsible for the formation of NS2, in a self-cleavage process and in concert with ER signal peptidases, the latter instead is delegated to the correct processing of the proteins NS3-NS4A-NS4B-NS5A-NS5B, aided in this role by its co-factor NS4A. Beside its function of protease, NS3 is also a viral helicase, capable of unwinding RNA-RNA duplex in a ATP-dependent manner, thus exploiting important function in viral RNA replication (Tai *et al*., 1996); the function of NS2 instead is still to be defined.

Another important protein for the HCV replicative events is NS4B. This protein in fact seems to trigger the modification of ER intracellular membranes leading to the formation of specialized structures. These membranous vesicles, forming the so-called membranous web, have been proposed by Egger *et al*. (2002) as the scaffold of the HCV replication complex, since viral RNA and all the viral proteins were found to be associated here in a multiprotein complex. It is NS5A that seems to bind and sequestrate the viral RNA in this area, protecting it from degradation by the cellular nucleases and preventing the activation of antiviral responses, such as Interferon pathways (Appel *et al*., 2006).

The last non-structural protein to be translated in the polyprotein sequence is the NS5B, whose function as RNA-dependent RNA polymerase (RdRp) makes it the catalytic core of the HCV replicative complex. NS5B assembles to the 3' end of the positive strand RNA to

initiate the synthesis of new negative strand RNAs. The newly generated RNAs (-) serves either as templates for the production of an excess of positive strand RNA to be translated in viral proteins, or for a new round of production of RNA plus strands to be packaged into native viral particles.

The mechanism by which mature, infectious HCV progeny is formed is still enigmatic. Blanchard *et al*. (2002) sustained that HCV core proteins self-assemble at the ER membrane to form a structured capsid, which acquires its envelope proteins E1 and E2 by budding into the lumen of the ER (Bartenschlager and Lohmann, 2000; Reed and Rice, 2000). Nevertheless, in a very recent work by Miyanari *et al*. (2007) it was highlighted the important role that core protein and lipid droplets (LDs) in the cytoplasm of infected cells might have in producing infectious viruses. What they stated is that core protein, mainly localized on the lipid monolayer that surrounds LDs, induces apposition of LDs to the ER membranes, recruiting NS proteins and replication complexes, which normally dwell in here. This recruitment of the NS protein, as well as the replication complexes, and the viral envelope proteins to the LD-associated membranes might serve to create a proper microenvironment for proteins association and virus assembly.
Once the virions have assembled they are probably released from cells through the exocytosis pathway (Mackenzie and Westaway, 2001) in intracellular vesicles, in analogy with what has been observed in other flaviviruses.

1.3 HCV receptors

A considerable number of receptors have already been proposed for HCV, and most of them have been isolated based on binding studies with the soluble truncated form of the envelope glycoprotein E2 (sE2), by the use of pseudoparticles (HCVpp), or cell-culture derived HCV (HCVcc).
By using sE2 as a probe to identify cell-surface proteins potentially involved in HCV entry, the human tetraspanin CD81 and the human scavenger receptor SR-BI (Pileri *et al*., 1998; Scarselli *et al*., 2002) were identified as potential HCV receptors (Fig. 3). Whether CD81 is involved in cell attachment, endocytosis, fusion, or another step remains unclear, but recent studies (Cormier *et al*., 2004b; McKeating *et al*., 2004) suggested that it operates after attachment of virions to the cell surface, acting as a co-receptor. Like all members of tetraspanin family CD81 is composed of four transmembrane passages, a small

extracellular loop (SEL) and a large extracellular loop (LEL) (Seigneuret, 2006). The involvement of CD81 in HCV entry has been confirmed in different models. For example, it has been demonstrated that hepatoma cells that have been silenced for CD81 expression are non longer permissive to HCVpp and HCVcc infection, and that susceptibility of cells to the virus is closely related to the CD81 expression level (Zhang *et al*., 2004; Akazawa *et al*., 2007; Koutsoudakis *et al*., 2007). Residues of CD81 involved in the interaction with the viral envelope, and in particular with E2, have been mapped in the Large Extracellular Loop (LEL) (Pileri *et al*., 1998; Zhang *et al*., 2004; Bertaux and Dragic, 2006), but although the E2 seems to have the main role in the receptor interaction, other studies demonstrated that E1E2 heterodimer have a stronger interaction with CD81 compared to the solely soluble form of E2 (sE2), suggesting that also E1 may play a key role, modulating the binding of E2 to CD81 (Cocquerel *et al*., 2003a). The expression of CD81 though, is not sufficient for a cell to become permissive to HCV infection. In fact, ectopic expression of this receptor in non-hepatic cells does not render them permissive to the infection (Hsu *et al*., 2003; Bartosch *et al*., 2003c), indicating that other molecules act in concert with CD81 to mediate HCV binding and entry into hepatocytes.

While CD81 may introduce conformational changes and influence HCV entry in an indirect fashion, it is not an obvious candidate to mediate endocytosis of the virus, as it is not strongly recycled from the surface into the cell interior, in contrast to SR-BI (Silver *et al*., 2001). SR-BI is expressed in the majority of mammalian cells, particularly in hepatocytes, and it is characterized by a C- and N- terminal cytoplasmic domains separated by a large extracellular domain. This glycoprotein of about 500 aminoacids is involved in cellular lipometabolism, thanks to its capability of binding high-density lipoproteins (HDLs) to its extracellluar domain and carrying them inside the cell (Connelly and Williams, 2003). The exact role of SR-BI in HCV entry is not well understood, but a couple of mechanisms have been proposed. Both Evans *et al.* (2007) and Catanese *et al*. (2007) suggest a direct interaction between the viral particle and SR-BI, whilst Maillard *et al.* (2006) suggested that the virus might indirectly interact with the receptor through its associated lipoproteins. In any case, the role of SR-BI seems to be essential for viral infectivity as Lavillette *et al*. (2005a,b) demonstrated in their study in which human hepatoma cells (Huh7) silenced for the expression of this receptor became of the 90% less permissive to HCVpp entry.

In studies on human hepatoma cell lines regarding the identification of receptors other than CD81 responsible for E2 binding, Scarselli *et al.* (2002) claimed that the high affinity between the hypervariable region HVR1 of this viral protein and SR-BI strongly support the hypothesis that this receptor plays a role in HCV infection. Further studies demonstrated that SR-BI represents a key host factors for HCV entry, and targets an entry step closely linked to HCV-CD81 interaction (Bartosch *et al*., 2003c; Zeisel *et al*., 2007). Although the importance of these two molecules for HCV-host cell interaction is no longer doubt, neither CD81 nor SR-BI has a liver-specific expression profile; on contrary, they are expressed on a number of cell lines of hepatic and non-hepatic origin. Because hepatic cell lines expressing CD81 and SR-BI are permissive to HCVpp (Bartosch *et al.*, 2003c), as well as HCVcc (Zeisel *et al*., 2007), whilst the non-hepatic cell line expressing the same receptors are not, researchers speculated that the missing entry factors for HCV are indeed liver specific molecules (Bartosch *et al*., 2003a, b, c; Lindenbach and Rice, 2005; Zhang *et al*., 2004).

Recently Evans *et al*. (2007) identified, among all cellular factors that might be involved in HCV entry, a tight junction protein that is highly expressed in liver cells, Claudin-1 (CLDN-1). The kinetics of its inhibition indicated that CLND-1 acts late in the entry process, after virus binding and interaction with the HCV co-receptor CD81. The engeneered expression of CLND-1 in non-hepatic cells, which naturally express both CD81 and SR-BI, rendered them permissive to HCVpp and HCVcc, leading to the identification of the first protein that actually confers susceptibility to HCV infection in non-hepatic cells. However, there are several cell lines which express CLND-1, CD81, and SR-BI that are resistant to HCV infection, and this proves that still other factors are required for HCV entry.

The strict localization of CLND-1 in the areas of cell-cell contact of polarized cells, where the tight junctions are, and the opposite diffuse localization of the other two main HCV receptors SR-BI and CD81, suggest a mechanism of apposition among these molecules during the binding and entry processes.

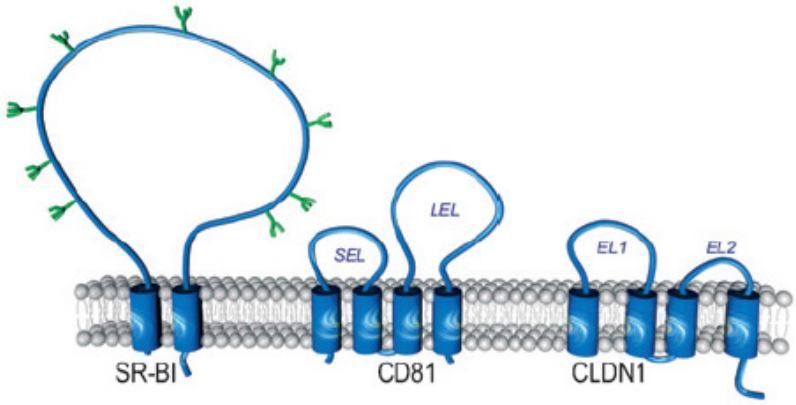

Fig. 3. Schematic representation of the three main HCV receptors. SR-BI (82 kD) is a 509-aa protein containing two transmembrane passages, two intracellular domains (of 11 and 45 aa respectively) and a large extracellular loop (411 aa) which contains nine potential glycosylation sites (in green), probably involved in cholesterol uptake. CD81 (26 kD) is composed of four transmembrane passages, two intracellular domains (each of 12 aa), a small intracellular loop (5 aa) and two extracellular loops, SEL (30 aa) and LEL (89 aa). It is in the Large Extracellular Loop that the residues of CD81 involved in the interaction with the HCV envelope protein E2 have been identified. CLDN-1 (22 kD) is composed of four transmembrane passages, a short intracellular N-terminal peptide(7 aa), an intracellular loop (13 aa), an intracellular C-terminal tail (27 aa) and two extracellular domains, EL1 (53 aa) and EL2 (27 aa), and is a structural component of the tight junctions in polarized cells.

Beside these three molecules so important for the HCV viral-host interactions, other receptor candidates have been identified in the Low Density Lipoprotein Receptor (LDLr), in glycosaminoglycans, and lectins. The previous, is normally involved in transport of cholesterol-containing lipoprotein particles from the extra cellular medium into cells, where lipoprotein particles proceed to lysosomes. In the context of native particles isolated from HCV-infected patients, the LDL receptor has been shown to mediate HCV internalization by binding to virion-associated LDL particles (Agnello *et al*., 1999), and this would lead to confer to the LDL receptor and to SR-BI a similar function. For LDL receptor though these results have not been confirmed by experiments using HCVpp (Bartosch *et al*, 2003a), and need further investigation. Glycosaminoglycans (GAGs) instead, are important chains on cell-surface proteoglycans that provide primary docking sites for the binding of various viruses to host cells (Villanueva *et al*., 2005). Barth *et al*. (2003), studiyng the interaction between the HCV envelope glycoprotein E2 and the GAG heparan sulfate, postulated that highly sulphated heparin sulphate my serve as the initial docking site for HCV attachment., but again these data did not find any support by HCVpp experiments (Callens *et al*., 2005).

It has also been shown that sE2 binds specifically to the mannose-binding lectins, DC-SIGN and L-SIGN, as HCVpp and native HCV particles (Gardner *et al*., 2003; Pöhlmann *et al*., 2003; Lozach *et al*., 2003; 2004). However these lectins are not expressed on hepatocytes and are therefore not receptors for these cells. DC-SIGN is expressed on dendritic cells, whereas L-SIGN is mainly expressed on sinusoidal endothelial cells in the liver and lymph nodes (Koppel *et al*., 2005) and both of them have been demonstrated to be capable of capturing and to transmitting HCVpp among adjacent cells (Cormier *et al*., 2004a; Lozach *et al*., 2004), so they may contribute to the establishment or persistence of HCV infection rather than being implicated in mechanisms of viral binding and entry.

1.4 Replicons and the JFH-1/HCVcc system

As I previously mentioned, the possibility to study the numerous aspects of the hepatitis C virus life cycle were, until very recently, seriously limited by the lack of a cell culture system for growing the virus under laboratory conditions. The method that represented a very important step forward in HCV research before the advent of the cell-culture derived HCV (HCVcc), and that is still largely used in studies on viral RNA replication and proteins maturation and processing, is based on replicon systems (Lohmann *et al*., 1999). These replicons are genetically engineered HCV genomes, that can contain either complete genomic RNA of HCV or shorter subgenomic fragments consisting in the complete non-structural region from NS2 to NS5B or part of it, when also NS2 is excluded. Since the HCV replication function is performed by the non-structural proteins, that naturally constitute the HCV replicative complex, the lack of the structural proteins as Core, E1 and E2 in replicons did not effect the amplification process, and this represent the most important characteristic of these replicons, which are self-amplifying elements. In the first prototype of subgenomic replicon, derived by HCV genotype 1b (Lohmann *et al*., 1999), the HCV structural region was replaced with two heterologous elements: the first was a sequence encoding for neomycin phosphotransferase (*neo*^{r}), conferring resistance to the antibiotic G418; the second was the internal ribosome entry site (IRES) of encephalomyocarditis virus (EMCV). These elements made the bi-cistronic RNA replicon selectable using G418, with the expression of *neo*^{r} gene directed by HCV IRES, whilst the second cistron encoded for the expression of the NS proteins directed by EMCV IRES.
Replicon RNA was generated by in vitro transcription from cDNA and transfected into human hepatoma cells Huh7. Replicons maintained in these cell clones subjected to G418

selection developed single-aminoacids substitutions that render their replication more efficient, and selected cells, that hosted them, resulted particularly permissive to viral RNA replication and protein expression, representing a new cell clone to be used in HCV experimental studies (Blight *et al*., 2000).

An important expansion of the replicon system was the development of full length genomic HCV replicons as a potential tool to generate viral particles in cell culture. But the main problem underlined by these systems was the lack of correlation between the level of the viral RNA replication and protein expression, that were highly detected in transfected cells, and the deficiency in formation of infectious virions (Bartenschlager *et al*., 2003; Brass *et al*., 2006; Ikeda *et al*., 2002; Pietschmann *et al*., 2002). To find a solution to this problem a large panel of different replicon systems has been generated (Blight *et al*., 2003; Kato *et al*., 2003), but although these systems presented a superior replication capacity, thanks to the development of adaptive mutations within the NS proteins, they were not able again to produce infectious viral particles. This limitation was overcome by the isolation by the group of Takaji Wakita of a peculiar HCV genotype 2a strain, designated as JFH-1 (Japanese Fulminant Hepatitis), from a patient with fulminant hepatitis (Kato *et al*., 2003). This isolate was able to replicate to high levels in human hepatoma cells without the requirement of cell culture adaptive mutations, and, primarily, it could produces infectious viral particles in cell culture (Lindenbach *et al*., 2005; Wakita *et al.* 2005; Zhong *et al.,* 2005). Using JFH-1 as a backbone, it was possible to generate chimeric genomes composed of the replicative portion of JFH-1 (NS encoding region), flanked by the sequence of structural proteins belonging to other genotypes, such as 1a, 1b, 2a, and 3a (Pietschmann *et al*., 2006). When the RNAs of these chimeric genomes were transfected in human hepatoma cells (Huh7) infectious viral particles were released in all cases, with the exclusion of the GT 3a/JFH chimera, although the low efficiencies of these systems, compared to the JFH-1 wt, did no allow the development of satisfactory cell culture HCV production.

Nowadays the highest yields of cell culture-derived HCVcc have been obtained using the JFH-1 genome and the intragenotypic HCV chimera J6/JFH, composed of the core to the NS2 region from the GT2a J6 HCV isolate substituting the analogous region in JFH-1. The establishment of cell-culture derived HCV (HCVcc) infectious particles in based on the *in vitro* transfection of human hepatoma cells Huh7 or derived permissive clones. The

infectious viruses produced with the HCVcc system are obtained from cell culture supernatants, and viral titers obtained so far correspond to 10^4-10^6 infectious units per ml. Different studies demonstrated that infectious particles are able to spread throughout the culture within a few days after inoculation, at low multiplicity of infection (moi), and that virus can be serially passaged without loss of infectivity (Lindenbach *et al*., 2005; Zhong *et al.,* 2005).

1.5 Additional model systems to study potential receptors: soluble E2, E1E2 complex and HCVpp

Although infectious viral particles produced with the HCVcc system represent a new important tool for the study of the full HCV life cycle *in vitro*, the yields obtained so far may not be sufficient to satisfy the requirement of all experimental designs.
It is for this reason that HCVcc are often replaced or supported by other tools, such as the soluble form of the recombinant protein E2, deleted of its ER anchoring transmembrane domain, the heterodimer E1E2 complex, or retroviral pseudoparticles set with HCV E1E2 envelope glycoproteins (HCVpp).

The soluble, truncated forms of HCV glycoprotein E2 (sE2: $E_{348\text{-}661}$, $E_{348\text{-}715}$) have been used by several laboratories around the world as a tool to search for cell-surface proteins potentially involved in HCV attachment end entry, and it is through the sE2 use that Pileri *et al.* (1998) were able to identify the cell surface protein CD81 as a potential receptor for HCV, involved in cell entry. The recombinant form of E2 binds with high affinity to human lymphoma and hepatocarcinoma cell lines, through the mediation of CD81 (Rosa *et al.*,1996), but some functional differences might exist between the sE2 and the full length E1E2 complex, as the presence of E1 might potentially affect the properties of E2 (Cocquerel *et al*., 2003a; Brazzoli *et al*., 2005) in its binding with HCV receptors. E1E2 heterodimer in fact, seems to be a more physiological ligand compared to the solely sE2, and this is the reason why in many cases experiments are run using both these forms. The availability of these ligands came when Choo *et al* (1994) were able to copurify E1 and E2 glycoproteins from mammalian cells; doing this they noticed that a fraction of this purified material existed in the form of a large E1E2 oligomeric complex, that could be as well extracted and used in experimental studies.

Another tool that has been used to study virus-host cell interactions is based on pseudoparticles HCVpp, which consist of unmodified HCV envelope glycoproteins assembled onto retroviral core particles (Bartosch *et al*., 2003b; Drummer *et al*., 2003; Hsu *et al*., 2003).

Characterization of HCVpp have shown that these mimic the early steps of HCV life cycle in terms of cell entry pathways, as well as attachment and receptor binding, and exhibit a preferential tropism for liver cells and a specific sensitivity towards anti-E2 monoclonal antibodies, which are able to neutralize their infectivity (Bartosch *et al*., 2003b; Hsu *et al*., 2003; Op De Beek *et al*., 2004).

HCV pseudotyped retroviral particles are usually engineered to contain a reporter gene transcript, such as the green fluorescent protein GFP, enclose in the retroviral capsid, that allow a quick detection of HCVpp infected cells using a common fluorescent microscopy. HCVpp are produced in 293T cells (human embryonic kidney-derived cells) after transfection of three independent DNA constructs containing the *gag* and *pol* genes of the retrovirus, a packaging/reporter gene construct, and HCV glycoproteins. Viral capsids engulfing the reporter gene (GFP) are assembled inside the transfected cells and acquire the HCV envelope proteins by budding at the host cell membranes (Bartosch *et al*., 2003b; Diedrich, 2006; Op De Beek and Dubuisson, 2003).

Althoug the HCVpp have been widely used for characterization of some candidate receptors for HCV (Cocquerel *et al*., 2006; McHutchinson *et al*., 2006) and their interaction with the E1E2 complex, it has to be considered that they differ from natural HCV virions. It has been demonstrated, for example, that HCVpp do not associate with lipoproteins as wild HCV virions do, leading to suppose that some of the mechanisms of that have been highlighted by the use of this system might required a parallel control to be validated. Nowadays, the system that for the most mimics the wild HCV particles is the HCVcc, and this is the reason why many laboratories are now working trying to improve its efficiency, to get the necessary viral yield for any typology of HCV experimental studies.

1.6 virus-host cell interactions

Viruses are obligatory intracellular parasites, and they evolved exploiting the behaviour and the physiology of their hosts to sustain their life cycle. At the cell level, this attitude is manifested in the activation of endogenous cellular responses that provide assistance to viruses during the entry and the replicative processes.

It is well known that many viruses make use of the cell signaling pathways during entry. This was first recognized for adenoviruses, which use CAR as primary receptor and integrins as co-receptors (Li et *al*., 1998; Nemerow and Stewart, 1999). Nemerow and Stewart demonstrated that the interaction between proteins forming the viral capsid and integrins activates phospatidylinositol 3-kinase (PI3K), which in turn activates Rac and Cdc42, resulting in the polymerization of actin- and clathrin-mediated endocytosis of the virus. Activation of many different signaling pathways has been described, with the involvement of a variety of factors, including serine/threonine, tyrosine, and PI kinases, and a variety of small GTPases, including Arf, Rab and Rho family members (Greber, 2002; Pelkmans *et al*., 2005). For example, the internalization of SV40 by caveolar/raft endocytosis is regulated by at least five different kinases (Pelkmans *et al*., 2005), and the inhibition of tyrosine kinase, in particular, blocks internalization and dramatically reduces infection (Chen and Norkin, 1999; Pelkmans *et al*., 2002). Cellular signals can be triggered by the virus in several ways, by direct binding to signaling molecules, using them as receptors, or by clustering specific cell-surface proteins or lipids that under these conditions can become associated to lipid rafts and initiate intracellular signaling (Coyne and Bergelson, 2006; Damm *et al*., 2005; Pelkmans *et al*., 2002; Sharma *et al*., 2004), but in all cases the dynamic changes of the actin cytoskeleton triggered by virus-induced signaling will lead to viral particles internalization, in most cases, by endocytosis.

In some cases, before being internalized the virus needs to induce its lateral movement along the cellular membrane, as it has been observed for murine leukemia virus and HIV-1 (Lehmann *et al*., 2005), and for coxsackieviruses. Coyne and Bergelson (2006) demonstrated that coxsackie B virus needs to trigger its lateral movement to reach the CAR receptor, which is a component of the tight junctions in epithelial cells and is inaccessible to virus approaching from the apical surface. This lateral movement occurs following the viral attachment to another receptor, DAF (glycosylphosphatidylinositol (GPI)-anchored decay-accelerating factor), abundant on the apical surface of polarized epithelial cells. This virus-receptor interaction activates the AbI kinase that triggers Rac-

dependent actin rearrangements that in turn permits virus movement to cell junctions, where the virus can interacts with its receptor CAR. A similar mechanisms of action during the binding and entry processes could be hypotized also for HCV, since the diffuse localization of the two main receptors SR-BI and CD81 on the cellular surface suggests a mechanism of apposition among these molecules and the third main HCV receptor CLND-1, localized at the cell junctions.

Detailed studies of the large number of pathogens that infect a wide variety of different cell types will lead to the descovery of currently unknown signaling pathways and to the dessecting of moleculat mechanisms that are underneath signaling networks. This will also facilitate the understanding of the molecular bases of the infection and could lead to the identification of new drug targets and to the developement of effective therapies to treat and prevent infection and desease.

§ 2. AIMS

HCV is a major cause of chronic liver diseases, but development of selectable drugs and efficient vaccines has been hampered by poor virus growth in cell culture; beside this it was difficult to unravel the HCV life cycle in absence of adequate cell culture system. Recently, a full length genotype 2a HCV genome, designated JFH-1 for Japanese Fulminant Hepatitis has been isolated. Genomic replicon of this clone is able to replicate to high levels in Huh7 cells independently from cell culture adaptations and produces viral particles that are infectious in cell culture In the present study we intended to exploit the ability of the JFH-1 system to replicate in cell culture to develop a new type of chimera in which only the encoding sequence for the envelope glycoproteins E1 and E2 of JFH-1 was substituted with the relative sequence belonging to other different genotypes. Our hypothesis is that cells carrying the information for E1E2 of GTs 2a (JFH-1), 1a (H77) and 1b (Con 1) transfected with the mRNA of JFH-1 backbone deleted of the relative envelope glycoproteins (JFHΔE1E2) are able to produce infectious viral particles. Such derived viral particles would represent a valid tool for characterization studies of the immune response, for example, in individuals vaccinated with the E1E2 subunit vaccine which Novartis Vaccines is currently testing, or in HCV positive patients. Their sera in fact could be tested to assess if they present antibodies that are able to neutralized viral particles from different genotypes.

Infectious viral particles obtained by transfecting permissive cells with the mRNA of the HCV isolate JFH-1 could also be used for experiments on the viral life cycle, as binding, attachment and entry. One of the main advantages of the cell culture-derived HCV (HCVcc) in fact is probably the mechanisms of interaction of these viral particles with the host cell receptors that closely mimic what happens in a natural infection event. In the present study we intended to further characterized the events related to the engagement of the HCV main receptor CD81 through the use of HCVcc or some surrogates, such as recombinant protein E2, the E1E2 heterodimer complex, or anti-CD81 antibody considering also the mechanisms of interactions that might exist between CD81 and the other recently discovered HCV receptor, Claudin-1 (CLDN-1). Since CD81 is normally expressed on the whole cytoplasmic membrane of polarized hepatic cell, whilst CLND-1 is

prevalently localized in the areas of cell-cell contact, one of the questions we intended to investigate involved the mechanisms and timings by which the virus interacts with both these receptors.

Beside these binding studies, we intended to identify and characterize the signal pathways that sequential interactions between viral proteins and cellular receptors might activate to promote viral infection.

§ 3. EXPERIMENTAL PROCEDURES & MATERIALS

3.1 Antibodies and reagents

Polyclonal chimpanzee antiserum Ch-L559 (Novartis Vaccines, CA) against E1E2, anti-human-FITC secondary Ab (SouthernBiotech)

Anti-CD81 (JS-81, Pharmigen, San Diego, CA), anti-β integrin (M-106, Santa Cruz Biotechnology), anti-HCV-Core antibody 3G1-1 (Novartis Vaccines, CA), anti-SR-BI (Affinity BioReagents), goat anti-mouse-IgG F(ab)2 fragments (Sigma), anti-HCV-E2 (291A2), screened for the ability to recognized E2 bound to target cells, polyclonal chimpanzee antiserum Ch-L559 (Tibi), able to recognize all forms of E1 and E2 both in immunoprecipitation and immunoblot analysis. Rabbit polyclonal Abs anti-Claudin-1, anti-ZO-1, and anti-Occludin (Zymed Laboratories); anti-Rac-1 (23A8), anti-Rho-A, anti-Cdc42 (Upstate Biothecnology); anti-actin mAb, C4 (Chemicon International). All secondary Abs conjugated to Alexa Fluor® dyes and to To-Pro-3 iodide are from Invitrogen/Molecular Probes.

Recombinant purified HCV-E1E2$_{193\text{-}746}$ and HCV-E2$_{384\text{-}715}$ from genotype 1a were obtained as described (Heile *et al.*, 2000; Spaete *et al.*, 1992). The exoenzyme C3 transferase from *Clostridium botulinum* was from Cytoskeleton; Jasplakinolide from Molecular Probes; Latrunculin A, Cytocalasin D , Rac1 inhibitor from Calbiochem, San Diego.

3.2 Cell culture

Eexperiments described in this study were performed using human hepatoma cells Huh-7 or a derived clone, highly permissive to HCV replication, S6.1. Embryonic human kidney cells (HEK 293T) were used in the production of lentiviral particles. All cell lines were grown at 37° C in 5% CO_2-air using Dulbecco's modified Eagles's medium (DMEM) containing 4.5 mg/ml of glucose and supplemented with 2 mM L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, 10% fetal calf serum.

All cell lines were periodically passaged as soon as they reach 80-90% of confluence, that is every two or three days depending on their rate of growth. Stressful overcrowded conditions were accurately avoided, and only low passage cells were used for our

experiments. Cells were always grown to confluence (48 h) before the experiments took place.

3.3 PCR reactions and synthesis of JFHΔE1E2 and fragments E1E2 (2a), E1E2 (1a), E1E2 (2b)

For the construction of JFHΔE1E2 we studied an in-frame deletion in the genome of JFH-1 Full Length, spanning the E1E2 coding region, from the aa 192 (Ala) to the aa 750 (Ala). This sequence, encoding the C terminus of core, fused directly to the N terminus of p7.
Three PCR reactions were designed on this purpose, using different sets of primers.
In PCR1 the reverse primer was designed to preserve the cleavage site between the core protein and E1. This primer sequence was partially homologous to the 3'end of Core, and partially complementary to p7, through an added nucleotide tail. The forward primer instead annealed to the 5' NCR region, upstream from a cloning restriction site (Age I). In PCR2 another couple of oligonucleotides amplified the region from 5'end of p7 to NS2, downstream from the other cloning restriction site (Not I).
In the third PCR two inner oligonucleotides were used to ligate the products of PCR1 and PCR2, producing the desired fragment deleted of the E1E2 segment.
Following the primer sequences used in these reactions:

PCR 1

Fwd: 5'-ACTCCGCCATGAATCACTCC (5' UTR, upstream from Age I, nt: 57-76)

Rew: 3'-CGTAACCTCTTCAACCCAAGGCCAGAGACGA (p7 5' end tail + Core 3' end, nt: 1033-1047)

PCR 2

Fwd: 5'-GCATTGGAGAAGTTGGTC (p7 5' end, nt: 2625-2642)

Rew: 3'-GCCCACACCACAAACTGT (on NS2 downstream to Not I, nt: 3031-3048)

PCR 3

Fwd: 5'-TTAGTATGAGTGTCGTAC (5' UTR upstream from Age I, nt: 124-141)

Rew: 3'-CGTAGCGCACCCGGCAGT (on NS2 downstream to Not I, nt: 3001-3018)

PCR reactions were run using 2 ng of template DNA in 50 µl of final volume reaction mix containing Pwo Taq Buffer 1x, dNTP mix 0.2 mM, fwd and rew primers 0.5 µM, and 1.25 U of PwoTaq Polimerase (Roche).

The three major steps that characterize a PCR reaction (denaturation, annealing and extension) were repeated for 30 cycles using the I-cycler (Bio-Rad).

Fragments obtained in the PCR reactions were isolated by electrophoresis in 1% agarose gel. and purified using QiAquick Gel Extraction kit (Qiagen) following operator instructions. The final product of PCR3 was then Age I/Not I (New England Biolabs) digested and cloned into the plasmid pUC-JFH-1 previously treated in the same way, to obtain the deleted mutant JFHΔE1E2.

To create the fragments E1E2 belonging to the three different HCV genotypes chosen three different PCR reactions were set as previously described using the full length genomes of JFH-1 (GT 2a; GenBank accession number AB047639) , H77 (GT 1a; GenBank accession number AF011751) and Con 1 (GT 1b; GenBank accession number AJ238799) as templates.

Forward oligonucleotides used for this purpose were designed with a poly A-tail added of an EcoRV cloning site plus a start codon Met (ATG) and a region homologous to the 3' end of core ER signal sequence, starting from the aminoacid tyrosine 164 (TAT).

Reverse primers instead were provided of a Kpn I cloning site inserted alongside a poly A tail, followed by a stop codon (TTA) and a region homologous to the 3' end of the envelope protein E2.

These characteristics allowed the PCR reaction to amplify the genome fragments E1E2 included between the start and the stop codon artificially provided and to insert a EcoRV cloning site at their 5'end and a Kpn I cloning site at their 3' end.

Following the primers sequences:

GT 2a:

Fwd: 5'-AAAAGAATATCATGTATGCAACAGGGAACC (poly A tail + EcoRV + Start codon-core ER signal sequence nt: 864-879)

Rev: 3'- GGTCCGGCTTCGTATTCCATGGAAAA (E2, 3' end nt: 2612-2624 + Stop codon + Kpn I + poly A tail)

GT 1a:

Fwd: 5'-AAAAGAATATCATGTATGCAACAGGGAACCTT (poly A tail + EcoRV + Start codon-core ER signal sequence nt: 1258-1275)

Rev: 3'-GGGTTCGCCTTCGCCGAATTCCATGGAAAA (E2, 3' end nt: 3993-4009 + Stop codon + Kpn I + poly A tail)

GT 1b:

Fwd: 5'-AAAAGAATATCATGTATGCAACAGGGAATCT (poly A tail + EcoRV + Start codon-core ER signal sequence nt: 2302-2318)

Rev: 3'-CGAGTTCGACTCCGGATTCCATGGAAAA (E2, 3' end nt: 4036-4051 + Stop codon + Kpn I + poly A tail)

Once the EcoRV-E1E2-Kpn I PCR fragments were obtained, they were isolated by gel electrophoresis as previously described, digested using these two restriction enzymes (New England Biolabs) and cloned in lentiviral transfer vectors.

3.4 Cloning E1E2 fragments into lentiviral transfer vectors

These three transfer vectors were obtained replacing the green fluorescent protein (GFP) of the self inactivating lentiviral vector pCCl.sin.PPT.hPGK.GFP.Wpre with the amplified E1E2 fragments.

The restriction enzyme BamHI (New England Biolabs) was chosen to linearize pCCl.sin.PPT.hPGK.eGFP.Wpre, cutting between the hPGK promoter and the 5' end of the GFP. A second digestion on the Kpn I site (GFP 3' end) would allow the extrusion of the fluorescent protein and the cloning of the E1E2 amplified fragments at its place, downstream from the human PGK promoter. But for this to be possible, the EcoRV blunt ends of the PCR fragments and the BamHI sticky ends of the vector clone had to become compatible, so the latter was first treated with Klenow esonuclease (New England Biolabs) to produce BamHI blunt ends. Finally, the vector could be also Kpn I digested, and the isolate derived from gel extraction could be ligated with the matching E1E2 PCR products. A this point the pCCl.sin.PPT.hPGK.E1E2.Wpre transfer vector could be used to produce lentiviral particles.

3.5 Transient transfection of 293T cells for the production of E1E2 lentiviral particles

Embryonic human kidney cells (293T) were seeded 24 h prior transfection in a 10 cm Petri dish ($4.5x10^6$ cells/dish), previously treated for 5 min at 37° C with gelatin at 0.1% to promote adhesion. Medium was renewed 2 h prior transfection. A plasmid DNA mix was prepared by putting together 3 µg of envelope plasmid ($pMD_2VSV.G$), 5 µg of packaging plasmid (pMDLg/pRRE), 2.5 µg of regulatory plasmid (pRSV.REV), and 10 µg of gene transfer plasmid (pCCl.sin.PPT.hPGK.E1E2.Wpre), to a final volume of 450 µl using 0.1x TE/dH_2O (2:1). Finally 50 µl of 2.5 M of $CaCl_2$ was added to the solution mix tube. After 5 min at RT, a DNA precipitate was promoted by a dropwise addition of 500 µl of a HEPES Buffer saline solution (2x HBS) under continuous vortexing of the mixture tube. The precipitate was then soon added onto 293T cells. The procedure was conduct in parallel using the three different transfer vectors previously obtained carrying the information for E1E2 of GTs 2a, 1a, and 1b).

Cells medium was renewed 24h post transfection, and 48 h p.t. supernatants were collected, clarified and lentiviral particles were isolated by ultracentrifugation for 3h at 52000 x g (26000 rpm, rotor type JA 25.50) at 4° C (Beckman Coulter, Optima™ L-90K). After that lentiviral particles were resuspended in PBS/BSA 0.5% and stored at -80° C.

The same procedure was also adopted to create Vesicular Stomatitis Virus and Moloney Murine Leukemia Virus pseudoparticles (MLVpp and VSVpp) to be used as controls in experiments on the effects of actin inhibitors on HCVcc infectivity. In these cases the transfer vector carried the information for the Green Fluorescent protein, GFP (pCCL.sin.PPT.hPGK.GFP), and a plasmid carrying the gene for the envelope proteins of the two viruses was added . Supernatants of transfected 293T cells containing the pseudotyped particles were harvested at 48 h post-transfection, filtered through 0.45 µm-pore-size filters, ultracentrifugated and used to infect Huh7 cells. Infected cells were defined trough GFP expression, as measured 72 h post-infection by FACS analysis.

3.6 Production of packaging cell lines S6.1/E1E2:2a, S6.1/E1E2:1a, S6.1/E1E2:1b

Lentiviral particles carrying the information for E1E2 of the three different HCV genotypes chosen were used to transduce S6.1 naïve cells. The day before the procedure took place, cells were seeded at a concentration of $2x10^4$ cells/well in a 24-well plate, as to

reach the 50% confluence at the moment of transduction. Before being put in contact with the lentiviral particles S6.1 cells were treated with 4 µg/ml (final volume) of polybrene whose role was to quinch the electrostatic repulsion among cellular and particles surfaces. After that, different aliquotes of lentiviral particles were added to the cells medium and left for the following 8 h at 37° C, before supernatants were removed and replaced with fresh medium.

Transduced cells were grown and expanded regularly during the following days until they could be kept in 75 cm^2 flasks. The efficiency of transduction was checked 3 days after cells infection by FACS analysis.

3.7 Staining for FACS analysis

To determine the expression of the viral envelope proteins in packaging cell line, aliquots of each one of them were seeded in a 96-well plate (10^5 cell/well), washed in PBS, fixed in 4% paraformaldehyde for 30 min, and permeabilized for intracellular staining using a solution of PBS-1% BSA-0.5% Saponine for 30 min at RT.

After that, cells were stained against the viral envelope proteins E1E2 using polyclonal chimpanzee antiserum Ch-L559 as primary Ab (10 µg/ml) for 1h at RT, washed in PBS and then treated with anti-human-FITC secondary Ab (10 µg/ml) for 1h at RT. Cells were washed again, resuspended in PBS and read for their fluorescent emission by FACS (Becton Dickinson, FACScan). Controls, represented by non transduced S6.1 naïve cells were stained with conjugated Ab alone or with both Ab I and Ab II and subjected to the same analysis.

3.8 *In vitro* transcription of JFHΔE1E2 and transfection of packaging cell lines

Plasmid pUC-JFHΔE1E2 DNA, controlled by a T7 promoter, was linearized at the 3' end by Xba I (New England Biolabs) digestion and after extraction with phenol and chloroform and precipitation with ethanol it was resuspended in RNase free water to be transcribed. *In vitro* transcription occurred using MEGAScript T7 Kit (Ambion) following manufacturer instructions; reaction solution contained 1 µg of linear template, the four ribonucleotide solutions, the Kit Reaction Buffer, and the RNA Polymerase Enzime Mix to a final volume of 20 µl. After 4 h at 37° C unincorporated nucleotides and proteins were precipitated using Lithium Chloride (LiCl) followed by phenol/chloroform extraction and isopropanol precipitation. The purified transcript was then resuspended in RNase free water and its

concentration was determined measuring the optical density at 260 nm, considering that for a single strand RNA 1 A_{260} unit corresponds to 40 µg/ml. The same procedure was used also to generate the genomic RNA of JFH full length and the RNA of the deleted mutant JFHΔE1E2, both inserted in pUC plasmids.

Packaging cell lines, S6.1/E1E2:2a, S6.1/E1E2:1a, S6.1/E1E2:1b, were then transfected by electroporating the JFHΔE1E2 RNA (10 µg of RNA : $6x10^6$ cells). Electroporation conditions were 960 µF and 270 V using a Gene Pulser System (Bio-Rad) and a cuvette with a gap of 0.4 cm (Bio-Rad). After electroporation cells were gently transferred in 12 ml of complete DMEM growing medium in a 75 cm^2 flask and grown in a BSL-3 environment; an aliquot of transfected cells was seeded in a 48-well plate to check for the efficiency of transfection 72 h later, through immunofluorescent staining against the viral core protein. Control cells used in trans-complementation experiments were represented by S6.1 naïve cells transfected with the RNA of JFH-1 FL or with the RNA of the deleted mutant following the protocol above described. Supernatants of all cells were regularly collected from day 3 post-transfection.

3.9 Production of cell-culture derived HCV, HCVcc

Packaging cell line transfected with JFHΔE1E2 RNA and S6.1 naïve cells transfected with JFH-1 FL or with JFHΔE1E2 were kept in culture in a BLS-3 environment for the production of cell culture-derived HCV particles (HCVcc). Cells were regularly passaged every two days and supernatants were collected three time a week or as cell layer become confluent and stored at 4° C. Fluids collected during the week were clarified by centrifugation at 4500 x g for 10 min to precipitate gross particulate and cellular debris and viral particles were collected by ultracentrifugation at 52000 x g (26000 rpm, rotor type JA 25.50) for 3 h at 4° C (Beckman Coulter, Optima™ L-90K). Pelletted viral particles were then resuspended (100x concentration) in PBS/BSA 0.1% and stored at 4° C or at -80° C, before being titrated.

3.10 HCVcc titration and use

For the titration of supernatants coming from packaging cell lines (S6.1/E1E2:2a, S6.1/E1E2:1a, S6.1/E1E2:1b) transfected with JFHΔE1E2 RNA, and from S6.1 naïve cell transfected with JFH-1 FL and with JFHΔE1E2, Huh7 naïve cells (10^4 cells/well) were

seeded in 96-well plates and inoculate with 10x viral suspension stocks in DMEM complete medium for eight replicas, to be passaged in 10 fold serial dilutions according to the Spearman and Kaerber fit. Cells were then grown at 37° C for the next 72h before being fixed, permeabilized and fluorescently stained against the viral core protein expressed in infected cells. Titer was expressed in particle forming units per ml (pfu/ml), enumerating the positive cells by the use of a fluorescent microscope.

After titration viral stocks were used for different experimental procedures.

For infection assays involving the study of the effects that actin inhibitors as Cytocalasin D (5 μM), Latrunculin A (1 μM) and Jasplakinolide (500 nM) had on HCVcc infectivity, Huh7 naïve cells, grown in 96-well plates at a density of 10^4 cells/well (four replicates for each treatment), were left in contact with 100 pfu/ml of HCVcc per well for 1h at 4° C to allow binding, at the continuous presence of the drug. After this period of time, unbound virions were washed away and cells were treated for additional 2 h with the mentioned drugs at 37° C; drug-containing medium was then removed and replace with fresh complete DMEM. At 72 h post-infection cells were fixed, permeabilized and fluorescently stained against the viral core protein and positive cells were enumerated using a fluorescent microscope.
Control viral particles, MLVpp and VSVpp, were subjected to the same procedure, but the efficiency of infection in this cases was determined by FACS analysis exploiting the expression of GFP in positive cells.

In time course experiments to study the kinetics of action of Lat A on HCVcc infectivity, cells prepared as previously described were inoculated with 100 pfu/ml of viral particles per well for 1h at 4° C and then put in contact with Lat A (1 μM) in concomitance with the viral inoculum at 4° C or at different time points upon temperature shift. Lat A treatment was extended for a total of three hours from the moment the drug was added onto cells. At 72 h post-infection cells were fixed, permeabilized and fluorescently stained against the viral core protein and positive cells were enumerated using a fluorescent microscope.

3.11 IFA Staining and immunofluorescent microscopy

Immunofluorescent staining against the core protein and against other viral structural (E1E2) and non structural (NS5a) proteins was performed 72 h post infection or transfection.

Cells were fixed with 4% paraformaldehyde for 30 min at RT, permeabilized with 0.1% Triton X-100 for 15 min at RT, and fluorescently stained for 1h at RT with different primary antibodies (10 µg/ml) followed by the respective secondary fluorescent Abs (10 µg/ml; 1h, RT). For the detection of core the anti-core 3G1-2 Ab followed by anti-mouse-AF-568 (red) were used; staining of E1E2 was performed using polyclonal chimpanzee antiserum Ch-L559 as Ab I followed by anti-Human-AF-488 (green); staining of NS5a using a rabbit polyclonal Ab I followed by anti-rabbit-AF-568 (red).

Infection was determined by counting core positive cells under a fluorescent microscope (Zeiss, Observer.A1), whilst colocalization of viral proteins was detected performing a double staining of the interested proteins exploiting different fluorochromes.

The same procedure was also adopted in experiments aimed to characterized the early events that follow HCV binding to target cells.

In relocalization experiments, Huh7 monolayers were exposed to anti-CD81 mAb JS-81 at 10 µg/ml or to soluble HCV-E2 or HCV-E1E2 (10 µg/ml) plus anti-E2 mAb (291A2) (10 µg/ml) for 30 min at 4° C, then shifted to 37° C. In all experiments cells were fixed and permeabilized before staining. Cells were then incubated with the indicated primary Abs for 1h at room temperature, washed, incubated with fluorochrome-conjugated secondary Abs for 1h at RT, washed and mounted with Vectashield (Vectors Laboratories). Images were captured with a Biorad 2100 confocal microscope using a 60x oil or 100x oil objectives. Image analysis was performed using the standard operating software provided with the microscope.

3.12 Immunoblot analysis

To assess the correct cleavage of E1 and E2 envelope proteins expressed by packaging cell lines S6.1/E1E2:2a, S6.1/E1E2:1a, and S6.1/E1E2:1b, cells extracts were resolved by SDS-PAGE and transferred to nitrocellulose membranes. Membranes were blocked in 5% nonfat dry milk, probed with polyclonal chimpanzee antiserum Ch-L559 as primary Ab

(10 µg/ml) and developed with horse-radish peroxidase-conjugated secondary antibody (anti-human-HRP, Jackson ImmunoResearch) and ECL reagents (Amersham Biosciences).

In cell signaling experiments, to assess the Rho GTPases response to CD81 engagement, Huh7 cells were incubated with anti-CD81 mAb (JS-81, 10 µg/ml) or 10 µg/ml sE2 plus anti-E2-mAb (291A2, 10 µg/ml) for 30 min at 4° C. After washing cells were shifted to 37° C in the presence of anti-mouse IgG F(ab)2 fragments (Sigma) (10 µg/ml) for the indicated time (0, 1, 3, 5, 10 min). Following stimulation cells were lysed in buffer containing 20 mM Tris-HCl (pH 7.4), 40 mM NaCl, 5 mM EDTA, 0.1% BSA, 1 mM NaF, 20 mM Na_3VO_4, x1 mM PMSF, 5 µg/ml aprotinin, 10 µg/ml leupeptin, 0.1% Triton X-100. Insoluble material was removed by centrifugation for 15 min at 15000 x g, and cell extracts were run on SDS-PAGE and immunoblotted as above described using specific antibodies against Rho, Cdc42 and Rac proteins.

3.13 Rho, Rac and Cdc42 activation assay. Affinity precipitation.

Assays were performed with reagents from Upstate Biotechnology, according to manufacture's instructions. Briefly, cells were lysed in magnesium lysis buffer (Upstate Biotechnology) supplemented with 1 mM NaF, 20 mM $Na_4P_2O_7$, 1 mM Na_3VO_4, 1 mM PMSF, 5 µg/ml aprotinin, 10 µg/ml leupeptin. Lysates were then incubated for 1h at 4° C with PAK-1 PBD-agarose to precipitate GTP-bound Rac and Cdc42, or with RBD-GST to precipitate GTP-bound Rho. After centrifugation, beads were washed in lysis buffer, resuspended in Laemmli sample buffer, run on SDS-PAGE, transfer to nitrocellulose membranes and immunoblotted. Total lysated were also immunoblotted to measure the total amount of GTP- and GDP-bound proteins.

3.14 siRNAs transfections

In experiments aimed to study the Rho GTPases response to CD81 engagement, double stranded siRNAs target against human Rac (Upstate Biotechnology) and Cdc42 (Santa Cruz Biotechnology) were transfected into Huh7 cells using the HiPerFect Transfection Reagent Kit (Qiagen) according to manufacturers instructions. 48 h post-transfection, cells were monitored for gene silencing by immunoblots and then used for HCVcc infection.

In experiments focused on the role of CD81 in the translocation process, Huh7 cells were silenced for CD81 expression using a siRNA sequence targeting human CD81 (nt: 138-156; target: 5'-ATCTGGAGCTGGGAGACAA-3'), chemically synthesize by Qiagen.

3.15 Statistical analysis

Data are presented as mean ± standard deviation. A two-tailed Student's t-Test was performed to analyze variance.

§ 4.1 RESULTS

Trans-complementation of gpE1/gpE2 allows recovery of infectious Hepatitis C virus

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HCV is a major cause of chronic liver diseases, but development of selectable drugs and efficient vaccines has been hampered by poor virus growth in cell culture. Beside this it was difficult to unravel the HCV life cycle in absence of adequate cell culture system. A major step forward was therefore the development of the replicon system as reported by Lohmann *et al*. (1999). This system allowed for the first time the efficient propagation of genetically modified HCV RNAs (replicons) in a human hepatoma cell line (Huh7), and since then it became a well defined tool to work with this virus

Work in the past few years revealed that the efficiency of the replicon system, and the enhancement of viral RNA replication, is determine by the selection for highly permissive host cells and by cell culture adaptive mutations in the replicon (Bartenschlager and Pietschmann, 2004). It appears that individual cells within a given Huh7 cell pool population vary dramatically in their ability to support high level of HCV RNA replication, and those cells that can support the highest levels are enriched during antibiotic selection, thanks to the presence in the replicon sequence of a gene coding for antibiotic resistance. Upon removal of HCV replicons from such established cell clones, for example by treatment with IFN-α, a cured cell clone is obtained which, upon reintroduction of the replicon, supports HCV RNA replication to a much higher level as compared to that of the parental (naïve) Huh7. Some highly permissive cell clones have been generated in this way as, for example, Huh7.5 and Huh7-lunet (Blight *et al*., 2002; Bartenschlager and Pietschmann, 2005; Koutsoudakis *et al.,* 2007). In our studies we used another highly permissive clone of Huh7 cells available in Novartis laboratories obtained as above described; this clone was defined as S6.1. The replicon system has been largely and successfully utilized for viral replication studies, but they did not allow the production of infectious viral particles *in vitro*.

Recently, a full length genotype 2a HCV genome, designated JFH-1 for Japanese Fulminant Hepatitis (Kato *et al.*, 2003) has been isolated. Genomic replicon of this clone is able to replicate to high levels in Huh7 cells independently from cell culture adaptations

and produces viral particles that are infectious in cell culture (Lindenbach *et al*., 2005; Wakita *et al*., 2005; Zhong *et al*., 2005). Using JFH-1 as a backbone, chimeric genomes were generated. These were composed of the core to NS2 regions of genotypes 1a, 1b, 2a, and 3a and the NS3 to NS5B from JFH-1 isolate (Pietschmann *et al*., 2006). Upon transfection of these genomes in Huh7 cells infectious virus particles were released, but in the case of GT 3a/JFH chimera.

In the present study we intended to exploit the ability of the JFH-1 system to replicate in cell culture to develop a new type of chimera in which only the encoding sequence for the envelope glycoproteins E1 and E2 of JFH-1 was substituted with the relative sequence belonging to other different genotypes. Our hypothesis is that cells carrying the information for E1E2 of GTs 2a (JFH-1), 1a (H77) and 1b (Con 1) transfected with the mRNA of JFH-1 backbone deleted of the relative envelope glycoproteins (JFHΔE1E2) are able to produce infectious viral particles. Such derived viral particles would represent a valid tool for characterization studies of the immune response, for example, in individuals vaccinated with the E1E2 subunit vaccine which Novartis Vaccines is currently testing, or in HCV positive patients. Their sera in fact could be tested to assess if they present antibodies that are able to neutralized viral particles from different genotypes.

For our aim to be achieved, we had firstly to verify that the deletion of the E1 and E2 sequence in JFH-1, to create the mutant JFHΔE1E2, did not impair its replicative capability. Secondly, since in our study the envelope proteins E1E2 would be provided *in trans* by packaging cell lines, whilst the rest of viral protein would be supplied by transfecting these cells with JFHΔE1E2 mRNA, there was the need of verifying a co-localization between the envelope and the backbone proteins. It has been reported in fact that for a correct HCV assembly and budding viral proteins co-localize in the perinuclear area, forming a multiprotein complex on ER-derived membranes (Hügle *et al.*, 2001; Brazzoli *et al*., 2007).

4.1.1 Synthesis of construct JFHΔE1E2, and PCR fragments E1E2(2a), E1E2(1a), E1E2(1b) to be cloned in pCCl.sin.PPT.hPGK plasmids for lentiviral system setting.

For the construction of JFHΔE1E2 we studied an in-frame deletion in the genome of JFH-1 Full Length, spanning the E1E2 coding region, from the aa 192 (Ala) to the aa 750 (Ala). This sequence, encoding the C terminus of core, fused directly to the N terminus of p7.

Three PCR reactions were designed on this purpose, using different sets of primers (Fig. 4).

In PCR1 the reverse primer was designed to preserve the cleavage site between the core protein and E1. This primer sequence was partially homologous to the 3'end of Core, and partially complementary to p7, through an added nucleotide tail. The forward primer instead annealed to the 5' NCR region, upstream from a cloning restriction site (Age I). In PCR2 another couple of oligonucleotides amplified the region from 5'end of p7 to NS2, downstream from the other cloning restriction site (Not I).

In the third PCR two inner oligonucleotides were used to ligate the products of PCR1 and PCR2, producing the desired fragment deleted of the E1E2 segment.

Eventually, this final product was cloned in JFH-1, previously treated with the same restriction enzymes.

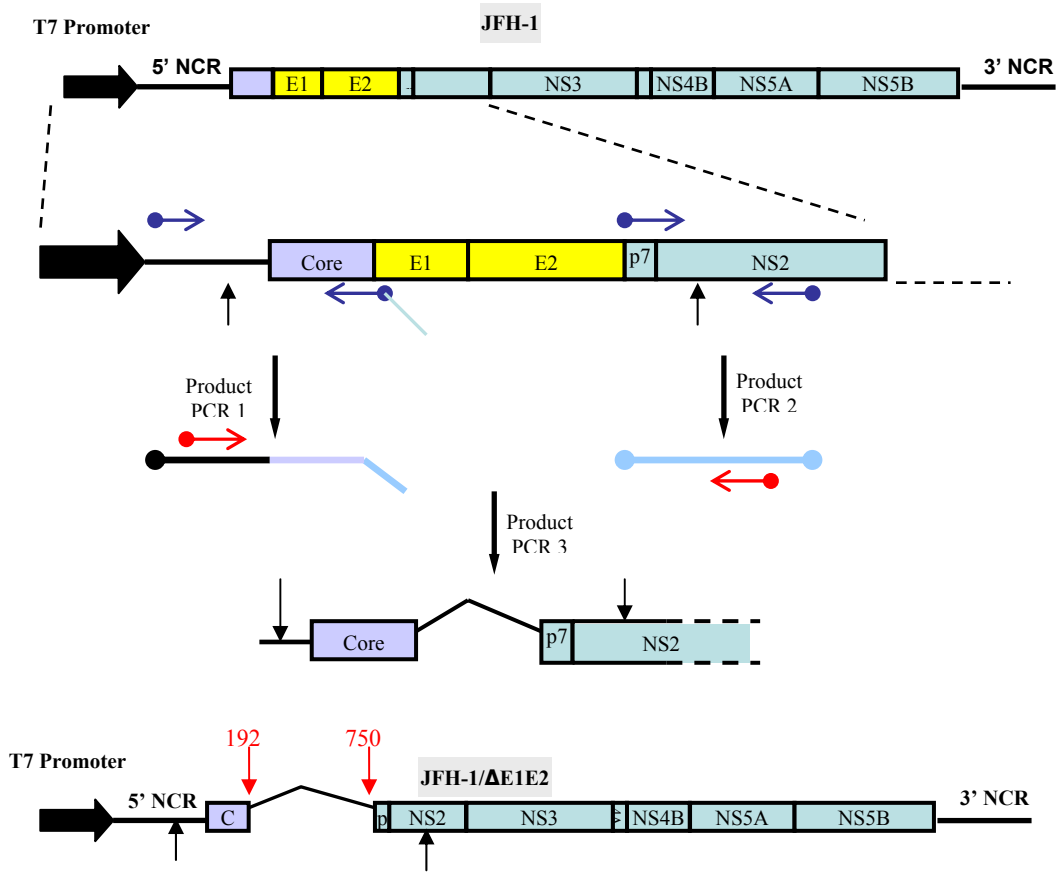


Fig. 4. Syntesis of the construct JFHΔE1E2 by nested PCR. Using the JFH-1 full length genome as template two couples of primers were designed firstly to amplify the 5'UTR-Core and the p7-NS regions (PCR 1 and 2), flanking the E1E2 coding sequence that had to be excised. In the PCR 3 inner primers were used to fuse the products previously obtained, creating the JFH-1 deleted mutant.

To generate stable cell lines expressing E1E2, a set of primers covering the envelope genome sequence from aa 192 to aa 750 were synthesized and use to amplify the glycoproteins from the three genotypes chosen: JFH-1 (GT2a), H77 (GT 1a),Con 1 (GT 1b). The design of the appropriate couples of oligonucleotides implied the insertion of a restriction site for EcoRV in the forward primers, and a restriction site for KpnI in the reverse. After the PCR products were obtained, these were digested with the restriction enzymes aforesaid, and were cloned in pCCl.sin.PPT.hPGK vectors.

At this point pCCl.sin.PPT.hPGK.E1E2.Wpre for genotypes 2a, 1a, and 1b were ready to be used to generate VSV-G pseudotyped particles. These were subsequentially used to transduce our target cells S6.1, exploiting the high efficiency of lentiviral system, to create three different packaging cell lines constitutively expressing the viral envelope proteins.

4.1.2 The lentiviral vectors system and the production of pseudo particles carrying the information for E1E2 of GTs 2a (JFH-1), 1a (H77)and 1b (Con1).

General transfer vectors derived from lentiviruses, such as human immunodeficiency virus 1 (HIV-1), can deliver large cDNA to a variety of dividing and non-dividing cells. Their ability to transduce and stably integrate their genomes into numerous types of cell lines, independently of their proliferation status, makes them an important tool in molecular studies.

Lentiviral vectors (LV) are hybrid viral particles made by the core protein and the enzymes of a lentivirus, and the envelope of a different virus, most often the Vesicular Stomatitis Virus (VSV), or the amphotropic envelope of Murine Leukemia Virus (MLV). The use of VSV.G usually present significant advantages in that it gives a vector a broad host-cell range and confers high yields and great stability of the vector particles (Dull *et al*., 1998; Follenzi and Naldini, 2002).

Lentiviral vectors are made defective for replication and are designed to be unable to continue to infect their host after they deliver their content; only the early steps of the lentivirus life cycle (attachment, entry, reverse transcription, nuclear transport, and integration) must be maintained. The reporter gene is cloned into a vector sequence that is flanked by LTRs and the Psi-sequence of HIV. The LTRs are necessary to integrate the reporter gene into the genome of the target cell, just as the LTRs in HIV integrate the dsDNA copy of the virus into its host chromosome (Fig. 5). The Psi-sequence (ψ) acts as a signal sequence and is necessary for packaging RNA with the reporter gene in virions. Viral proteins which make virus shells are provided in the packaging cell line, but are not in context of the LTRs and Psi-sequences and so are not packaged into virions.

Replication-defective retroviral particles are produced transiently co-transfecting human embryonic kidney cells 293T with four plasmids:

1. *packaging plasmid*, pMDLg/pRRE: is a CMV-driven expression plasmid encoding the viral capsid, which contains the *gag* and *pol* coding sequences and a 374-bp Rev-responsive element (RRE)-containing sequence from HIV-1 downstream of the *pol* coding sequences (Dull *et al*.,1998). In primate lentiviruses the *gag* open reading frame directs the synthesis of structural viral proteins which control the encapsidation of genomic viral RNA, so in the LV system it controls the encapsidation of the reporter gene. The *pol* open reading frame instead encodes the viral enzymes which are

involved in synthesis of viral cDNA and which direct the integration of viral into cellular RNA.

2. pRSV.REV: a non overlapping construct expressing the cDNA of *rev*, an HIV regulatory gene essential for viral replication, under the control of the Rous Sarcoma Virus U3 (RSV) promoter.

3. *self-inactivating (SIN) transfer vector plasmid*, pCCl.sin.PPT.hPGK.E1E2.Wpre, containing HIV-1 cis-acting sequences and an expression cassette for the transgene of interest, in this case the HCV envelope glycoproteins E1 and E2, driven by the internal promoter hPGK (human posphoglycerate kinase). The polypurine tract PPT strongly increases the nuclear transport and the total amount of genome integrated into the DNA of target cells, whilst the insertion of the Post-transcriptional Regulatory Element of Woodchuck hepatitis virus, Wpre, promotes nuclear export of transcripts and increases the efficiency of polyadenylation of the nascent mRNA. The transfer vector plasmid contains cis-acting genetic sequences necessary for the vector to infect the target cell and for transfer of the reporter gene and contains restriction sites for insertion of desired genes.(Naldini *et al*., 1996).

4. $pMD_2VSV.G$: construct encoding the heterologous envelope G of vesicular stomatitis virus, under the control of the CMV promoter, for packaging the vector genome carrying the gene of interest (pCCl.sin.PPT.hPGK.E1E2.Wpre) into hybrid replication-defective viral particles

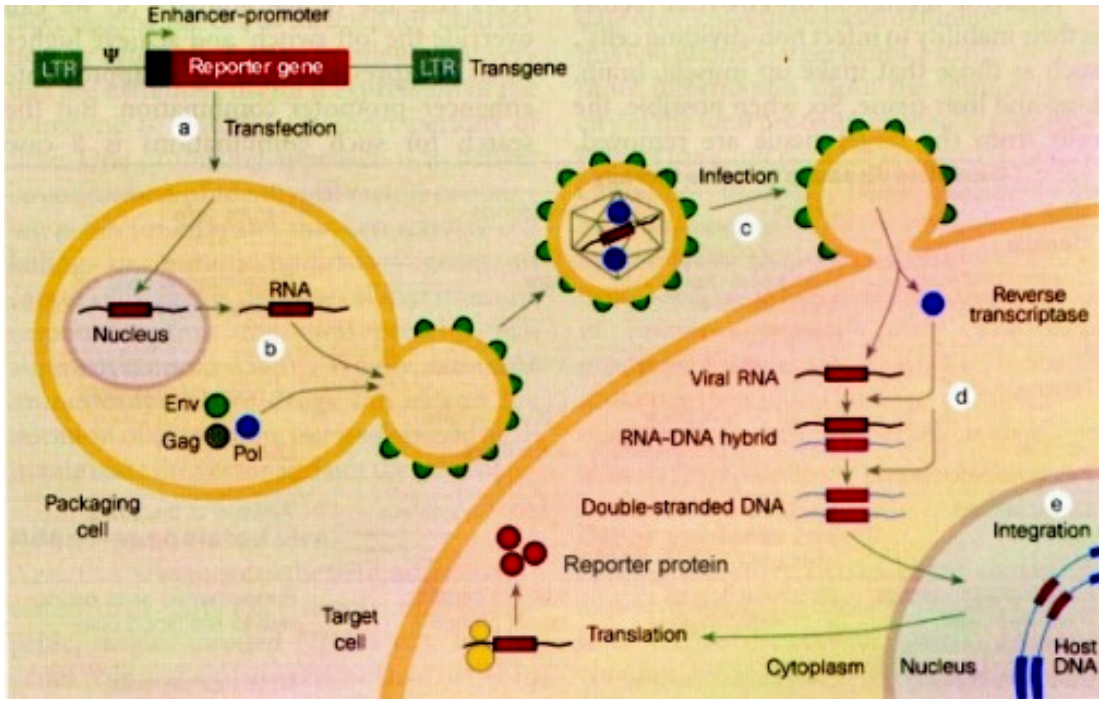


Fig. 5. Schematic rapresentation of lentiviral particles formation. (a). The cDNA of a transfer vector plasmid carrying the information for a reporter gene, as the GFP, or another gene of interest, is transfected in 293T cells together with a packaging plasmid (gag, pol), a regulatory plasmid controlling viral replication (rev), and one coding for the expression of the lentiviral particles envelope (env) proteins. (b) Inside the transfected cells lentiviral particles self assemble and ingulf the RNA of gene of interest thanks to the presence of a signal sequence. (c, d, e). Lentiviral particles released are then ready to infect target cells leading to the stable integration in the host chomosome of a dsDNA copy of the reporter gene. This will allow the constitutively expression of the exogenuos protein by the target cells.

After the three the self-inactivating lentiviral vectors pCCl.sin.PPT.hPGK.E1E2.Wpre carrying the information for E1E2 of the genotypes 2a, 1a, and 1b were created, these were used to transfect three different pools of 293T cells, together with the other plasmids.

To collect relative lentiviral pseudo particles produced by transfected 293T cell, supernatants were collected 48 and 72 hours post transfection, clarified from cellular debris, and concentrated by ultracentrifugation. Pelletted particles, resuspended 200x in PBS/BSA, were now ready to be used to transduce S6.1 cells.

4.1.3 Transduction of S6.1 cells with lentiviral pseudo particles carrying E1E2, and production of packaging cell lines: S6.1/E1E2:2a, S6.1/E1E2:1a, S6.1/E1E2:1b.

The lentiviral particles previously obtained were used to transduce S6.1 cells, highly permissive for HCV replication, to create three different packaging cell lines, constitutively expressing the envelope glycoproteins of JFH-1 (GT 2a), H77 (GT 1a) and

Con 1 (GT 1b). This three packaging cell lines were defined as follows: S6.1/E1E2:2a, S6.1/E1E2:1a, and S6.1/E1E2:1b (Fig. 6).

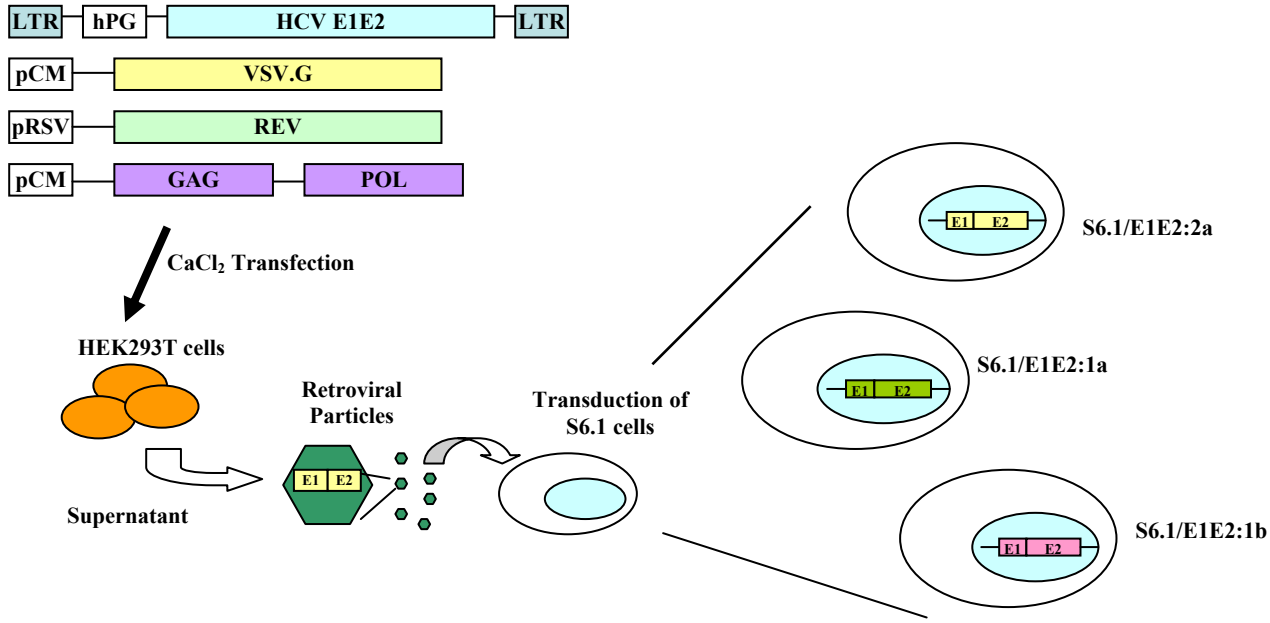


Fig. 6. Creation of packaging cell lines expressing HCV E1E2 through lentiviral system. Lentiviral particles carrying the information for the HCV envelope proteins of GTs 2a, 1a and 1b were obtained transfecting 293T cells with proper lentiviral vectors. 48 hs p.t supernatants were collected, lentiviral particles precipitated by ultracentrifugation, and then used to transduce target cells S6.1. The stable integration of the E1E2 dsDNA into the genome of S6.1 cells led to the creation of packaging cell lines constitutively expressing the envelope glycoproteins of the three HCV genotypes chosen.

To evaluate the success and the efficiency of transduction, transduced S6.1 were tested by FACS analysis for the expression of the relative viral envelope glycoproteins (Fig. 7, A). Cells were intracellularly stained using as primary antibody a polyclonal chimpanzee antiserum, Ch-L559 (Tibi), able to recognize all forms of E1 and E2 both in immunoprecipitation and in Western Blot analysis (Choo *et al*., 1994; Merola *et al*., 2001, Rosa *et al*., 1996). A fluorescent secondary antibody (α-Human-FITC) was then utilized for detection. Non-infected naïve cells were used as negative control and stained with the conjugated antibody alone, and then with both the primary and the secondary antibodies, to detect the background staining. As shown in the following diagram, a well defined population of S6.1 expressing the viral proteins is present in all three cases, in respect to

control cells, confirming the high efficacy of the technique chosen to constitutively transduce our target cells.

To assess the correct cleavage of E1 and E2 by the packaging cell lines, cell extracts were analyzed by Western Blot (Fig. 7, B). In natural conditions, the cleavage in the structural region of the polyprotein relative to E1/E2 is catalyzed by a host signal peptidase localized in the endoplasmic reticulum (Reed and Rice, 2000), and produces two mature proteins of about 30 kD and 60 kD.

Our Western Blot analysis on cell extracts derived from S6.1/E1E2:2a, S6.1/E1E2:1a, and S6.1/E1E2:1b showed the presence of two bands relative to E1 and E2 of the correct molecular weight that were not detected in the lysates of the mock cells used as negative control.

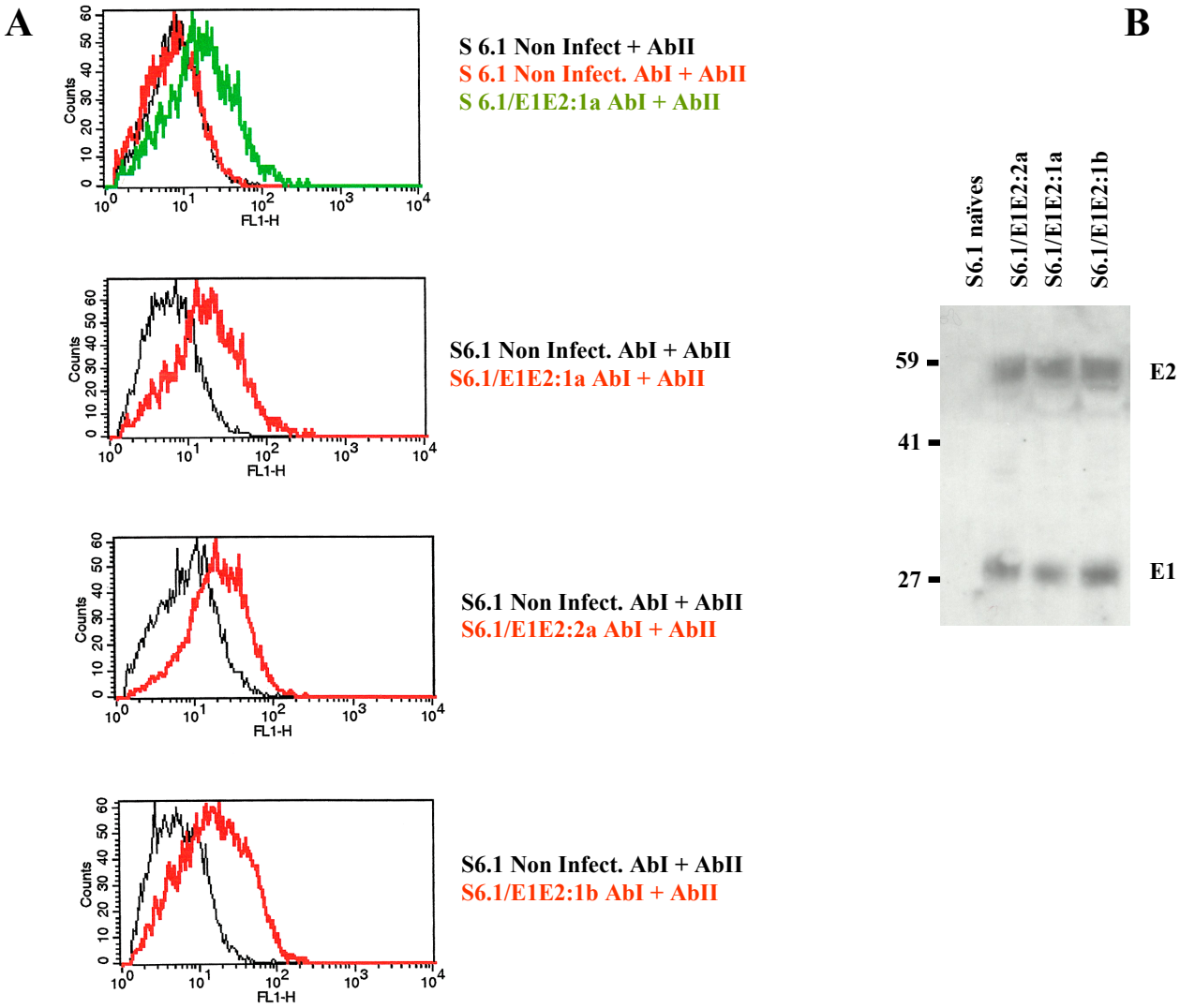


Fig. 7. (A). Efficiency of transduction analysed by FACS analysis. An aliquot of each packaging cell line was checked for the expression HCV E1E2 proteins through intracellular staining using polyclonal chimpanzee antiserum Ch-L559 as primary Ab, followed by anti-Human-FITC secondary Ab. Controls are represented by non transduced naïve cells stained with conjugated Ab alone or with both Ab I and Ab II. The analysis showed the clear presence of neat populations expressing the HCV glycoproteins (red line, in the three lower panels), compared to non-transduced cells used as control (black line). (B). Assessing the correct cleavage of E1 and E2. Western Blot analysis on cell lysates using polyclonal Ab Ch-L559 followed by anti-human-HRP revealed the presence of two bands relative to E1 and E2 of the correct molecular weight (30 and 60 kD respectively) in S6.1/E1E2:2a, S6.1/E1E2:1a, and S6.1/E1E2:1b cell lines, that were not present in non-transduced cells (S6.1 naives).

4.1.4 Transfection of packaging cell lines with JFHΔE1E2 mRNA

To determine whether it was possible to obtain HCV viral particles budding and release when E1 and E2 were provided *in trans*, S6.1/E1E2:2a, S6.1/E1E2:1a, and S6.1/E1E2:1b were transfected with the mRNA of JFH-1 deleted of the envelope proteins (JFHΔE1E2).

As positive control we transfected S6.1 naïve cells with the mRNA of the full length JFH-1 genome, capable of replicating in the host cell and able to induce the formation of infectious viral particles (Cai *et al*., 2005; Lindenbach *et al*., 2005; Wakita *et al*., 2005; Zhong *et al.*, 2005). As negative control for particle production, S6.1 naïve cells were transfected with the mRNA backbone of JFH- 1 deleted of the envelope proteins E1 and E2 (JFHΔE1E2). This genome modification allows the expression of Core and of the Non Structural (NS) viral proteins inside the host cell, but the lack of E1 and E2 does not permit the formation of complete and functional virions (Wakita *et al*., 2005; MinKyung *et al.*, 2006). The capability of JFHΔE1E2 mRNA to autonomously replicate was verified through Northern Blot analysis 5 days after transfection (Fig. 8). As expected, viral mRNA was detected in S6.1 cells transfected with JFHΔE1E2, consistently with what we found in naïve cells transfected with JFH-1 FL, used as positive control. Non-transfected naïve cells represented our negative control.

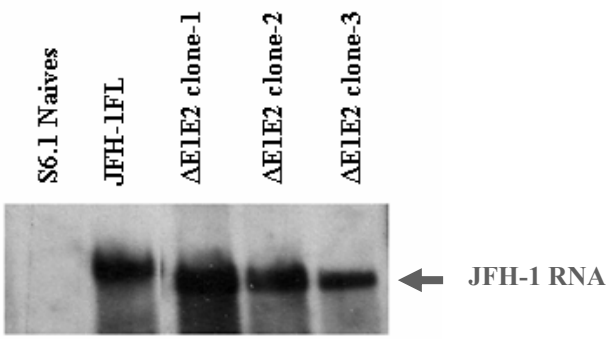


Fig. 8. Assessment of the replicative functionality of JFHΔE1E2. Total RNA was extracted from cells transfected with JFH-1 FL and the deleted mutant JFHΔE1E2, 5 days p.t.. RNA was then run on an agarose gel, blotted and hybridized with a probe on the 3' portion of the HCV genome labelled with UTP-DIG, followed by a treatment with an anti-Digoxigenin-AP solution. The deletion of the envelope proteins E1E2 did not affect the replicative capability of the JFH-1 mutant, compared to JFH-1 FL. No viral mRNA was detected in non transfected S6.1 naïve cells used as negative control.

Each of our packaging cell line transfected with JFHΔE1E2 was checked for the presence of viral proteins and efficiency of transfection 72h later, by immunofluorescence (IFA)

staining of core. This protein was detected in cells transfected with JFH-1 FL and in those transfected with JFHΔE1E2, indicating that the deletion does not impair the ability of the deleted JFH-1 backbone to replicate. The core protein was also detected in each packaging cell line transfected with JFHΔE1E2 (Fig. 9). In all cases, transfection appeared to be efficient in about 80% of cells.

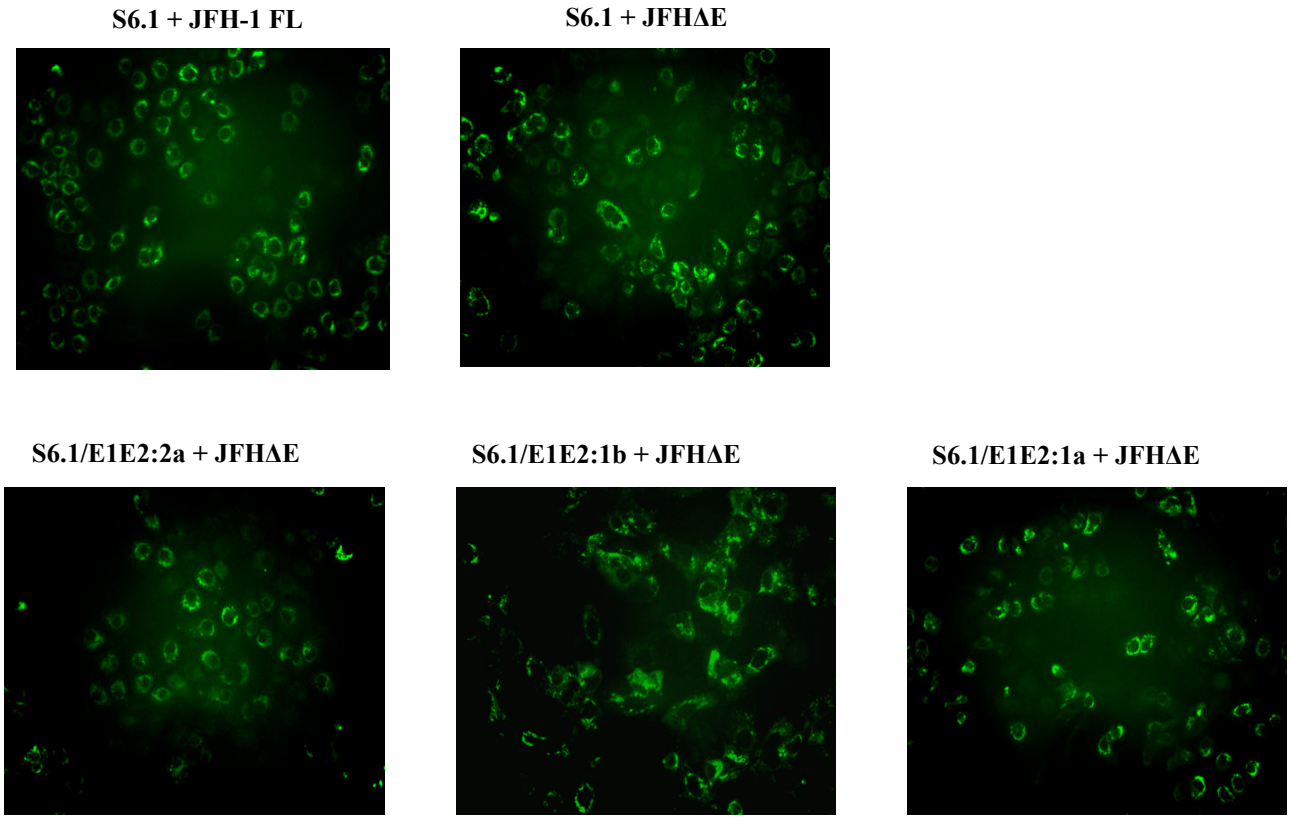


Fig. 9. Detection of viral protein expression in transfected cells. S6.1 cells transfected with the mRNA of JFH-1 FL and JFHΔE1E2, and packaging cell lines transfected with JFH-1 mutant were fixed permeabilized and checked for the presence of viral proteins and efficiency of transfection 72h p.t. by immunofluorescence (IFA) staining of core, using the anti-HCV core mAb 3G1-1 followed by an anti-mouse-AF-568 secondary Ab (green). Positive cells were visualized using a fluorescent microscope.

Although these analyses indicated the presence of viral protein in the transfected cells, they are not directly linked to the ability to produce infectious viral particles. This process indeed is probably connected with the formation in the perinuclear area of a multiprotein complex on ER-derived membranes, where all structural and non-structural HCV proteins co-localize (Hügle *et al.*, 2001). This protein complex, and in particular NS4B, is involved in intracellular membrane changes, to form a tight structure defined as membranous web,

that Egger and coworkers (2002) proposed as site where the HCV replication complex might be comprised. More recently Brazzoli and coworkers (2007) stated that cells supporting HCV RNA replication accumulates all viral products in huge structures that could correspond to pre-budding areas.

On this basis, it was important to detect a co-localization between the envelope proteins provided *in trans* by our packaging cell lines and one structural or non-structural protein derived by the translation of JFHΔE1E2 mRNA.

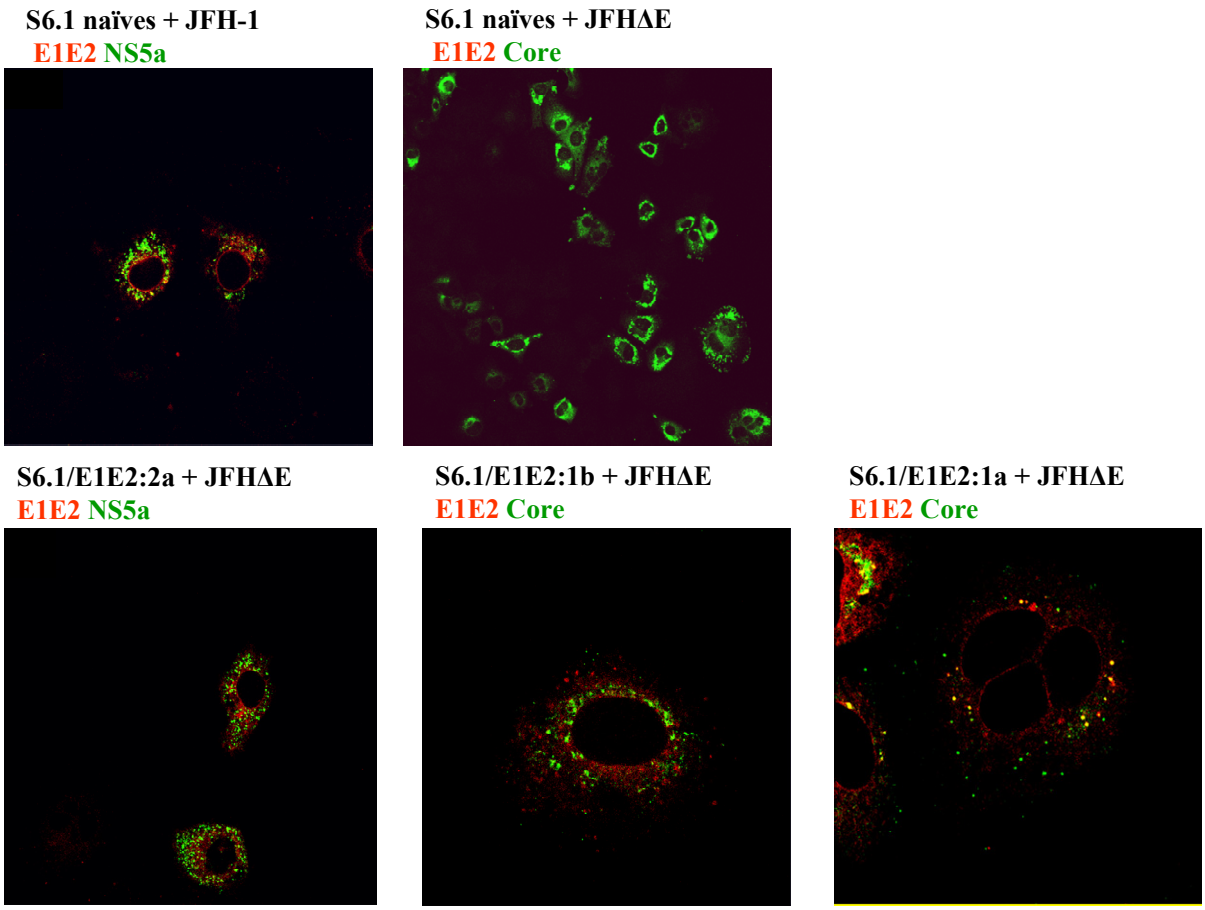
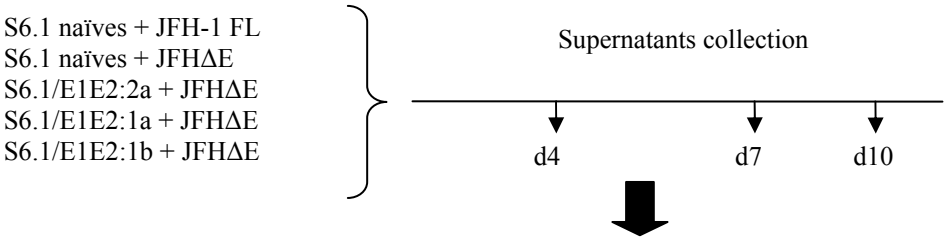


Fig. 10. Co-localization of E1E2 with structural proteins. Naïve cells transfected with JFH-1 FL and with the deleted mutant, and packaging cell lines transfected with JFHΔE1E2 were fixed, permeabilized and stained for the envelope proteins E1E2 and for core or NS5a, 72 h p.t.. Immunofluorescence (IFA) staining of core was performed using the anti-HCV core mAb 3G1-1 primary Ab followed by anti-mouse-AF-568 secondary Ab (Red); staining of E1E2 using polyclonal chimpanzee antiserum Ch-L559 as Ab I followed by anti-Human-AF-488 (green); staining of NS5a using a rabbit polyclonal Ab I followed by anti-rabbit-AF-568 (red). Positive cells were visualized using a fluorescent microscope. Bar 10 µm.

Confocal microscopy analysis showed a perinuclear co-localization of the envelope proteins with core or with the non-structural protein NS5a both in the positive control (S6.1 naïves/FL) and in each packaging cell line transfected with JFHΔE1E2 (Fig. 10) suggesting a potential capability of virus assembly. Only core and not E1E2 protein was detected in the S6.1 naïves/JFH ΔE1E2, as expected.

To verify the production of infectious viral particles by these cells, supernatant fluids were collected at day 4, 7, and 10 post-transfection. Clarified fluids were ultracentrifugated and pelletted virions were resuspended 100x in PSB/BSA and tested for the presence of infectious particles by inoculation onto Huh7 naïve cells. After 72 hours from infection these cells were fixed, permeabilized, and stained for the presence of core and NS5a antigens. Titration was possible solely for the supernatant of S6.1/FL cells, the positive control, since only a few cells resulted to be infected by the other fluids (Table. 1).



Infectious titer (pfu/ml)

	FL	ΔE	2a/2a	2a/1a	2a/1b
d4	10exp3	-	-	-	-
d7	10exp4	-	+	?	-
d10	10exp4	-	+	?	-

Table 1. Experimental procedure for viral particle collection and titration. Viral particles were isolated from supernatant fluids derived from packaging cell lines and naïve cells transfected with JFHΔE1E2, and from naïve cells transfected with JFH-1 FL, collected at d4, d7 and d10 p.t. Supernatants were clarified and viral particles concentrated by ultracentrifugation. Their presence was checked by infectivity tests on Huh7 naïve cells. 72 h post infection cells were fixed, permeabilized and stained for core protein using anti-HCV core mAb 3G1-1 Ab I followed by anti-mouse-AF-568 Ab II (Red). Supernatants of transfected cell were titrated on Huh 7 naïve cells following the Spearman and Kaerber fit. 72 hours later cells were fixed, permeabilized and stained for core protein, using the anti-HCV core mAb 3G1-1 followed by an anti-mouse-AF-568 secondary Ab (red). Positive cells were counted under a fluorescent microscope, and the infectious titer was expressed as particles forming unit per ml (pfu/ml).

Infectivity tests on Huh7 naïve cells showed that core and NS5a were present in numerous cells inoculated with the JFH-1 supernatant fluid, consistent with the infectious JFH-1 virus, whilst fluids coming from JFHΔE1E2 transfected cells did not produce any infection, as expected. Importantly, we also observed core and NS5a antigens in a smaller number of cells inoculated with fluids of d7 and d10 of S6.1/E1E2:2a + JFHΔE1E2.

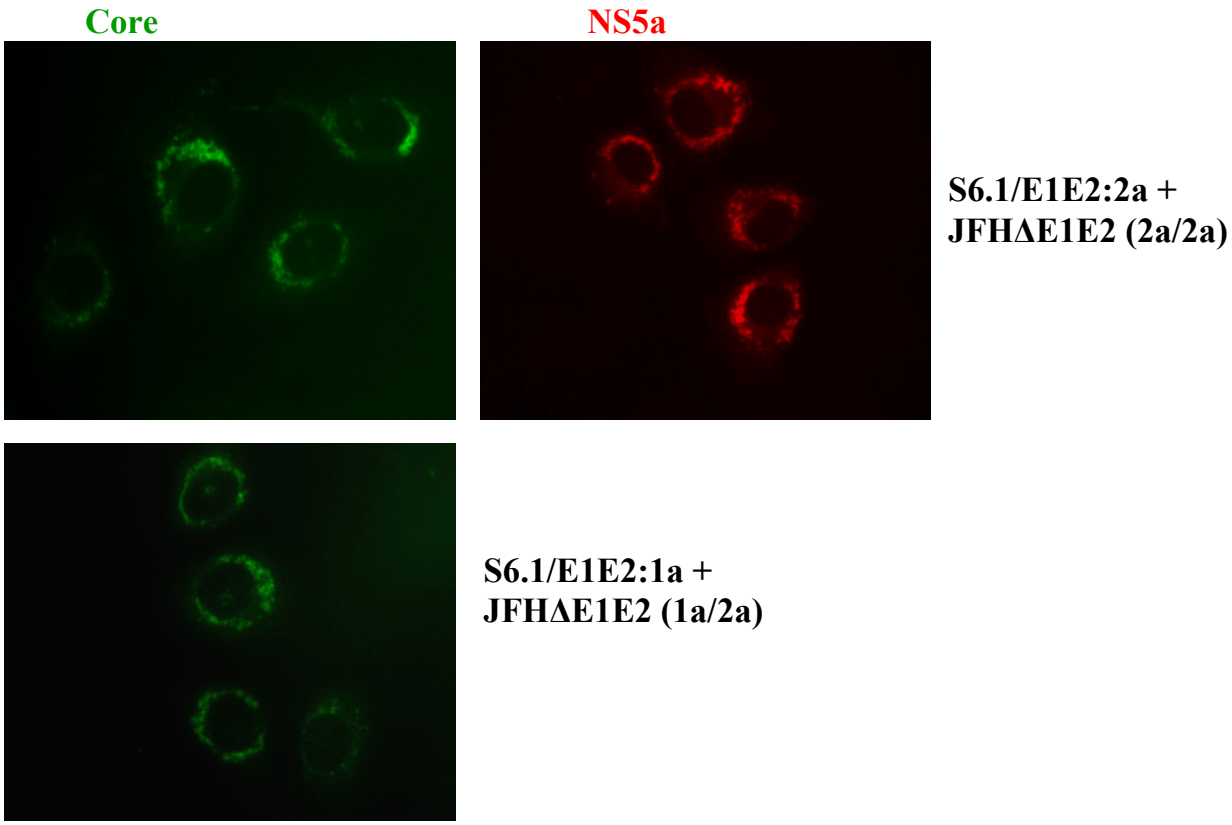


Fig. 11. IFA on infected cells. Immunofluorescent analysis was used to detect core (green) and NS5a (red) proteins on Huh7 cells inoculated with supernatants of S6.1/E1E2:1a + JFHΔE1E2, S6.1/E1E2:1b + JFHΔE1E2 and S6.1/E1E2:2a + JFHΔE1E2. Staining for core was performed using the anti-HCV core mAb 3G1-1 Ab I followed by an anti-mouse-AF-568 secondary Ab (red); staining NS5a using a rabbit polyclonal Ab I followed by anti-rabbit-AF-568 (red). Confocal microscopy analysis.

No positive results were obtained on cells inoculated with d4 fluids and with supernatant fluids derived from S6.1/E1E2:1a + JFHΔE1E2, and S6.1/E1E2:1b + JFHΔE1E2, although the former case was at the beginning not well defined; immunofluorescence analysis in fact could detect only core protein but not NS5a (Fig. 11). Further studies led us to exclude the positiveness of these data, and to consider the presence of what we could identify as the core protein as an artifact.

4.1.5 In Summary

- Northern Blot analysis on the lysates of S6.1 naïve cells transfected with JFHΔE1E2 revealed the presence of viral mRNA even 5 days after transfection. This indicates that this viral mutant is able to autonomously replicate and that the deletion of the envelope proteins E1 and E2 did not impairs its functionality.

- In about 80% of each packaging cell line transfected with JFHΔE1E2, as well as in the positive (S6.1 naïve/FL) and negative (S6.1 naïve/JFHΔE1E2) controls, immunofluorescent analysis detected the presence of core, to indicate the capability by these cells to express viral proteins.

- IFA analysis also showed a co-localization among the envelope proteins and some of the proteins provided by the JFHΔE1E2 backbone (Core, NS5a) in each packaging cell line, consistently with what we found in S6.1 naïve cells transfected with JFH-1 FL. This led us to speculate a correct addressing to the intracellular membranes, relative to the viral proteins provided *in trans* by the host cell and those provided by transfection of the viral backbone.

- When the mRNA of JFHΔE1E2 was transfected into our packaging cell lines, S6.1/E1E2:2a cells were able to release HCV infectious particles, consistently with the results obtained transfecting S6.1 naïve cells with JFH-1 FL. This indicates that for successful virus assembly, budding and release the structural glycoproteins E1 and E2 can be provided *in trans*.

  This is the first time that such a complementation has been demonstrated for the HCV structural glycoproteins.

- Unfortunately the initial aim to achieve trans-complementation using the gpE1/gpE2 from heterologous strains (1a, 1b) in order to produce HCV chimeras from different genotypes could not be satisfied.

§ 4.2 RESULTS

Characterization of early events that follow HCV binding to target cells

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As we have previously seen, by transfecting permissive cells with the mRNA of the HCV isolate JFH-1 it is possible to produce infectious viral particles that might be used for experiments on the viral life cycle, as binding, attachment and entry. One of the main advantages of the cell culture-derived HCV (HCVcc) in fact is probably the mechanisms of interaction of these viral particles with the host cell receptors that closely mimic what happens in a natural infection event.

Besides the two main HCV receptors identified so far, CD81 and SR-BI (Pileri *et al*., 1998; Scarselli *et al*., 2002), many cellular factors act in concert to mediate HCV binding and entry into hepatocytes. Recently Evans *et al*. (2007) identified a tight junction protein that is highly expressed in liver cells, Claudin-1 (CLDN-1), which seems to act late in the entry process, after virus binding and interaction with the HCV co-receptor CD81.

Since CD81 is normally expressed on the whole cytoplasmic membrane of hepatic cell, whilst CLND-1 is prevalently localized in the areas of cell-cell contact, one of the questions we intended to investigate involved the mechanisms and timings by which the virus interacts with both these receptors. On the basis of the recent discovery on the role of CNDN-1 in HCV entry, we hypothesized that once the virus has bound to its co-receptor CD81, and to SR-BI, the entire complex is translocated to the areas of cell-cell contact, where Claudin-1 dwells, and here the virus is internalized.

Beside these binding studies, we intended to identify and characterize the signal pathways that sequential interactions between viral proteins and cellular receptors might activate to promote viral infection.

We designed a pool of experiments triggering the principal HCV receptor CD81 under different conditions, and we analyzed outputs by the mean of biochemical or silencing RNA techniques acted to identify the signal pathways that are activated upon virus-receptor interaction. Once signal pathways were identified, we investigated their relevance in viral life cycle using the siRNAs and other pharmacological inhibitors in time-course

experiments, in which the establishment of a productive infection by cell-culture derived HCV (HCVcc) on Huh7 cells was monitored over time.

The use of HCVcc was not possible in biochemical experiments and in confocal fluorescent microscopy, because the yield of virus obtained so far would not allow following the virus binding to target cells or to induce an activation of cellular signal pathways. Therefore, in these cases we took advantage of some surrogates of the viral particles as anti-CD81 mAb, the soluble truncated form that lack the transmembrane domain of the recombinant protein E2 (E_{715}) or the E1E2 heterodimer complex.

4.2.1 Stimulation of Huh7 with a-CD81 mAb, $E2_{715}$ and E1E2: engagement of CD81 and lateral migration of the ligand-receptor complex to areas of cell-cell contact

To investigate the response that the main HCV receptor CD81 might have when stimulated with specific ligands, we designed some experiments using the commercially available anti-CD81 mAb (purified anti-human CD81, JS-81) and then two surrogates of HCV viral particles: the soluble form of the recombinant protein E2 (sE2), and the full-length heterodimer E1E2.

Rosa *et al.* (1996) showed that the recombinant form of the major envelope protein of HCV, E2, binds with high affinity to human lymphoma and hepatocarcinoma cell lines and that this binding is mediated by CD81; for this reason E2 and its truncated soluble forms sE2 ($E_{348\text{-}661}$, $E_{348\text{-}715}$) have been used in many studies as tool to search for cell-surface proteins potentially involved in HCV entry, comprising the one that identified CD81 as putative receptor (Pileri *et al*., 1998). Some functional differences might exist between the sE2 and the full length E1E2 complex, as the presence of E1 might potentially affect the properties of E2 (Cocquerel *et al*., 2003a; Brazzoli *et al*., 2005) in its binding with HCV receptors, and it is for this reason that we performed our main experiments, connected to the study of CD81, using both these ligands. E1E2 heterodimer in fact, seems to be a more physiological ligand compared to the solely sE2. The availability of these ligands came when Choo *et al* (1994) were able to copurify E1 and E2 glycoproteins from mammalian cells; doing this they noticed that a fraction of this purified material existed in the form of a large E1E2 oligomeric complex, that could be as well extracted and used in experimental studies. E1E2 heterodimer, produced as recombinant protein in CHO (Chinese Hamster Ovary) cells and purified from ER membranes, is now also used as antigen, adjuvated with

MF59, for Novartis vaccine against HCV, in therapeutics (in chronic infected patients) and preventive clinical trials (in at high risk healthy donor).

Since in previous experiments we noticed that the stimulus of CD81 in Huh7 cell with the solely sE2 or E1E2 was not sufficient to trigger any cellular response, in the following studies we decided to treat cells simultaneously using $E2_{715}$ or E1E2 and a primary antibody, called 291A2 (anti-HCV-291A2), that was screened for the ability to recognize E2 bound to target cells. This induces a lateral crosslink reaction among different CD81 molecules that enhanced the cellular response.

In our experimental design, human hepatoma cells (Huh7) were seeded on 24-well cover slips as to reach a confluent stage at the moment of the experiment. To allow the binding of the ligands to CD81 without triggering any cellular response, cell plates were kept at 4° C for 30 minutes during the treatment with α-CD81, and with the recombinant protein $E2_{715}$/Ab291A2 and the E1E2/Ab291A2 heterodimer. After that, control cells were fixed, permeabilized and stained with a fluorescent secondary antibody, whilst sample cells were shifted at 37° C for 1h to stimulate a cellular response, before being fixed, permeabilized and fluorescently stained.
Results observed by confocal fluorescent microscopy showed that in control cells the receptor CD81 appeared to have a uniform distribution on the whole cellular surface, and this pattern was consistent in all the three situations considered (anti-CD81, $E2_{715}$/Ab291A2, E1E2/Ab291A2). After 1h of stimulation at 37° C instead, the receptor distribution appeared markedly different, being mostly localized in areas of cell-cell contact, either after binding of the monoclonal antibody and the recombinant proteins (Fig. 12).
To assess that this mechanism of action was specific for the CD81 receptor, we designed a parallel control. We performed the same experiment above described treating Huh7 cells with an antibody against β1-integrin, a trans-membrane protein that mediates cell adhesion and cytoskeletal organization, functioning as receptor for physiological ligands and transducer of intracellular signaling. β1-integrin, that at 0 min presents an uniform distribution on the whole cellular surface, after 1h of stimulus at 37° C showed a patch distribution, with the formation of clusters, that completely differed from the one we

noticed for CD81. This was a further indication to assess the specific involvement of CD81 in the translocation process.

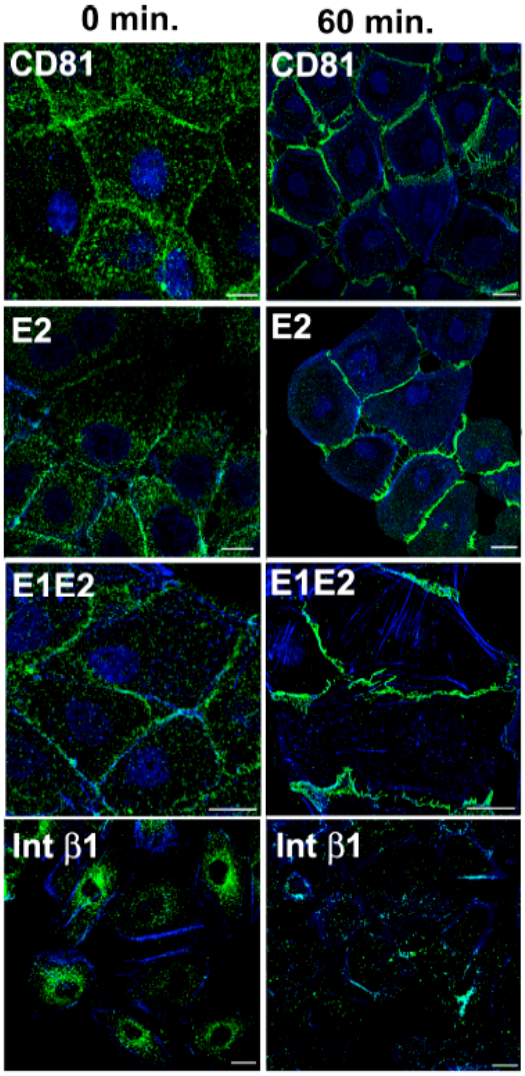

Fig. 12. Translocation of stimulated CD81 to the areas of cell-cell contact. 0 min: Huh7 cells were treated with an anti-CD81 mAb (JS-81) at 4° C, or with the recombinant proteins E2 or E1E2 plus the mAb 291A2 to allow binding of these ligands to CD81 and then shifted to 37°C for the indicated time. Cells were then fixed, permeabilized and stained with Alexa-Fluor-488 secondary Ab (green). Confocal fluorescent microscopy showed a diffuse distribution of the receptor CD81 on the whole cellular surface in all the three cases considered. 60 min: After 1h at 37° C the distribution of CD81 appeared markedly localized in areas of cell-cell contact. As control experiment, cells were stained with an anti β1-integrin, followed by the secondary Ab fluorocrome-conjugated (green). Confocal microscopy analysis showed a diffuse distribution of this protein on the cellular surface at 0 min, and a patch distribution after 1h at 37° C, 60 min, to confirm that, under the same conditions, the translocation process specifically involve CD81 and not other receptors. The actin cytoskeleton was stained by phalloidin (blue) and nuclei were visualized with To-Pro3 Iodide (blue). Bar 10 μm.

The translocation of CD81 in the areas of cell-cell contact led us to further analyze its localization, regarding the connections that might exist between the receptor and putative cell junctions. In fact, the hepatocytes within the liver, which are the HCV main natural target cells, are known to be highly polarized cells, with tight junctions (TJs) separating the basolateral domain (sinusoidal) from the apical (canalicular) surface. These TJs create a barrier regulating the paracellular movement of water and solutes across the epithelium, thanks to continuous rows of transmembrane proteins belonging to three main groups: the Junction Adhesion Molecules (JAM), the Claudins, and the Occludins (Fig. 13, A). Beside these, the peripherally associated scaffolding proteins Zonula Occludens (ZO-1) appear to organize the transmembrane proteins and couple them with other cytoplasmic proteins and to actin microfilaments (Andreson, 2001).

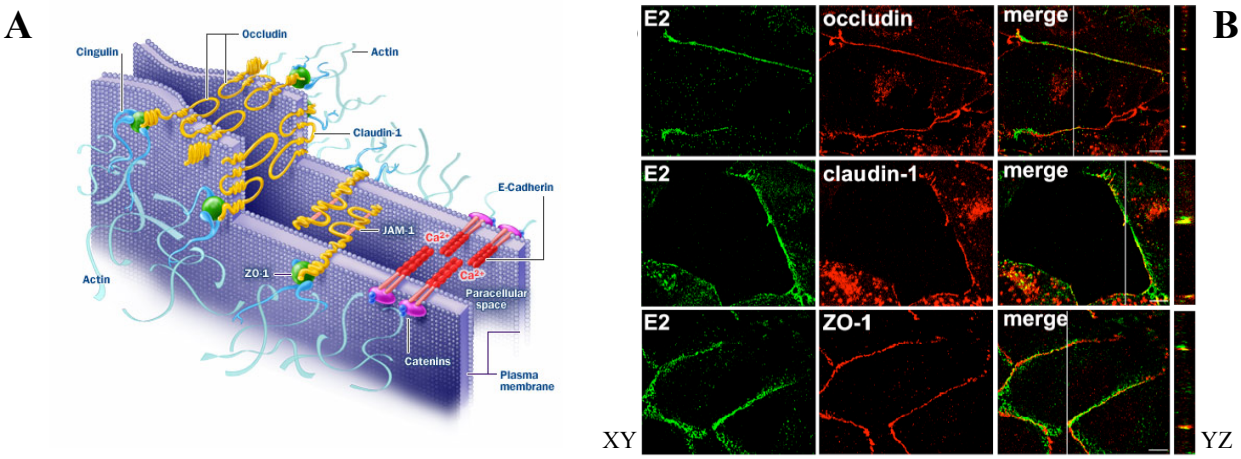

Fig. 13. (A). Schematic structure of a cellular tight junction (TJ). The main structural transmembrane proteins as JAM, Claudins and Occludins, and their relative connections with the peripherally scaffolding proteins Zonula Occludens (ZO-1) and the actin filaments are shown. (B). Co-localization of the recombinant protein E2 and three TJs markers. Monolayers of Huh7 cells were incubated at 4° C with E2 plus the anti-E2 mAb 291A2 to allow binding, and then shifted at 37° C for 1h. Cells were then fixed, permeabilized and stained for E2 (green) using Alexa-Fluor-488 secondary Ab, and for some TJ proteins as Occludin, Claudin-1 and ZO-1 (red), using rabbit pAbs against these proteins followed by anti-rabbit-AF-568 secondary antibody. Confocal fluorescent microscopy detected a marked co-localization between the recombinant proteins and each one of these TJ markers, visible in yellow in merged pictures, in which the white line indicates the position of the YZ cross-section. Details of this co-localization are clearly visible on the YZ axis. Bar 10 µm

To mimic as close as possible the natural structure of the liver epithelium, we performed our experiments on human hepatoma cells that were let to grow until they form a confluent monolayer. In this way we intended to promote the natural formation of cell junctions.

Some commercially available rabbit polyclonal antibodies against the TJs proteins claudin-1, occludin and ZO-1 were utilized to carry on the following experiment.
Monolayers of Huh7 cells were inoculated with $E2_{715}$/Ab291A2 for 30 minutes at 4° C to allow binding, then temperature was raised at 37° C for 1h, to trigger cellular response. After this period of time cells were fixed, permeabilized and stained using the rabbit pAbs against Claudin-1, occludin, and ZO-1 followed by the secondary anti-rabbit fluorescent antibody-568 (Red), to detect the position of the TJs proteins. The same was done also for CD81, staining the bound $E2_{715}$/Ab291A2 with an anti-mouse-488 secondary antibody (green). Merging the two images obtained at confocal fluorescent microscopy we could noticed a marked co-localization of the receptor with the TJs proteins that we chose as markers (Fig. 13, B). Moreover, considering the XZ layer of the picture, we could detect two bigger dot like structures indicating the punctiform colocalization at the level of TJs of E2/ZO-1, E2/Cla-1, E2/Occludin; in this latter case this pattern seemed to diffuse also inside the cell.

The role of CD81 in this translocation process was further analyzed suppressing its activity by the use of silencing RNA (siRNA). In this technique short dsRNA, called small interfering RNA (siRNA), are transfected into target cells. When inside the cells, dsRNAs are unwind by cellular proteins and, as single stranded, they are free to bind to a target mRNA in a sequence-specific manner. This binding mediates the cleavage of the target mRNA by the cellular endonucleases, that recognized it as aberrant, and prevents the translation of the relative protein.
In our study, we used Huh7 CD81(–) cells that were kindly provided by Takashi Harada, Novartis Vaccines. These cells were created using a chemically synthesized siRNA sequence targeting human CD81 (nt: 138-156). The cDNA of the silencing CD81 sequence was carried in a plasmid that could stably integrate into the genome of the target cells, and that contained a resistance gene for puromicin. Positive clones of transfected cells were isolated by selection with this antibiotic and store for future needs.

Control cells, Huh7CD81(+), were transfected with a mock siRNA, and used as well for an isotype control, to estimate the background non-specific binding of the primary antibody to cell surface antigens. To check the presence of CD81, Huh7 CD81(–) and Huh7 CD81(+) were fixed and superficially stained with a fluorescent primary Ab against CD81 (anti-CD81-PE); beside this Huh7 CD81(+) were also stained with an isotype non-correlated Ab. FACS analysis outputs showed that the surface expression of CD81 in silenced Huh7 cells was almost null, compared to what could be found on the surface of Huh7 CD81(+) (Fig. 14).

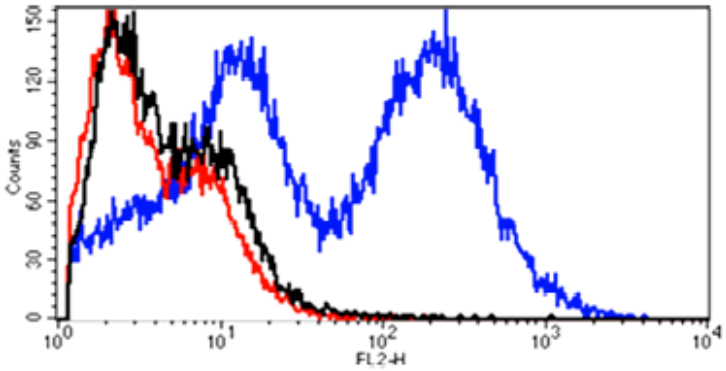

Fig. 14. Silencing of CD81 in Huh7 cells through siRNA technique: flow cytometry analysis shows the expression of CD81 on the cellular surface of silenced Huh7 (red line) compared to Huh7 cells normally expressing the receptor protein (blue line). The black line indicates the isotype control.

Once these silenced Huh7 for CD81 were obtained, they were incubated with $E2_{715}$/Ab291A2 for 30 min at 4° C (binding). After that, control cells were shifted for 5 minutes at 37° C before being fixed, permeabilized and double stained with a rabbit polyclonal antibody against SR-BI, the second main receptor for HCV, followed by an anti-rabbit-568 secondary Ab (Red), and with an anti-mouse-488 secondary Ab against $E2_{715}$/Ab291A2 (green) (Fig. 15, 0 min). Sample cells instead were shifted at 37° C for 1h, as to stimulate a cellular response, and then fixed, permeabilized and stained as we did for the control (Fig. 15, 60 min). As the fluorescent microscopy showed, stimulation of Huh7 cells with the recombinant protein did no lead to translocation of any receptor bound to $E2_{715}$/Ab291A2, to the areas of cell-cell contact, on contrary of what we previously observed in CD81(+) cells. Moreover, SR-BI appeared to have the same distribution pattern on the cellular surface before and after stimulating Huh7 cells with $E2_{715}$/Ab291A2. This made us to postulate that the HCV receptor SR-BI is not involved in

such a mechanism of translocation, which in our experiment appeared to be mediated solely by CD81.

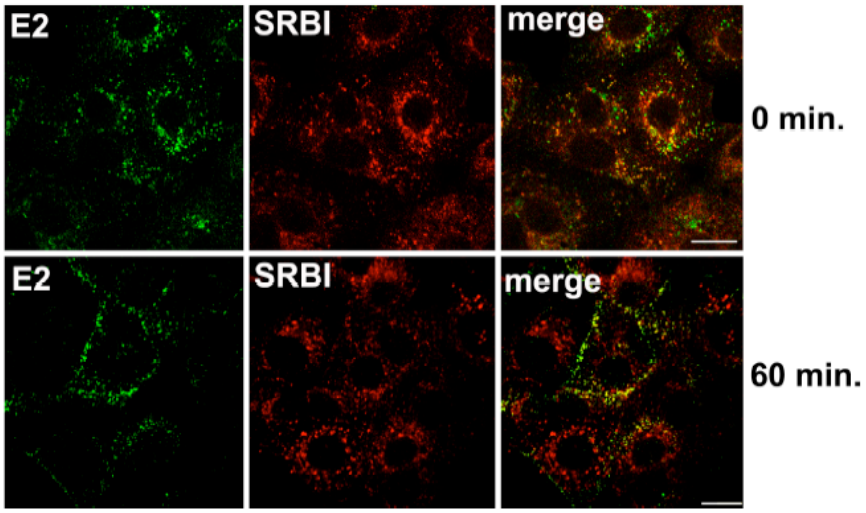

Fig. 15. In stimulated Huh7 CD81(-) the re-localization event do not occur. Huh7 cells silenced for CD81 are inoculated with the recombinant protein E2 plus the anti-E2 mAb 291A2 for 30 min at 4° C and then shifted at 37° C for 60 min. Cells were then fixed, permeabilized and double stained for E2 and for the HCV receptor SR-BI using anti-mouse-AF-488 secondary antibody (green) to detect the recombinant protein, and anti-SR-BI rabbit pAb followed by anti-rabbit-AF-568 (red) for the receptor. Merging the two images it is possible to observe yellow dots where the recombinant protein and the receptor co-localize. Confocal fluorescent analysis revealed the same diffuse distribution on the whole cellular surface of E2 (green) and SR-BI (red) before and after stimulation. Bar 10 µm.

On the basis of these results we formulate the hypothesis that once the HCV viral particles reach their main receptor CD81 on the hepatocytes cellular surface, the entire viral-receptor complex is translocated to the tight junctions, and here the virions come in contact with the co-receptor claudin-1.

4.2.2 A functional actin cytoskeleton is required for the relocalization process, and for the establishment of a productive infection.

What we saw in the previous experiments led us to assume an involvement of the actin cytoskeleton in the translocation process of CD81. To investigate this theory, human hepatoma cells were pre-treated for 1h at 4° C with chemical inhibitors of actin polymerization, Latrunculin A (LatA, 1 µM) and Jasplakinolide (Jasp, 500 nM). These two drugs act directly on cytoskeleton, the former sequestering the G-actin subunits, and

preventing actin polymerization, and the latter inducing stabilization of the actin filaments, without depolymerizing them. After this hour of pre-treatment cells were simultaneously incubated for 60 min at 4° C with $E2_{715}$/Ab291A2 and with LatA or Jasp, before being shifted to 37° C for 1h, always in continuous presence of the compounds. After that, cells were fixed, permeabilized and stained with an anti-mouse-488 secondary Ab to detect $E2_{715}$/Ab291A2, bound to CD81. Control cells, mock treated with the inert solvent DMSO (dimethyl sulfoxide) were subjected to the same procedure.

As shown in confocal analysis, the usual localization of E2 (bound to CD81) to the areas of cell-cell contact was detected only in the control cells, but it was not in cells treated with LatA, nor in those treated with Jasp (Fig. 16). In cells subject to these treatments in fact, E2 appeared in a disorganized pattern that in some degrees reflected the actin impairment. Details of this analysis can be detected considering the XZ layer of the picture: in control cells in fact, E2 (green) have a dot like localization, in correspondence to the TJs structures, whilst in treated cells E2 appear to have a more equal and diffuse distribution on the whole cellular surface.
Results of this experiment suggest that an integral and functional actin cytoskeleton might play an important role in the relocalization process of CD81.

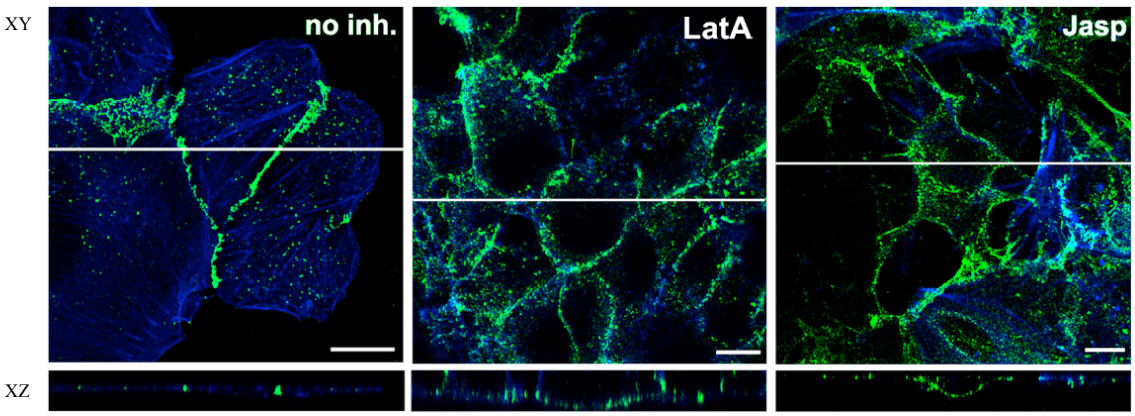

Fig. 16. Treatment of Huh7 cells with inhibitors of actin polymerization blocks the re-localization process. Cells were pretreated at 4° C for 1h with actin destabilizing drugs such as Latrunculin A (LatA, 1 μM) and Jasplakinolide (Jasp, 500 nM) or mock treated. After that, Huh7 cells were allowed to bind E2 plus the anti-E2 mAb 291A2 again for 1h at 4° C, before being shifted at 37° C for 1h in the continuous presence of the compounds. Cells were then fixed, permeabilized and stained with an anti-mouse-AF-488 secondary Ab (green) to detect E2. Phalloidin (blue) was used to detect actin filaments. Control cells (no inh.), mock treated with the inert solvent DMSO, were subjected to the same procedure. LatA or Jasp have a detrimental effect on the actin fibers, that appear in a patch and disorganized distribution, besides the lost of E2 peripheral localization. White lines in the pictures indicate the position of the XZ cross-section in which details are visible: E2 relocalized on areas of cell-cell contact in non treated cells (green), whilst it presents a diffuse distribution in cells subjected to drug treatments. Bar 10 μm.

As a consequence of these results we intended to evaluate the physiological relevance that actin inhibitors could have on viral infection.

We designed a set of experiments in which Huh7 cells, treated with different drugs, were infected with cell-culture derived HCV (HCVcc). These drugs were the previously described LatA and Jasp, and another compound, the cytochalasin D (CytD), that destructs the cytoskeleton depolymerizing the actin filaments.

Monolayers of human hepatoma cells were exposed to viral particles for 1h at 4° C, to allow binding but not the entry of the virions into the cells; at the same time cells were mock treated (control) or incubated with medium containing 1 μM LatA, 5 μM CytD or 500 nM Jasp. After this period of time, the unbound virions were washed away, and drug treatment was continued for other 2h at 37° C. Synchronous infection of cells was triggered by the simultaneous temperature shift. After this period of time, drugs were removed and substituted with fresh medium.

Cells were left for 72h in medium alone, before being fixed, permeabilized and checked for infection by immunofluorescence using antibodies against the viral core protein. This was

done treating cells with a mouse primary antibody against core (3G1-1) followed by an anti-mouse-568 secondary Ab (Red). Using a fluorescent microscopy we determined the efficiency of infection by counting those cells that presented the viral protein, and we reported the relative results as percentage, in comparison with values obtained in the absence of drugs (mean ± SD for four replicates; * $p < 0.05$; * * < 0.01)

As shown in the diagram of Fig. 17, treatments with these drugs significantly reduced the infectivity of the HCVcc, compared to the relative mock-treated cells and to the two types of viral particles selected as controls. In particular, MLV pseudo particles (MLVpp) where chosen to mimic viruses that fuse at the plasma membrane, and VSV pseudo particles (VSVpp) were chosen for viruses that require to be delivered to early endosomes to enter the host cells, as HCV does.

To create these two sets of lentiviral particles to be used in control experiments, 293T cells were transiently transfected as described in paragraph 4.1.3. In this case though, the self-inactivating (SIN) transfer vector plasmid, pCCl.sin.PPT.hPGK.GFP.Wpre, carried the information for the Green Fluorescent Protein, and the envelope of the lentiviral particles derived from pMD2.VSVG, with the G protein of the Vesicular Stomatitis Virus, or from pSV-A-MLV-env, coding for the envelope protein of Moloney Murine Leukemia Virus. Lentiviral particles produced by 293T transfected cells were used to infect Huh7 treated with LatA, CytD, Jasp, following the procedure of the experiment above described. Outputs could then be red by FACS exploiting the expression of GFP in the infected cells.

Actin inhibitors had a minor effect on both VSVpp and, to a lesser extent, on MLVpp compared to the one related to HCVcc. This might be due to the fact that these pseudoparticles do not need a relocalization process before entering into the host cells, whilst HCV seems to be firstly tanslocated to the TJs and then internalized through clathrin-coated vesicles. (Blanchard *et al*., 2006; Meertens *et al*., 2006; Mousavi *et al*., 2004).

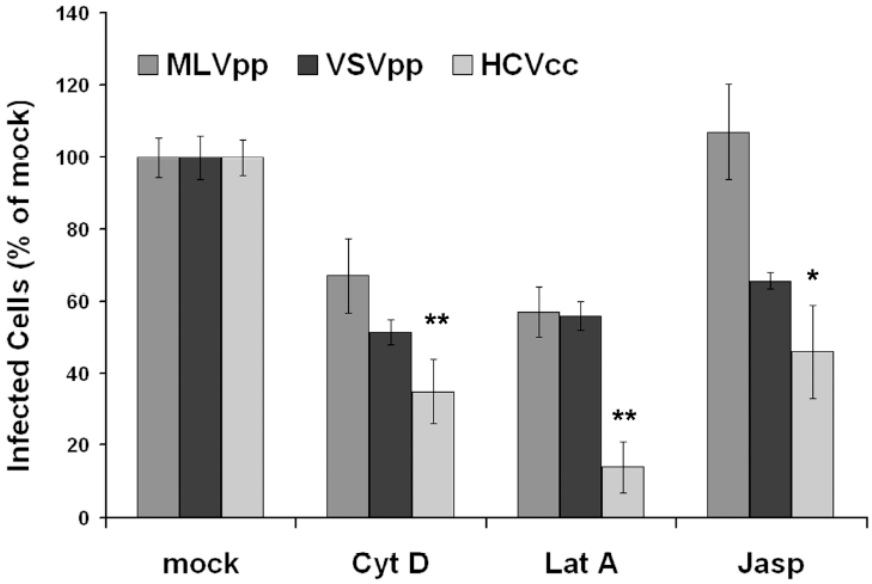

Fig. 17. Effect of actin inhibitors on HCVcc infectivity. Huh7 cells mock treated or incubated with Cytocalasin D (5 μM), Latrunculin A (1 μM) or Jasplakinolide (500 nM) were left in contact with HCVcc for 1h at 4° C to allow binding, before being shifted at 37° C for other 2h of treatment. The efficiency of infection was detected 72 hours later on fixed and permeabilized cells by immunofluorescent staining of core protein, using the anti-HCV core mAb 3G1-1 followed by an anti-mouse-AF-568 secondary Ab (red). Positive cells were counted under a fluorescent microscope; infectivity is expressed as percentage relative to the values obtained in non-treated cells. Controls are represented by MLV pseudoparticles (MLVpp), that fuse at the plasma membrane, and VSV pseudoparticles (VSVpp) that, as HCV, required to be delivered to early endosomes to enter cells. In controls, carrying the information for GFP, the efficiency of infection was detected by FACS exploiting the expression of the fluorescent protein in infected cells The detrimental effect that this inhibitors have on actin cytoskeleton markedly reduced the efficiency of infection of cell-culture derived HCV particles (mean ± SD; * $p < 0.05$; * * $p < 0.01$), but had a minor effect on both VSVpp and on MLVpp.

At this point it was clear that the detrimental effect that these drugs had on the actin cytosckeleton could markedly reduce cells susceptibility to HCV viral infection. The next step would be studying the time course of these events.

To do this we designed an experiment in which Huh7 cells, inoculated with HCVcc, came in contact with the inhibitor of the F-actin polymerization LatA (1 μM), at different time points.

Virus binding but not entry was achieved by inoculating cells at 4° C for 1h, then the unbound virion were washed away and cells were shifted at 37° C, to restore the infection process. In two cases LatA was added to the growing medium in concomitance with the viral inoculum at 4° C, and then it was washed off (-60 min + w/o) or left for the remaining time (-60 min). In all conditions, after 1h of binding at 4° C, the unbound virion were

removed and cells were shifted at 37° C. LatA was soon added (0 min), or after 20, 60 and 90 minutes following temperature raising. In all cases the drug was left onto cells for three hours from the moment it was added to the medium, before being removed and substituted with fresh medium. Cells were left in medium alone for the next 72 h and then they were fixed, permeabilized and fluorescently stained for the viral core protein (AbI: 3G1-1, AbII: α-mouse-568, Red). By the use of a fluorescent microscopy we determined the number of infected cells, in which core was present, and we compared these values with those of the control cells, in which the infection was performed in the same way but always without the drug.

As shown in the diagram below (Fig. 18), LatA had no effect on viral binding to the cells because it did not cause any significant reduction in infectivity when added only during the incubation at 4° C (-60 min + w/o), but there was a significant drop of infectivity when drug was added during virus binding and left for the following 2h (-60 min), or soon after binding and left for three hours (0 min) (mean ± SD for four replicates; * $p < 0.05$; * * < 0.01). As treatment was postponed from the binding stage, the number of infected cells progressively increased, and when the drug was added at 90 minutes after temperature shift, we observed that almost the 60% of cells could be infected, compared to the approximate 20% of those treated from the very beginning (-60 min).
This kinetics of inhibition led us to presume that an intact and functional cytoskeleton is required in the infection process at a very early stage, and it is probably involved in the relocalization of CD81, that we previously reported, and/or in the entry of the HCV virions into the hepatoma cells as expected by the fact that HCV entry is a clathrin dependent mechanism (Blanchard *et al*., 2006; Meertens *et al*., 2006) that exploit the endocytic pathway for viral internalization.

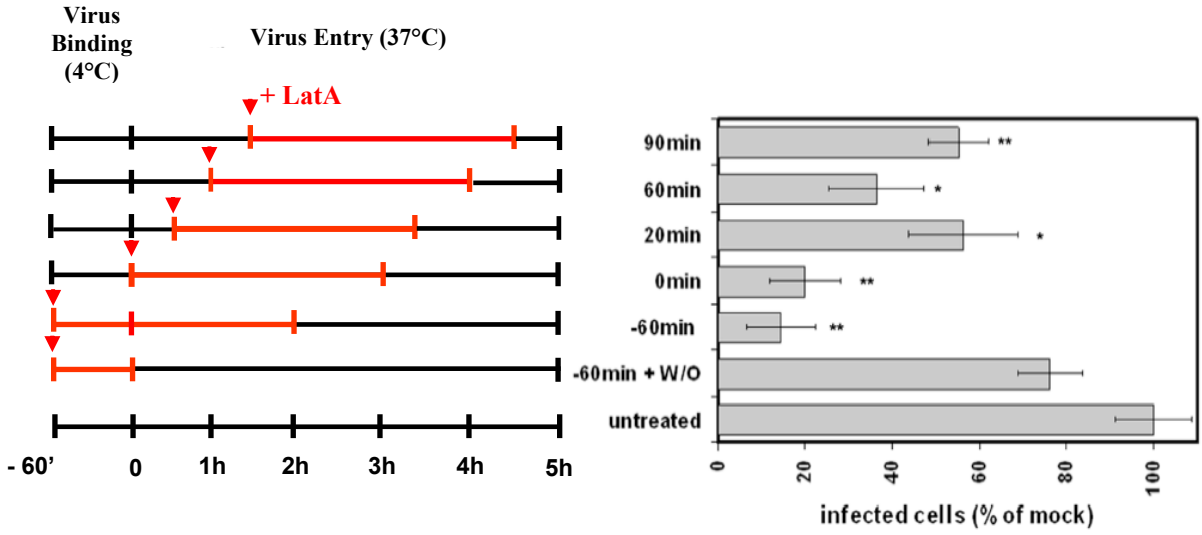

Fig. 18. Inhibitory activity of LatA on HCVcc infectivity. Huh7 cells, inoculated with HCVcc for 1h at 4° C to allow binding, were put in contact with 1 µM Lat A in concomitance with the viral inoculum at 4° C or at different time points upon temperature shift to 37° C (red arrows). In all cases but one (-60 + w/o) LatA treatment has been extended for 3hs from the moment it was added onto cells. After that, cells were provided of fresh medium for the next 72 hrs, before being fixed, permeabilized and stained against the core protein, using the anti-HCV core mAb 3G1-1 followed by an anti-mouse-AF-568 secondary Ab (red). Positive cells were counted under a fluorescent microscope. Efficiency of infection was determined as percentage of positive cells relative to the values obtained in non-treated cells. The kinetic of inhibition suggest that a functional cytoskeleton is required at a very early stage in the infection process (mean ± SD; * $p < 0.05$; * * $p < 0.01$).

So, if an intact actin cytoskeleton is required for the translocation process of stimulated CD81 from cellular surface to the tight junctions, what are the signal pathways that trigger the actin cytoskeleton rearrangement leading to this relocalization?

4.2.3 CD81 triggers Rho GTPase-mediated actin rearrangements

In a recent study on Coxsackievirus, Coyne and Bergelson (2006) demonstrated that the transport of this virus from the apical surface of polarized cells to the TJs required actin cytoskeleton reorganization, and they stated that this rearrangement is modulated by Rac GTPase activation.

It is well known in fact that members of the Rho GTPases family, as Rho, Rac and Cdc42, are key regulatory molecules that link surface receptors to the organization of the actin cytoskeleton (Ridley and Hall, 1992; Ridley *et al.*,1992; Nobes and Hall, 1995), and that the activation of Rac and Cdc42 induces polymerization of monomeric actin, resulting in

the formation of a dense meshwork of actin filaments underlying the plasma membrane (Li *et al*., 1998). Inside the cell proteins Rho, Rac and Cdc42 act as molecular switch, cycling between an active GTP-bound to an inactive GDP-bound state. In the GTP-bound form they are able to interact with effectors or target molecules to initiate a downstream response, while an intrinsic GTPase activity returns the proteins to a GDP-bound state, to complete the cycle and terminate signal transduction.

Based on these knowledge, we assumed that members of the Rho GTPases family could represent a good point to start our investigations on signaling linked to CD81 relocalization, and in our experiment design we observed the effect that CD81 engagement could have on the most characterized members of the Rho GTPases family: Rho, Rac and Cdc42.

Human hepatoma cells Huh7 were exposed to anti-CD81 antibody (mouse) at 4°C for 30 min to allow binding, before being stimulated with a goat-anti-mouse secondary Ab (GαM) at 37° C for different times, as indicated in Fig. 19. The cross-linking derived from binding of the secondary Ab to the receptor was able to enhance and trigger the signaling response as soon as the temperature was raised. Cells were then lysed and the active-GTP-bound forms of the Rho family proteins were detected by affinity precipitation using agarose-associate PAK-1 PBD (p-21 binding domain) for Cdc42 and Rac, and agarose-associated Rhotekin RBD (Rho binding domain) for Rho, which were able to bind and to specifically precipitate Rac-GTP, Cdc42-GTP and Rho-GTP complex from cell lysates. The precipitated proteins were then separated by SDS-PAGE and immunoblotted with antibodies specific for Rho, Rac1 or Cdc42. Equal amounts of whole cell lysates were also blotted to evaluate total proteins, both in the GTP activated and in the GDP inactivated forms. Results shown a marked increase of the GTP-bound forms of the three proteins after the first few minutes (1 to 5).

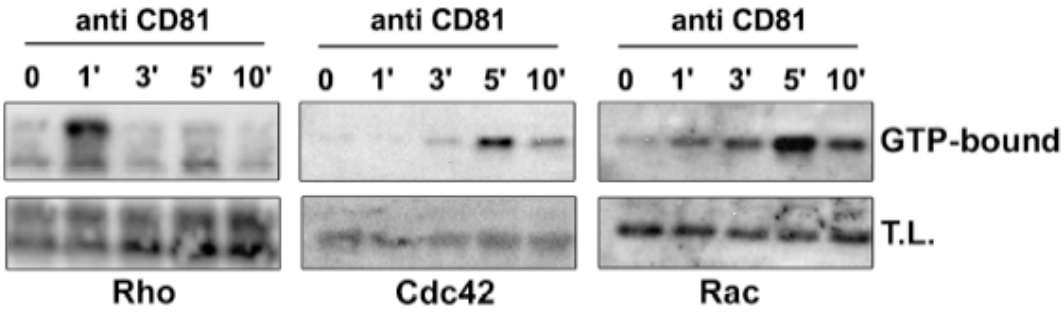

Fig. 19. Rho GTPases response to CD81 engagement. Huh7 were incubated with anti-CD81 for 30 min at 4° C and then cross-linked with a goat anti-mouse secondary Ab for the indicated times at 37° C. Cells were then lysed, and GTP-bound activated forms of Rho, Cdc42, and Rac were isolated by affinity precipitation and immunoblotted using specific antibodies. TL: Total Lysate was also blotted to evaluate total GTP- GDP-bound proteins.

To further investigate the importance of Rho, Cdc42, and Rac for HCV infection we decided to knockdown the activity of these proteins either by using siRNA technique, or some specific inhibitors, and to observed the effects that this would have on HCVcc infectivity.

In the former case, Huh7 cells were transfected with a commercially available siRNA targeting Cdc42 or Rac, whilst control cells were transfected with a non specific siRNA. The achievement of a significant silencing of the target proteins was checked 48h post transfection, when cell lysates were immunoblotted to detect Cdc42 and Rac, using specific antibodies against these proteins. The actin protein was also blotted, as parallel control (Fig. 20).

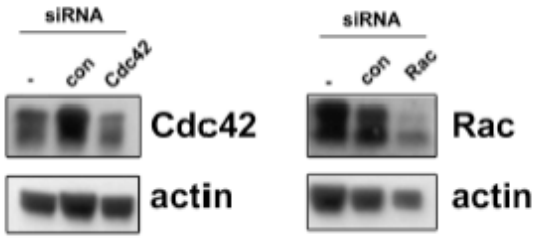

Fig. 20. Silencing of Cdc42 and Rac. Huh7 cells transfected with Cdc42 and Rac siRNA and with a control siRNA were lysated 48h p.t. and immunoblotted using specific antibodies against Cdc42, Rac and the actin protein. The expression of the two proteins was markedly reduced in silenced cells compared to non-transfected cells (-) or to cells transfected with a control siRNA (con).

Huh7 cells mock transfected and Cdc42- and Rac- silenced Huh7 cells were then used for HCVcc infectivity tests. Cells, incubated with 100 pfu/ml of viral particles for 1h at 4° C to allow binding, were then shifted at 37° C. Function of Rho GTPases proteins was also analyzed using specific inhibitors, such as the NSC23766 (100μM) for Rac1 and the exoenzyme C3 transferase from *Clostridium botulinum* (5 μg/ml) for Rho. The former acts inhibiting the GDP/GTP exchange of Rac1 by interfering with the interaction between this protein and some Rac-specific GEFs (guanine nucleotide exchange factors) that normally regulate the cycling between the active and the inactive forms, the latter, through the C3 Transferase, inhibits Rho proteins by ADP-ribosylation on asparagine 41 in the effector binding domain of the GTPase. Huh7 naïve cells were inoculated with the HCVcc for 1h at 4° C, then shifted at 37° C and left for 3 hours in a medium with presence or absence of the drug. After that, treatment was removed and substituted with fresh medium. After 72 hours all cells were fixed, permeabilized and stained against the viral core protein, and infected cells were counted using a fluorescent microscopy.

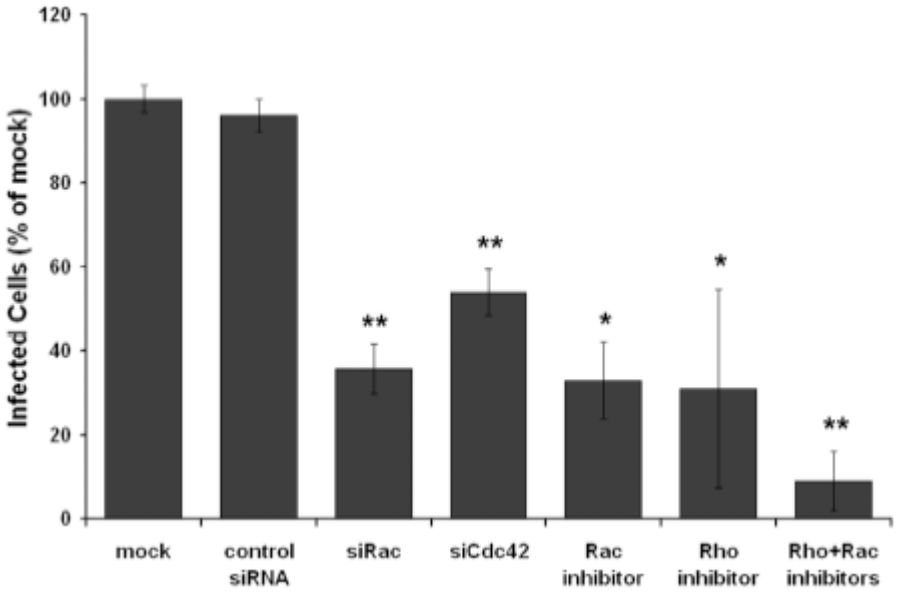

Fig. 21. Effect of inhibition or silencing of Rho GTPases on HCVcc infectivity. Huh7 cells silenced for the expression of Rac and Cdc42, cells treated with the indicated drug, and not treated controls were incubated with HCVcc for 1h at 4° C and then shifted at 37° C. After 72 hs cells were fixed, permeabilized and stained against the viral core protein using an anti-HCV core mAb 3G1-1 followed by an anti-mouse-AF-568 secondary Ab (red). Positive cells were counted using a fluorescent microscopy. The effect that both silencing and inhibitors had on HCVcc infectivity was a marked drop of it, compared to control cells, mock treated or transfected with a non-specific siRNA. Infectivity is expressed relative to the values obtained in mock treated cells (mean ± SD, * $p < 0.05$; * * $p < 0.01$).

As shown in Fig. 21, results highlighted that blocking the activity of Rho, Rac1 and Cdc42 using specific inhibitors as well as siRNA the infectivity of HCVcc is reduced. The drop of infected cells that we observed in response to Rac and Cdc42 silencing corresponded to 65 ± 5% and to 50 ± 5% respectively, compared to what was observed in mock treated cells or in cells transfected with a control siRNA (four replicates; mean ± SD, * $p < 0.05$; * * $p < 0.01$). We could detect the same effect on HCVcc infectivity also after Huh7 treatment with Rac and Rho inhibitors, with an enhancement of the impact when these drugs were used simultaneously.

These outputs led us to assert that Rac1, Cdc42 and Rho are actually involved in mechanisms of HCVcc cell entry, and open the discussion on what other signal pathways may be activated by the engagement of CD81 and the others HCV receptors.

4.2.4 In summary

- Confocal microscopy analysis revealed that after Huh7 cells were stimulated with anti-CD81 Ab or with the recombinant protein $E2_{715}$ and the E1E2 heterodimer, the distribution of CD81 changed from a diffuse pattern that involved the whole cellular surface to a strict localized distribution in the areas of cell-cell contact.

- Further analysis using pAbs against Claudin-1, Occludin-1 and ZO-1, chosen as markers of the cellular tight junctions, showed that in stimulated Huh7 cells the presence of CD81 in areas of cell-cell contact corresponded to the localization of the TJ structural proteins.

- The mechanism of translocation visible in stimulated Huh7 cells is mediate by the solely CD81, since E2 relocalization does not occur in cells in which CD81 has been silenced

On the basis of these results we formulate the hypothesis that once the HCV viral particles reach their main receptor CD81 on the hepatocytes cellular surface, the entire viral-receptor complex is translocated to the tight junctions, and here the virions come in contact with the co-receptor claudin-1.

- The involvement of the actin cytoskeleton in the translocation process of CD81 was proven by the use of chemical inhibitors of actin polymerization. The usual localization of E2 (bound to CD81) in the areas of cell-cell contact was not detected in stimulated Huh7 cells treated with Latrunculin A (1 μM) and Jasplakinolide (500 nM).

- The detrimental effect of LatA, Jasp and Cytochalasin D (5 μM) on actin cytoskeleton had also a physiological relevance on viral infection, markedly reducing the infectivity of HCVcc on Huh7 cells.

- Time course experiments with LatA led us to assume that an intact and functional cytoskeleton is required in the infection process at a very early stage, and it is probably involved in the relocalization of CD81.

- CD81 engagement is able to trigger the activation of members of the Rho GTPases family, such as Rho, Rac1 and Cdc42. Experiments aimed at blocking the activity of these proteins using siRNA technique or specific inhibitors showed a significant reduction of HCVcc infectivity leading us to assert that these proteins are actually involved in mechanisms of HCVcc cell entry.

§ 5.1 DISCUSSION

Trans-complementation of gpE1/gpE2 allows recovery of infectious Hepatitis C virus

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All positive-strand RNA viruses, as HCV, replicate their genomes in virus-induced vesicular membrane compartments, which are rather enclosed structures with a limited possibility for exchange of viral RNA or proteins by trans-complementation. Although observed for some members of the Flaviviridae family, including bovine diarrea virus (Harada *et al*., 2000; Grassmann *et al*., 2001; Reimann *et al*., 2003) and Kunjin virus (Khromykh *et al*., 1998; 1999a, b; 2000), trans-complementation of structural as well as non structural proteins as been generally inefficient and limited to only a few viral defects. In the case of hepatitis C virus (HCV), in which non structural proteins seem to remain associated to their respective complexes and may not be able to access other complexes (Evans *et al*., 2004), the possibility of recovering the effects of lethal mutations on non structural genes through trans-complementation was observed only recently by Appel and coworkers (2005), and only in the case of NS5a mutants.

In the present work we demonstrated for the first time for HCV that when the structural proteins E1 and E2 are provided *in trans* by the use of complementing cell lines constitutively expressing them it is possible to achieve production of infectious viral particles.

This has been observed when the structural envelope proteins and non structural proteins belong to the same genotype (GT 2a), whilst no productive complementation has been detected between heterologous strains, when the envelope proteins come from GTs 1a and 1b and the NS proteins from the JFH-1 (GT 2a) backbone. Immunofluorescent analysis of the three packaging cell lines transfected with JFHΔE1E2 mRNA detected the presence of the viral core protein in the cellular cytoplasm to indicate the capability of the delete viral genome to replicate. Moreover IFA analysis also showed a co-localization in the perinuclear area among the envelope proteins and some of the proteins provided by the JFHΔE1E2 backbone (Core, NS5a) in each packaging cell line, consistently with what we

found in S6.1 cells transfected with JFH-1 FL. This led us to speculate a correct addressing to the intracellular membranes, relative to the viral proteins provided *in trans* by the host cell and those provided by transfection of the viral backbone; however, the release of infectious viral particles was detected only in homologous trans-complementation (S6.1/E1E2:2a + JFHΔE1E2).

Probably, two main steps of viral particle production have been affected in the heterologous trans-complementation experiments, such as viral assembly or/and particles budding and release from the host cell.

An inefficient viral assembly might have been due to a genetic incompatibility between GT 1a and GT 1b structural proteins and GT 2a non structural proteins. HCV in fact is an envelope virus, and its maturation likely requires the interaction between its core and the envelope proteins. Previous studies demonstrated that the capsid protein can interact both with itself (Lo and Ou, 1998; Matsumoto *et al*., 1996) and with E1 and E2 (Baumert *et al*., 1998; Lo *et al*., 1996), but in particular it has been shown that are the C-terminal sequences of both core and E1 proteins that are important for their interaction (Lo *et al.*; 1996). The two hydrophobic domains of E1, H1 and H2, spanning from aa 261 to 291 and from aa 329 to 383, would have two important functions: binding the capsid protein, probably through an interaction between H1 and the hydrophobic domain of core (Ma *et al*., 2002), representing its ER signal sequence, and anchoring the envelope protein E1 to the ER membranes, through a retention signal sequence (Cocquerel *et al.*, 1999). Moreover the correct folding of the E1 glycoprotein depends on the presence of the E2 protein, so it is possible to speculate that during the intracellular assembly of HCV particles, E2 might help the correct folding of E1 by interacting with H2 domain and subsequently facilitating the H1 domain to interact with the capsid protein, so E2 would indirectly interacts with the core protein through its interaction with E1. However the H1 domain of the HCV E1 is not the most conserved region of this protein (Bukh *et al*., 1993) nor is the hydrophobic domain of core the most conserved region of the capsid protein (Bukh *et al*., 1994), and this may imply that when the envelope protein E1 and the capsid protein core belong to different genotypes there might be an inefficient interaction between their relative hydrophobic stretches that do not permit a correct assembly of viral particles. In support of this thesis is the fact that in homologous complementation S6.1/E1E2:2a + JFHΔE1E2 we were able to recover infectious viral particles from cell supernatants, demonstrating that

the interaction among the envelope proteins and the core protein belonging to the genotype 2a (JFH-1) is possible even when their genes are provided *in trans*.

On the other hand, viral particles could have assembled inside the cell in our experiments of heterologous trans-complementation but they could have found difficulties in budding from the intracellular membranes and in being released in the extracellular fluids.

On this regard, very recent studies demonstrated the essential function that the little p7 protein have for efficient assembly and release of infectious virions across divergent HCV virus strains, and demonstrated that p7 promotes virus particle production in a genotype-specific manner most likely due to interaction with other viral factors (Jones *et al*., 2007; Steinmann *et al*., 2007). In experiment with different HCV chimeras Jones and co-workers demonstrated that the interchange of the solely p7 protein profoundly altered the efficiency of viral production while it did not affect viral RNA replication. In particular intergenotypic transfer of p7 of the isolate J6CF (GT 2a) to the chimera Con1/C3 (E1E2 1b) (Pietschmann *et al*., 2006) suppressed viral production, probably for a genetic incompatibility between GT 2a p7 and GT 1b structural proteins. On this basis, we can hypotize that in the case of heterologous trans-complementation, genetic incompatibility between JFH-1 backbone and the structural proteins of GT 1a and GT 1b and potential changes in what is the processing of the E2-p7 junction in wt compared to what could it be when the two proteins are provided *in trans* could have totally or partially affected the production of viral particles in our system. Obviously, these are only speculative hypothesis that need to be verified by characterization studies of p7 in new homologous and heterologous experiments of trans-complementation, determining also the relationship that this protein might have with other viral factors.

§ 5.2 DISCUSSION

Characterization of early events that follow HCV binding to target cells

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Initialization of infection by HCV virus is determined by its attachment to specific receptors present on the cellular surface of human hepatocytes. Current evidence suggest that at least three host cell molecules are important for HCV entry *in vitro*: the tetraspanin CD81 (Pileri *et al*., 1998; Zhang *et al*., 2004; Bertaux and Dragic, 2006), the Scavenger Receptor Class B type I (SR-BI) (Scarselli *et al*., 2002; Grove *et al*., 2007; Kapadia *et al*., 2007) and the recently identified Claudin-1 (CLDN-1) (Evans *et al*., 2007). In hepatocytes as well as in most tissues CD81 and SR-BI have a diffuse distribution on the whole cellular surface, whilst CLDN-1, being a structural component of the cell tight junctions (TJs), is strictly localized in areas of cell-cell contact and is particularly highly expressed in liver cells.

The relative localization of these main HCV receptors lead to hypotize an interaction among the three during the HCV entry process that might involve a lateral migration of the virus-receptor complex to the tight junctions, where virions come in contact with CLND-1 before being internalized.

The external stimulation of human hepatoma cells with ligands that mimic HCV binding to target cells revealed that the receptor CD81 not only is involved in the HCV binding process but most likely plays a key role in the early steps of viral entry into the host cell. According to what was recently observed for Coxsackievirus by Coyne and Bergelson (2006), ligand attachment to the main HCV receptor CD81 provokes its lateral migration on the cellular surface, mostly dependent on actin rearrangements that permit ligand-receptor complex movement to the tight junctions. The key role that actin filaments have on the entry process of certain envelope viruses in polarized cells (Gottlieb *et al*., 1993) as well as their involvement in cellular uptake of Adenovirus particles (Patterson and Russel, 1983) have longly been demonstrated, but none of these studies have been done for HCV so far. The use of chemical inhibitors of actin polymerization, such as Latrunculin A and

Jasplakinolide, proved the involvement of the actin cytoskeleton in CD81 translocation process since no relocalization of CD81 in the areas of cell-cell contact was detected in treated cells. Time course experiments with LatA demonstrated that an intact and functional cytoskeleton is required at a very early stage in the relocalization process, beside having also a relevant physiological importance on viral infection.

In the present study we also demonstrates that the Rho GTPases Rac, Rho and Cdc42 are involved in the translocation process of CD81 to the areas of cell-cell contact as well as in HCVcc internalization. According to what has been previously observed in different studies these Rho GTPases are key regulatory molecules that link surface receptors to the organization of the actin cytoskeleton (Ridley and Hall, 1992; Ridley *et al*., 1992; Nobes and Hall, 1995) and are also involved in receptor-mediated endocytosis regulating clathrin-coated vesicles formation (Lamaze *et al*., 1996). Engagement of CD81 led to the activation of Rho, Rac and Cdc42 as we demonstrated in affinity precipitation experiments and when human hepatoma cells were treated with inhibitors specific for these Rho GTPases or were silenced for their expression both the CD81 translocation to the cellular tight junctions and HCVcc infectivity were reduced.

To fully understand the important role that this mechanism of virus-receptor translocation might have on HCV entry in host cells it is necessary to consider the peculiar organization of the hepatic parenchyma. The architecture of this tissue in fact is unique when compared to other epithelia, and it is composed of multiple and interconnected cords of hepatocytes, in which each hepatocyte is attach to its neighbors in a two-dimensional sheet. On either side of the cord each hepatocyte communicates with sinusoids, where the bloodstream flows. On contrary of a normal polarized epithelial cell, that sits on its basal surface and has an apical surface exposed to the external space, the hepatocyte presents three distinct membrane domains: sinusoidal (basal), lateral and canalicular (apical). Lateral plasma membranes fuse alongside bile canaliculi to form tight junctions that occlude the apical domain from the basolateral surface. The CD81 mediated lateral migration of bound virus could explain how HCV present in human blood can be relocalized from the basolateral surface of polarized hepatocytes to the areas of cell-cell contact, where tight junctions and Claudin-1 are. Internalization would then occur by clathrin- mediated endocytosis

(Blanchard *et al*., 2006), through endosomal, low pH compartments (Koutsoudakis *et al*., 2006; Tscherne *et al*., 2006).

We need to consider that in the present study the complex structure of the hepatic parenchyma has been mimic by the use oh human hepatoma cells (Huh7) that were grown to create a uniform monolayer before they could be used in our experiments. While isolated Huh7 cells are relatively non polarized, if they are grown on lysine-coated plates, the contact between each other trigger canalicular differentiation and the acquisition of membrane polarity (Lian *et al.*, 1999). Under this conditions in fact we were able to observe the appearance of tight junction-like structures, where the TJ components Occludin, ZO-1, and Claudin-1 preferentially accumulated, and where the receptor CD81 relocalized after stimulation.

In conclusion, our approach allowed to elucidate the events that follows the interaction between the HCV receptors CD81 and its ligands and helped to better understand the mechanism of HCV infection.

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