HEPATITIS C: MOLECULAR VIROLOGY AND ANTIVIRAL TARGETS

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Hepatitis C virus (HCV) is now the major cause of chronic hepatitis, liver cirrhosis and hepatocellular carcinoma (HCC) in the western world. In addition, end-stage liver disease due to chronic hepatitis C is the leading indication to liver transplantation. A protective vaccine is not available yet and therapeutic modalities are still limited. As a consequence, the number of patients presenting with long-term sequelae of chronic hepatitis C, including HCC, is expected to further increase for the next two decades even if the incidence of new cases has diminished since the introduction of anti-HCV screening of blood and blood products in 1990 [1]. Given this scenario, there is an urgent need to develop more effective and better tolerated therapies for chronic hepatitis C. A detailed understanding of the viral life cycle underpins these efforts.

HEPATITIS C

Epidemiology

It is estimated that 120–180 million people worldwide are infected with HCV [2], which corresponds to roughly 4 times the number of individuals infected with HIV and about half the number of persons infected with the hepatitis B virus (HBV). The seroprevalence rate is 1–2% in western Europe and North America, 3–4% in some Mediterranean and Asian countries and up to 20% in Egypt and parts of Central Africa. HCV is parenterally transmitted. With the introduction of anti-HCV screening, new cases of posttransfusion hepatitis C have virtually disappeared. Indeed, over the last 20 years the risk of posttransfusion hepatitis C could be reduced from about 1 per 100 blood units transfused to 1 per 2,000,000–10,000,000 [3]. Unfortunately, the lack of systematic screening of blood donors continues to result in HCV transmission in countries with developing or transitional economies. In these

countries, large-scale immunization and parenteral therapy programs (e.g., for the treatment of schistosomiasis in Egypt or leishmaniosis in India) as well as surgical and dental procedures with inadequately sterilized equipment have also been important routes of transmission. In the western world, intravenous drug use is now the major identifiable mode of HCV transmission. In addition, HCV transmission has been described in the nosocomial setting and as a consequence of occupational exposure. Sexual transmission is rare. The risk of perinatal transmission is probably less than 5% unless the mother is co-infected with HIV. Intriguingly, in clinical practice no epidemiologic risk factor can be identified in up to one third of patients with hepatitis C («sporadic hepatitis C»).

Natural history

After an incubation period of 3–12 weeks HCV infection is usually followed by a clinically inapparent hepatitis [4]. Only about 25% of patients are symptomatic. Fulminant hepatitis is very uncommon. One of the most important clinical features of hepatitis C is its progression to chronicity in 50–80% (Fig. 1). Typically, patients with chronic hepatitis C have few if any symptoms and these are usually nonspecific, intermittent, and mild.
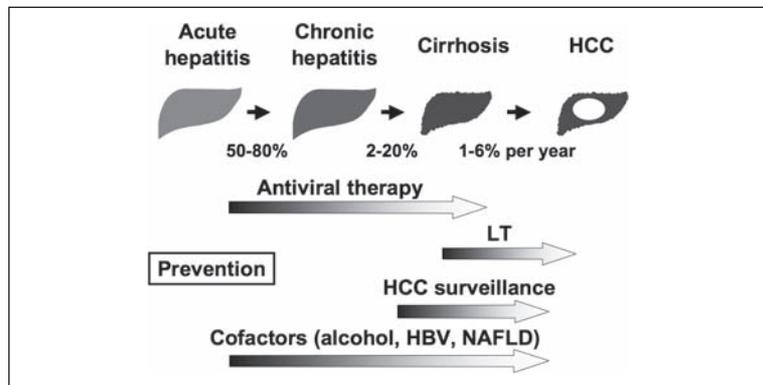

Figure 1
Natural history and management of hepatitis C. HBV, hepatitis B virus; HCC, hepatocellular carcinoma; LT, liver transplantation; NAFLD, nonalcoholic fatty liver disease.

The natural history of chronic hepatitis C has been analyzed in several retro- and prospective studies. Overall, 2–20% of patients with chronic hepatitis C will develop liver cirrhosis within 20 years. Factors associated with more frequent and rapid progression to cirrhosis are higher age at the time of infection, male sex, alcohol consumption, coinfections with HBV or HIV, nonalcoholic fatty liver disease (NAFLD), hepatic iron overload, smoking and immunosuppression. Comprehensive management of chronic hepatitis C takes these factors into account and aims at eliminating or improving the ones that can be modified. In this context, patients with chronic hepatitis C should avoid alcohol consumption and smoking, should be vaccinated against hepatitis A and B, and should receive counseling for overweight.

Once cirrhosis is established, the rate of HCC development is 1–6% per year. Indeed, HCV infection is responsible for a substantial proportion of the increase in HCC incidence and mortality recently observed in most western countries.

Diagnosis
Diagnosis of hepatitis C is based on serological assays which detect HCV-specific antibodies (anti-HCV) and on molecular assays which detect HCV RNA. Current enzyme immunoassays (EIAs) are highly sensitive as well as specific and represent the primary diagnostic tool. HCV RNA detection by real-time RT-PCR is now standardized, reliable and reproducible, and offers a broad dynamic quantitation range. HCV becomes positive by RT-PCR as early as 1–2 weeks after infection and 4–6 weeks before anti-HCV seroconversion. HCV RNA testing is used to confirm active infection in anti-HCV-positive individuals and to diagnose acute hepatitis C or chronic hepatitis C in the rare immunocompromised patients that do not develop anti-HCV antibodies. However, the principal role of HCV RNA testing is in the tailoring and monitoring of antiviral therapy. Determination of HCV genotype is important for the selection of the optimal antiviral regimen.

Liver biopsy allows to determine the necro-inflammatory activity (grading) and the degree of fibrosis (staging) as well as to recognize or exclude coexisting liver pathology (such as alcoholic liver disease, iron

18

overload or NAFLD). Non-invasive tests are currently being explored to predict liver fibrosis. These are based on different combinations of blood tests, transient elastography or magnetic resonance imaging [5]. While promising, current prediction methods remain limited with respect to the differentiation of intermediate fibrosis stages and may be able to replace liver biopsy only in selected patients. Moreover, they will miss the opportunity of molecular profiling as a novel means to predict treatment outcome [6, 7].

Current therapy
Current standard therapy of chronic hepatitis C consists of pegylated interferon-α (PEG-IFN-α), administered once weekly by subcutaneous injection, combined with ribavirin, which is taken orally on a daily basis [8]. Both drugs operate through incompletely understood, likely direct antiviral and immunomodulatory mechanisms. There are a number of contraindications, and adverse effects, sometimes serious, are frequent. Standard treatment duration is 48 weeks for HCV genotype 1 and 24 weeks for genotypes 2 and 3. With this treatment, 40–50% of genotype 1- and about 80% of genotype 2- and 3-infected patients achieve a sustained virological response (SVR). Current efforts are aimed at tailoring doses and treatment duration to the individual patient based on baseline parameters (e.g., genotype, viremia, fibrosis stage) and on-treatment viral kinetics (viremia at 4, 12 and 24 weeks). Hence, therapy may be abbreviated in selected patients with favorable baseline parameters and a rapid virological response (i.e., negative HCV RNA after 4 weeks of treatment) while others may benefit from prolonged treatment.

Comprehensive management of hepatitis C includes, besides antiviral therapy, efforts to prevent the infection, HCC surveillance in patients with cirrhosis, liver transplantation for selected patients with end-stage liver disease, and, as pointed out above, the elimination or improvement of cofactors of disease progression (Fig. 1).

Liver transplantation for end-stage chronic hepatitis C is inevitably followed by recurrent infection of the graft. Unfortunately, two thirds of patients will develop recurrent hepatitis C and one third will rapidly

develop graft cirrhosis. The scarcity of cadaveric donor organs represents an immense problem. Living donor liver transplantation is a possible alternative. However, this necessitates careful consideration of the potential risks for the donor and of complex ethical issues and, therefore, will remain reserved for a limited proportion of patients.

Overall, about 50% of patients with chronic hepatitis C can be cured with the current treatment. For these patients, ongoing efforts are aimed at tailoring treatment to the individual needs in order to improve tolerability. For the other patients and for the important proportion of patients who cannot tolerate current treatment, there is an urgent need to develop more effective and better tolerated therapies. Advances in the molecular virology and pathogenesis of hepatitis C form the basis for these efforts.

THE HEPATITIS C VIRUS AND ITS LIFE CYCLE

HCV was identified in 1989 by immunoscreening of an expression library with serum from a patient with post-transfusion non-A, non-B hepatitis [9]. However, the virus was not visualized conclusively, the low titres in serum and liver tissue precluded biochemical characterization of native viral products, and, most importantly, it was not possible to culture HCV efficiently *in vitro*, impeding elucidation of the viral life cycle as well as the development of specific antiviral agents and preventive vaccines. Despite these obstacles, great progress has been made in the study of HCV over the past almost 20 years using heterologous expression systems, functional cDNA clones that are infectious *in vivo* in chimpanzees, pseudoparticles that enable the study of viral entry and, most recently, complete cell culture systems (reviewed in [10]). These and other milestones in HCV research are listed in Table 1. With these advances, the entire life cycle of HCV can now be studied under reproducible conditions in cell culture.

Table 1 Milestones in HCV research

1975	Description of non-A, non-B hepatitis
1989	Identification of HCV
1993	Delineation of HCV genome organization and polyprotein processing
1996	First three-dimensional structure of an HCV protein (NS3 serine protease)
1997	First infectious clone of HCV constructed
1998	Interferon-α and ribavirin combination therapy
1999	Replicon system established
2003	Functional HCV pseudoparticles described
2003	Proof-of-concept clinical studies of an HCV protease inhibitor
2005	Production of recombinant infectious HCV in cell culture

HCV is classified in the *Hepacivirus* genus within the *Flaviviridae* family. It contains a 9.6-kb positive-strand RNA genome composed of a 5' noncoding region (5' NCR) which includes an internal ribosome entry site (IRES), a long open reading frame encoding a polyprotein precursor of about 3,000 amino acids, and a 3' NCR. The polyprotein precursor is co- and posttranslationally processed by cellular and viral proteases into the mature structural and nonstructural proteins. The structural proteins include the core protein, which forms the viral nucleocapsid, and the envelope glycoproteins E1 and E2. These are released from the polyprotein precursor by the signal peptidase and the signal peptide peptidase of the endoplasmic reticulum (ER). The nonstructural proteins include the p7 ion channel, the NS2-3 and NS3-4A proteases, which are essential for polyprotein processing in the nonstructural region, an RNA helicase located in the C-terminal two-thirds of NS3, the NS4B and NS5A proteins, and the NS5B RNA-dependent RNA polymerase (RdRp). NS4B induces a specific membrane alteration that serves as a scaffold for the HCV replication complex (see below). NS5A is an RNA-binding zinc phosphoprotein that plays a role in regulating HCV RNA replication vs. translation vs. packaging, but its precise function remains to be established. The viral enzymes are essential for viral replication and have emerged as major targets for the design of novel antiviral agents [11] (reviewed in [12]).

HCV infection is a highly dynamic process with a viral half-life of only a few hours and production and clearance of an estimated 10^{12} virions per day in a given individual [13]. This high replicative activity, together with the lack of a proof-reading function of the viral RdRp, is the basis of the high genetic variability of HCV. These properties are similar to those of HIV infection and provide a strong rationale for the development and implementation of antiviral combination therapies.

HCV isolates can be classified into 6 major genotypes and numerous subtypes [14]. As stated above, patients infected with genotype 1 do not respond as well to IFN-α-based therapy as those infected with genotype 2 or 3. The term quasispecies refers to the genetic heterogeneity of the population of HCV genomes coexisting in an infected individual.

Virion structure
While exciting progress has been made towards determining virion structures of related viruses, e.g., dengue virus [15], HCV has not been definitively visualized and its structure remains to be elucidated. Based on filtration and electron microscopic studies, HCV particles are 40–70 nm in diameter ([16] and references therein). E1 and E2 are presumably anchored to a host cell-derived double-layer lipid envelope that surrounds a nucleocapsid composed of multiple copies of the core protein and the genomic RNA. HCV circulates in various forms in the infected host and can be associated with low-density (LDL) and very-low-density lipoproteins (VLDL), which appears to represent the infectious fraction, virions bound to immunoglobulins, and free virions. This feature may explain its unusually heterogenous and low buoyant density (peak infectivity near 1.10 g/ml).

Viral entry
The HCV life cycle begins with binding to the cell surface and internalization, as schematically illustrated in Figure 2. Hepatocytes are the main target cells but infection of B cells, dendritic cells, and other cell types has also been reported. CD81, a tetraspanin protein found on the surface of many cell types including hepatocytes, the low density lipoprotein receptor (LDLR), scavenger receptor class B type I (SR-BI), and, most recently, claudin-1 [17] have, among others, been proposed
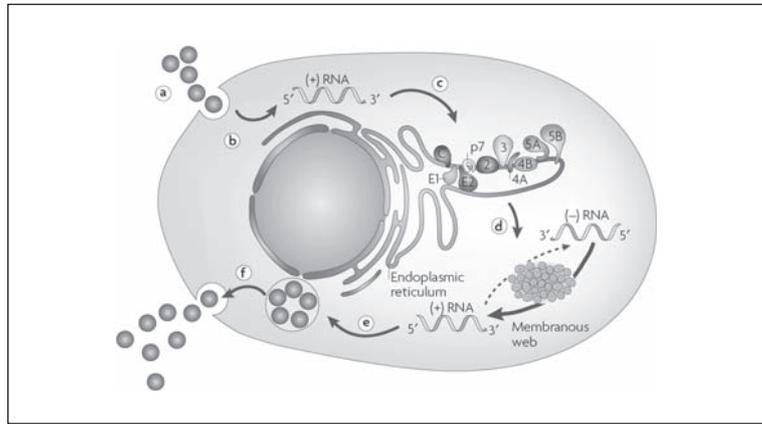
22

Figure 2
Life cycle of HCV. 1) Virus binding and internalization, 2) cytoplasmic release and un-coating, 3) internal ribosome entry site (IRES)-mediated translation and polyprotein processing, 4) RNA replication, 5) packaging and assembly, 6) virion maturation and release. The topology of HCV structural and nonstructural proteins at the endoplasmic reti-culum (ER) membrane is shown schematically. HCV RNA replication occurs in a specific membrane alteration, the membranous web. Note that IRES-mediated translation and poly-protein processing as well as membranous web formation and RNA replication, illustrated here as separate steps for simplicity, may occur in a tightly coupled fashion (from ref. [10]).

as HCV receptors or components of a receptor complex (reviewed in [10]). CD81, SR-BI and claudin-1 have been found to be required for HCV entry. However, certain cell types were found to be nonpermissive despite expression of CD81, SR-BI and claudin-1, indicating that one or more additional HCV entry factor(s) remain to be discovered.

HCV enters *via* clathrin-mediated endocytosis, with transit through an endosomal, low pH compartment and presumed endosomal membrane fusion. The structural basis for low pH-induced membrane fusion has been elucidated for related viruses [18, 19]. The envelope proteins of these viruses have an internal fusion peptide that is exposed during low pH-mediated domain rearrangement and trimerization of the protein. The scaffolds of these so called class II fusion proteins are remarkably similar, suggesting that entry of all viruses in the *Flaviviridae* family,

including HCV, may include a class II fusion step. However, the mechanisms involved in activating HCV for low pH-induced fusion, the fusion step, and the identity of the fusion peptide(s) have not yet been characterized.

Formation of a membrane-associated replication complex
Once liberated into the cytosol, the viral genome is translated by an IRES-mediated mechanism, yielding a polyprotein precursor that is co- and posttranslationally processed by cellular and viral proteases. A characteristic feature of HCV proteins is their association with intracellular membranes. Indeed, each HCV protein contains a specific determinant for membrane association. These hydrophobic segments have been difficult to study and have commonly been deleted in recombinant proteins used for biochemical and structural studies. A primary interest in our laboratory, therefore, was to identify and characterize the determinants for membrane association of the HCV nonstructural proteins 3-5B, which are required for RNA replication (reviewed in [10]). Figure 3 illustrates available three-dimensional structures and our current understanding of the membrane association of HCV proteins.
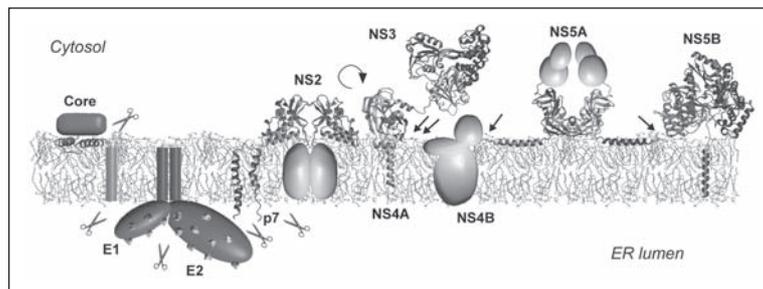

Figure 3
Structure and membrane association of HCV proteins. Scissors indicate cleavages by the endoplasmic reticulum (ER) signal peptidase, except on the cytosolic side where it indicates processing of core by signal peptide peptidase. The cyclic arrow denotes cleavage by the NS2-3 protease. Black arrows indicate processing by the NS3-4A protease complex. Known protein structures are shown as ribbon diagrams. The structures and the membrane bilayer are shown at the same scale. Proteins or protein segments of unresolved structure are represented as colored spheres or cylinders with their approximate sizes. See ref. [10] for details.

24

As an example, we have recently explored the mechanism of membrane association of the HCV NS3-4A complex [20]. NS3-4A is a multifunctional protein harboring serine protease and RNA helicase activities. It is an essential component of the HCV replication complex and a prime target for antiviral intervention. We were able to show through biochemical assays, site directed mutagenesis, HCV RNA replication assays and circular dichroism as well as nuclear magnetic resonance structural analyses that membrane association and structural organization of HCV NS3-4A are ensured in a cooperative manner by two membrane binding determinants. We demonstrated that the N-terminal 21 amino acids of NS4A form a transmembrane α-helix that is likely involved in intramembrane protein-protein interactions essential for the assembly of a functional replication complex. In addition, we demonstrated that amphipathic helix α_0, formed by NS3 residues 12–23, serves as a second essential determinant for membrane association of NS3-4A, allowing proper positioning of the serine protease active site on the membrane. These results allowed us to propose a dynamic model for the membrane association and structural organization of NS3-4A on the membrane (Figure 4).

An important consequence of our model relates to the proteolytic targeting of host factors whereby the strict positioning of the protease active site with respect to the membrane confers a high degree of selectivity to potential cellular trans-cleavage substrates. In this context, it has recently been shown that the NS3-4A protease cleaves and thereby inactivates two crucial adaptor proteins in innate immune sensing, namely Trif [21] and Cardif [22] (also known as MAVS, IPS-1 and VISA), thereby blocking interferon production. Cardif cleavage by NS3-4A was observed in various experimental systems and in the liver from patients with hepatitis C ([23] and our unpublished data). Indeed, the Cardif cleavage site is located close to the membrane surface and fits well with our structural model of the membrane-associated trans-cleavage conformation of NS3-4A (Fig. 4, Step 5).

Formation of a membrane-associated replication complex, composed of viral proteins, replicating RNA, altered cellular membranes, and additional host components, is a hallmark of all positive-strand RNA viruses
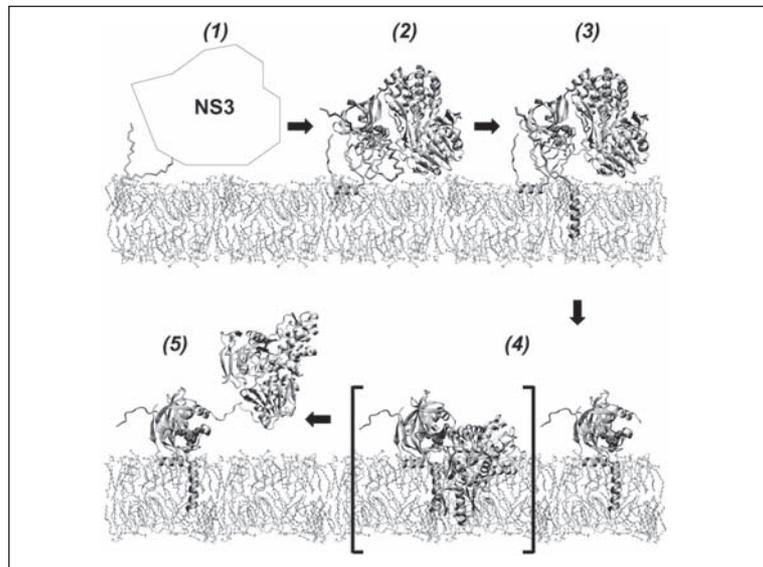
Figure 4
Dynamic model for the membrane association and structural organization of NS3-4A on the membrane. In the HCV polyprotein context, translation of NS3 occurs at the membrane, as the preceeding NS2 protein is associated with membranes through its N-terminal domain (Step 1). Induced folding of amphipathic helix α_0 due to its interaction with the membrane interface is presumably a cotranslational event, followed by folding of the NS3 serine protease and helicase domains (Step 2). A low affinity interaction of the central segment of NS4A with NS3 serine protease β-strands A_1 and A_0 is likely at this point, keeping the central segment of NS4A close to the serine protease domain before cleavage. Note that at this stage the in-plane membrane association of helix α_0 does not impose constraints on the positioning of NS3. Hence, a forward movement of NS3 would bring the hydrophobic N-terminal segment of NS4A into close contact with the membrane, thereby facilitating its posttranslational insertion into the membrane after processing at the NS3/NS4A site (Step 3). Final incorporation of the central segment of NS4A into the N-terminal β-barrel stabilizes the interaction of helix α_0 with the NS3 serine protease. This complete folding as well as membrane association by amphipathic helix α_0 and the transmembrane segment of NS4A lock the serine protease in a strictly defined position onto the membrane (Step 4). As shown in the brackets, the hydrophilic helicase domain would be immersed into the membrane at this stage in the known NS3-4A cis-cleavage conformation. Hence, the helicase domain has to move away from the serine protease domain in the final membrane-associated stage through a rotation of the linker segment connecting the two domains (Step 5). See [20] for details.
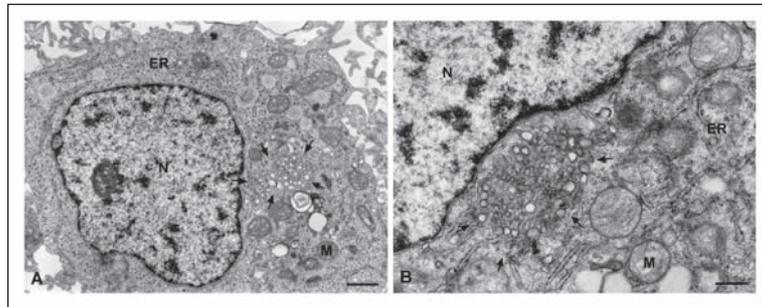
26

Figure 5
HCV replication complex. (A) Low-power overview of a Huh-7 cell harboring a sub-
genomic HCV replicon. A distinct membrane alteration, designated as membranous web
(arrows), is found in the juxtanuclear region. Note the circumscript nature of this speci-
fic membrane alteration and the otherwise unaltered cellular organelles. Bar, 1 μm. (B)
Higher magnification of a membranous web (arrows) composed of small vesicles embed-
ded in a membrane matrix. Note the close association of the membranous web with the
rough endoplasmic reticulum. Bar, 500 nm. The membranous web harbors all HCV non-
structural proteins and nascent viral RNA in Huh-7 cells harboring subgenomic replicons
[24], and therefore represents the subcellular site of HCV RNA replication. N, nucleus;
ER, endoplasmic reticulum; M, mitochondria.

investigated thus far. Depending on the virus, replication may occur on altered membranes derived from the ER, Golgi apparatus, mitochondria or even lysosomes. The role of membranes in viral RNA synthesis is not well understood. It may include (i) the physical support and organization of the RNA replication complex, (ii) the compartimentalization and local concentration of viral products, (iii) tethering of the viral RNA during unwinding, (iv) provision of lipid constituents important for replication, and (v) protection of the viral RNA from double-strand RNA-mediated host defenses or RNA interference.

A specific membrane alteration, designated as membranous web, was identified as the site of RNA replication in Huh-7 cells containing subgenomic HCV replicons [24] (Fig. 5). Formation of the membranous web was induced by NS4B alone and was very similar to the "sponge-like inclusions" previously found by electron microscopy in the liver of HCV-infected chimpanzees [25]. The membranous web is likely derived

from ER membranes. Ongoing studies are aimed at characterizing the host factors and cellular processes involved in formation of the HCV replication complex. In this context, we have developed a system that allows to visualize functional replication complexes in living cells [26, 27].

Recent studies demonstrate a complex interaction between HCV RNA replication and the cellular lipid metabolism, presumably *via* the trafficking and association of viral and host proteins with intracellular membranes. Such observations suggest that pharmacologic manipulation of lipid metabolism may have therapeutic potential in hepatitis C. Additional host factors, including cyclophilins, have been found to be involved in HCV RNA replication, opening new angles for therapeutic intervention.

Packaging, assembly and release
Little is known about the late steps of the viral life cycle, as these have only recently become amenable to systematic study. Interestingly, the nonstructural proteins p7, NS2 and NS5A as well yet to be defined RNA structures are involved in these processes (reviewed in [28]). Exciting new findings indicate an important role for lipid droplets and the VLDL secretory pathway in HCV assembly and release [29, 30]. Virions persumably form by budding into the ER or an ER-derived compartment and leave the cell through the secretory pathway.

IMPLICATIONS FOR THE DEVELOPMENT OF NEW THERAPEUTIC STRATEGIES

In principle, each step of the HCV life cycle illustrated in Figure 2 represents a target for antiviral intervention [12]. Specific inhibitors of the biochemically and structurally well-characterized NS3-4A serine protease and NS5B RdRp are currently being developed as antiviral agents, and the first candidates have already been evaluated in clinical trials. Serine protease inhibitors seem particularly promising, as they not only block viral polyprotein processing but may also reverse the inhibition of innate immune sensing by HCV (see above). In addition,

new targets have been uncovered by the recent studies highlighted above, including, among others, the HCV 5' NCR, viral entry and fusion, the p7 ion channel, the NS2-3 protease and NS5A. Moreover, drugs affecting host factors involved in HCV replication are being explored as antiviral agents. Already at this early stage it is evident that the genetic variability of HCV, allowing the rapid development of anti-viral resistance, represents a major challenge to the clinical develop-ment of specific inhibitors and that, in common with HIV infection, combination therapy will be necessary for therapeutic success.

CONCLUSIONS AND PERSPECTIVES

The development of powerful model systems enables dissection of the HCV life cycle. Much work remains to be done with respect to the early and late steps, the virion assembly and structure, the mechanism and regulation of RNA replication, and the pathogenesis of HCV-induced liver disease. Ultimately, these efforts should result in innovative thera-peutic and preventive strategies for one of the most common causes of chronic hepatitis, liver cirrhosis, and HCC worldwide.

ACKNOWLEDGMENTS

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