

Network: Infection and Inflammation: from Pathogen-induced Signatures to Therapeutic Target Genes

Project: Identification of Host Cell Factors Involved in Hepatitis C Virus Replication as Potential Targets for HCV and Flavivirus Therapy

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Introduction

An estimated 170 million people are infected worldwide with Hepatitis C Virus (HCV), leading in many cases to severe liver damage. The only available therapy involves interferon in combination with ribavirin, resulting in about 50% of patients showing sustained virological response. Several specific drugs targeting viral enzymes are in development but it is not clear, how efficient the virus will be eliminated by these drugs and it is likely that drug resistance will be a problem as in case of HIV. The aim of our studies is the identification and evaluation of host cell factors involved in HCV replication providing attractive targets for an alternative anti HCV therapy.

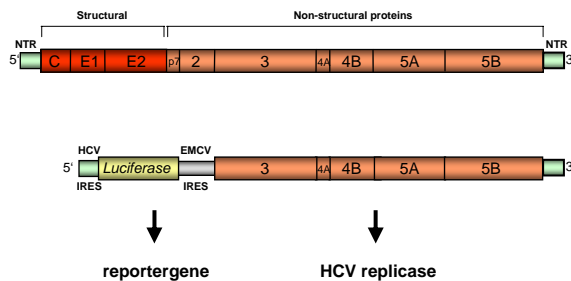


Fig 1: Schematic structure of the HCV genome and a subgenomic replicon encoding firefly luciferase instead of the structural proteins. The structural proteins Core (C), E1 and E2 are components of virus particles, the nonstructural (NS) proteins have diverse functions in viral replication, particle production and infection. NS3, 4A, 4B, 5A, 5B and the nontranslated regions (NTR) are necessary and sufficient for intracellular RNA replication. IRES, internal ribosome entry site.

HCV is a positive strand RNA virus belonging to the hepacivirus group of the Flaviviridae. Studies on HCV have been hampered for many years by the lack of efficient and convenient cell culture systems and animal models. The development of subgenomic replicons in our lab allowed for the first time a detailed study of HCV RNA replication in cell culture¹. The original system was based on HCV genomes lacking the structural proteins but harboring the gene encoding neomycin phosphotransferase, conferring resistance to G418. Upon transfection of in vitro transcripts of subgenomic replicons into the human hepatoma cell line Huh-7 and selection with G418, cell clones were generated showing a high level of autonomous, persistent HCV replication. Two major determinants accounted for the efficient replication in G418 selected Huh-7 replicon cell clones compared to other cell culture systems: (i) The original HCV isolate, which was used to generate the replicons, replicated poorly in Huh-7 cells and had to acquire adaptive mutations enhancing replication efficiency to a level that allowed the host cell to survive G418 selection^{2,3}. (ii) Only a minor subpopulation of the Huh-7 cells supported persistent high level HCV replication being robust enough to permit cell growth under the applied selective pressure⁴. Based on these results we were able to setup a transient HCV replication assay with subgenomic replicons encoding the firefly luciferase gene (Fig. 1), which enabled us to further characterize and quantify the permissiveness of different Huh-7 populations. We found that various Huh-7

passages, all generated in our laboratory from the same original population differed up to 100fold in their ability to support HCV replication in our transient assay⁴. We also tested replicon cells that were “cured” from HCV by interferon-treatment or by application of HCV specific drugs, since we assumed that those cells might have been selected for efficient HCV replication and found some of them showing an extremely high permissiveness. Variations in translation efficiency and RNA stability did not account for these different phenotypes, therefore it seems likely that fluctuating concentrations of cellular factors required for or inhibiting HCV replication might influence permissiveness. The correlation of permissiveness with the expression profile of cellular genes and proteins by genomic and proteomic screening might be the clue to identify relevant host cell factors involved in HCV replication. Those candidate proteins correlating best with permissiveness for HCV replication in a panel of Huh-7 passages will be analyzed for their effects on HCV replication by silencing and overexpression in permissive and nonpermissive cells.

Results/Project Status

We followed two strategies to identify host factors involved in HCV replication: comparative genomic and proteomic analysis of permissive and nonpermissive Huh-7 cells and the biochemical purification of HCV replication complexes. Furthermore we addressed factors that have been described to be critical for HCV replication but would not be apparent in our analyses because the underlying mechanisms would not lead to a detectable differential gene expression. Control of the IRF3 pathway of the host cells innate immune response has been shown to play an important role for HCV replication and defects in this pathway have been attributed to be critical for permissiveness of Huh- cells⁵, however, we did not find any correlation between the strength of IRF3 activation and permissiveness of our cell populations. Therefore we further focused on the identification of candidate cDNAs/proteins that correlate in their expression level with permissiveness for HCV replication.

Genomic and proteomic analysis of permissive and nonpermissive Huh-7 cells

As a first step we carefully characterized a number of naive Huh-7 passages and clones for their permissiveness and selected the panel shown in Fig 2. We were able to identify a clone with outstanding ability to replicate HCV RNA, designated Huh7-Lunet, and received by chance a variant of this clone, Huh7-Lunet NT, which was almost nonpermissive for HCV replication. Since both of these cell populations refer to the same cell clone, we assumed that a comparative analysis of this pair should be an ideal tool to identify host factors that are associated with permissiveness. We also included two nonpermissive passages of naive Huh-7 cells and several cell populations with intermediate phenotypes.

The genomic analysis relied on affymetrix genechip arrays and included two sets of experiments: In one set we focused on an extensive analysis of two extreme phenotypes, Lunet and p28 (Fig 2, arrows), using HCV transfected and non-transfected cell in replicates to screen for factors upregulated in Lunet cells or induced by expression of HCV proteins with statistical means. In a second set of experiments we analysed the whole panel of cell populations shown in Fig. 2 to evaluate the data from the first experiment. The overall

gene expression profiles were very similar between the different cell populations. By combination of the data of both experiments we ended up with five candidate cDNAs that seemed to be positively associated with permissiveness and two candidates that were induced by HCV in nonpermissive cells. We were able to evaluate these candidates by quantitative PCR and are currently generating Huh-7 based cell lines by retrovirally transduced sh-RNAs to knock down the expression of the relevant proteins.

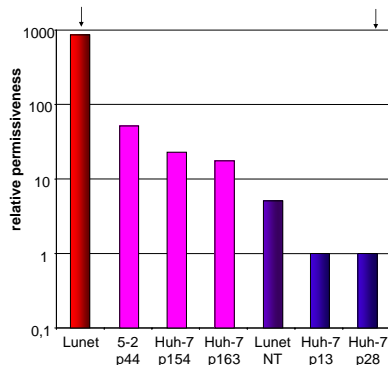


Fig 2: Panel of Huh-7 cell passages and clones that were subjected to genomic and proteomic analysis. Permissiveness of each cell population is given in fold relative to nonpermissive cells (Huh-7 p13 and p28). Lunet and Clone 5-2 represent highly permissive Huh-7 clones derived from replicon cell clones “cured” from HCV by Interferon treatment. Lunet NT is a derivative of the Lunet-cells that has lost by chance the permissive phenotype.

The proteomic analysis focused on the analysis of the complete proteome of Huh7-Lunet cells in comparison to either nonpermissive naive cells or to Lunet NT. In all settings, the proteome was very similar and we obtained a well reproducible pattern of spots. We also found a small number of differential spots in each individual experiment, however up to know we were not able to identify candidates reproducibly correlating with permissiveness yet. Therefore we extended the proteomic part of the project on the analysis of purified replication complexes.

Alternate Strategy: Purification of the HCV replication complex

A more direct approach to identify host factors involved in HCV replication relies on the biochemical purification of HCV replication complexes. HCV replication takes place in distinct vesicular structures designated the “membranous web”⁶. We found that these HCV replication complexes isolated from replicon cells are highly resistant to nuclease and protease treatment and analyzed the stoichiometry of HCV RNA and proteins in these structures⁷. Based on our data and on previously published EM-studies⁶ we propose a tentative model of the HCV replication complex (Fig. 3):

Multiple copies of HCV nonstructural protein complexes encompassing NS3 to NS5B build up a vesicular membrane structure, which mediates the protection against nucleases and proteases. Each vesicle should have a connection to the cytoplasm allowing the constant supply with nucleotides for RNA synthesis, but preventing the access of molecules larger than 16 kD, e.g. S7 nuclease and proteinase K. A number of these vesicles accumulate at distinct sites in the cytoplasm and form the membranous web. Within every vesicle that contains an active replicase complex we find at least one negative-strand RNA, several positive-strand RNAs and up to one thousand copies of each of the nonstructural proteins⁷. In addition, HCV replication complexes will most likely contain host factors that are therefore also protected from protease digest. We employed the protease resistance of HCV replication complexes into a purification scheme that

allows a comparative proteomic analysis of material from HCV replicon cells and naive Huh-7 cells, with a limited number of protein spots. Using this method we already identified several candidate proteins that will be further analyzed for potential roles in the HCV life-cycle.

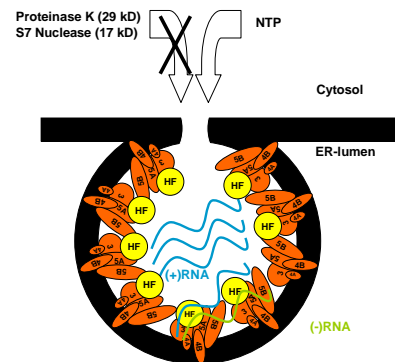


Fig 3: Tentative model of the HCV replication complex. HCV positive strand RNA is shown in blue, negative strand RNA in green, nonstructural proteins in orange and potential host factors (HF) in yellow.

Outlook

After the identification of a number of candidate proteins either by comparative genomic and proteomic analysis of permissive and nonpermissive Huh-7 cells or by purification of HCV replication complexes from replicon cells, we are currently evaluating potential functions of these proteins in viral replication by silencing and overexpression in permissive and nonpermissive cells. Proteins with a proven role in the HCV life-cycle will be further characterized for the underlying molecular mechanism and based on these results, a concept for inhibitor development will be established. Furthermore, the candidate genes will be tested for possible roles in the replication of the related flavivirus Dengue type 2 to develop broadly active antivirals against the whole group of flaviviruses.

Lit.: 1. Lohmann V et al. Replication of subgenomic hepatitis C virus RNAs in a hepatoma cell line. Science 1999; 285:110-113. 2. Lohmann V et al. Mutations in hepatitis C virus RNAs conferring cell culture adaptation. J. Virol. 2001; 75:1437-1449. 3. Krieger N et al. Enhancement of Hepatitis C Virus RNA Replication by Cell Culture- Adaptive Mutations. J. Virol. 2001; 75:4614-4624. 4. Lohmann V et al. Viral and cellular determinants of hepatitis C virus RNA replication in cell culture. J. Virol. 2003; 77:3007-3019. 5. Sumpter R. et al. Regulating intracellular antiviral defense and permissiveness to hepatitis C virus RNA replication through a cellular RNA helicase, RIG-I. J Virol. 2005 Mar;79(5):2689-99. 6. Gosert R et al. Identification of the hepatitis C virus RNA replication complex in huh-7 cells harboring subgenomic replicons. J. Virol.2003; 77:5487-5492. 7. Quinkert D et al. Quantitative Analysis of the Hepatitis C Virus Replication Complex. J.Virol, in press.