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Review

The HCV Replicase Interactome

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Abstract: Viruses are obligate parasites and can only reproduce within host cells because they lack metabolic pathways to complete their replication cycles. Host factors required in viral replication are mainly those involved in lipid metabolism, cell cycle control and apoptosis, cell-to-cell interactions, immune system regulation, etc. Several inhibitors targeting viral polymerases have been designed. However, the rapid appearance of resistant mutants, as a direct consequence of the viral population structure, diminishes the efficacy of this kind of molecules. To elude the rapid loss of treatment efficiency due to the appearance of resistance mutations, cellular factors have been proposed as a promising therapeutic target to inhibit RNA(+) virus replication. In this review, we focus on those interactions between host factors and HCV replicase, to modulate either cellular metabolism or HCV polymerase activity.

Keywords: Hepatitis C Virus, NS5B, Protein-Protein Interactions, Interactome

Introduction

Viruses are obligate parasites and can only reproduce within host cells because they lack metabolic pathways to complete their replication cycles. Host factors required in viral replication are mainly those involved in lipid metabolism, cell cycle control and apoptosis, cell-to-cell interactions, immune system regulation, etc. Viruses may infect a cell only if the cellular factors that virus needs to replicate are present in the cell (Flint *et al*., 2015; König and Stertz, 2015).

Positive strand RNA viruses (RNA(+) virus) are classified in the group IV of the Baltimore's classification of viruses. They are the greatest group of pathogenic viruses affecting human and animal health (Flint *et al*., 2015). RNA(+) include viruses from well-known families as *Coronaviridae* (Alpha Coronavirus 1, SARS-related coronavirus, MERS-related coronavirus), *Picornaviridae* (Hepatitis A virus, Human Rhinovirus, Enterovirus including poliovirus), *Flaviviridae* (Dengue virus, Yellow Fever virus, Hepatitis C virus), among others (Flint *et al*., 2015). RNA(+) viruses replicate their RNA genomes through a negative strand intermediate and this reaction is catalyzed by a viral RNA dependent RNA Polymerase (RdRP) (Ferrer-Orta *et al*., 2015). Consequently, RdRP plays a key role in virus replication cycle (Verdaguer *et al*., 2014). RNA(+) genome replication is an error prone process and thereby genomic copies will carry mutations that could be selected in the viral offspring following Darwinian forces. Furthermore, RNA(+) virus replicate at large population size, reaching 10^{10}-10^{12} viruses in an infected individual. Putting these two factors together, error prone replication and population size, RNA(+) viral populations consist of mutant spectra (or mutant clouds) rather than genomes with the same nucleotide sequence. Mutant spectra, usually referred as viral quasispecies and not individual viral particles are the target of evolutionary events (Más *et al*., 2010).

Several inhibitors targeting viral polymerases have been designed. However, the rapid appearance of resistant mutants, as a direct consequence of the viral population structure, diminishes the efficacy of these kind of molecules (Más *et al*., 2010). To elude the rapid loss of treatment efficiency due to the appearance of resistance mutations, cellular factors have been proposed as a promising therapeutic target to inhibit RNA(+) virus replication (Lou *et al*., 2014). Factors of cellular origin cannot mutate and be selected to escape antiviral pressure at the same rate as virus factors. Therefore, host-targeted antivirals show high genetic barrier to escape (Plummer *et al*., 2015).

Hepatitis C Virus (HCV) is RNA(+) virus with a high-titer and error-prone replication rate leading to the generation of viral populations in which mixtures of almost infinite different variants called quasispecies may coexist (Más *et al*., 2010). HCV infection is widespread worldwide, showing geographical differences in terms of genetic identity with seven well defined genotypes

(Baumert *et al*., 2016; Clemente-Casares *et al*., 2011). Independently of the infecting genotype, HCV infection is the main cause for cirrhosis and hepatocellular carcinoma (Westbrook and Dusheiko, 2014). HCV entry into the cell is mediated by the interaction of the glycoproteins from the viral envelope with receptors on the surface of the hepatocyte such as CD81, CLDN1 and OCLN among others (Ding *et al*., 2014). HCV entry is a complex process governed by viral and cellular factors and several of them contribute to liver tropism and limit host range of this virus. Once the virus has entered the cell, RNA(+) HCV genome is released into the cytoplasm where it is translated at the rough Endoplasmic Reticulum (ER) as a polyprotein (Paul *et al*., 2014). HCV polyprotein is about 3000 amino acids in length and is co- and post-translationally processed by proteases from cellular and viral origin to give ten mature viral proteins. HCV proteins are structural (core C and envelope proteins E1 and E2) and nonstructural (p7, NS2, NS3, NS4A, NS4B, NS5A and NS5B) proteins. Proteins C, E1 and E2 are main constituents of the virus particle. The p7 viroporin and NS2 participate in virus assembly. Finally, NS3, NS4A, NS4B, NS5A and NS5B form the replicase complex that is sufficient for viral RNA replication (Paul *et al*., 2014). RNA(+) replication product may be either used for translation, for synthesis of new negative strands, or can be packaged into virus particles that exit the cell via the secretory pathway. Translation and replication take place in opposite directions on the RNA(+) and cannot occur simultaneously. A rigorous control by cis-acting elements in the HCV genome and antigenome as well as cellular proteins and miRNAs mediates the transition from translation to replication (Sagan *et al*., 2015).

HCV replication takes place in microvesicles derived from ER where replication complex is located. Viral replicase is composed of at least viral proteins NS3, NS4A, NS4B, NS5A and NS5B. NS3 is composed of two domains located at N-terminal and C-terminal ends, showing serine-protease and helicase activities, respectively (Moradpour and Penin, 2013). The serine-protease domain is responsible for polyprotein cleavage in complex with the NS4A protease cofactor, whereas the helicase domain is important for RNA replication because of its RNA unwinding activity. NS4B is a poorly characterized protein with a complex transmembrane topology involved in inducing membrane alterations (Egger *et al*., 2002). NS5A is a RNA-binding phosphoprotein that exists as both a basal and a hyperphosphorylated form. The phosphorylation status of NS5A appears to be determined by several cellular kinases, including Glycogen Synthase Kinase 3 beta (GSK3β), Protein Kinase A (PKA), Casein Kinases (CK) I and II, polo-like kinase 1 and Mitogen-Activated Protein Kinases (MAPKs) (Colpitts *et al*., 2015). NS5A function seems to be to interact with other viral replicase components as well as cellular factors (Ross-Thriepland and Harris, 2015). Proteomics and molecular systematics approaches have been reported that more than one hundred proteins interact with NS5A (Tripathi *et al*., 2013; Li *et al*., 2014a). Affinity capture was also used for identifying host factors interacting with HCV RNA positive strand (Upadhyay *et al*., 2013). Some of them have been described above and comprises La protein (Kumar *et al*., 2013), Heterogeneous Nuclear Ribonucleoprotein L (hnRNP L) (Li *et al*., 2014b), Nuclear Factor 90 (NF90) (Li *et al*., 2014b), Vesicle-associated membrane protein-associated protein A and B (VAPA and VAPB) (Evans *et al*., 2004; Gao *et al*., 2004; Hamamoto *et al*., 2005), Polo-like Kinase 1 (Chen *et al*., 2010), TBC1 domain family member 20 (TBC1D20) (Nevo-Yassaf *et al*., 2012), Amphiphysin II (Zech *et al*., 2003), Reticulon 1 and 3 (RTN1 and RTN3) (Tripathi *et al*., 2013), Protein Phosphatase 2A (Georgopoulou *et al*., 2006), cyclophilin A (Liu *et al*., 2009), F-Box and Leucine-rich repeat protein 2 (FBXL2) (Wang *et al*., 2005), stress granule components (Pène *et al*., 2015) and the lipid kinase phosphatidylinositol-4 kinase III (Harak *et al*., 2014) among many others. Some of these are cellular kinases with well known roles in HCV infection *in vivo* (Reed *et al*., 1997).

NS5B is the viral RNA-dependent RNA Polymerase (RdRP) responsible for the synthesis of the (+) strand progeny through a (-) strand intermediate (Sesmero and Thorpe, 2015). NS5B X-ray crystal structures have revealed a polymerase-typical right-hand shape with fingers, palm and thumb subdomains (Verdaguer *et al*., 2014). The catalytic site is totally encircled, as other viral RdRP, with extensive interactions by loops connecting fingers and thumb subdomains (Verdaguer *et al*., 2014). The C-terminal end has a very hydrophobic peptide that allows NS5B to be anchored to ER membrane. This peptide can be removed to increase recombinant NS5B purification yields without affecting NS5B RdRP activity (López-Jiménez *et al*., 2014). *In vitro* RNA synthesis by NS5B can be induced in the presence of a template-primer or initiated by a *de novo* mechanism (López-Jiménez *et al*., 2014), the latter being the most likely to occur *in vivo*. A beta-hairpin from the thumb subdomain protrudes into the catalytic center preventing primer-dependent RNA synthesis (Lesburg *et al*., 1999). Residues in the tip of this structure act as a platform to initiate RNA synthesis by a de novo mechanism. Once the first phosphodiester bond is formed the beta-hairpin is removed and NS5B can complete genome replication (Appleby *et al*., 2015).

HCV replicates its genome in replication complexes where viral and cellular proteins co-localize. A large excess of each HCV non-structural protein with respect

to (+) and (-) strand HCV RNA has been observed (Quinkert *et al*., 2005), suggesting extensive protein-protein interactions and molecular crowding phenomena. Actually, HCV NS5B interacts with itself, affecting RNA synthesis activity in a cooperative way (López-Jiménez *et al*., 2014). Furthermore, HCV NS5B interacts with other HCV proteins and interactions with NS3, NS4A, NS4B and NS5A have been described (Ishido *et al*., 1998; Piccininni *et al*., 2002; Shimakami *et al*., 2004).

Also, HCV polymerase directly interacts with host factors. NS5B activity can be regulated by phosphorylation. Actually, one of the first cellular proteins with a confirmed interaction with HCV polymerase was the PKC-Related Kinase 2 (PRK2) (Kim *et al*., 2004). This Serine/Threonine protein kinase regulates viral polymerase activity by phosphorylation of NS5B residues Ser29 and Ser42 (Han *et al*., 2014). Other cellular proteins with which NS5B interacts include ELAV like RNA binding protein 1 (ELAVL1 or HuR) (Shwetha *et al*., 2015), BCL2 Interacting Killer (BIK) (Aweya *et al*., 2015), Vesicle-Associated Membrane Protein (VAMP)-associated proteins A, B and C (VAPA, VAPB and VAPC) (Hamamoto *et al*., 2005; Tu *et al*., 1999; Goyal *et al*., 2012), Nucleolin (Hirano *et al*., 2003; Kusakawa *et al*., 2007), human Eukaryotic Initiation Factor 4A2 (hEIF42) (Kyono *et al*., 2002), ubiquilin 1 (UBQLN1 o hPLIC1) (Gao *et al*., 2003), Alpha-actinin (Lan *et al*., 2003) and chaperonin TRiC/CCT (Inoue *et al*., 2011).

The cytoplasmic double-stranded RNA binding protein Staufen 1 (Stau1) coimmunoprecipitates HCV NS5B and the host factor Protein Kinase R (PKR), which is critical for interferon-induced cellular antiviral and antiproliferative responses (Dixit *et al*., 2016). Protein Kinase R (PKR) inhibits translation via eIF2α phosphorylation (Donnelly *et al*., 2013) and regulation of PKR activity is central for the control of cellular translation by several viruses (Flint *et al*., 2015). HCV may appropriate Stau1 to its advantage to prevent PKR-mediated inhibition of eIF2α, which is required for the synthesis of HCV proteins and also for translocation of viral RNA genome to the polysomes for efficient translation and replication (Dixit *et al*., 2016).

Our laboratory has recently described the interaction of NS5B with the Ser/Thr kinase Akt (Llanos Valero *et al*., 2016). This interaction has been confirmed by *in vitro* kinase assays, coimmunoprecipitation of NS5B and Akt, either expressed ectopically or from HCVcc infected cells. The interaction of HCV NS5B with this cellular kinase of the PI3K/Akt/mTOR pathway leads to a subcellular relocalization of Akt from a cytoplasmic to a perinuclear region in a clear colocalization with HCV polymerase. Relocalization was observed in cells transfected with plasmids encoding NS5B and Akt as well as in cells carrying a subgenomic replicon or HCVcc infected cells. NS5A is susceptible to be phosphorylated by Akt and relocalization of Akt with NS5B could drive NS5A phosphorylation at this subcellular region.

Relationship between HCV infection and sex hormones has been previously documented (Giannitrapani *et al*., 2006; Baden *et al*., 2014; White *et al*., 2014). Some estrogen-related drugs inhibits the production of HCV virus particles in an Estrogen Receptor alpha (ER1)-dependent manner (Hayashida *et al*., 2010). It has been also shown that ER1 may recruit NS5B to the HCV replication complex (Watashi *et al*., 2007) and our laboratory has described the interaction between HCV NS5B and ER1 *in vitro*, showing that this protein-protein interaction depends on NS5B oligomerization (Hillung *et al*., 2012). Cellular DEAD-box helicase 5 (DDX5 or p68) also interacts with HCV NS5B (Goh *et al*., 2004). DDX5 is a RNA-dependent ATPase and it is implicated in cellular processes involving alteration of RNA secondary structure, such as translation initiation. DDX5 has been involved in HCV translation (Ríos-Marco *et al*., 2016) as well as in replication of other RNA(+) viruses as Japanese Encephalitis virus (Li *et al*., 2013) and retrovirus (Sithole *et al*., 2015) and negative strand RNA viruses as influenza virus (Jorba *et al*., 2008). DDX5 also interacts with Estrogen Receptor 1 (ER1) (Fujita *et al*., 2003) and with Akt (Zhu *et al*., 2011). Therefore, it seems to be a complex network comprising interactions among HCV replicase, Akt, DDX5 and ER1 in association with ER membrane that are important for HCV replication. However, experiments to demonstrate a clear localization of these host factors into the HCV replication complex have to be done. Once the mechanism governing these interactions will be decoyed we explore use of host factor inhibitors to treat viral infections. Currently, some inhibitors directed against ER1 (Riggs and Hartmann, 2003; Cuzick *et al*., 2013) and Akt (Brown, 2016; Nitulescu *et al*., 2016) are in clinical use or in development for treating other diseases.

Therefore, HCV polymerase interacts with several host factors that are important not only for viral replication process but also to control cell cycle, cell metabolism, etc (Lee *et al*., 2006). By these interactions, NS5B not only replicates HCV genome but also controls several cellular functions important for virus-cell relationship. Under these premises NS5B is a multifunctional protein so NS5B direct inhibition could lead to HCV replication inhibition by affecting several steps in the replicative cycle of the virus. However, the great genetic diversity of RNA(+) viruses make the appearance of resistant mutants a definite possibility. Targeting one or more of the interactions described above could also blockade HCV replication making it more difficult for the selection of resistant viruses.

Finally, several cellular pathways are shared by different RNA(+) viruses and targeting host factors could be useful for inhibiting viral infections from different viruses.

Conclusion

Viruses needs to replicate inside the cells usurping cellular functions. HCV NS5B, the main component of the viral replicase, not only replicates HCV RNA but also interacts with host factors to subjugate cellular metabolism. A deeper knowledge about NS5B-host interactions will be useful in the design of new, strongest and panviral antiviral strategies with limited side effects.

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Author's Contributions

All authors equally contributed in this work.

Ethics

This article is original and contains unpublished material. The corresponding author confirms that all of the other authors have read and approved the manuscript and no ethical issues involved.

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