Virology 475 (2015) 139–149

Contents lists available at ScienceDirect

Virology

journal homepage: www.elsevier.com/locate/yviro

The interaction between the Hepatitis C proteins NS4B and NS5A is involved in viral replication

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ARTICLE INFO

Article history:
Received 17 July 2014
Returned to author for revisions
7 August 2014
Accepted 22 October 2014

Keywords:
Hepatitis C virus
Protein–protein interaction
Viral replication
Fluorescence resonance energy transfer (FRET)
Endoplasmic reticulum (ER)

ABSTRACT

Hepatitis C virus (HCV) replicates in membrane associated, highly ordered replication complexes (RCs). These complexes include viral and host proteins necessary for viral RNA genome replication. The interaction network among viral and host proteins underlying the formation of these RCs is yet to be thoroughly characterized. Here, we investigated the association between NS4B and NS5A, two critical RC components. We characterized the interaction between these proteins using fluorescence resonance energy transfer and a mammalian two-hybrid system. Specific tryptophan residues within the C-terminal domain (CTD) of NS4B were shown to mediate this interaction. Domain I of NS5A, was sufficient to mediate its interaction with NS4B. Mutations in the NS4B CTD tryptophan residues abolished viral replication. Moreover, one of these mutations also affected NS5A hyperphosphorylation. These findings provide new insights into the importance of the NS4B–NS5A interaction and serve as a starting point for studying the complex interactions between the replicase subunits.

Introduction

Hepatitis C virus (HCV) infection affects millions worldwide and is one of the leading causes of chronic liver disease. Prolonged HCV infection may lead to liver cirrhosis and liver failure and is associated with the development of liver cancer (El-Serag, 2011). HCV is a positive strand RNA virus, that like other members of this group, replicates in membrane associated replication complexes (RC). These altered membranes are thought to be endoplasmic reticulum (ER) derived and to contain in addition to the viral genome and proteins, a variety of host factors necessary for viral replication (Miller and Krijnse-Locker, 2008).

Once the positive strand RNA genome enters the host cell, its single open reading frame is translated into a polyprotein. This polyprotein is co- and post-translationally processed to yield three structural proteins (core, E1 and E2), an ion-channel (p7), and six non-structural proteins (NS2, NS3, NS4A, NS4B, NS5A, NS5B) (Grakoui et al., 1993b). The minimal set of proteins essential for viral replication consists of NS3, NS4A, NS4B, NS5A and NS5B (Lohmann et al., 1999). Most of these proteins have known activities: NS3 is a helicase and a serine protease that uses NS4A as a cofactor. NS5B is the viral RNA-dependent RNA polymerase (Ago et al., 1999; Bressanelli et al., 1999; Grakoui et al., 1993a; Hijikata et al., 1993; Lesburg et al., 1999). The exact role of NS4B and NS5A, which are essential for viral replication and have a role in assembly, is still not fully understood.

NS4B is a 27 kDa, ER localized, small, hydrophobic transmembrane protein thought to induce the membranous web: modified membrane elements that comprise the viral RC (Egger et al., 2002). NS4B contains three major segments: a cytoplasmic N-terminal part (a.a. 1 to ~69), shown to contain two amphipathic helices (AH1 and AH2) (Elazar et al., 2004; Gouttenoire et al., 2010); a central domain that contains four putative transmembrane segments (a.a. ~70 to ~191) and a cytoplasmic C-terminal part (a.a. ~191 to 261) that contains two conserved helices (H1 and H2) (Gouttenoire et al., 2009; Jones et al., 2009; Lundin et al., 2003). Three main features of NS4B are thought to contribute to its activity in the induction of the membranous web formation: The first is its ability to form oligomers, mediated by several domains of the protein, mainly AH2 and elements at the C-terminus (Gouttenoire et al., 2010; Paul et al., 2011; Yu et al., 2006). The

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http://dx.doi.org/10.1016/j.virol.2014.10.021
0042-6822/

second feature is the ability NS4B's AH2 domain to promote lipid vesicle aggregation (Cho et al., 2010). The third feature is the ability NS4B's N-terminal domain to post-translationally translocate from the cytosolic face of the ER into its lumen. NS5A was found to negatively affect this translocation, suggesting that NS5A influences the topology of NS4B (Lundin et al., 2006).

NS5A is a 56–58 kDa phosphorylated protein, occurring in different phosphorylation states, the exact role of these different phosphorylation states in the viral life cycle is still unclear. Adaptive mutations reducing the amount of hyperphosphorylated NS5A increase viral replication (Appel et al., 2005b; Blight et al., 2000; Katze et al., 2000; Tanji et al., 1995b). Furthermore, inhibition of NS5A hyperphosphorylation by kinase inhibitors yields similar consequences (Neddermann et al., 2004). Yet, low levels of hyperphosphorylated NS5A seem to be essential for viral replication since viral replication is inhibited upon its complete loss (Appel et al., 2005a; Fridell et al., 2011). The increase in viral replication caused by reduced levels of the NS5A hyperphosphorylated form inversely correlates with viral production, leading to the notion that the hyperphosphorylated form of NS5A has adverse effects on viral replication but is required for viral assembly (Masaki et al., 2008; Miyanari et al., 2007; Pietschmann et al., 2009; Tellinghuisen et al., 2008a). It was also proposed to regulate the interface between replication and assembly (Tellinghuisen et al., 2008a). Several nonstructural proteins including NS4B were found to facilitate NS5A phosphorylation (Koch and Bartenschlager, 1999; Neddermann et al., 1999). The N-terminal domain of NS5A contains an amphipathic helix that mediates its membrane association (Elazar et al., 2003). The rest of the protein is organized in three domains (I, II, and III), which are separated by repetitive low-complexity sequences (Tellinghuisen et al., 2004). Domain I has been attributed to the replicase activity of NS5A (Tellinghuisen et al., 2005). Although a large part of domain II could be deleted without effecting replication or production of the virus (Appel et al., 2008), deletion of a specific region or deletion of the whole domain abolished viral replication (Appel et al., 2008; Tellinghuisen et al., 2008b). In contrast, domain III has been shown to be dispensable for RNA replication (Appel et al., 2008; Tellinghuisen et al., 2008a). Mutations and insertions in domain III showed impact on virus particle assembly (Appel et al., 2008). Domain I was crystallized as a dimer, leading to the notion that NS5A functions as a multimer (Love et al., 2009; Tellinghuisen et al., 2005).

Traditionally, NS4B was thought to be the sole driving force for the induction of the membrane alterations that harbor the RCs (Egger et al., 2002). It was recently shown that double membrane vesicles that appear in infected cells upon the initiation of viral replication are induced by NS5A, highlighting a critical role for both NS4B and NS5A in this initial step in the establishment of the viral RC (Romero-Brey et al., 2012). A genetic interaction between NS4B and NS5A was reported, where a mutation in the C-terminal domain of NS4B that abolished viral replication was rescued by a pseudoreversion in NS4B and an additional mutation in domain I of NS5A (Paul et al., 2011). Similarly, replacement of the NS4B C-terminal domain from a genotype 2a genome with sequences from genotype 1b attenuated virus production. The phenotype was rescued by two adaptive mutations one in the C-terminal of NS4B and the other in NS5A domain III (Han et al., 2013).

NS4B and NS5A were previously shown to physically interact (Aligo et al., 2009; Dimitrova et al., 2003; Gao et al., 2004), however, in spite of their aforementioned functional association, this interaction was never characterized, most likely due to the hydrophobic nature of NS4B that complicates biochemical analysis. Using immunofluorescence, fluorescence resonance energy transfer (FRET) and a mammalian two-hybrid system approach, we confirmed this interaction. The C-terminal domain of NS4B and domain I of NS5A, were found to contain the major determinants that mediate the NS4B–NS5A interaction. Furthermore, mutations in NS4B's C-terminal domain that affected this interaction abolished viral replication. One of these mutations also inhibited NS5A phosphorylation. These results may provide additional insights into organization of the viral RC, towards an informed model of its functional mechanism.

Results

NS4B and NS5A are both known to be essential components of HCVs replicase machinery and as such are expected to colocalize within RCs (Lohmann et al., 1999). As a baseline for our forthcoming experiments, their localization in replicon cells was confirmed. To this end, Bart-HA cells (Huh7 cells harboring a subgenomic 1b replicon with an HA tag insertion in NS5A) were used for immunofluorescence analysis with specific antibodies. Both proteins displayed a typical reticular ER and a speckle-like pattern, thought to represent the membrane rearrangements harboring the viral RCs (Egger et al., 2002; Ide et al., 1996; Polyak et al., 1999; Tanji et al., 1995a). NS4B and NS5A colocalized in these structures (Fig. S1). NS4B is a highly hydrophobic transmembrane protein, previous studies reported unsuccessful attempts to study its interaction with NS5A using immunoprecipitation of ectopically expressed proteins or from infected cells (Blight, 2011; Han et al., 2013). Thus, we used an experimental approach that allows analysis of proteins in their native intact membranes namely, Fluorescence Resonance Energy Transfer (FRET). FRET occurs between two fluorophores with partially overlapping emission of one (the donor) and excitation of the other (the acceptor) when the two molecules are very close to each other (less then 10 nm). The efficiency of the FRET is inversely proportional to the distance to the power of 6. Thus, FRET between two fluorescently tagged molecules is a strong indication for interaction (Kenworthy, 2001). In acceptor photobleaching, occurrence of FRET is detected as the increase in the fluorescence emission of the dequenched donor fluorophore following photobleaching of the acceptor. Here FRET was carried out using repetitive partial acceptor photobleaching to demonstrate a correlation between acceptor photobleaching and donor fluorescence increase (Fig. S2). Full length NS4B or NS5A from HCV genotype 1b or 2a were each fused to GFP or mCherry that were then used as a donor-acceptor pair for FRET analysis, following their coexpression in Huh7 cells (Fig. 1A). GFP-tagged Pendrin (PDS) a plasma membrane iodide transporter that upon overexpression is essentially retained and accumulated in the ER (Shepshelovich et al., 2005) was used as a negative control. NS4B (genotype 1b), previously shown by FRET to oligomerize, was used as a positive control (Gouttenoire et al., 2010). As expected, a significant FRET signal (mean E_F 6.6 ± 3.5, $p < P \leq 0.03$) was observed in cells coexpressing GFP- and mCherry-tagged NS4B, while insignificant FRET was detected in cells expressing PDS together with NS4B or NS5A. A significant FRET signal, comparable to the NS4B positive control, was observed in cells coexpressing NS5A-GFP and NS4B-mCherry from genotype 2a (mean E_F 5.5 ± 7.2, $p < 0.001$). A lower, yet noticeable FRET signal was detected in cells coexpressing NS5A-mCherry and NS4B-GFP from genotype 1b (mean E_F 6.0 ± 3.8). Chimeras containing NS4B of genotype 1b and NS5A of genotype 2a have been shown to replicate (Han et al., 2013). Thus, we tested if NS4B and NS5A could interact intergenotypically. Indeed FRET was observed between NS5A from genotype 2a and NS4B from genotype 1b (mean E_F 6.5 ± 3.2, $p < 0.005$), with FRET efficiency levels comparable to those preformed using NS4B and NS5A from the same genotype as a FRET pair. The proper and equivalent co-expression levels of the above-mentioned proteins were assessed using western blot analysis with primary anti-GFP and mCherry antibodies (Fig. 1B). To further confirm the physical interaction between these proteins, a mammalian two-hybrid system was used. In this system one of potential