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Membrane association of hepatitis C virus nonstructural proteins and identification of the membrane alteration that harbors the viral replication complex

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Abstract

Hepatitis C virus (HCV) replicates its genome in a membrane-associated complex composed of viral proteins, replicating RNA, and altered cellular membranes. Determinants for membrane association of the HCV nonstructural proteins involved in genome replication have been defined. In addition, a specific membrane alteration, designated membranous web, was recently identified as the site of viral RNA synthesis and, therefore, represents the HCV replication complex. These findings add to our current understanding of the HCV life cycle and may ultimately allow to design novel antiviral strategies against hepatitis C.

Keywords: HCV replication complex; Nonstructural proteins; Membranous web

Formation of a membrane-associated replication complex, composed of viral proteins, replicating RNA, and altered cellular membranes, is a hallmark of all plus-strand RNA viruses investigated thus far (Bienz et al., 1992; Schaad et al., 1997; Pedersen et al., 1999; Westaway et al., 1999; Gosert et al., 2002; Schwartz et al., 2002; reviewed in Egger et al., 2002a). Depending on the virus, replication may occur on altered membranes derived from the endoplasmic reticulum (ER) (Restrepo-Hartwig and Ahlquist, 1996; Schaad et al., 1997; van der Meer et al., 1998; Rust et al., 2001; Ritzenthaler et al., 2002), Golgi apparatus (Mackenzie et al., 1999; Gazina et al., 2002; Krogerus et al., 2003), mitochondria (Miller et al., 2001) or even lysosomes (Kujala et al., 2001). 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 (Lyle et al., 2002), (ii) the compartimentalization and local concentration of viral products (Schwartz et al., 2002), (iii) tethering of the viral RNA during unwinding (Egger et al., 2002a), (iv) provision of lipid constituents important for replication (Wu et al., 1992; Ahola et al., 1999), and (v) protection of the viral RNA from double-strand RNA-mediated host defenses or RNA interference.

Hepatitis C virus (HCV) contains a plus-strand RNA genome of approximately 9600 nucleotides that encodes a polyprotein precursor of about 3000 amino acids (reviewed in Lindenbach and Rice, 2001; Moradpour et al., 2002). The polyprotein is co- and posttranslationally processed by cellular and viral proteases into the mature structural and nonstructural proteins. The structural proteins include the core protein and the envelope glycoproteins, E1 and E2. The structural proteins are released from the polyprotein precursor by the ER signal peptidase. The nonstructural proteins 2–5B include the NS2–3 autoprotease, the NS3 serine protease, a NTPase/RNA helicase domain in the carboxyterminal two-thirds of NS3, the NS4A polypeptide, the NS4B and NS5A proteins, and the NS5B RNA-dependent RNA polymerase (RdRp).

Investigation of the HCV life cycle has been limited by the lack of an efficient cell culture system permissive for HCV infection and replication. Nevertheless, considerable progress has been made using heterologous expression systems (Hijikata et al., 1993; Grakoui et al., 1993; Bartenschlager et al., 1994; Moradpour et al., 1998), functional cDNA clones (Kolykhalov et al., 1997), and, more recently, the replicon system (Lohmann et al., 1999; Blight

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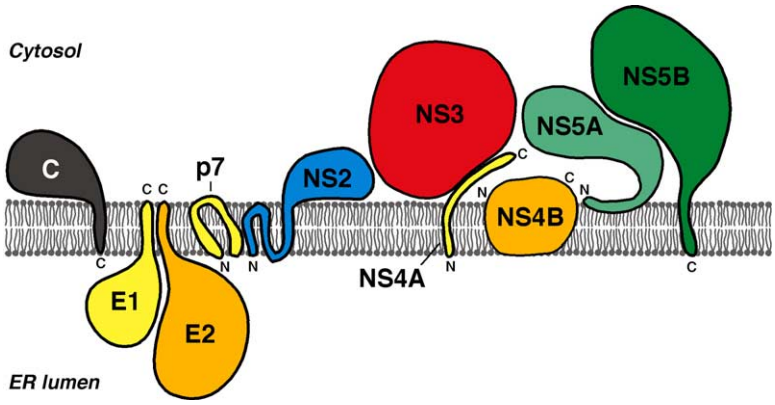

Fig. 1. Membrane association of HCV proteins. Note that the topologies of NS2, NS4A and NS4B are currently under investigation and are only schematically illustrated. A recent study indicated that the C-terminus of NS2 may be localized in the ER lumen, resulting in four transmembrane domains (Yamaga and Ou, 2002). Also, it was recently reported that the N-terminus of NS4B can be at least partially translocated into the ER lumen (Lundin et al., 2003).

et al., 2000; Ikeda et al., 2002; Pietschmann et al., 2002) (reviewed in Lindenbach and Rice, 2001; Bartenschlager, 2002; Moradpour et al., 2002). This system has allowed, for the first time, to study efficient and genuine HCV RNA replication in Huh-7 human hepatoma cells in vitro. Moreover, the replicon system has clearly shown that the HCV nonstructural proteins 3–5B form an independent module sufficient for autonomous HCV RNA replication.

Fig. 1 schematically illustrates the membrane association of HCV proteins. In the following, we will summarize our current understanding of the determinants and mechanisms of membrane association of the HCV nonstructural proteins 3–5B which are involved in genome replication. For a more comprehensive review on the interactions of HCV proteins, including the structural proteins, with host cell membranes the reader is referred to Dubuisson et al. (2002). In the second part of this review, the recent identification of the membrane alteration harboring the HCV RNA replication complex will be discussed.

1. Membrane association of HCV nonstructural proteins

1.1. NS3–4A complex

NS3 is a multifunctional protein with a serine protease domain located in the N-terminal one-third and a NTPase/RNA helicase domain in the C-terminal two-thirds. NS3 by itself has no membrane anchor, but it forms a noncovalent complex with NS4A which is a membrane-anchored polypeptide. NS4A is a 54-amino acid cofactor for the serine protease, and cofactor activity requires stable complex formation between NS3 and the central domain of NS4A, an interaction that also serves to stabilize NS3 (Tanji et al., 1995; Wölk et al., 2000). NS3 expressed alone is diffusely distributed in the cytoplasm and nucleus (Wölk et al., 2000). In contrast, when coexpressed with NS4A, NS3 is found in association with membranes of the ER or an ER-like modified compartment. Deletion analyses revealed that the hydrophobic N-terminal domain of NS4A is required for ER targeting of NS3 (Wölk et al., 2000). The N-terminal domain of NS4A is strongly predicted to form a transmembrane α-helix involved in membrane anchorage of the NS3–4A complex.

1.2. NS4B

NS4B, a hydrophobic protein of 27 kDa, is the least characterized HCV protein. Immunofluorescence analyses, subcellular fractionation and membrane extraction experiments indicate that NS4B is an integral ER membrane protein both when expressed alone or in the context of the HCV polyprotein (Hügle et al., 2001). More recently, it was found by electron microscopy (EM) that expression of NS4B induces the formation of a seemingly ER-derived membranous web that is able to harbor all HCV structural and nonstructural proteins (Egger et al., 2002b). Thus, a function of NS4B may be to induce a specific membrane alteration that serves as a scaffold for the HCV replication complex.

When NS4B is expressed alone, its association with the ER membrane occurs cotranslationally, presumably via engagement of the signal recognition particle (SRP) by an internal signal peptide (Hügle et al., 2001), but no canonical signal peptide has been identified so far. NS4B is predicted to be a polytopic membrane protein with a cytoplasmic N-terminal region followed by, depending on the prediction program, 4 or 6 transmembrane segments and a C-terminal region in the cytosol (Hügle et al., 2001; Qu et al., 2001). It has been shown experimentally that the bulk of the protein is cytosolically oriented (Hügle et al., 2001). Introduction of glycosylation acceptor sites at various positions of NS4B recently confirmed the presence of a predicted ER luminal loop around amino acid position 161 (Lundin et al., 2003). Surprisingly, the N-terminus of NS4B was found to

be translocated into the ER lumen at least partially, presumably by a posttranslational mechanism (Lundin et al., 2003). It will be interesting to examine whether this unusual translocation occurs also when NS4B is expressed in context of the HCV polyprotein.

1.3. NS5A

NS5A is a phosphoprotein of unknown structure and function. It has attracted considerable interest because of its potential role in modulating the interferon response and various other host cell pathways (Enomoto et al., 1996; Tan and Katze, 2001).

Membrane association of NS5A is independent of the expression of other HCV proteins. Deletion studies and green fluorescent protein (GFP) fusion analyses have allowed the membrane anchor to be mapped to the N-terminal 30-amino acid residues of NS5A (Brass et al., 2002; Elazar et al., 2003) (Fig. 2). When expressed alone, membrane association of NS5A occurs by a posttranslational mechanism and, presumably, *via* an SRP-independent pathway. Sequence analyses and secondary structure predictions indicated the presence of an amphipathic α-helix in the N-terminal portion of NS5A (Brass et al., 2002) (Fig. 3). This was confirmed by circular dichroism analyses of a synthetic peptide representing the N-terminal 31-amino acids of NS5A. In addition, detergent binding properties of this helical peptide together with the nature and location of its amino acids suggest a mechanism of insertion *via* the hydrophobic side of the helix, yielding a topology parallel to the lipid bilayer in the cytoplasmic leaflet of the ER membrane (Brass et al., 2002). Such a topology, termed monotopic, has now been confirmed by NMR spectroscopy (Ramboarina et al., unpublished data). This helix exhibits fully conserved polar residues at the membrane surface which define a unique platform that is likely involved in specific protein–protein interactions thought to be essential for HCV replication complex assembly.

Interestingly, a synthetic peptide representing the N-terminal amphipathic α-helix of NS5A was recently found to block membrane association of NS5A in vitro in a dose-dependent and sequence-specific fashion (Elazar et al., 2003). The mechanism of inhibition remains to be determined, but this observation raises the interesting possibility of interfering with membrane association of HCV proteins as a novel therapeutic strategy.

1.4. NS5B

HCV replication proceeds *via* synthesis of a complementary minus-strand RNA using the viral genome as a template and the subsequent synthesis of genomic plus-strand RNA from this minus-strand RNA template. The key enzyme responsible for both of these steps is the NS5B RdRp. This essential viral enzyme has been extensively characterized at the biochemical (Behrens et al., 1996; Lohmann et al., 1997; Yamashita et al., 1998) and structural level (Lesburg et al., 1999; Bressanelli et al., 2002) and has emerged as a major target for antiviral intervention.

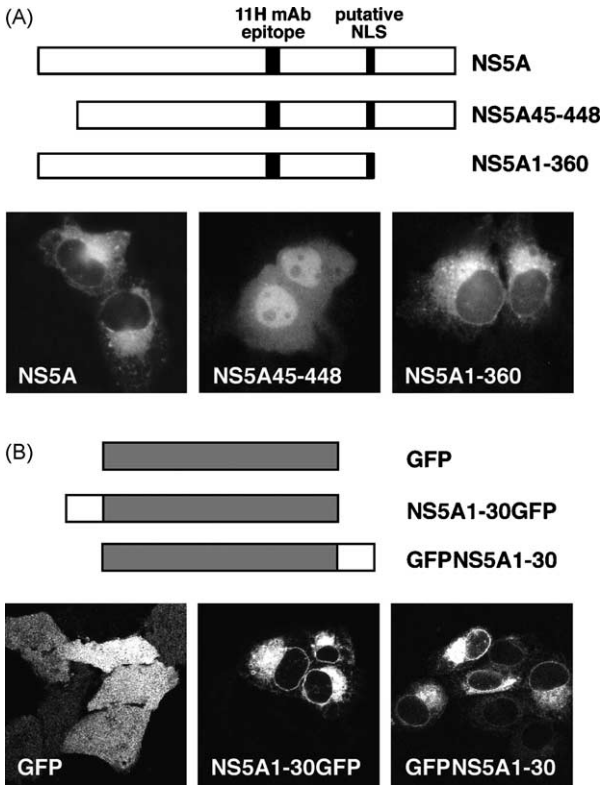

Fig. 2. The N-terminal 30-amino acid residues of NS5A serve as a membrane anchor. (A) Subcellular localization of amino- and carboxyterminal NS5A deletion constructs. U-2 OS cells were transiently transfected with expression constructs coding for full-length NS5A (NS5A), amino acid residues 45–448 of NS5A (NS5A45–448), or amino acid residues 1–360 of NS5A (NS5A1–360), as indicated by the captions. Cells were subsequently processed for indirect immunofluorescence microscopy using a monoclonal antibody against NS5A. (B) Subcellular localization of GFP fusion constructs. U-2 OS cells were transiently transfected with GFP as well as with expression constructs with the N-terminal 30-amino acid residues of NS5A fused to the N-terminus (NS5A1–30GFP) or the C-terminus of GFP (GFPNS5A1–30), as indicated by the captions. Slides were analyzed by confocal laser scanning microscopy. Adapted from Brass et al. (2002) with permission.

Membrane association of the HCV RdRp is independent of other membrane proteins. Indeed, the C-terminal 21-amino acid residues of NS5B are necessary and sufficient to target NS5B or heterologous fusion proteins to the cytosolic side of the ER membrane (Schmidt-Mende et al., 2001). Membrane association of NS5B occurs by a posttranslational mechanism and results in integral membrane association (Schmidt-Mende et al., 2001). These features, namely (i) posttranslational membrane targeting *via* a hydrophobic C-terminal insertion sequence, (ii) integral membrane association, and (iii) cytosolic orientation of the functional protein domain, define the HCV RdRp as a new member of a relatively small class of membrane proteins termed tail-anchored proteins (reviewed in Kutay et al., 1993; Wattenberg and Lithgow, 2001). More recently, two

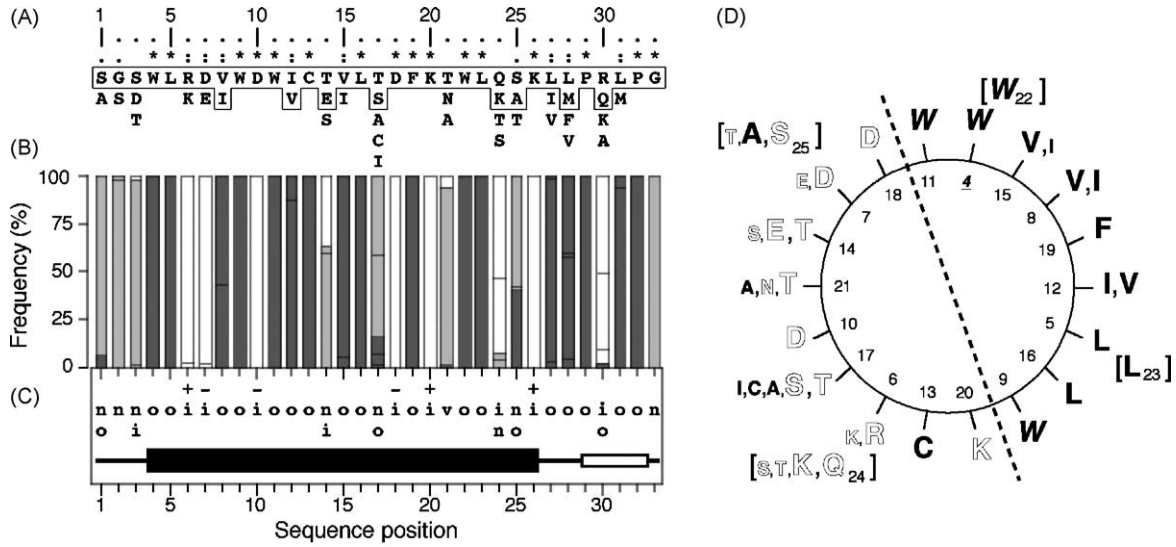

Fig. 3. An N-terminal amphipathic α-helix mediates membrane association of NS5A. (A) Repertoire of amino acid residues at each position in 280 HCV isolates of various genotypes. Amino acids are listed in decreasing order of observed frequency, from top to bottom. Fully conserved, conserved and similar residues at each position are symbolized by an asterisk (∗), a colon (:), and a dot (.), respectively. (B) Histogram showing the hydropathic character of residues at each NS5A position. The height of each box in each bar indicates the number of sequences observed with a given residue at a given position. The boxes are presented in order of decreasing hydrophobicity, from bottom to top. Each box is colored according to the hydrophobic character of the residue: dark gray for hydrophobic (F, I, W, Y, L, V, M, P, C, A), light gray for neutral (G, T, S), and white for hydrophilic (K, Q, N, H, E, D, R). (C) Consensus hydropathic pattern and secondary structure prediction. The consensus hydropathic pattern was deduced from (C) o, hydrophobic residue; n, neutral residue; i, hydrophilic residue; v, variable residue. + and −, positions where fully conserved positively or negatively charged residues were observed, respectively. The large black box indicates the consensus α-helix deduced from the analysis of various NS5A sequences using a large set of secondary structure prediction methods; the small white box indicates turn prediction. (D) Ideal α-helix projection of segment 4–25 of NS5A. The variability of residues at each position is included, according to panel (A). The larger characters indicate the most frequently observed residues. Outline and bold letters correspond to polar and hydrophobic residues, respectively. Adapted from Brass et al. (2002) with permission.

independent experimental strategies demonstrated that the HCV RdRp insertion sequence crosses the membrane phospholipid bilayer as a transmembrane segment (Ivashkina et al., 2002). The HCV RdRp was found to be retained in the ER or an ER-derived modified compartment both following transient transfection and in the context of a subgenomic replicon. Interestingly, an absolutely conserved GVG motif in the RdRp insertion sequence was not essential for membrane insertion but possibly provides a docking site for transmembrane protein–protein interactions. These observations and additional data from our laboratories indicate that the HCV nonstructural protein membrane segments serve not only as membrane anchors but exhibit additional functions in the context of the HCV replication complex.

2. HCV replication complex

As outlined above, the HCV nonstructural proteins form a membrane-associated replication complex together with viral RNA, altered cellular membranes, and additional as yet unidentified host cell components. In this context, physical interactions among nonstructural proteins have been described (Dimitrova et al., 2003). However, the protein–protein interactions and their hierarchy and dynamics within a functional replication complex are poorly defined.

Membrane alterations induced by the expression of HCV proteins were studied systematically using a panel of tetracycline-regulated cell lines inducibly expressing the entire HCV polyprotein or individual HCV proteins or their combination (Egger et al., 2002b). In this study, it was found that expression of the HCV polyprotein induces distinct membrane alterations. A prominent alteration, designated membranous web, was found to contain all viral structural and nonstructural proteins and, therefore, was proposed as a candidate replication complex (Egger et al., 2002b). The membranous web could be induced by NS4B alone and was very similar to the ‘sponge-like inclusions’ previously observed by EM in the liver of HCV-infected chimpanzees (Pfeifer et al., 1980).

More recently, these studies were extended to Huh-7 cells harboring autonomously replicating HCV RNAs (Gosert et al., 2003). The membranous web could be identified in these cells as well and was found to contain HCV nonstructural proteins as well as viral plus-strand RNA (Fig. 4). Metabolic labeling of the nascent viral RNA with BrUTP in the presence of actinomycin D revealed that the membranous web is the site of viral RNA synthesis and, therefore, represents the replication complex of HCV (Gosert et al., 2003) (Fig. 5).

The similarity of the membranous web identified in the replicon cells with that previously found in tetracycline-

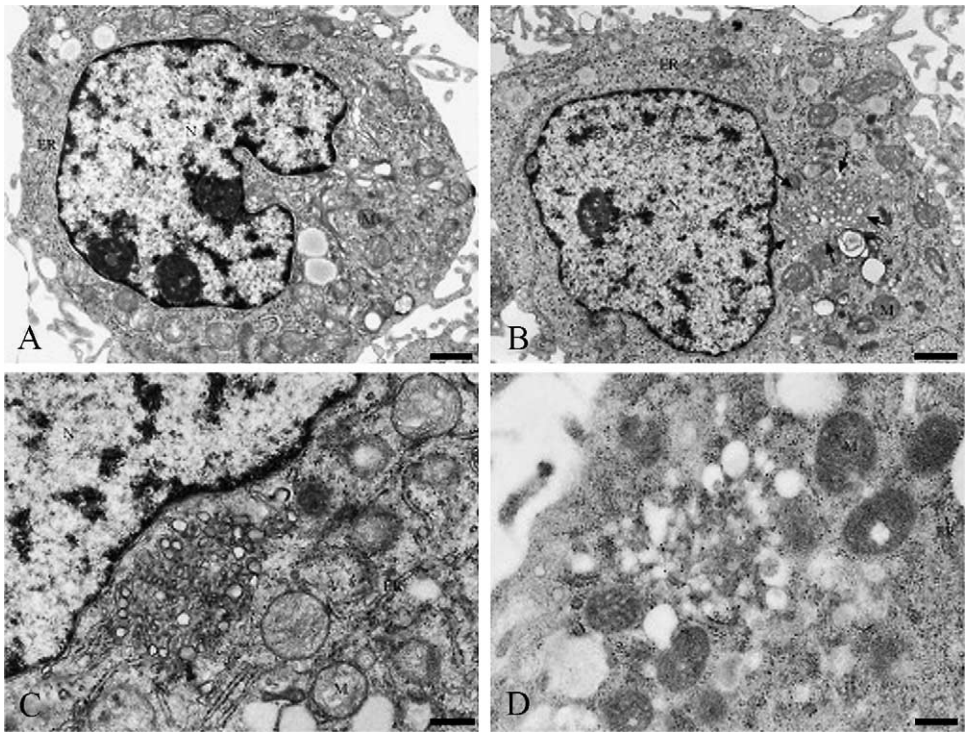

Fig. 4. Identification of the membranous web in Huh-7 cells harboring subgenomic replicons. (A) Low-power overview of a naïve Huh-7 cell. The cell displays a characteristic morphology with unaltered organelles. Bar, 1 μm. (B) Low-power overview of a Huh-7 cell harboring a subgenomic HCV replicon. A distinct membrane alteration, designated membranous web (arrows), is found in the juxtanuclear region. Note the circumscript nature of this specific membrane alteration and the otherwise unaltered cellular organelles. Bar, 1 μm. (C) Higher magnification of a membranous web composed of small vesicles embedded in a membrane matrix. Note the close association of the membranous web with the rough ER. Bar, 500 nm. (D) Localization of NS5A by immuno electron microscopy in Huh-7 cells harboring a subgenomic replicon. Replicon cells were labeled with a monoclonal antibody specific for NS5A. Gold particles accumulate on the membranous web. In addition, some label is found on the ER. Bar, 500 nm. N, nucleus; ER, endoplasmic reticulum; M, mitochondria. Adapted from Gosert et al. (2003) with permission.

regulated cell lines indicates that expression of HCV proteins is sufficient to induce formation of the membranous web which accommodates active RNA replication. This is consistent with results obtained with other plus-strand RNA viruses where single proteins expressed alone or in the context of the entire open reading frame induced membrane alterations very similar to membrane changes found during natural infection (Gosert et al., 2000; Snijder et al., 2001; Teterina et al., 2001).

As in the tetracycline-regulated cell lines, the membranous web was often found closely associated with the rough ER. Based on this observation, together with earlier studies

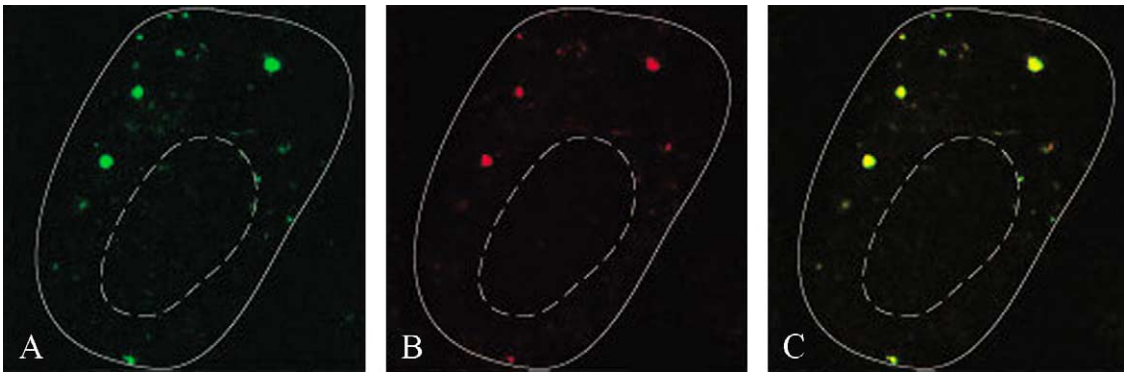

Fig. 5. Detection of nascent HCV RNA in Huh-7 cells harboring a subgenomic replicon. Replicon cells were metabolically labeled with BrUTP in the presence of actinomycin D, followed by double-labeling confocal laser scanning microscopy. (A) Detection of NS5A with a polyclonal antiserum and a FITC-conjugated secondary antibody. (B) Detection of newly synthesized, BrU-labeled viral RNA with a monoclonal antibody against BrdU and a Texas Red-conjugated secondary antibody. (C) The overlay demonstrates colocalization (yellow) of NS5A, which serves as a marker of the membranous web (see Fig. 4), and nascent viral RNA. Lines denote the border of the cell and the nucleus. Each image covers an area of 37 μm × 37 μm. Adapted from Gosert et al. (2003) with permission.

demonstrating the colocalization of individually expressed HCV proteins with membranes of the ER (Wölk et al., 2000; Hügle et al., 2001; Schmidt-Mende et al., 2001; Brass et al., 2002) and recent data indicating that HCV RNA replication takes place in a compartment that sustains endoglycosidase H-sensitive glycosylation (Ivashkina et al., 2002), we speculate that the membranous web is derived from membranes of the ER. Ongoing studies are aimed at isolating and further characterizing this complex and at defining the viral and cellular processes involved in formation of the membranous web. Elucidation of these processes will in all likelihood allow to define novel targets for antiviral intervention against hepatitis C.

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