Hepatitis C Virus Structural Proteins Reside in the Endoplasmic Reticulum as Well as in the Intermediate Compartment/cis-Golgi Complex Region of Stably Transfected Cells

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The intracellular localization of hepatitis C virus structural proteins was analyzed by confocal immunofluorescence microscopy, cell fractionation, and immunoelectron microscopy in stably transfected cells that do not overexpress the viral proteins. The results strongly suggest that at steady state the structural proteins reside not only in the endoplasmic reticulum but also in the intermediate compartment and cis-Golgi complex region. By analogy with other viral systems, this finding raises the possibility that the intermediate compartment and cis-Golgi complex play a role in the assembly and budding of hepatitis C virus.

INTRODUCTION

Hepatitis C virus does not replicate in tissue-cultured cells, thus very little is known about the assembly and budding of the viral progeny. Several reports of transfection experiments have documented that the three structural proteins (core and glycoproteins E1 and E2) do not reach the plasma membrane but accumulate in internal membranes, identified as ER, and that the glycoproteins form variable amounts of unfolded/misfolded aggregates (Choukhi et al., 1998; Dubuisson et al., 1994; Ralston et al., 1993; Santolini et al., 1994; Selby et al., 1993). Both features probably contribute to the low level of assembly of the progeny virus in the infected individuals. In addition, the absence of surface exposure of the glycoproteins may play a role in impairment of an efficient response of the host immune system, thus favoring the development of persistent infection. However, most of the studies mentioned above were performed after transient transfections in which the glycoproteins were overexpressed with strong promoters. This situation enhances the amount of unfolded/misfolded aggregates destined to be retained in the ER because of the inability of the protein to pass the quality control process (Ellgaard et al., 1999). Therefore, to determine the intracellular localization of HCV structural proteins, we have chosen as model system the stably transfected clone HCV1b-4 (Santolini et al., 1994), isolated from CV1 cells. In this clone, the expression of the viral structural proteins coded by the HCV-BK strain is under the control of an inducible promoter, which allows the modulation of the amount of protein synthesized. In this paper, we present results from morphological and cell fractionation studies showing that HCV structural proteins are not confined to the ER but are also present in the IC and cis-Golgi complex of HCV1b-4 cells.

RESULTS AND DISCUSSION

First we analyzed by immunofluorescence microscopy the biosynthesis of HCV structural proteins in HCV1b-4 cells. These cells have integrated the pHMT(HCV1b) plasmid that contains the metal-inducible human metallothionein IIa promoter to drive the transcription of a truncated mRNA expressing the first 861 aa residues of HCV polyprotein (Santolini et al., 1994; Schmidt et al., 1985). HCV proteins were not detectable in the absence of induction: they started to appear about 6 h postinduction with 5 μM CdCl₂, to reach maximum expression at 12–14 h (data not shown). Thus, we chose 9 h of induction, a time point that showed suboptimal synthesis in the absence of morphological alteration of the cells. At this time of induction, E2 and core proteins appeared in Western blot analysis after SDS–PAGE as single bands of the expected molecular weight of about 70 and 20 kDa, respectively (Fig. 1A). E1 protein was not detected by E1a antibody, which worked poorly in Western blot and immunoprecipitation analyses. However, the protein order on the HCV polyprotein precursor (core–E1–E2 from the aminoterminal), the results described in Fig. 1, and the immunofluorescence evidence (see below) make it likely that E1 glycoprotein was synthesized by these cells.
Several reports have documented that HCV structural proteins tend to form covalently and noncovalently bound oligomeric aggregates in highly expressing cells (Choukhi et al., 1998; Dubuisson et al., 1994; Ralston et al., 1993; Selby et al., 1993), so we addressed this point in induced HCV1b-4 cells. When the reduction step in the SDS–PAGE analysis was omitted (Fig. 1A), core protein migration was unchanged and E2 glycoprotein migrated as a single band of about 140 kDa, in the absence of higher molecular weight aggregates. This band most likely represents E2 homodimers stabilized by disulfide bridges. When the HCV proteins were analyzed by sucrose gradient (see Material and Methods) and the content of the collected fractions analyzed by 12.5% SDS–PAGE and Western blot. The nitrocellulose filter was cut at the position of the 52-kDa marker and the two halves were incubated with the R21 and C1 antibodies to detect E2 and core protein, respectively. The positions of molecular weight markers (in kDa) run in a parallel gradient are indicated at the top. (C) The amount of each protein in the fractions shown in B was quantified by laser scanning and reported as percentage of total. The profile of the sucrose gradient is indicated.

FIG. 1. Biosynthesis of HCV structural proteins in HCV1b-4 cells. The cells were induced with CdCl₂, for 9 h and lysed as detailed under Material and Methods. (A) Parallel aliquots of the lysate were processed for SDS–PAGE, in the presence or absence of reducing agents (DTT), followed by Western blot analysis developed with anti-core or anti-E2 glycoprotein antibody. The positions of molecular weight markers (in kDa) are indicated on the left. E2 and core protein were analyzed on 10 and 12.5% gels, respectively. (B) The lysate was centrifuged on a velocity sucrose gradient and the content of the collected fractions analyzed by 12.5% SDS–PAGE and Western blot. The nitrocellulose filter was cut at the position of the 52-kDa marker and the two halves were incubated with the R21 and C1 antibodies to detect E2 and core protein, respectively. The positions of molecular weight markers (in kDa) run in a parallel gradient are indicated at the top. (C) The amount of each protein in the fractions shown in B was quantified by laser scanning and reported as percentage of total. The profile of the sucrose gradient is indicated.

Next we studied the distribution of glycoprotein E2 by cell fractionation (Erra et al., 1999). As shown in Fig. 3, the majority of E2 glycoprotein cosedimented with the
ER, but a significant portion trailed in the Golgi and IC region, indicated by ERGIC-P53 and GS28, prototype protein markers for the IC and cis-Golgi complex, respectively (Schweizer et al., 1988; Subramanian et al., 1996). Therefore, to establish if a fraction of HCV structural proteins is indeed present in the IC and cis-Golgi complex, we performed additional confocal light microscopy assays. As shown in Fig. 4, a fraction of E2 and core proteins did colocalize in the perinuclear region with ERGIC-P53 and GS28 proteins. Conversely, calnexin and ERGIC-P53 did not colocalize (Fig. 4c), indicating that ER and IC were not altered in these cells. As expected, ERGIC-P53 and GS28 showed only a minor overlap (not shown), and all the results were confirmed by analysis of single optical sections (Fig. 4, SS). In addition, E2 and GS28 protein did show a partial colocalization in cells induced for only 7.5 h (data not shown). Given that only a very small amount of viral proteins is accumulated in cells induced for 7.5 h compared to 9 h, the localization of HCV structural proteins in the IC/cis-Golgi region does not appear to depend on their expression levels.

In order to obtain definitive evidence on the presence of HCV structural proteins in the IC/cis-Golgi region, we analyzed the intracellular localization of E2 glycoprotein by immuno-electron microscopy of ultrathin cryosections. As shown in Fig. 5, the immunogold labeling of E2 glycoprotein appeared mostly present on ER membranes (Figs. 5a and 5b), but specific immunolabeling was also observed on several vesicles and small tubules of the IC as well as over cis-Golgi cisternae and associated vesicles (Figs. 5c and 5d). Plasma membranes and endosomal compartments appeared unlabeled (not shown), and no viruslike particles were detected in the cells (Baumert et al., 1998). Thus, although it was not possible to extend these results to core and E1 protein because the specific antibodies worked poorly in immuno-electron microscopy, all the results strongly indicated that a fraction of HCV structural proteins is present in the IC/cis-Golgi region of HCV1b-4 cells. Finally, in a first attempt to compare the structural proteins present in the IC/cis-Golgi region with those contained in the ER, we analyzed by velocity sucrose gradient the oligomerization condition of the E2 glycoprotein forms present in the ER- and IC/cis-Golgi-enriched regions of the cell fractionation gradient. The two profiles were very similar to each other and to the one reported in Fig. 1C (data not shown). However, the aggregated material sedimenting in the bottom of the gradient was enriched in the ER pool.
suggesting that the E2 forms that leave the ER have successfully undergone the quality control process (data not shown).

It is well documented that HCV glycoproteins do not acquire Endo H resistance (Deleersnyder et al., 1997; Dubuisson et al., 1994; Fournillier-Jacob et al., 1996;Ral-
ston et al., 1993), suggesting that they do not reach the medial/trans-Golgi regions of the secretory pathway. Recently, this finding has been extended by analysis of the N-glycans of HCV glycoproteins (Duvet et al., 1998; Mottola et al., 2000). No modifications due to Golgi-resident enzymes were detected, and this led to the proposal that HCV glycoproteins are tightly retained in the ER (Duvet et al., 1998), thanks also to the presence of specific retention signals (Duvet et al., 1998; Mottola et al., 2000), and do not recycle from the Golgi complex as other ER proteins (Martire et al., 1996; Lotti et al., 1999). However, there is no compelling evidence in the literature that N-glycans-modifying enzymes reside in the IC/cis-Golgi complex; thus, the results we present herein do not conflict with the previously mentioned results. On the other hand, it is well known that different viruses start

FIG. 4. Localization of a fraction of E2 and core proteins in the IC and cis-Golgi complex. HCV1b-4 cells were processed for immunofluorescence analysis and viewed with a laser confocal microscope as described for Fig. 2. The results are presented as in Fig. 2. The F8B12 and R21 antibodies were used for detecting core and E2 proteins, respectively.
their assembly in the IC/cis-Golgi region (Griffiths and Rottier, 1992; Klumpermann et al., 1994; Peterson, 1991). Therefore, an interesting alternative hypothesis is that the HCV structural proteins that go beyond the ER in the secretory pathway are the ones that start the low level of virus assembly and budding which is responsible for HCV spreading in the natural host. The fact that at steady state only a fraction of the proteins is in the IC/cis-Golgi region, while the majority stays in the ER, must be considered with caution: it is concentration rather than absolute quantity to drive viral assembly and budding, and it is known that the ER has a much larger surface area than the IC and cis-Golgi complex (Griffiths, 1993; Lotti et al., 1999). Obviously, further work is needed to test the role of the IC and cis-Golgi complex in HCV assembly. It will be important to compare the concentration of HCV structural proteins in the ER and IC/cis-Golgi region, to verify if the HCV-H strain reproduces the results obtained with the BK strain, and to determine if the structural proteins that are present in the IC/cis-Golgi region are different from their counterparts present in the ER. Finally, an interesting model system has been recently described for studying the replication of a subgenomic HCV RNA that codes for all the nonstructural proteins in stably transfected cells (Lohmann et al., 1999). Hopefully, this approach will be developed to provide a model system for the replication of the entire HCV genome. In the meantime, although the structural protein portion is not present in the subgenomic RNA, it would be interesting to see if ER and IC/cis-Golgi region membranes play a role in this replication process.

MATERIAL AND METHODS

Materials

All culture reagents were supplied by Sigma–Aldrich (Milan, Italy). Solid chemicals and liquid reagents were obtained from E. Merck (Darmstadt, Germany), Farmitalia Carlo Erba (Milan, Italy), Serva Feinbiochema (Heidelberg, Germany), and BDH (Poole, United Kingdom). The ECL kit was from Amersham–Pharmacia Biotech (Milan, Italy). The following antibodies were used: mouse mAb E1A (anti-E1 glycoprotein; Dubuisson et al., 1994), rabbit polyclonal R21 and mouse mAb 185A (anti-E2 glycoprotein; Santolini et al., 1994), mouse mAb C1 (anti-core protein), F8B12 human mAb F8B12 (anti-core protein; Santolini et al., 1994), mouse mAb anti-GS28 protein (Subramanian et al., 1996), mouse mAb G1/93 (anti-ER-GIC-53 protein; Schweitzer et al., 1988), rabbit polyclonal anti-calnexin (Lotti et al., 1999), peroxidase-conjugated anti-mouse and anti-rabbit IgG (Sigma-Aldrich), and Texas red-conjugated anti-mouse IgG and fluorescein-conjugated anti-rabbit IgG (Jackson Immunoresearch Laboratories, West Grove, PA).

Cell culture, preparation of cells extracts, SDS–PAGE and Western blotting, and velocity sucrose gradient analysis

HCV1b-4 cells were cultured as described previously (Santolini et al., 1994) and lysed with 10 mM Tris–HCl, pH 7.4, 150 mM NaCl, 1% Triton X-100, 1 mM EDTA, 20 mM NEM; aliquots of the lysate were processed for SDS–PAGE and Western blotting as detailed elsewhere (Marti et al., 1996; Montola et al., 2000). Alternatively, the cell lysates (1 ml) were analyzed on an 11-ml linear 5–20% sucrose gradient made up in lysis buffer (without NEM) and run in a Beckman SW 41 rotor for 19 h at 4°C. Fractions were collected from the bottom, concentrated by TCA precipitation, and analyzed by SDS–PAGE followed by Western blot.

Cell fractionation

Cell fractionation was performed exactly as detailed previously (Erra et al., 1999). The single fractions were

FIG. 5. Immunogold electron microscopical analysis of the localization of HCV E2 glycoprotein in HCV1b-4 cells. After induction, the cells were processed for cryosectioning and immunogold analysis. The immunolabeling of E2 glycoprotein is mostly associated with ER membranes (a, b, arrows); gold particles are also observed on vesicles and small tubules on the IC (c, arrows), as well as on Golgi cisternae (cis-Golgi by morphological criteria) and peripheral Golgi-associated vesicles (d, arrows). The anti-E2 R21 antibody was used. er, endoplasmic reticulum; G, Golgi complex; bars, 0.2 mm.
concentrated by TCA precipitation and analyzed by SDS-PAGE followed by Western blot.

**Indirect immunofluorescence and immunoelectron microscopy**

Cells grown on glass coverslips were fixed with 4% formaldehyde for 20 min at room temperature and made permeable with 0.1% Triton X-100 in phosphate-buffered saline. Cells were labeled with the appropriate antibodies and with fluorescein and Texas red-conjugated secondary antibodies. Coverslips were mounted in Moviol and viewed by epifluorescence on a Zeiss Axiosom photomicroscope or a laser confocal microscope (LCM Zeiss 310) with a 63X planar objective. Cryosectioning and immunogold analysis were performed as detailed elsewhere (Liou et al., 1996).

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