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Research paper

Green fluorescent protein – Tagged HCV non-enveloped capsid like particles: Development of a new tool for tracking HCV core uptake

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ABSTRACT

Circulating 'free' non-enveloped Hepatitis C virus (HCV) core protein has been demonstrated in HCV-infected patients, and HCV subgenomes with deletions of the envelope proteins have been previously identified. Initial studies from our laboratory, previously published, indicated that expression of HCV core in insect cells can direct the formation of capsid-like particles lacking the envelope glycoproteins. These protein nanospheres, morphologically similar to natural capsids, were shown to be taken up by human hepatic cells and to produce cell-signalling events. To follow the intracellular fate of these particles we fused the core protein to eGFP. We demonstrate that the chimeric proteins core$_{173}$-eGFP, eGFP-core$_{191}$ and eGFP-core$_{173}$ can be efficiently expressed, self-assembled, and form fluorescent non-enveloped capsid-like particles. By using confocal microscopy and FACS analysis, we provide evidence that the fluorescent nanospheres can not only enter human hepatic cells – the main target of HCV – but also human immune cells such as T and B lymphocytes, as well as human myeloid leukaemia cells differentiated along the monocyte/macrophage-like pathway. The fluorescent particles might thus be used to trace the intracellular trafficking of naked HCV capsids as showed by live microscopy and to further understand their biological significance.

1. Introduction

Hepatitis C virus (HCV) is an enveloped virus belonging to the Flaviviridae family [1]. Its nucleocapsid is surrounded by a lipid bilayer containing the glycoproteins E1 and E2. However, different forms of HCV particles have been found in the circulation of infected individuals, among them naked capsids [2,3]. A plausible explanation for the presence of non-enveloped particles in the blood is their release into the circulation by the lysis of infected hepatocytes that accompanies liver inflammation. Non-enveloped particles have also been detected as viral inclusions in the cytoplasm of liver cells of infected patients [40]. Furthermore, HCV subgenomes with in-frame deletions of both envelope proteins were identified with relatively high abundance in the liver as well as in the serum of HCV infected individuals. The biological significance of these findings remains unclear although recently it was suggested that defective HCV clones might be associated with poor response to combination therapy [4–6]. Defective RNA viral genomes lacking the envelope coding sequence or possessing a natural stop codon have also been recently reported for other flaviviruses with important implications for their evolutionary dynamics and viral persistence [35].

Recently, Tsitoura et al. [7] reported the generation of recombinant non-enveloped HCV core particles in the absence of other HCV proteins and, more importantly, demonstrated that these naked capsids can be taken up by cells and induce cell-signalling phenomena. These intriguing properties of HCV core protein can be of great interest as a recent report highlights the possibility that subgenomic RNA resembling natural occurring deletion mutants are efficiently trans-packaged into virus-like particles by helper virus or helper cell lines [36].

For these reasons we aimed at developing a strategy for the labelling of the HCV non-enveloped capsid by using enhanced

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green fluorescent protein (eGFP). GFP and derivatives from it such as eGFP have been fused to a great number of proteins in order to study their intracellular trafficking and localization; this technique provides an attractive tool for viral tracking [8–13].

We here demonstrate that chimeric proteins $core_{173}$-eGFP, eGFP-$core_{191}$ and eGFP-$core_{173}$ can be efficiently expressed and self assembled in the baculovirus system. The three fusion proteins all form fluorescent non-enveloped capsid-like particles that can efficiently be taken up by human cells, allowing for the study of their intracellular trafficking to further understand the biological significance of naked HCV capsids.

2. Materials and methods

2.1. Plasmids

The HCV genotype 1a (H) cDNA [14] was used to generate the plasmid constructs used in this study. Core sequences encoding the full-length C_{191} and the truncated form C_{173} (this form is generated after cleavage by signal peptide peptidase [15]) were amplified by PCR using pHPI 1327 [7] as a template with the following primers: C_{191} forward 5′ ggaagatct**atg**acgaatcctaaacct 3′, C_{191} reverse 5′ ggaagatct**tta**gcctgaagcgggcacggtcaggca 3′, C_{173} reverse 5′ ggaagatct**tta**agagcaaccaggaaggttccctgt 3′ (BglII sites underlined, start and stop codons in bold). The PCR fragments were digested with BglII and ligated with BamHI-digested pEGFP/C1 to generate plasmid pHPI 1739 with the eGFP sequence fused in frame with C_{173} and pHPI 1738 with the C_{191} fused to the C-terminus of eGFP. $Core_{173}$ was fused to the N-terminus of eGFP via PCR by using pHPI 1327 as template with the following primers: $C_{173\text{-}c}$ forward 5′ cgcggatcc**atg**acgaatcctaaacctcaa 3′ and $C_{173\text{-}c}$ reverse 5′ gcgggatccagagcaaccaggaaggttccctgt 3′ (BamHI underlined and start codon in bold). The PCR fragments were digested with BamHI and ligated with BamHI-digested pEGFP/N3 to generate plasmid pHPI 1735 with C_{173} fused in frame to the amino terminus of eGFP.

To generate the recombinant baculoviruses (Bac 1736, Bac1740 and Bac 1741) expressing the HCV core-eGFP fusions under the polyhedrin promoter, the BacPAK8 (Clonetech) vector was used. The NotI-EcoRI C_{173}-eGFP cassette obtained from pHPI 1735 was cloned into the NotI – EcoRI site of BacPaK8 yielding pHPI 1736. Similarly, NheI – EcoRI fragments released from pHPI 1738 and pHPI 1739 (eGFP-C_{191} and eGFP-C_{173} respectively) were cloned into the XbaI – EcoRI sites of BacPAK8 yielding pHPI1740 and pHPI1741, respectively. For generation of the recombinant pHPI 1746 control baculovirus, the eGFP coding region was isolated from pEGFP/N3 as EcoRI–NotI fragment and subcloned into the EcoRI–NotI sites of the BacPAK8 plasmid.

The GFP-ERF plasmid [44] was used for transient expression of ERF, and transfection in Huh7 cells was performed as previously described [7].

2.2. Cells and construction of recombinant baculoviruses

The *Spodoptera frugiperda* Sf9 cell line was used for the generation and propagation of recombinant baculoviruses Bac1736, Bac1740, Bac 1741 and Bac 1746. Cells were maintained in Sf900II SFM medium (Gibco) supplemented with 5% fetal bovine serum at 27 °C. Recombinant baculoviruses were obtained following standard protocols using Baculogold linearized DNA (BD Biosciences), and viruses were propagated in Sf900II SFM medium supplemented with 5% fetal bovine serum and 50 μg/ml gentamicin (Gibco). The titers of the viral stocks were determined by limited dilution on Sf9 cells. Bac 1432 was previously described [7].

2.3. SDS-PAGE and immunoblotting analysis

The time-course of expression of the different forms of the HCV core protein fused to GFP in Sf9 cells infected with the recombinant baculovirus Bac1736, Bac 1741 and Bac 1740 (or Bac1746 as a negative control) was determined by immunoblotting with polyclonal [7] or monoclonal (Alexis Biochemicals) anti-core and anti-GFP (Santa Cruz Biotechnology Inc.) antibodies. Cells grown in 6-well plates were infected at a multiplicity of infection (MOI) of ~7 and harvested at different times post infection (p.i.). An aliquot of the lysates was subjected to 12% SDS-polyacrylamide gel electrophoresis followed by electroblotting to nitrocellulose membrane (Schleicher & Schuell). Membranes were stained with Ponceau S (Sigma) and blocked with 5% nonfat dry milk in phosphate-buffered saline (PBS), 0.01% Tween 20 (Sigma) for 1 h, before the addition of the primary antibodies. The blots were then probed with horseradish peroxidase-conjugated goat anti-rabbit immunoglobulins (Chemicon) and were finally visualized with the ChemiLucent detection system (Chemicon).

2.4. Sucrose density gradient analysis

Sf9 cells (approximately 4×10^7) were infected with the Bac1736, Bac 1740 and Bac 1741, respectively, at MOI = 5 and harvested at 2 day p.i. The capsid-like particles were isolated from cell lysates, according to the procedure described before [7,16]. Briefly, the cleared cell lysate, after treatment with RNAse (Sigma) and DNAse (Promega Corp.) for 30 min, was pelleted through a 30% w/v sucrose cushion for 3h at 30,000 rpm. The pellet was resuspended in 50 mM Tris, 100 mM NaCl and layered onto a discontinuous 20–60% w/w sucrose gradient and ultracentrifuged at 35,000 rpm for 22 h (SW41 Beckman rotor).

Fractions (600 μl each) were collected from the top of the gradient, the density was determined by refractometry, and HCV antigen was analyzed both with the Ortho HCV core antigen ELISA test system (dilution 1:1000 in PBS), and by SDS-PAGE followed by immunoblotting. Total protein concentrations were measured with the Bio-Rad protein assay. The fractions were stored at −80 °C. Lysates from cells infected with the Bac 1746 control virus were fractionated by the same procedure.

2.5. Visualization of chimeric recombinant capsids

2.5.1. Immunofluorescence

Immunofluorescence microscopy was used to visualize putative HCV capsids, to estimate their diameter, and to confirm their antigenic nature. As standard for calibration, we used red fluorescent microspheres (Molecular Probes), 0.1 μm in diameter at 1:10,000 dilution.

A mixture of the different GFP positive fractions (1 μl) and of properly diluted microspheres (5 μl) was spread and air-dried on a glass slide. After mounting with mowiol, the slides were examined by confocal microscopy.

For colocalization of the GFP-tagged particles with core antigen a small quantity of the GFP positive gradient fraction from the various baculovirus extracts was spread and air-dried on a glass slide and processed for immunostaining as described before [7]. After fixation, the sample was incubated with polyclonal anti-core IgG followed by incubation with the anti-rabbit Alexa Fluor 568-conjugated secondary antibody (Molecular Probes). After mounting, the slides were examined with a Leica TCS-SP5 confocal microscope equipped with a 63× objective. Quantification was proceed with Image-Pro plus (Media Cybernetics).