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Functional expression, purification, characterization, and membrane reconstitution of non-structural protein 2 from hepatitis C virus

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ABSTRACT

Non-structural protein 2 (NS2) of the hepatitis C virus (HCV) is an integral membrane protein that contains a cysteine protease and that plays a central organizing role in assembly of infectious progeny virions. While the crystal structure of the protease domain has been solved, the NS2 full-length form remains biochemically and structurally uncharacterized because recombinant NS2 could not be prepared in sufficient quantities from cell-based systems. We show here that functional NS2 in the context of the NS2-NS3pro precursor protein, ensuring NS2–NS3 cleavage, can be efficiently expressed by using a wheat germ cell-free expression system. In this same system, we subsequently successfully produce and purify milligram amounts of a detergent-solubilized form of full-length NS2 exhibiting the expected secondary structure content. Furthermore, immuno-electron microscopy analyses of reconstituted proteoliposomes demonstrate NS2 association with model membranes.

1. Introduction

The HCV non-structural protein 2 (NS2) is critical for both HCV polyprotein processing and virus particle formation [1–3]. The C-terminal domain functions as a cysteine protease [4] that cleaves the NS2–NS3 junction to liberate fully functional NS3 thus promoting HCV RNA replication [5–7]. In addition, NS2 is thought to play a central organizing role during HCV assembly, and is likely involved in a complex network of interactions including E2, p7, NS3, and NS5A [8–12]. NS2 is a 217-amino-acid integral membrane protein that is bound to intracellular membranes *via* its hydrophobic N-terminal integral membrane domain (residues 1 to about 100). This domain is believed to comprise 3 transmembrane segments whose high resolution structures have been studied separately by NMR (nuclear magnetic resonance) spectroscopy, allowing us to propose a topology model of full-length, membrane-associated NS2 [8,13]. The X-ray crystal structure of the C-terminal protease domain displays a domain-swapped homodimer with composite catalytic triads including residues from the two chains [4]. This domain was shown to contribute to membrane association via two α-helices [14]. Although a wealth of *in cellulo* and functional studies has been carried out for NS2 [8,13,14], its biochemical analysis is hampered by the very low expression level of recombinant full-length NS2 using classical cell-based expression approaches [15]. We here present successful cell-free expression of the protein using wheat germ extracts (WGE) in the presence of detergent [16–20]. Moreover, we demonstrate that the protein can be obtained in a detergent-solubilized, well-folded, homogenous and functional form, and that it interacts with model membranes.

Abbreviations: CD, circular dichroism; CFS, cell-free sample; DDM, n-dodecyl β-D-maltoside; HCV, hepatitis C virus; MNG-3, lauryl maltose neopentyl glycol; NMR, nuclear magnetic resonance; NS2, non-structural protein 2; NS3, non-structural protein 3; PC, L-α-phosphatidylcholine; PCR, polymerase chain reaction; WGE, wheat germ extract.

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2. Results

2.1. NS2 can be efficiently produced in an active form using a wheat germ extract (WGE) cell-free expression system

Expression of NS2 with a *Strep*-tag II (ST) fused at its N-terminus in the WGE cell-free expression system relies on the production of synthetic, *in vitro* transcribed RNA encoding the protein sequence, which is added to a reaction mixture containing the WGE, together with energy-sources, energy-recycling enzymes and amino acids needed for protein synthesis [16,21,22]. Using this approach, NS2 from different HCV genotypes comprising isolates Con1 (genotype 1b), H77 (genotype 1a), JFH-1 (genotype 2a) and 452 (genotype 3a) was successfully expressed (Fig. 1A). In the absence of detergent, the protein is mainly insoluble as indicated by its localization in the pellet fraction. Only a minor portion of NS2 protein was found in the supernatant (denoted "SN-beads" since the tagged protein is concentrated by binding of the NS2 N-terminal *Strep*-tag II to magnetic *Strep*-Tactin beads). However, in the presence of 0.1% lauryl maltose neopentyl glycol (MNG-3) detergent, fully solubilized JFH-1 and Con1 NS2 were obtained (see also reference [16]) while H77 and 452 NS2 partially remained in the pellet. In order to evaluate functionality of NS2 obtained in this expression system, we monitored its cysteine-protease activity. To this end we expressed a JFH-1 NS2-NS3pro *Strep*-tagged precursor construct (denoted NS2-NS3pro-ST) that includes the 213 N-terminal amino acids of NS3, and detected the cleavage products using antibodies directed either against the NS3pro C-terminal *Strep*-tag II (denoted NS3pro-ST), or an antibody against NS2. Note

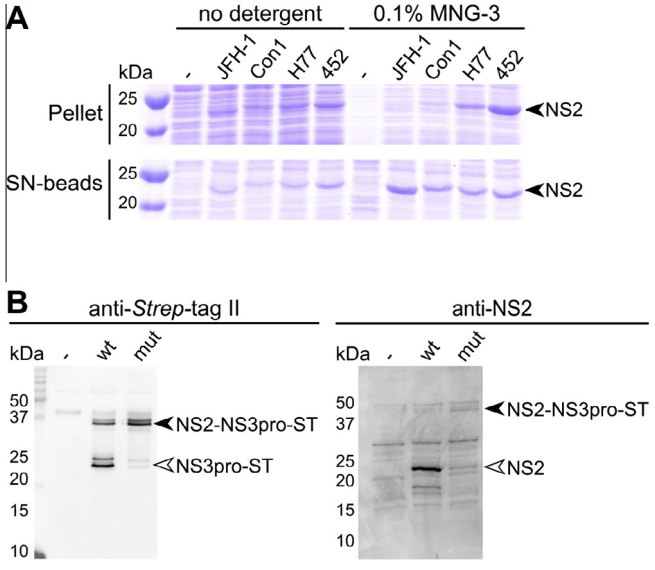

Fig. 1. Expression and protease activity of full-length NS2 in the wheat-germ extract cell-free system. (A) Full-length NS2 from different HCV strains were expressed in the absence of detergent, (left) or in the presence of 0.1% MNG-3 (right) as described previously [16]. Protein samples were analyzed by SDS–PAGE followed by Coomassie blue staining. –, negative control (no NS2 expression); Pellet, pellet obtained after centrifugation of total cell-free sample (CFS); SN-beads, supernatant obtained after centrifugation of CFS and incubated with *Strep*-Tactin magnetic beads to capture tagged NS2. Comparable amounts were loaded on the gel for Pellet and SN-beads. Bands corresponding to full-length NS2 are indicated by black arrowheads. (B) Wild type and mutant NS2-NS3pro-ST precursors (strain JFH-1) were expressed in the presence of 0.1% MNG-3. Total CFS were analyzed by immunoblotting using antibodies either against the *Strep*-tag II fused at the C-terminus of NS3pro-ST (left panel) or against the C-terminal domain of NS2 [13] (right panel). –, negative control (no NS2-NS3pro-ST expression); wt, wild type NS2; mut, NS2 with C184A mutation. Bands corresponding to uncleaved NS2-NS3pro-ST protein precursor are indicated by black arrowheads while cleaved NS2 and NS3pro-ST are indicated by empty arrowheads. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

that NS3pro-ST was inactivated (S139A mutation) to avoid unspecific cleavages by this protease. The immunoblot displayed in Figure 1B shows that both cleavage products, being NS3pro-ST and NS2, could be detected in the reaction mixture. Cleavage specificity was confirmed by using the inactive NS2 mutant bearing the C184A mutation in its catalytic site, for which accumulation of uncleaved NS2-NS3pro-ST precursor was observed.

2.2. Solubilized NS2 is obtained in a pure and homogenous form

A major challenge in the expression of membrane proteins is to isolate the protein in a pure form, and to avoid formation of aggregates. The N-terminal *Strep*-tag II allowed us to obtain pure NS2 protein using a single affinity-purification step starting from the supernatant fraction containing the solubilized protein (Figure 2A). It is worth mentioning that the addition of 0.25% DDM before the affinity-purification step not only allowed buffer exchange from MNG-3 to DDM, but resulted in improved NS2 purity and yield. Indeed, the purity of NS2 obtained in the presence of MNG-3 during this step was not satisfactory [16], as also observed in the presence of lower amounts of DDM (data not shown). While purity was good in the presence of even higher amounts of DDM (0.5–1%), the protein yield was substantially reduced under this condition since the high detergent concentration decreases NS2-*Strep*-tag II binding to the *Strep*-Tactin column [23]. The yield of purified NS2 was about 1 mg per mL of WGE. To further characterize purified NS2 protein, we performed size exclusion chromatography. A major peak containing NS2 was observed (Fig. 2B), which is slightly asymmetric, possibly due to some non-specific binding interaction of NS2 with the SEC media. This leads to trace amounts of NS2 in the following minor peak, which is mainly due to reagents present in the *Strep*-Tactin elution buffer. NS2 protein-detergent complexes eluted with an apparent molecular mass of about 150 kDa (Fig. 2C). From the ratio DDM-protein reported for membrane proteins in Moller and Le maire [24], one can estimate that for small monomeric membrane proteins including one, two or three transmembrane passages (such as NS2), the molecular mass of the protein-DDM micelle should be around the sum of molecular masses of the protein and a pure DDM micelle (i.e., 75 kDa taking into account a maximum aggregation number of 140 and a molecular weight of 511 for DDM). As the MW of NS2 is 25 kDa, one can infer that the size of the DDM micelles containing a monomer of NS2 would be around 100 kDa. Hence, the fact that the apparent MW of NS2-containing micelles measured by gel filtration is much higher (150 kDa) suggests an oligomeric arrangement of NS2. It is worth mentioning that the isolated NS2pro domain has been determined by X-ray crystallography to be a dimer [4].

2.3. NS2 secondary structure

The far UV circular dichroism (CD) spectrum of full-length NS2 eluted from size exclusion chromatography in 0.1% n-dodecyl β-D-maltoside (DDM) is typical of a well-folded protein (Fig. 3). The two minima at 208 and 222 nm, together with a maximum at 192 nm, are typical of α-helical folding. Spectral deconvolution methods yield an α-helix content of 52–58%, while turn content is 12–17%, and β-sheet content is limited to 8–9%. The latter values are consistent with the content in β-sheet deduced from the crystal structure of the cytosolic ectodomain of NS2 [4]. The limited number of residues folded into α-helices in this crystal structure indicates that the main contribution to helix content in full-length NS2 is due to its membrane domain. This is in agreement with our previous structural analyses of this membrane domain using synthetic peptides, which mostly revealed the presence of α-helical segments [8,13].