Mobility of the hepatitis C virus NS4B protein on the endoplasmic reticulum membrane and membrane-associated foci

Sarah N. Gretton,† Annette I. Taylor‡ and John McLauchlan

MRC Virology Unit, Institute of Virology, Church Street, Glasgow G11 5JR, UK

The hepatitis C virus (HCV) non-structural protein NS4B induces morphological changes in the endoplasmic reticulum (ER) membrane that may have a direct role in viral RNA replication. A chimeric GFP–NS4B fusion protein located to the ER membrane and to foci that were attached to the ER. These membrane-associated foci (MAFs) could be related to the membrane alterations observed in cells that replicate HCV RNA. The relationship of MAFs to pre-existing cellular structures is not known. Indirect immunofluorescence analysis demonstrated that they did not contain a cellular marker for vesicles, which have been implicated in the replication of other viruses. From photobleaching studies to examine diffusion of NS4B, the GFP-tagged protein had reduced mobility on MAFs compared with on the ER membrane. This slower mobility suggested that NS4B is likely to form different interactions on MAFs and the ER.

Hepatitis C virus (HCV) RNA replication requires a minimum of five viral proteins (NS3, NS4A, NS4B, NS5A and NS5B) (Lohmann et al., 1999; Blight et al., 2000; Ikeda et al., 2002). Viral RNA synthesis is considered to occur in foci, which are novel intracellular structures generated by morphological alteration of the endoplasmic reticulum (ER) membrane (Gosert et al., 2003; Moradpour et al., 2004). The appearance of foci correlates with the presence of a membranous web that is detected by electron microscopy (Egger et al., 2002; Gosert et al., 2003) and it is probable that the foci and membranous web are related, if not identical, structures. Expression of NS4B alone is sufficient for induction of these morphological changes (Egger et al., 2002; Lundin et al., 2003). However, the mechanisms that drive their formation and the nature of the association of NS4B with these structures have not been elucidated. Here, we examined the behaviour of NS4B in live cells to determine the characteristics of the morphological alterations produced by the protein and to analyse its mobility on membranes.

To examine NS4B in live cells, the polypeptide was attached to the C terminus of EGFP. A PCR product encoding NS4B (aa 1712–1973 of HCV 1a strain H77) was amplified from pcV-H77C (kindly supplied by Dr J. Bukh; Yanagi et al., 1997) using forward and reverse primers (5′-GGGAGAATTCTAGATCAGCCTACATCATCAGG-3′ and 5′-GGGAAAGCTTCTAGAGATCGCTTAGATGGAGTTCACACTCCGAGC-3′, respectively) that incorporated restriction enzyme sites for cloning purposes and a stop codon (underlined). The PCR product was introduced into pGEM-1 following digestion with EcoRI and XbaI, to generate plasmid pGEM-1/NS4B. A BglII/XbaI DNA fragment from pGEM-1/NS4B was inserted into pEGFP-C1 (Clontech) to create pGFP-NS4B. pGFP-NS4B was transfected into tissue culture cells estimated to be between 30 and 60 % confluent using Lipofectamine 2000 (Invitrogen). To express untagged protein, an oligonucleotide (5′-AAATTCATGCCTACATTGATGA-3′) that incorporated an initiator methionine codon was introduced between the EcoRI and BglII sites in pGEM-1/NS4B to produce plasmid pGEM-1/atg-NS4B. A BamHI DNA fragment from pGEM-1/atg-NS4B was inserted into the BamHI site in the Semliki Forest virus vector pSFV-1, generating pSFV/NS4B. RNA was transcribed from pSFV/NS4B and electroporated into tissue culture cells as described previously (Patel et al., 1999; Hope & McLauchlan, 2000). Untagged NS4B was detected using a polyclonal antiserum called R1061, which was raised against two synthetic peptides, LAEQKFWKALQFLT and QTNWQKLEVFVAKH, derived from aa 13–28 and 40–53, respectively, of the strain H77 polypeptide (NCBI accession number NP_751926). For live studies, cells were seeded onto glass-bottomed 35 mm Petri dishes (MatTek). Photobleaching was performed in Dulbecco’s modified Eagle’s medium lacking phenol red (Invitrogen), supplemented with 1 % fetal calf serum and 30 mM HEPES at 37 °C on a heated stage. Confocal microscopy was carried out with a Zeiss LSM510 META microscope.

Transfection of pGFP-NS4B into cells followed by examination under live conditions revealed that the fusion

†These authors contributed equally to this work.
‡Present address: Ingenza Ltd, King’s Buildings, Edinburgh EH9 3JJ, UK.
protein was distributed in a thread-like pattern, consistent with ER localization, and at small foci. This distribution was evident in BHK, HuH-7 [Fig. 1a, panels (i), (ii), (iv) and (v)] and Vero cells (data not shown) and therefore was not dependent on cell origin, although we did observe greater numbers of foci in BHK cells compared with HuH-7 cells. Using a specific NS4B antiserum, R1061, indirect immunofluorescence of cells that had been electroporated with RNA transcribed from pSFV/NS4B confirmed that both distributions were indicative of NS4B localization and did not arise from fusion to GFP [Fig. 1a, panels (iii) and (vi)]. We also verified that the thread-like pattern denoted association of NS4B with the ER membrane using an anti-calnexin antibody (kindly supplied by B. Martoglio, ETH, ETH Hoenggserberg, 8093 Zurich, Switzerland; Fig. 1b). These data agree with previously published studies (Lundin et al., 2003). At higher magnification, the foci were coincident with the ER membrane [Fig. 1a, panels (ii) and (v)], which demonstrated their close connection with the ER network. We propose that these NS4B-induced structures are referred to as membrane-associated foci (MAFs).

It is not known whether MAFs represent previously characterized sites on the ER. In poliovirus-infected cells, viral RNA replication occurs at vesicles induced by the virus protein 2BC. These vesicles include cellular proteins that coat COPII vesicles, which are ER exit sites (Rust et al., 2001). It was possible that MAFs also marked the position of COPII vesicles or recruited COPII proteins. However, analysis with an antibody specific for sec23 (E-19; Santa Cruz; Stephens et al., 2000), a component of COPII vesicles, showed that MAFs did not co-localize with these structures in either BHK [Fig. 1c, panels (iii) and (vi)] or HuH-7 cells (data not shown). Thus, the origin of MAFs remains unclear.

Analysis of GFP-tagged proteins in live cells enables monitoring not only of the movement of subcellular structures but also of protein diffusion within an organelle by applying a method termed fluorescence recovery after photobleaching (FRAP) (Reits & Neefjes, 2001). In FRAP experiments, fluorescent molecules in pre-defined areas are irreversibly photobleached by a high-power focused laser beam. Subsequent diffusion of non-bleached molecules into the bleached area leads to a recovery of fluorescence. To test whether NS4B was mobile in ER membranes and MAFs, fluorescence intensity in selected regions in live cells expressing the GFP-tagged protein was measured before and after photobleaching [Fig. 2a, panels (i) and (ii)]. For comparison, cells expressing GFP-tagged DNase X were examined under identical conditions [Fig. 2a, panels (iii) and (iv)]. GFP–DNase X targets the ER membrane (http://gfp-cdna.embl.de/loc-html/P49184.html) and has been used previously in live-cell studies (Targett-Adams et al., 2003).

Conditions were selected such that cells were exposed to the minimum number of bleaching iterations \((n=3, 100\ \text{%} \text{ laser power, 488 nm laser line})\) that reduced fluorescence intensity by 70–85 \% in a circular area of 38 \(\mu\text{m}^2\). Before and after bleaching, images were taken with 2 \% laser power. For GFP–DNase X, fluorescence recovered rapidly in BHK and HuH-7 cells to a mean of 82 and 88 \%, respectively, of the intensity prior to bleaching (Fig. 2b). In the case of GFP–NS4B, there was slower fluorescence recovery that was particularly evident in BHK cells and the intensity attained at the end of the monitoring period (64 and 75 \% in BHK and HuH-7 cells, respectively) was reduced compared with GFP–DNase X. These differences in recovery between the two proteins in both cell types were significant to a level of 1 \% by the Mann–Whitney U-test. In addition, there was a greater range of fluorescence recovery for GFP–NS4B compared with GFP–DNase X (see error bars in Fig. 2b). Therefore, diffusion of GFP–NS4B was slower and more variable than GFP–DNase X. Closer inspection of images during the recovery phase after photobleaching revealed that GFP–NS4B fluorescence returned to the ER membrane but not to MAFs (Fig. 3a). From re-evaluation of the GFP–NS4B data (Fig. 2b and c), it was noted that bleached regions with lower fluorescence recovery contained higher numbers of MAFs than areas with greater recovery. Therefore, we postulated that GFP–NS4B attached to MAFs had reduced mobility compared with fluorescent protein on the ER membrane. Indeed, photobleached areas that contained high numbers of MAFs gave levels of recovery reaching only 25 \% (Fig. 3b). To demonstrate that this property of GFP–NS4B associated with MAFs was not a general feature of vesicular structures attached to the ER, photobleaching studies were conducted in parallel with yellow fluorescent protein (YFP)–sec23A, which associates with COPII vesicles (Stephens, 2003). Using bleaching conditions identical to those for GFP–NS4B, fluorescence rapidly recovered to >85 \% of pre-bleach values for YFP–sec23A in both BHK and HuH-7 cells (Fig. 3c). Thus,
GFP–NS4B in MAFs and YFP–sec23A in foci attached to the ER membrane displayed distinct diffusion properties, with the viral protein having lower mobility.

In agreement with others (Egger et al., 2002; Konan et al., 2003; Lundin et al., 2003), our data showed that expression of NS4B alone induced morphological alterations at the ER membrane, which we have termed MAFs. Alterations also appeared as a membranous web in electron microscopic ultrastructural analysis (Egger et al., 2002; Gosert et al., 2003). MAFs and the membranous web may be identical features that represent sites of viral RNA synthesis (Gosert et al., 2003); however, their relationship to pre-existing regions of the ER is not known. MAFs do not co-localize with markers for either early endosomes or the intermediate compartment (Lundin et al., 2003). We showed that they also did not coincide with sec23, a component of COPII vesicles. Therefore, we concluded that HCV RNA replication is unlikely to occur at sites that contain or recruit COPII proteins. The number of MAFs was greater in BHK compared with HuH-7 cells. MAFs may be derived directly from the ER membrane and it is possible that their induction is dependent on achieving a critical concentration of NS4B at the ER membrane. BHK cells may have a less extensive ER network than HuH-7 cells and hence provide an environment where the NS4B concentration needed for MAF induction can be achieved more readily, leading to a greater likelihood that membrane alterations will occur.

Among the strategies that enable biophysical analysis of intracellular events, FRAP has emerged as a powerful method for monitoring the kinetics of protein movement (Reits & Neefjes, 2001). NS4B is mobile in the ER membrane, although its diffusion is slower compared with DNase X. Both NS4B (Lundin et al., 2003) and DNase X (data not shown) are predicted to contain transmembrane domains and therefore are topologically related. More importantly, we have demonstrated that NS4B has reduced mobility in MAFs compared with the ER membrane. These characteristics for NS4B were identical, irrespective of whether GFP was located at the N or C terminus of the protein (data not shown). We concluded that there are two populations of NS4B with distinct relative mobilities in the ER membrane and MAFs.

Several factors may account for the reduced diffusion of NS4B in MAFs. NS4B is an integral membrane protein (Hugle et al., 2001), predicted to contain at least four...
transmembrane segments (Lundin et al., 2003). Transmembrane regions behave as cylindrical molecules in a lipid environment and contribute considerably to diffusional movement. NS4B could form an oligomeric complex in MAFs that would effectively increase the bulk of transmembrane regions embedded in the membrane. Movement of an oligomeric complex in membranes would be retarded, although it should be noted that diffusion rate is proportional to the logarithm of the inverse of the radius of transmembrane segments (Saffman & Delbruck, 1975; Vaz

Fig. 3. FRAP analysis of GFP–NS4B and YFP–sec23A in BHK and HuH-7 cells. Cells were transfected with plasmids for 8–10 h prior to photobleaching. Circled areas in the panels in (b) and (c) denote regions selected for photobleaching. (a, b) Fluorescence recovery in BHK cells expressing GFP–NS4B. MAFs in the photobleached region are indicated by an arrow in (a). Images were recorded at the times indicated before and after photobleaching. In (b), the percentage fluorescence intensity is shown at 2 s intervals prior to and after photobleaching (indicated by an arrow). (c) Fluorescence recovery in BHK and HuH-7 cells expressing YFP–sec23A. The panels show images from a BHK cell recorded 0–2 s prior to (left panel) and 1 s after (right panel) photobleaching. Images were recorded at approximately 2 s intervals before and after bleaching (indicated by an arrow) and the percentage fluorescence intensity in photobleached regions was determined. Mean values for the percentage fluorescence intensity and standard errors were calculated from data obtained from several cells (n > 5).
et al., 1982). Thus, increasing the radius of transmembrane regions through oligomerization has less of an impact on diffusion than would be predicted intuitively. Alternatively, interactions between NS4B and cellular proteins in MAFs could be different from those in the ER membrane. Studies with GFP-tagged H2Ld, an MHC class I molecule involved in peptide presentation, have shown that two populations of the protein exist (Marguet et al., 1999). One form diffused rapidly in membranes and apparently was not associated with the transporter associated with antigen processing (TAP) complex. The second form had a much lower capacity to diffuse and was associated with TAP. These slower GFP–H2Ld molecules had a diffusion coefficient similar to TAP, leading to the conclusion that interactions between the two proteins constrained the mobility of H2Ld. Little is known about the interactions of NS4B with cellular components. If such interactions are established, it will be important to determine whether they could also influence the mobility of NS4B. Finally, interactions between NS4B and lipids present in MAFs could reduce its diffusion. For the cellular protein H-Ras, lateral diffusion is restricted through interaction with lipid rafts on the plasma membrane (Niv et al., 2002). NS4B also associates with lipid rafts (Gao et al., 2004), although we do not know whether NS4B in MAFs is indicative of its association with such membrane domains. None the less, MAFs could represent specialized regions in the ER membrane with a particular lipid composition that influences NS4B mobility.

The MAFs generated by NS4B alone could be equivalent to the foci present in cells harbouring the HCV replicon. Since MAFs and the ER membrane are apparently contiguous, we consider it likely that NS4B in MAFs originates from the pool of protein attached to the ER membrane that has greater mobility. However, after incorporation into MAFs, the movement of NS4B molecules may be blocked by the interactions that occur at these sites. Therefore, our results may have some bearing on the interpretation of the sites for viral RNA synthesis (Gosert et al., 2003). HCV RNA is detected in foci, suggesting that these structures represent locations of active replication complexes. Alternatively, replication complexes that are functional for RNA synthesis could diffuse rapidly along the ER membrane. Upon incorporation into foci, viral proteins and any associated viral RNA in these complexes would be restricted in their movement, resulting in accumulation at foci. Thus, foci may be sites at which synthesized RNA accumulates but not necessarily the locations for replication. Such a distinction is important for our understanding of HCV replication, and further experimental evidence is needed to test these possibilities.

Acknowledgements

We thank Drs R. Pepperkok, D. Stephens, A. Girod and J. Rietdorf for invaluable advice and the generous gift of pYFP–sec23A. We are grateful also to Dr J. Coy for pGFP–DNase X. We also thank Drs G. Hope and S. Graham for production of antisera R1061 and Dr D. Gatherer for statistical analysis. This work was supported by short-term fellowships to A. I. T. (from the Society for General Microbiology) and J. McL. (from the European Union under the Transnational Access to Major Research Infrastructures programme) for use of microscopy facilities at EMBL, Heidelberg, Germany.

References


Mobility of HCV NS4B protein in live cells


