Investigation of a role for lysine residues in non-structural proteins 2 and 2/3 of the hepatitis C virus for their degradation and virus assembly

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It has been demonstrated that both uncleaved, enzymatically inactive NS2/3 and cleaved NS2 proteins are rapidly degraded upon expression in cells, phenomena described to be blocked by the addition of proteasome inhibitors. As this degradation and its regulation potentially constitute an important strategy of the hepatitis C virus (HCV) to regulate the levels of its non-structural proteins, we further investigated the turnover of these proteins in relevant RNA replication systems. A lysine-mutagenesis approach was used in an effort to prevent protein degradation and determine any effect on various steps of the viral replication cycle. We show that, while NS2-lysine mutagenesis of protease-inactive NS2/3 results in a partial stabilization of this protein, the increased NS2/3 levels do not rescue the inability of NS2/3 protease inactive replicons to replicate, suggesting that uncleaved NS2/3 is unable to functionally replace NS3 in RNA replication. Furthermore, we show that the cleaved NS2 protein is rapidly degraded in several transient and stable RNA replicon systems and that NS2 from several different genotypes also has a short half-life, highlighting the potential importance of the regulation of NS2 levels for the viral life cycle. However, in contrast to uncleaved NS2/3, neither ubiquitin nor proteasomal degradation appear to be significantly involved in NS2 degradation. Finally, although NS2 lysine-to-arginine mutagenesis does not affect this protein’s levels in a JFH-1 cell culture infection system, several of these residues are identified to be involved in virion assembly, further substantiating the importance of regions of this protein for production of infectious virus.

INTRODUCTION

Hepatitis C virus (HCV) is a single-stranded positive-sense RNA virus of the family Flaviviridae (Choo et al., 1991; Miller & Purcell, 1990) and is an important cause of chronic liver disease. The RNA genome encodes a single polyprotein that comprises both structural as well as non-structural proteins. While host signal peptidases cleave the polyprotein in the structural region, the non-structural proteins are cleaved by two virally encoded proteases: NS2/3 and NS3. NS3 is a serine protease that cleaves at the NS3/4A and downstream junctions (Hijikata et al., 1993b) and also harbours C-terminal ATPase and helicase activities (Kim et al., 1995).

The HCV NS2/3 protease is an autocatalytic cysteine protease that is responsible for the cleavage between NS2 and NS3 and whose activity has been shown to be required for both viral infectivity and RNA genome replication (Grakoui et al., 1993; Hijikata et al., 1993a; Kolykhalov et al., 2000; Welbourn et al., 2005). With the recent introduction of the JFH-1 HCV cell culture infection system, the presence of the cleaved NS2 protein has been shown to be required for HCV virion assembly and viral infectivity, although its mechanism of action in this process remains unclear (Jirasko et al., 2008; Jones et al., 2007).

Cellular protein levels are tightly regulated by the balance between transcription/translation and degradation. Interestingly, many virally encoded proteins have also been demonstrated to be quickly degraded, suggesting that control of their levels could regulate their role in the viral life cycle (Mousnier et al., 2007; Schlax et al., 2007). Furthermore, levels of several HCV proteins such as core, E2 and NS5B have been suggested to be regulated at the
level of protein stability (Gao et al., 2003; Pavio et al., 2002; Suzuki et al., 2001), which may in turn modulate viral replication. Indeed, core degradation by the ubiquitin-proteasome pathway has recently been shown to involve interaction with E6AP. When levels of this ubiquitin ligase were increased, a decrease in core levels was observed as well as a decrease in viral titres in a cell culture system (Shirakura et al., 2007).

Most cellular proteins are degraded via the ubiquitin-proteasome pathway. In this system, poly-ubiquitin chains are attached to lysine residues of the target protein, which is then recognized for degradation by the 26S proteasome (for review see Hershko & Ciechanover, 1998).

Interestingly, both uncleaved mutant NS2/3 and processed NS2 have been described by us and others to be rapidly degraded in cells, phenomena which were inhibited by the addition of proteasome inhibitors (Franck et al., 2005; Welbourn et al., 2005). The degradation of these proteins could constitute an important way for the virus to regulate the levels of its own proteins and therefore its own replication or assembly. However, most of the work on NS2 degradation was performed using tagged NS2 expressed alone (Franck et al., 2005) which could differ from what occurs with the untagged protein in its viral RNA context. We decided to further investigate the degradation of these proteins in more relevant replication and infection systems. We used a lysine-mutagenesis approach in an effort to prevent protein degradation and determine any effect on various aspects of the viral life cycle.

We report here that, while NS2-lysine mutagenesis of protease inactive NS2/3 results in a partial stabilization of this protein, the increased levels are unable to rescue the inability of NS2/3 protease inactive replicons to replicate, suggesting uncleaved NS2/3 is unable to functionally replace NS3 in genome replication. Furthermore, using several different DNA and RNA replicon cell systems, we show that the cleaved NS2 protein from several relevant HCV genotypes is rapidly degraded, highlighting the potential importance of the regulation of NS2 levels for the viral life cycle. Although NS2 lysine mutagenesis is found not to affect this protein’s levels, we identified several residues in NS2 to be required for infectious HCV virion assembly in a JFH-1 cell culture infection system, further substantiating the importance of regions of this protein for viral infectivity.

**METHODS**

**Constructs.** To generate pcDNA32xFNS2 DNA constructs and to insert NS2 mutations into pNeo/2-5B and pFK-repPI-luc/NS2-3’ (Welbourn et al., 2005), subgenomic Con1 replicons containing NS2–NS5B, pFK-l38neo/Con3’/5.1 (Pietschmann et al., 2002) and pJFH-1 (Wakita et al., 2005) standard subcloning techniques and Quick Change mutagenesis (Stratagene) were used. See supplementary material, available in JGV Online, for details.

**Cell culture.** Huh-7, Huh7.5 (generously provided by C. Rice, Rockefeller University, NY, USA) and Huh7-Lunet cells were grown in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal bovine serum, 100 U penicillin ml⁻¹, 100 µg streptomycin ml⁻¹ and 100 µM non-essential amino acids. G418 (500 µg ml⁻¹) was also added to stable replicon cell lines.

**In vitro transcription.** See supplementary material, available in JGV Online.

**Generation of stable replicon cell lines.** Subconfluent Huh-7 (pNeo/2-5B WT), Huh-7.5 (pNeo/2-5B WT, pNeo/2-5B K-6-R, core-3’/5.1 WT, core-3’/5.1 K-6-R) or Huh7-Lunet (pNeo/2-5B WT) cells were electroporated with 1 µg RNA and selected with 500 µg G418 ml⁻¹ as described previously (Lohmann et al., 2001). Individual clones were expanded except in the case of the full-length constructs where pools were generated.

**Transient replication assays and production of infectious HCV.** Transient replication assays were performed as described previously (Welbourn et al., 2005). For production of infectious HCV, Huh7.5 cells were transfected with 5 µg of a given _in vitro_ transcript and processed as described.

**Transient transfection for pulse–chase analysis.** For DNA constructs, Huh7.5 cells were transfected using lipofectamine 2000 (Invitrogen), whereas luciferase replicons (20 µg RNA) were electroporated into Huh7-Lunet cells as described above. Transfected cells were used for pulse–chase analysis 48 or 72 h after transfection as described below.

**Pulse–chase analysis.** For analysis of protein half-life, the relevant cells were methionine-starved for 1 h using methionine and cysteine-free DMEM supplemented with 10% serum, penicillin–streptomycin and L-glutamine. Cells were then pulsed in the same medium containing 250 µCi [³⁵S]methionine/cysteine (1.29 MBq ml⁻¹; Redivue Pro-mix, GE Healthcare) for 3 h. After washing with PBS, complete media supplemented with 0.05% cold methionine was added for the relevant chase periods after which the cells were harvested and lysed using lysis buffer (50 mM Tris/HC1 pH 8.0, 150 mM NaCl, 1% NP-40) with addition of protease inhibitors. Cleared cell lysates were immunoprecipitated by incubation with NS2 antibody for 2 h followed by addition of 50/50 protein A/G coupled agarose beads (Upstate Cell Signalling Solutions) for an additional 2 h. NS3 immunoprecipitation was then performed in the same way. For FLAG-tagged proteins, lysates were incubated for 3 h with EZView Red ANTI-FLAG M2 Affinity Gel (Sigma). The beads were then washed four times with wash buffer (50 mM Tris/HC1 pH 8.0, 900 mM NaCl, 1% NP-40) and once with lysis buffer. Bound proteins were eluted with SDS-sample buffer, run on SDS-PAGE, and visualized by autoradiography. Relative band intensity was measured using a Fuji X BAS 2000 phosphorimager.

**Immunoblotting.** For immunoblot analysis of protein levels after transient electroporation, 20 µg RNA (5 µg for JFH-1 and derivatives) was electroporated as described above. For proteasome inhibitor treatment, cells were seeded and 10 µM MG132 (Sigma) or vehicle control (Me₂SO) were added 4 h after plating. Cells were harvested at the indicated time points by scraping in PBS, lysed in lysis buffer and cleared by centrifugation. Following quantification using Bio–Rad protein assay (Bio–Rad), total cellular proteins were separated by SDS-PAGE, transferred to nitrocellulose membrane and immunoblotted as described (Welbourn et al., 2005). Cell extracts from stable cell lines were processed similarly.

**Antibodies.** The antibodies used in this study were: a rabbit polyclonal anti-NS3 antibody generated against the NS3 Con1
protease domain (Welbourn et al., 2005), a rabbit polyclonal anti-NS2 antibody generated against NS2 Con1 residues 904–1026, anti-luciferase (Chemicon International), anti-Flag M2 (Sigma) and anti-actin (AC40, Sigma). For the immunoblot in Fig. 6 a rabbit polyclonal antibody raised against the helicase domain of NS3 Con1, a rabbit polyclonal antibody raised against two NS2-derived peptides spanning residues 148–163 and 202–217 of JFH-1 NS2 and the core-specific polyclonal antibody C-830 (Shavinskaya et al., 2007) were used.

Core ELISA. Quantitative detection of intracellular and extracellular HCV core protein was done using the Trak-C Core ELISA assay (Ortho Clinical Diagnostics) as recommended by the manufacturer and as described previously (Jirasko et al., 2008).

Infectivity assays. Cell-associated infectivity was determined as described previously (Gastaminza et al., 2006). Infectivity titres of cell lysates, and cell culture supernatants, were determined by limiting dilution assay on HuH7.5 cells as described previously (Koutsoudakis et al., 2007) and as described in supplementary material (available in JGV Online).

RESULTS

Lysine mutagenesis of NS2/3H952A replicons increases NS2/3 stability, but fails to rescue genome replication

We have previously shown that an NS2/3 protease active site mutant abolishes HCV RNA genome replication in the replicon system and that this uncleaved NS2/3 is rapidly degraded after transient electroporation into HuH7 cells (Welbourn et al., 2005). However, the question remained as to whether the mutant NS2/3 replicons were unable to replicate because of insufficient NS3 levels (as a component of the rapidly degraded NS2/3) or because the NS2/3 protein can simply not functionally replace the role of NS3 in the replication complex. The initial goal was therefore to increase the levels of uncleaved NS2/3 protein sufficiently to determine whether there was any rescue in replication of the NS2/3 protease inactive replicons. As NS2 itself was previously suggested to have a short half-life compared with NS3 (Franck et al., 2005) and levels of both NS2 and mutant uncleaved NS2/3 could be increased by treatment with the proteasome inhibitor MG132, the role of the ubiquitin/proteasome pathway in the degradation of NS2/3 was investigated. Because of the inability to use the MG132 inhibitor in replication assays due to toxicity at the timescales involved, and as ubiquitin chains are generally attached to lysine residues, a mutation analysis of the lysine residues conserved amongst all HCV genotypes. (see Fig. 1). Single lysine to arginine mutations were introduced into PI-luc/NS2-3/H952A luciferase reporter replicons and electroporated into HuH7-Lunet cells, a highly permissive subclone of the HuH-7 cell line (Binder et al., 2007). After attachment (4 h post-electroporation), 10 μM MG132 was added for an additional 5 h. Fig. 2(a) shows that, while levels vary, none of the single mutants is able to prevent NS2/3 degradation, with all constructs showing very low levels prior to MG132 treatment, similar to what is observed with the H952A mutation alone. As seen previously (Welbourn et al., 2005), mutant NS2/3 was visualized as multiple bands, all three of which reacted with both anti-NS3 and anti-NS2 antibodies (data not shown). As it has been shown with other proteins that mutation of a single lysine is often not sufficient to prevent ubiquitination and targeting for degradation (Wang et al., 2005), a construct was also generated in which all six NS2 lysines were converted to arginines. As seen in Fig. 2(b), this NS2-lysine-free NS2/3 (K-6-R) is partially stabilized and is readily detectable without MG132 treatment at levels comparable to the treated H952A mutant alone. Luciferase levels were also found to be similar, indicating that the stabilization seen is in fact due to the different mutations, not varying RNA levels.

Transient luciferase replication assays were performed to determine whether the additional stabilization observed was sufficient to rescue replication. Fig. 2(c) reveals that both the single-lysine mutants and the NS2-lysine-free H952A constructs fail to replicate, showing a similar effect as the H952A mutation alone or a GND polymerase-inactive negative control. Furthermore, when the same mutations were introduced into pNeo/2-5B neomycin-selectable replicons, no rescue of replication was seen by colony-formation assay (data not shown). The stabilization of NS2/3 is therefore not sufficient to rescue viral replication, suggesting that the uncleaved NS2/3 fusion protein cannot replace NS3 in the replication complex.

NS2 is rapidly degraded in the replicon system

Fig. 2 indicates that an NS2-lysine-free NS2/3 H952A mutant is stabilized compared to the H952A protein alone. It was therefore interesting to investigate whether the same
effect is seen with the mature NS2 protein. NS2 itself was previously shown to be rapidly degraded in a proteasome-dependent manner (Franck et al., 2005). However, as these studies were mostly performed using tagged DNA constructs in HepG2 cells, we wanted to investigate this degradation in the more relevant context of RNA replicons in Huh-7 based cells. A stable cell line containing pNeo/2-5B was established to investigate degradation of NS2 in the context of the replicating viral genome. A pulse-chase analysis was performed in which these cells were pulsed with [35S]methionine/cysteine and then chased for the indicated time periods. Labelled proteins were immunoprecipitated with anti-NS2 antibody followed by anti-NS3 immunoprecipitation. Fig. 3(a) shows rapid decrease in the levels of NS2 as compared with the slower decrease in NS3 levels. Quantification of the amounts relative to the initial levels at time 0 is shown in Fig. 3(b). Over several experiments the half-life of NS2 is found to be between 3 and 6 h, slightly longer than what had been reported using DNA constructs, but much quicker than what is seen with NS3 and what has been reported for other non-structural proteins (Pietschmann et al., 2001). Therefore these experiments confirm the short half-life of NS2 in the context of a replicating viral RNA genome.

**Lysine mutagenesis of NS2 does not increase its stability in replicon systems**

Since we observed stabilization of uncleaved NS2/3 by replacement of NS2 lysines with arginine, we wanted to determine if any of these mutations would increase levels of the cleaved protein in a subgenomic replicon system. Lysine to arginine changes were therefore generated in pFK-PI-luc/NS2-3 luciferase replicons. These RNA constructs were electroporated into Huh7-Lunet cells and after

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**Fig. 2.** Effect of NS2 lysine mutations on uncleaved NS2/3 levels and replication of subgenomic NS2/3 protease inactive replicons. Single (a) and multiple (b) NS2 lysine-to-arginine mutations were introduced into pFK-repPI-luc/NS2-3/H952A replicons and electroporated into Huh-7-Lunet cells. After attachment, cells were treated with 10 μM MG132 or Me2SO control for 5 h. NS2/3 and NS3 levels were visualized by immunoblot analysis using an anti-NS3-specific antibody. Luciferase detection was also included as a control for RNA levels. (c) Indicated pFK-repPI-luc/NS2-3’ luciferase constructs were electroporated into Huh-Lunet cells, harvested at the indicated time points, and luciferase activity measured. Values are reported as a percentage of the counts obtained 4 h post-transfection. ET, Adapted wild-type replicon; GND, polymerase inactive negative control. Error bars indicate standard deviation of a minimum of two independent experiments.

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**Fig. 3.** Degradation of NS2 in replicon stable cell lines. (a) Pulse-chase labelling of pNeo/2-5B replicon cell lines was performed for the time points indicated followed by anti-NS2 and anti-NS3 immunoprecipitation. Labelled protein levels were visualized by autoradography and quantified (b) by phosphorimaging. Initial protein level at time 0 was set at 100%.
attachment, 10 μM MG132 was added. The NS2 levels after 8 h are shown in Fig. 4(a). No significant increase in levels compared with wild-type NS2 is seen with any of the lysine single mutants, with the effect of MG132 also being consistent in most cases: a very slight but reproducible increase. NS3 levels are also shown to be similar, indicating similar RNA levels in all cases. Interestingly, unlike the NS2/3H952A protein which was stabilized by the addition of NS2 lysine mutations, when all three conserved NS2 lysines (K836, K890, K982) were replaced by arginine (K-3-R) or when all six lysines in NS2 were replaced together (K-6-R), no effect was seen on levels of cleaved NS2 compared with wild-type (Fig. 4b). Furthermore, a S977A mutation, suggested to stabilize genotype 1a NS2 in DNA-transfected cells by preventing phosphorylation of this site by CKII (casein kinase II; Franck et al., 2005), also had no effect in this system (Fig. 4c). Interestingly, a recent report showed that use of a specific CKII inhibitor also did not increase levels of a genotype 2a NS2 protein (Tellinghuisen et al., 2008), suggesting that perhaps the stability of NS2 proteins from different genotypes could be regulated in different ways. In addition, the similar levels seen here of both NS2 and NS3 for all constructs indicate that the mutations are not interfering with NS2/3 cleavage. As this cleavage is required for genome replication, this was also confirmed by a transient luciferase replication assay where the NS2-lysine-free replicon was found to replicate to very similar levels as the wild-type control (Fig. 4d).

In order to determine more precisely any effect of the lysine mutations on NS2 stability, a pulse–chase analysis of NS2 half-life was performed after transient transfection of both the wild-type (ET) and NS2-lysine-free (K-6-R) luciferase replicons. NS2 levels shown in Fig. 4(e) and quantified in

Fig. 4. Effect of lysine mutations on NS2 stability. (a, b and c) The indicated pFK-repPF-luc/NS2-3′ luciferase replicons were electroporated into Huh7-Lunet cells. After attachment, cells were treated with 10 μM MG132 or Me2SO control for 8 h. NS2, NS3 and actin levels were then visualized by immunoblot analysis using specific antibodies. (d) The indicated pFK-repPF-luc/NS2-3′ luciferase replicons were electroporated into Huh7-Lunet cells and luciferase activity measured after 4, 24, 48 and 72 h. Values are reported as a percentage of the counts obtained after 4 h. Error bars indicate standard deviation of a minimum of two independent experiments. (e) Pulse–chase analysis of NS2 half-life was determined 72 h after electroporation of wild-type (ET) and NS2-lysine-free (K-6-R) luciferase replicons into Huh7-Lunet cells. Labelled proteins were isolated by anti-NS2 immunoprecipitation at the chase times indicated and visualized by autoradiography. (f) Quantification of NS2 levels in (e) as determined by phosphorimaging and expressed as a percentage of NS2 present at time 0. (g) Full-length core-3/5.1 WT and NS2-lysine-free core-3/5.1 K-6-R cell lines were treated with 10 μM MG132 for 8 h and the levels of NS2, NS3 and actin visualized using specific antibodies.
Fig. 4(f) show that indeed the lysine mutations are not sufficient to stabilize NS2 in this system. Furthermore, stable cell lines were generated containing wild-type and NS2-lysine-free pNeo/2-5B neomycin-selectable replicons in Huh7.5 cells. Pulse–chase analysis of NS2 half-life in these subgenomic cell lines also shows little difference in the degradation of the wild-type or lysine-free NS2 proteins (data not shown), therefore confirming in the context of stably replicating viral genomes the results obtained above.

NS2 lysine mutations were also introduced in combination into pFK-core-3’/5.1 full-length genotype 1b neomycin replicons and stable cell lines generated from Huh-7.5 cells. This allowed us to determine any additional effect the presence of structural proteins could have on NS2 levels. As recent genetic evidence suggests that HCV structural proteins may interact with NS2 (Murray et al., 2007; Pietschmann et al., 2006), it is also conceivable that their presence could affect its stability. Wild-type and NS2-lysine-free full-length replicon cells were therefore treated with MG132 or DMSO for 8 h and NS2 levels visualized (Fig. 4g). Compared with actin (loading control) and NS3 (RNA replication level control), the levels of NS2 do not increase in the lysine-free mutant, and as is seen with subgenomic cell lines, proteasome inhibition has very little effect. These results are in contrast to what was reported by Franck et al. (2005), who showed very low amounts of NS2 in genotype 1a full-length replicon cells, levels which were greatly increased by MG132 treatment. One possibility to explain this discrepancy is the difference in genotypes used as mentioned above.

**Effect of MG132 on NS2 levels**

Despite the fact that MG132 increases levels of uncleaved mutant NS2/3, the effect of this proteasome inhibitor on cleaved NS2 levels is less obvious in the systems described above. This was further confirmed with cell lines containing pNeo/2-5B in either Huh-7, Huh-7.5 or Huh7-Lunet cells which were treated with MG132 for 8 h and the effect on NS2 levels visualized (Fig. 5a). Again, in contrast to what is shown with NS2/3 and what has been observed with cleaved NS2 using transient DNA systems (Franck et al., 2005 and data not shown), proteasome inhibition shows only a very slight increase in NS2 levels in these cell lines. Interestingly, when MG132-dependent induction of a non-related protein (Hifα) is measured as a control for proteasome inhibition, the parental Huh-7 cell line (with and without replicon) shows a much larger increase than what is observed with cured cell lines (data not shown), suggesting there could be differences in these Huh-7-based cells with respect to MG132-induced proteasome inhibition. However, when MG132 is added to pulse–chase analysis of pNeo/2-5B-containing Huh-7 cell lines, no significant stabilization of NS2 is observed (Fig. 5b and c), suggesting that NS2 degradation could be at least partly proteasome-independent.

**Effect of lysine mutations in the genotype 2a JFH-1 infection system**

Stability analysis of NS2 derived from other strains (H77c, Con1, J6, JFH-1) was performed in DNA expression systems and they were all found to be rapidly degraded (Supplementary Fig. S1, available in JGV Online), suggesting the short half-life of NS2 is not unique to genotype 1. As JFH-1 NS2 was rapidly degraded in this DNA-based system, NS2 levels were also investigated in the genotype 2a cell culture infection system. Single NS2 lysine mutations, as well as a seven mutant lysine-free NS2 sequence, were introduced into pJFH-1. After transfection, very similar levels of core and NS3 proteins were achieved with all constructs, indicating similar replication capability of all
mutants compared with wild-type (Fig. 6a). Furthermore, NS2 is easily detected at similar levels, indicating no difference in production or stability of the protein in this system, consistent with what is described above with genotype 1b replicons. Consistent with the comparable replication efficiency of the mutants, accumulation of intracellular core protein was comparable between them and the wild-type (Fig. 6b). However, in the case of the K894R and K-7-R mutants, core release was reduced, indicating that these mutants were impaired in either virus assembly or release. In support of this, release of infectious virus particles was reduced up to 100-fold with these two mutants (Fig. 6c). This reduction was, however, much stronger than the effect on core release, indicating that primary infectivity of the virus particles rather than their release is affected. In support of this assumption, intracellular infectivity titres were also reduced by almost 2 logs for K894R (Fig. 6d), thus showing that this residue plays a critical role in assembly of infectious HCV particles. Interestingly, the K1028R substitution resulted in an adaptive phenotype with release of infectious HCV enhanced more than tenfold at 24 h post-transfection (Fig. 6c), even though the overall infectivity titres achieved at later time points were not higher than wild-type. A kinetic analysis at even earlier time points showed that while neither wild-type nor K1028R show significant infectious virus release before 20 h, core release from the mutant genome appears at least 4 h before that of the wild-type and infectivity levels continue to be higher with the mutant until about 48 h (Supplementary Fig. S2, available in JGV Online). As this residue is close to the NS2–NS3 cleavage site (Fig. 1), its mutation could be modulating NS2/3 processing kinetics. However, no obvious difference in cleavage kinetics at the NS2/3 junction was seen with this mutant compared with wild-type in an in vitro...
translation system (Supplementary Fig. S3, available in JGV Online). All other lysine mutants were comparable to the wild-type, suggesting that they do not play an important role in viral replication and assembly.

Overall, while NS2 is easily detectable in this system and lysine mutations have no effect on its levels, some of these mutations affect steps in viral assembly and infectivity, an interesting observation that will require further study.

**DISCUSSION**

One goal of this study was to further investigate the mechanism by which HCV NS2/3 mutant replicons are rendered inactive for RNA replication. Our previous work (Welbourn et al., 2005) looked at the NS3 catalytic activities of mutant uncleaved NS2/3, and while a small decrease in NS3 protease activity was seen in vitro, no significant effect on polyprotein processing was seen in cells (see also Supplementary Fig. S4, available in JGV Online) and therefore for this study we decided to focus on the observation that the resulting NS2/3 protein was rapidly degraded. We therefore performed a lysine-mutagenesis study to determine if a stabilized NS2/3 protein would be functional for RNA replication. While an NS2-lysine-free protein was found to be substantially stabilized, we show here that the longer-lived mutant H952A NS2/3-containing replicon is still unable to replicate, suggesting that uncleaved NS2/3 may not be able to functionally replace NS3 for genome replication.

This is in line with recent evidence from bovine viral diarrhea virus (BVDV) suggesting that even at high levels, uncleaved NS2/3 may simply not be able to functionally replace NS3 in viral genome replication. For this pestivirus, the uncleaved NS2/3 protein is stable and required for viral infectivity (Agapov et al., 2004) while viral RNA levels have been shown to correlate with levels of cleaved NS3 (Lackner et al., 2004). For BVDV therefore, it would appear that uncleaved NS2/3, while present, simply cannot functionally replace cleaved NS3 in genome replication and this could also be the case for HCV NS2/3. It is important, however, to point out several differences between the NS2/3 proteins of these two viruses. Any potential role for uncleaved NS2/3 has yet to be established in HCV infection as cleavage at the 2/3 junction appears to be very efficient in cell systems, without the identification of an uncleaved NS2/3 precursor. Indeed, recent evidence argues against a role for such a precursor. It was reported that, using bicistronic constructs to physically separate NS2 from NS3 in a viral system, no effect was seen on infectious virus production (Jirasko et al., 2008; Jones et al., 2007), unlike with BVDV where uncleaved NS2/3 is required for infectivity (Agapov et al., 2004).

Rapid degradation of several viral proteins has been reported and could constitute an important way for either the host cell or the virus itself to regulate the various events of the viral life cycle. While a previous study focussed on NS2 degradation in DNA-transfected cells using tagged constructs, we show here that NS2 is also a short-lived protein in various RNA-based replication systems. This was important to investigate, as expressing untagged NS2 in the context of the other viral proteins could have important differences in terms of its function, localization or degradation. Moreover, our results differ from what was previously reported by Franck et al. (2005), in that in all systems used here, NS2 was readily detectable without proteasome inhibition, likely due to differences with respect to the genotypes used. The relevance of this degradation for the virus remains elusive, as does the mechanism of degradation.

Lysine mutagenesis, which would abolish potential ubiquitination sites, had no effect on NS2 levels. Several proteins have been reported to be degraded by both ubiquitin-dependent and -independent pathways. Some were suggested to interact directly with the proteasome for degradation, such as IkBz (Alvarez-Castelao & Castano, 2005). Ornithine decarboxylase is degraded in a similar fashion via recognition by the proteasome after binding to an antizyme co-factor (Murakami et al., 1992). However, our observation that MG132 has only a very slight effect on NS2 levels suggests that other, non-proteasomal pathways could be involved in the degradation of this protein. While further study is required, it is possible that NS2 is being targeted for degradation via lysosomal pathways or that targeted proteolytic cleavage may be involved. Indeed, although its significance remains unknown, Jirasko et al. (2008) have recently shown the generation of a truncated form of NS2 in infectious cell systems.

Interestingly, while no change in NS2 levels is seen upon introduction of lysine mutations into the JFH-1 infectious construct, several of the mutants themselves affect viral infectivity, despite showing no defect in NS2/3 processing or RNA replication. Recently, it was reported that the NS2 protein is required for HCV infectivity by functioning at a stage prior to virion assembly (Jirasko et al., 2008; Jones et al., 2007), although its exact role has yet to be fully defined. This is consistent with the K894R mutation observed here to block the formation of intracellular infectious viral particles. Indeed, this mutation likely disturbs some intra- and/or intermolecular interactions within or between NS2 or its biological partners. Recent genetic evidence has suggested that NS2 may function through interaction with other structural and non-structural HCV proteins. The generation of intergenotypic chimeras showed an ideal junction site to be within NS2, suggesting one part of this protein could interact with structural proteins and/or p7, while its C terminus required compatibility with the other non-structural proteins (Pietzschmann et al., 2006). This is further substantiated by the appearance of compensatory mutations in these proteins upon culture of an H77c/JFH-1 chimera (Yi et al., 2007). These mutations significantly increased viral titres, potentially by correcting incompatibilities between interaction sites of the proteins from different genotypes.
Interestingly, K890R was identified in that study, and we show here that this residue (K894 in JFH-1, see below) is indeed affecting viral assembly. Similarly, compensatory mutations in NS2 and p7 were also identified to increase viral assembly of defective constructs containing core mutations, suggesting many interactions between the structural proteins p7 and NS2 could be required for virion assembly (Murray et al., 2007). Alternatively, it is also possible that NS2 interacts with other, yet to be identified, cellular proteins during the assembly process and the mutation identified here could also be affecting this process.

In order to investigate the relationship between the location of the mutated lysines and their effect on viral infectivity, a model structure of the JFH-1 NS2 ectodomain (Fig. 6e) was constructed by homology modelling using the three-dimensional X-ray structure of the genotypel 1a NS2 variant (Lorenz et al., 2006). As shown in Fig. 6(e), four of the lysines belonging to the NS2 ectodomain are located at the protein surface (K930, K985, K986, K1025). This could explain why their mutation to arginine has no detectable effect, as they would be unlikely to alter NS2 structure. Interestingly, K1028R resulted in a stimulation of viral infectivity titres at early time points. This residue is located close to the NS2–NS3 cleavage site, and while we detected no significant effect on NS2/3 cleavage, it could also likely be acting by modulating intra- and/or intermolecular interactions.

In contrast, K840 and K894 reside in the membrane domain of NS2. While K840 belongs to the putative cytosolic loop connecting TM1 and TM2, K894 is located within predicted TM3 (Fig. 6e). The large decrease of infectious virus particle release induced by the mild mutation of K894 to arginine is therefore consistent with the critical role of the NS2 membrane domain in the processes of viral assembly, as reported previously (Jirasko et al., 2008; Jones et al., 2007).

Overall, although much work is still required to understand NS2’s role in the viral life cycle, we show in this study that NS2 levels could play an important part in this process and confirm that NS2 is a critical component of the viral assembly mechanisms by identifying residues in NS2 required for virus assembly.

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