Mutations in hepatitis C virus p7 reduce both the egress and infectivity of assembled particles via impaired proton channel function

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Hepatitis C virus (HCV) p7 protein is critical for the efficient production of infectious virions in culture. p7 undergoes genotype-specific protein–protein interactions as well as displaying channel-forming activity, making it unclear whether the phenotypes of deleterious p7 mutations result from the disruption of one or both of these functions. Here, we showed that proton channel activity alone, provided in trans by either influenza virus M2 or genotype 1b HCV p7, was both necessary and sufficient to restore infectious particle production to genotype 2a HCV (JFH-1 isolate) carrying deleterious p7 alanine substitutions within the p7 dibasic loop (R33A, R35A), and the N-terminal trans-membrane region (N15 : C16 : H17/AAA). Both mutations markedly reduced mature p7 abundance, with those in the dibasic loop also significantly reducing levels of mature E2 and NS2. Interestingly, whilst M2 and genotype 1b p7 restored the same level of intracellular infectivity as JFH-1 p7, supplementing with the isogenic protein led to a further increase in secreted infectivity, suggesting a late-acting role for genotype-specific p7 protein interactions. Finally, cells infected by viruses carrying p7 mutations contained non-infectious core-containing particles with densities equivalent to WT HCV, indicating a requirement for p7 proton channel activity in conferring an infectious phenotype to virions.

INTRODUCTION

Hepatitis C virus (HCV) represents a major global health challenge, chronically infecting 3% of the population and causing severe liver disease. HCV is genetically diverse and is separated into seven genotypes, yet only a single genotype 2a isolate, termed JFH-1 (Japanese fulminant hepatitis 1), has been identified that recapitulates the complete virus life cycle in hepatoma cells (Wakita et al., 2005). Many infectious JFH-1 chimaeras have been generated containing allogeneic core, E1, E2, p7 and NS2 proteins, yet the assembly, trafficking and release of infectious virions remains poorly understood. However, it is increasingly clear that several viral and host factors are involved in addition to the canonical viral structural proteins. One such factor is the p7 protein, which is essential for both infectious virion production in culture (Jones et al., 2007; Steinmann et al., 2007a) and infection in vivo (Sakai et al., 2003).

p7 is a 63 aa integral membrane protein (Carrère-Kremer et al., 2002; StGelais et al., 2009), composed of two amphipathic trans-membrane α-helices separated by a conserved cytosolic ‘dibasic loop’ domain (Lin et al., 1994). p7 oligomerizes and exhibits cation channel activity in vitro sensitive to several prototype compounds (Griffin et al., 2003; Pavlović et al., 2003; Premkumar et al., 2004), with patterns of susceptibility depending on virus sub/genotype (Griffin et al., 2008). p7 activity equilibrates proton gradients within cell membrane compartments, protecting nascent virions from reduced pH during secretion (Wozniak et al., 2010). However, p7 also undergoes essential interactions with NS2, targeting NS2 to foci where it interacts with other HCV proteins (Boson et al., 2011; Jirasko et al., 2010; Ma et al., 2011; Popescu et al., 2011; Stapleford & Lindenbach, 2011; Tedbury et al., 2011). Disrupting interactions via genotype differences or mutations eliminates both foci formation and particle infectivity, supporting a hypothesized role coordinating virion assembly.

p7 has been proposed to act both at assembly and later secretory phases of particle production (Brohm et al., 2009; Jones et al., 2007; Steinmann et al., 2007a), and several point mutations severely impair both intracellular and secreted infectivity. Alanine substitutions of the p7 dibasic...
loop are commonly utilized, preventing genotype 1b p7 channel activity in surrogate cellular assays (Griffin et al., 2004) and severely hampering infectious virion production in multiple JFH-1-based chimaeras; glutamine substitutions display less-severe phenotypes (Jones et al., 2007; Steinmann et al., 2007a). However, dibasic loop mutations within highly efficient infectious particle-producing contexts (J6/JFH-1 genotype 2a/2a chimaeras, sometimes referred to as JFH-chimaera 1 or JC-1) retain measurable infectivity, albeit with markedly reduced titres.

It is unclear whether dibasic loop substitutions disrupt channel activity, p7–NS2 interactions, or both, and phenotypes are further obscured by effects on E2-p7-NS2 precursor processing (Steinmann et al., 2007a), NS2 localization (Tedbury et al., 2011) and p7 membrane insertion in vitro (Pérez-Berná et al., 2008; StGelais et al., 2009). Dibasic loop mutant phenotypes resemble p7 deletions by disrupting both intracellular and secreted infectivity, yet, unlike deletions, infectivity can be partially rescued by influenza virus M2 or bafilomycin A treatment (Brohm et al., 2009; Wozniak et al., 2010). p7–NS2 interactions persist in the context of a haemagglutinin (HA)-tagged dibasic p7 loop glutamine substitution (Vieyres et al., 2013), yet small-molecule inhibitors of p7 channel activity specifically prevent the accumulation of secreted but not intracellular infectivity (Foster et al., 2011; Gottwein et al., 2011; Griffin et al., 2008; Steinmann et al., 2007b). It therefore remains unclear precisely which stages of the HCV life cycle are affected by dibasic loop substitutions.

Here, we showed by trans-complementation and the detection of non-infectious core-containing HCV particles that infectivity defects, caused by both a dibasic loop mutant and another deleterious mutation (N15, C16, H17/AAA, termed NCH) in JFH-1 p7, resulted primarily from a lack of proton channel activity. We concluded that p7 channel activity is required for conferring infectivity to assembled HCV particles as well as mediating their efficient secretion.

RESULTS

p7 associates with other HCV particle production factors

Overexpression studies have identified endoplasmic reticulum (ER)-localized p7, with some plasma membrane (Carrère-Kremer et al., 2002) and mitochondrion-associated ‘heavy’ ER membrane (Griffin et al., 2005) association. Epitope-tagged p7 in full-length genomes is also ER localized, although tagging eliminated infectious particle production (Haqshenas et al., 2007). Recently, an HA-tagged JC-1 p7 preserved particle production and co-localized with E2 on the ER, as well as associating with both the core and NS5A at foci (Vieyres et al., 2013). However, direct detection of native p7 protein is desirable to confirm these associations.

Polyclonal sera specific to both genotype 1b (Griffin et al., 2005) and 2a (Griffin et al., 2008; Jirasko et al., 2010; Luik et al., 2009; StGelais et al., 2009) p7 proteins are available, but only the 1b serum is suitable for immunofluorescence. Therefore, we altered the C terminus of JFH-1 p7 to incorporate the 1b epitope [-PRQAYA(CO2H) to -PPRAYA(CO2H)], generating JFH-C3. This mutation reduced secreted infectivity by ~1 log10 yet had only a minimal effect on intracellular infectivity and did not affect the processing of viral proteins (Fig. 1a). Specific detection of the 1b epitope in JFH-C3, but not in JFH-1, was confirmed by both Western blotting and indirect immunofluorescence (Fig. 1a, middle and right panels).

Immunodetection of JFH-C3 p7 revealed a punctate, ER-associated distribution, co-localizing at foci with the more broadly ER-distributed E2 and concanavalin A (an ER/Golgi marker) (Fig. 1b). This agrees with a predominant ER localization for p7, but additional staining also suggested distribution within endosomal compartments, consistent with a role in vesicle alkalization (Griffin et al., 2004; Wozniak et al., 2010). Furthermore, as described for HA-tagged JC-1 p7 (Vieyres et al., 2013), p7 was also associated with both NS5A and core, co-localizing at punctae around circular structures reminiscent of lipid droplets (Fig. 1b, bottom right zoomed panels, white arrows). This most likely represents areas of ER contiguous with these organelles, rather than distribution of p7 within the droplets themselves.

Characterization of defects caused by p7 basic loop mutations

Whilst the phenotype of dibasic loop mutations is often attributed to disrupting channel activity, the precise consequences for p7 itself are unknown. We characterized alanine substitution mutants within both JFH-1 (R33A/R35A, termed JFH-RR) and JC-1 (K33A/R35A, termed JC1-KR). Consistent with previous studies (Steinmann et al., 2007a), JFH-RR reduced both intracellular and secreted infectivity to nearly undetectable levels and caused a marked reduction in secreted, sucrose-cushion-purified core protein (Fig. 2a), whereas JC1-KR continued to produce secreted infectious virions over a linear time course (Fig. 2b).

Comparison of dibasic loop mutant protein expression over time with WT virus revealed accumulation of E2-p7-NS2 precursors (Fig. 2b, top panel), as well as a significant reduction in all three mature proteins, most notably p7 itself. Less WT JC-1 p7 was detectable compared with the JFH-1 protein, despite equivalent core/E2 expression and both p7 proteins sharing identical N-terminal epitopes, suggesting that mature p7 proteins may display varying turnover rates within cells. Reductions in both cellular and viral proteins over time indicated a potential enhanced cytopathic effect in cells harbouring JC1-KR, making it possible that cell lysis contributes directly to infectivity within the secreted compartment by releasing intracellular virions.
We next assessed whether dibasic loop mutations affected the folding of E2 or E2-p7-NS2 precursors by comparing binding to the CD81 large extracellular loop E2 ligand, which involves a discontinuous binding site within E2 (Chan-Fook, 2000; Owsianka, 2006) (Fig. 2c). Both precursors and mature E2 bound the large extracellular loop proportionately to their relative abundance, suggesting that glycoprotein misfolding is not a direct consequence of p7 dibasic loop substitutions. Finally, we assessed the localization of core, E2 and NS5A in a dibasic loop mutant context, revealing no significant differences in patterns compared with WT JFH-1 (Fig. 2d). Consistent with Western blot analysis, dibasic loop mutant p7 was not detectable in the context of a JFH-C3 chimera (data not shown).

Identification of a p7 null mutant without effects on E2 or NS2

Effects on processing made p7 loop mutant phenotypes potentially attributable to a lack of mature E2, p7 or NS2. We therefore sought alternative p7 alanine substitutions with infectivity-null phenotypes. Positions in JFH-1 p7 were selected for mutagenesis based on key residues identified in genotype 1b p7 (StGelais et al., 2009): His17Ala (H17A), an adjacent Asn15:Cys16:His17/Ala triplet (NCH/AAA, termed NCH) and Pro49Ala (P49A).

Both the H17A and NCH mutations markedly reduced the abundance of mature JFH-1 p7 protein, although, unlike dibasic loop mutations, levels of E2 and NS2 were not affected (Fig. 3a). However, whereas JFH-NCH exhibited severely impaired particle infectivity phenotype equivalent to dibasic loop mutations, JFH-H17A produced near-WT infectivity, suggesting that p7 abundance does not necessarily correlate with infectivity (Fig. 3b). This was confirmed by additional alanine substitutions within the NCH triplet (Fig. 3c, e.g. compare lanes 3 and 5). JFH-P49A displayed an intermediate infectivity phenotype without altered p7 levels compared with WT (Fig. 3b). Unexpectedly, levels of sucrose-cushion-purified core protein present in p7 mutant transfection supernatants also did not correlate with infectivity, suggesting an effect on particle-specific infectivity (Fig. 3b).

Lack of proton channel activity determines p7 null mutant phenotypes

It remained unclear whether p7–protein interactions, channel activity or both were adversely affected in either dibasic loop or NCH mutants. We therefore utilized trans-complementation to distinguish the relative contribution of each aspect of p7 function, based on two assumptions: first, that p7–NS2 interactions only occurred efficiently between proteins of the same genotype (Jirasko et al., 2010), and secondly, that the influenza virus M2 protein would exhibit proton channel activity entirely independently of interactions with HCV proteins. Baculovirus transduction was used to express M2, genotype 1b p7 or JFH-1 p7, the latter either as core-E1-E2-p7 [bac-c-p7(JFH-1)] or core-E1-E2-p7-NS2 [bac-c-NS2(JFH-1)]. Baculovirus-expressed tetracycline trans-activator protein served as a negative control, and genotype 1b p7 with a dibasic loop mutation [p7-K33A/R35A, termed p7KR(1b)] controlled for p7 functionality. The results obtained for genotype 1b baculoviruses were independent of expression in a polyprotein or individual context (data not shown).

Provision of M2 in trans restored intracellular infectivity to both dibasic loop (JFH-RR) and NCH mutant (JFH-NCH) viruses to within the same order of magnitude as providing the native JFH-1 p7 protein, strongly supporting the
(a) Graph showing log₁₀ infectivity (f.f.u.) for GND, JFH-1, and JFH-RR.

(b) Western blots showing proteins at different molecular weights for GND, JFH-1, JFH-RR, JC-1, and JC1-KR.

(c) Western blots showing E2-p7-NS2 and E2 for MBP-CD81 and input samples.

(d) Immunofluorescence images showing α-core, α-E2, and α-NS5A for JFH-1 and JFH-RR.
suggestion that a lack of proton channel activity alone, rather than a lack of p7–NS2 interactions, underpinned the particle production phenotypes. Accordingly, trans-expression of WT, but not dibasic loop mutant genotype 1b p7, also led to a similar level of intracellular infectivity being present. A small but statistically significant increase in titre occurred where JFH-1 p7 was provided to mutants in trans, suggesting a moderate genotype-specific enhancement in the rescue of infectious particle production. Interestingly, both JFH-1 p7 baculoviruses rescued infectivity to the same degree, again suggesting that p7 expression levels do not necessarily correlate with infectivity (Fig. 4b). In contrast to levels of intracellular infectivity, restoration of secreted infectivity was more favourable where JFH-1 p7 was expressed compared with either genotype 1b p7 or M2, with a >1 log₁₀ increase in each case (Fig. 4a, bottom graphs). Again, this appeared to be independent of the level of p7 expression, as bacC-NS2(JFH-1) and bacC-p7(JFH-1) achieved similar levels of rescue (Fig. 4b). Thus, whilst restoration of proton channel activity alone via, for example, M2 can restore secreted infectivity, genotype-dependent p7 functions lead to a marked enhancement in released infectious virions.

Finally, to demonstrate that the rescue of p7 mutants was specifically dependent on proton channel activity, we capitalized on genotype-dependent p7 small-molecule sensitivity (Griffin et al., 2008). Amantadine (50 μM), to which JFH-1 p7 activity and associated particle production are resistant (Fig. 4c; Griffin et al., 2008), instead caused a significant decrease in secreted infectivity where an amantadine-sensitive influenza virus M2 was used to rescue JFH-1 with a dibasic loop mutation (JFH-RR).

**Non-infectious intracellular particles are present within cells harbouring HCV with deleterious p7 mutations**

As M2 and genotype 1b p7 were able to restore intracellular infectivity to both JFH-RR and JFH-NCH, we hypothesized that pre-assembled particles existed within cells, to which proton channel activity conferred an infectious phenotype. We therefore investigated whether core-containing particles of appropriate density were present within freeze–thaw Huh7 cell lysates harbouring JFH-RR and JFH-NCH.

Lysates were purified through a sucrose cushion prior to separation by density using continuous isopycnic iodixanol gradients. Consistent with previous studies (Gastaminza et al., 2006), a broad peak of WT infectivity was observed within a density range of 1.125–1.157 g ml⁻¹ (Fig. 5a). Both JFH-H17A and JFH-P49A p7 mutants displayed a similar distribution to WT particles (Fig. 5a), albeit with reduced overall infectivity (see Fig. 3b) suggesting that mutations did not alter the physical characteristics of intracellular particles. No infectivity was detectable in JFH-RR or JFH-NCH gradients.

Gradient fractions were assessed by direct core protein ELISA following detergent lysis (Fig. 5b). Baselines were established using polymerase-null JFH-1 transfected Huh7 lysates (GDD>GND active site mutant; Fig. 5b). To control for migration of non-particulate core protein, lysates from JFH-ΔE1/E2 transfected Huh7 cells were also analysed. These contained measurable core protein towards the top of the gradient, yet this reduced towards the middle and lower regions of the gradient with no discernible peaks. However, a statistically significant accumulation of core protein was present within gradient fraction 7 of JFH-1 lysates, overlapping with the observed peaks of infectivity (Fig. 5b). This was also the case for both JFH-RR and JFH-NCH lysates, indicating that, whilst infectivity was absent, the lack of p7 channel activity caused by both dibasic loop and NCH mutations did not prevent the assembly of core-containing intracellular particles with densities identical to infectious virions.

**DISCUSSION**

This study sought to define the mechanisms by which point mutations within the HCV p7 protein interfere with infectious virion production. We confirmed that proton channel activity alone is sufficient to restore infectivity to
(a) 

(b) 

(c) 

(d)
et al. disrupt particle infectivity (Brohm et al., 2009). Several studies have identified p7 point mutations that confer an infectious phenotype to HCV particles and acts at a post-assembly stage of infectious virion production, enabling their secretion. Whilst proton channel activity restored a proportion of secreted infectivity, genotype-dependent p7 functions further enhanced this process, suggestive of a late role for p7–protein interactions. We conclude that p7 channel activity acts at a post-assembly stage of infectious virion production, conferring an infectious phenotype to HCV particles and enabling their secretion.

Several studies have identified p7 point mutations that disrupt particle infectivity (Brohm et al., 2009; Jones et al., 2007; Meshkat et al., 2009; Steinmann et al., 2007a; Vieyres et al., 2013; Wozniak et al., 2010), although whether phenotypes relate to the disruption of either channel activity or genotype-dependent interactions with NS2 (Jirasko et al., 2010; Ma et al., 2011; Popescu et al., 2011; Stapleford & Lindenbach, 2011; Tedbury et al., 2011) was unclear. Dibasic loop mutations also cause defects in proteolytic processing (Steinmann et al., 2007a), membrane insertion (Pérez-Berná et al., 2008; StGelais et al., 2009) and, as shown herein, the abundance of p7, E2 and NS2. However, as an equivalent level of intracellular infectivity was restored by trans-expression of channels that either should (JFH-1 p7), or should not (influenza M2, genotype 1b p7) undergo NS2 interactions, a lack of proton channel activity underpins deleterious infectious phenotypes for both dibasic loop mutants and NCH alanine substitutions in the N-terminal helix. Furthermore, the NCH mutation does not affect E2-p7-NS2 processing, excluding effects on other proteins.

Consistent with studies of tagged protein (Vieyres et al., 2013), fluorescence staining of native p7 revealed an ER-associated punctate distribution, partially co-localizing with E2, core and NS5A. Whilst mutated p7 proteins were not detectable by fluorescence due to their abundance (distribution in the intermediate phenotype P49A mutant was identical to WT; data not shown), localization of other viral factors in cells harbouring mutant viruses appeared unaffected. This is in contrast to recent reports where JC-1 K33Q/R35Q HCV induced enlargement of lipid droplets as well as accumulation of core protein (Gentzsch et al., 2013). It is possible that such changes are specific to either glutamine dibasic loop substitutions or to the JC-1 genetic background. Deleterious mutants markedly affected p7 abundance. However, p7 abundance did not directly correlate with either infectivity levels (Fig. 3b, c), the amount of core protein secreted into the supernatant (Fig. 3b), or the level of trans-complementation achieved by JFH-1 p7 expression (Fig. 4). Thus, it seems likely that p7 is ordinarily present in excess within transfected Huh7 cells. Whilst dibasic loop mutant p7 abundance could be restored by proteasome inhibitors, this was not the case for the NCH mutant, indicating separate turnover mechanisms for each context (Fig. 3d). As E2 and NS2 levels are also reduced by p7 dibasic loop mutations, we hypothesize that turnover occurs at the level of E2-p7-NS2 precursors, which accumulate concomitantly. As E2 retained its ability to bind CD81 (Fig. 2c), we propose that precursor degradation may be induced by an inability of p7 carrying a dibasic loop mutation to insert into membranes, as observed in vitro (Pérez-Berná et al., 2008; StGelais et al., 2009). In this scenario, p7 inserts into membranes spontaneously via its conserved basic residues following translation of E2-p7-NS2, in a similar fashion to picornavirus 2B proteins (Agirre et al., 2002). This would not affect the topology of either E2 or NS2, and might explain how HCV carrying in-frame deletions of the p7 N terminus remain viable, rather than altering the membrane topologies of the downstream polyprotein (Brohm et al., 2009). It may also help explain why glutamine substitutions cause less-severe phenotypes compared with alanine (Steinmann et al., 2007a) due to their less-hydrophobic amide side chains. Thus, p7 proton channel activity would be disrupted by dibasic loop mutations due to a lack of membrane-inserted channel complexes, rather than aberrant channel gating. This membrane insertion mechanism may also be linked to delayed E2-p7-NS2 precursor processing by signal peptidase (Carrière-Kremer et al., 2004; Isherwood & Patel, 2005; Lin et al., 1994; Mizushima et al., 1994).

Reduced intracellular infectivity is often attributed to particle assembly defects, yet our identification of non-infectious core-containing particles in cells harbouring p7 mutants suggests that proton channel activity is necessary to confer an infectious phenotype to pre-assembled virions. As the majority of intracellular HCV virions reside within the ER and so have not experienced acidic conditions.
(Gastaminza et al., 2008), the way in which proton channel activity confers infectivity is probably distinct from the ability of p7 to equilibrate pH gradients within secretory vesicles (Wozniak et al., 2010). Accordingly, p7 channel inhibitors prevent the accumulation of secreted rather than intracellular infectivity (Foster et al., 2011). One explanation is that p7 complexes reside within virions and mediate stages of cell entry, although this is controversial (Griffin et al., 2008; Saunier et al., 2003; Steinmann et al., 2007a; Vieyres et al., 2013).

**Fig. 4.** Trans-complementation of p7 null mutants. (a) Individual electroporations were split among 12-well plates and infected with various baculoviruses (two wells per condition) at an m.o.i. of 10, at 16 h post-transfection. Secreted and intracellular titres were determined by a focus-forming assay after 72 h. Titres are derived from three separate experiments unless indicated and error bars show SEM. ND, Not done. Asterisks represent statistically significant differences (*P < 0.05, **P < 0.01) using Student’s t-test. (b) Western blot analysis was performed at 72 h on whole-cell lysates from (a) for HCV and baculovirus-expressed proteins as indicated. (c) Secreted infectivity from cells harbouring JFH-1 or JFH-RR transduced with M2-expressing baculovirus was assessed in the presence (light grey bars) or absence (black bars) of amantadine (Ama, 50 μM). Results are the mean of three experiments and error bars show SEM. The P value is shown for the difference between untreated and treated JFH-RR cells. NS, Not significant.
Fig. 5. Detection of intracellular HCV particles. Huh7 cells were harvested at 72 h post-electroporation with polymerase-null (GND), WT JFH-1, p7 null mutant (JFH-RR, NCH) or other p7 mutant (H17A, P49A) RNAs, via freeze–thaw. Particles were then pelleted through a 20% sucrose cushion, prior to loading onto a 10–40% iodixanol/PBS gradient. Following ultracentrifugation, 12 equal fractions were collected and assessed for infectivity and core protein content. (a) Line graph showing infectivity determined by a focus-forming assay from representative intracellular gradients along with corresponding densities (g ml⁻¹) on secondary axis. Results are the mean of four separate experiments (except for P49A and H17A where two were performed) and error bars show SEM where n ≥ 2. (b) HCV core protein ELISAs from gradient fractions represented as line graphs of absorbance readings at 450 nm (A₄₅₀) following o-phenylenediamine dihydrochloride colorimetric change). Baseline readings were obtained from gradients containing a GND negative control (□) and compared with gradients from JFH-1 (top left), JFH-RR (top right), JFH-NCH (bottom left) and JFH-ΔE1/E2 (bottom right) (○). Results are the mean of at least three experiments and error bars show SEM. Statistically significant differences in the peak fraction (fraction 7) compared with GND controls are shown as P values calculated using Student’s t-test.
Unlike the case of intracellular infectivity, trans-complementation with p7 from the same genetic background to either JFH-1 dIb1sc loop or NCH mutants led to a marked increase in secreted infectivity (Fig. 4). This suggests that genotype-dependent aspects of p7 function appear more important for particle secretion than for the assembly/inf ectivity of virions within a trans-complementation setting. As the only known genotype-dependent aspect of p7 function corresponds to p7–NS2 interactions, this would seemingly contradict an early role for these events during particle assembly. However, studies have also demonstrated late-acting functions of NS2 during particle secretion, which may be similarly p7 dependent and explain an enhancement of secreted infectivity (de la Fuente et al., 2013; Yi et al., 2009). Interestingly, whilst p7–NS2 interactions persist in Jc-1 dIb1sc loop glutamate substitution mutants (Vieyres et al., 2013), targeting of NS2 to foci is disrupted (Ma et al., 2011; Popescu et al., 2011; Tedbury et al., 2011), suggesting that the two processes may be distinct. By contrast, exogenously expressed p7–NS2 constructs containing dIb1sc loop mutations retain their ability to modulate core protein localization (Boson et al., 2011), although it is noted that mutant processing defects and reduced protein abundance do not occur when expressed in a non-polyprotein context (Griffin et al., 2004; Wozniak et al., 2010). However, early roles for p7 are supported by the observation that particle infectivity of in-frame p7 deletion mutants cannot be rescued by either M2 or by bafilomycin A treatment, as can point mutants (Brohm et al., 2009; Wozniak et al., 2010). Whether non-infectious particles exist in this scenario is not known, but they have been observed in cells harbouring HCV with deleterious NS2 mutations (Yi et al., 2009). Accordingly, p7 deletion phenotype defects may be attributable to either interactions with NS2 (Jirasko et al., 2010; Ma et al., 2011; Popescu et al., 2011) or to a recently described role for p7 during capsid assembly (Gentzsch et al., 2013). Thus, a greater understanding is required to create a unifying hypothesis explaining how both p7 and NS2 influence protein localization, virion assembly, secretion and infectivity during HCV particle production.

In summary, we have demonstrated that infectivity defects associated with deleterious p7 point mutations result primarily from a lack of proton channel activity. This activity is critical for conveying an infectious phenotype to assembled HCV particles and also enables secretion of infectious virions.

METHODS

Plasmids, viral clones and recombinant baculoviruses. pJFH-1, pJFH-1c3 (JC-1 and K33A, R35A mutant) have been described previously (Pietzschmann et al., 2006; Steinmann et al., 2007a; Watakai et al., 2005). For JFH-1 mutation, a unique BsiWI–KpnI fragment was ligated into pLitmus 28i (NEB), termed pLitJFH-B/K. A silent AvrII site was then introduced adjacent to the 5’ region of p7 using Kunkel mutagenesis (Kunkel et al., 1987, 1991), generating pLitJFH-B/K(A). This was then reintroduced into the pJFH-1 backbone, termed JFH(A). JFH(A) replicated and produced particles with identical kinetics to WT JFH-1 following transfection into Huh7 cells, indicating no adverse effects from silent mutagenesis (data not shown). p7 mutations were generated by overlap PCR using specific primers and flanking primers containing the AvrII and KpnI sites. Digested amplimers were digested with PstI (JFH-1) or SalI (pJFH-1), and the resulting PstI–SalI fragment was then transferred into the JFH-1 backbone. All constructs were confirmed by dSNA sequencing. Detailed methods and primer sequences are available upon request.

Recombinant baculoviruses expressing the JFH-1 C-p7 and C-NS2 proteins have been described (Adair et al., 2009). Viruses expressing genotype 1b J4 isolate p7 protein and the highly pathogenic avian influenza A/HongKong/156/97 (H5N1) M2 protein (amantadine sensitive) were provided by Thomas Zurcher and Helen Bright (formerly of GlaxoSmithKline, Middlesex, UK).

Cell and virus culture. Huh7 cells were maintained and transfected with in vitro-transcribed full-length HCV RNA as described previously (Griffin et al., 2008). Transfections were resuspended in 7 ml HEPES-buffered Dulbecco’s minimal essential medium and seeded into a T75 flask. Typically, cells and supernatants were harvested at 72 h post-transfection. For time-course experiments, individual transfections were split into three T25 flasks, each containing 2.5 ml medium. For baculovirus transduction, transfections were split among 24-well culture dishes (300 µl per well) and then infected with baculovirus at an m.o.i. of 10 after 16 h. Secreted and intracellular infectivity was quantified by a focus-forming assay (Griffin et al., 2008).

Small molecules. Proteasome inhibitors MG132 (Sigma) and lactacytin (Calbiochem) were prepared as 10 mM stocks in DMSO and used at 5 µM. Lysosome inhibitors chloroquine (Sigma) and leupeptin (Calbiochem) were prepared as 50 and 10 mM stocks in water and used at 50 and 10 µM, respectively. Amantadine hydrochloride (Sigma) was applied to transfected cells at 50 µM, as described previously (Griffin et al., 2008).

Purification of HCV particles. Extracellular virions were purified from 4 ml clarified culture supernatant by layering over a 1 ml cushion (20 % sucrose, w/v, in PBS) followed by ultracentrifugation at 150,000 g for 4 h at 4 °C. The resulting pellet was resuspended in either PBS for titre determination or in 35 µl of EBC lysate buffer for Western blot analysis (Griffin et al., 2008). Intracellular virions were released from cells by four freeze–thaw cycles in PBS (250 µl per 175) and 200 µl was diluted to 1 ml final volume in PBS prior to layering over a 1 ml cushion as above, followed by centrifugation at 150,000 g for 3 h at 4 °C. Resuspended pellets (250 µl PBS) were layered onto a preformed 2 ml iodixanol (10–40 % w/v in PBS) gradient and centrifuged as above. Twelve equal fractions were taken, 10 µl of each of which was used to infect naïve Huh7 cells for focus-forming assays. A further 50 µl was added to 150 µl PBS containing 0.133 % SDS in an ELISA plate, giving a final concentration of 0.1 % detergent.

Protein analysis. The antibodies used were: rabbit anti-core polyclonal serum (308 or R412), mouse anti-core mAb 215 (Biogenes), mouse anti-E2 mAb (AP33, genotype 2a rabbit anti-p7 polyclonal serum 2716 (N terminus) (Griffin et al., 2008; Jirasko et al., 2010; Luik et al., 2009; StGelais et al., 2009), or genotype 1b 1055 (C terminus) (Griffin et al., 2005), rabbit anti-NS2 polyclonal (R3), sheep anti-NS5A polyclonal serum and mouse anti-GAPDH mAb (6C5; Abcam). Supplies of 1055 are now exhausted; we are attempting to develop new p7 antisera. Appropriate HRP-conjugated secondary antibodies were obtained from Sigma. Alexa Fluor-conjugated secondary antibodies were obtained from Invitrogen, as was fluorescent-conjugate concanavalin A. For Western blot analysis, Huh7 cells were lysed in EBC lysis buffer and subjected to SDS-PAGE.
and Western blot analysis as described previously (Griffin et al., 2008). Fluorescence studies of transfected HuH7 cells were performed as described previously (Griffin et al., 2005).

Direct ELISAs were carried out on plates incubated with diluted gradient fractions overnight. Wells were then blocked for 2 h with 5% fat-free milk in PBS containing 0.5% (v/v) Tween 20 (PBS-T), washed five times in PBS-T and then incubated with 308/R4210 rabbit anti-core serum diluted 1:1000 in PBS-T for 1 h at room temperature. Plates were washed again prior to incubation with goat anti-rabbit HRP (1:1000 in PBS-T, 1 h at room temperature). ELISAs were quantified using α-phenylenediamine dihydrochloride substrate (Sigma) with A405 nm readings taken after 1 h at 37 °C without stopping the reaction.

MBP–CD81 large extracellular loop fusion protein was purified on maltose resin according to the manufacturer’s instructions (NEB). Protein (50 μg) on the resin was then added to clarified Glasgow lysis buffer lysate (Hughes et al., 2009) from transfected HuH7 cells (T75 in 500 μl) and incubated at 4 °C for 1 h. Resin was washed three times in Glasgow lysis buffer prior to resuspension in 20 μl Laemmli buffer. Samples were submitted to SDS-PAGE/Western blot analysis alongside input lysate.

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REFERENCES


