Brief Communication

The stability of secreted, acid-labile H77/JFH-1 hepatitis C virus (HCV) particles is altered by patient isolate genotype 1a p7 sequences

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Secreted infectious particles generated by the genotype 2a JFH-1 hepatitis C virus infectious clone are resistant to acidic pH, whereas intracellular virions remain acid-labile. Thus, JFH-1 particles are thought to undergo pH maturation as they are secreted from the cell. Here, we demonstrate that both infectious intracellular and secreted genotype 1a (H77)/JFH-1 chimaeric particles display enhanced acid sensitivity compared with JFH-1, although pH maturation still occurs upon release. Introduction of p7 sequences from genotype 1a infected HCV patients into the H77/JFH-1 background yielded variable effects on infectious particle production and sensitivity to small molecule inhibitors. However, two selected patient p7 sequences increased the acid stability of secreted, but not intracellular H77/JFH-1 particles, suggesting that p7 directly influences particle pH maturation via an as yet undefined mechanism. We propose that HCV particles vary in acid stability, and that this may be dictated by variations in both canonical structural proteins and p7.

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Introduction

Hepatitis C virus (HCV) infects over 170 million individuals, causing severe liver disease and hepatocellular carcinoma. Despite the development of infectious HCV culture over recent years, the processes underpinning infectious particle production are poorly understood and the properties of infectious particles are yet to be fully characterised. Studies of genotype 2a JFH-1 HCV revealed intracellular particles to be acid-labile, whereas secreted particles are resistant to transient pH reductions following release from the cell (Tscherne et al., 2006; Wozniak et al., 2010). Thus, virion pH maturation is thought to occur during/following secretion, counteracting the fusogenic effects of reduced pH on particle-associated glycoproteins (Haid et al., 2009; Lavillette et al., 2006). pH maturation is subsequently reversed during productive virus entry (Tscherne et al., 2006), yet the mechanisms underpinning this process are unclear and the acid sensitivity of other HCV isolates has not been established.

Several HCV proteins contribute to infectious virion production in addition to canonical core and envelope (E1/2) glycoproteins. Amongst these, the 63 amino acid integral membrane protein, p7, performs at least two essential functions, namely: interactions with HCV NS52 that target it to hypothetical sites of virion morphogenesis (Boson et al., 2011; Jirasko et al., 2008; Jirasko et al., 2010; Ma et al., 2011; Popescu et al., 2011; Stapleford and Lindenbach, 2011; Tedbury et al., 2011), as well as oligomerisation to form membrane channel complexes (Griffin et al., 2003; Pavlovic et al., 2003; Premkumar et al., 2004). p7 channel activity within cells can equilibrate proton gradients and potentially protect acid-labile intracellular particles within acidifying secretory vesicles (Wozniak et al., 2010). p7 channel function is therefore reminiscent of influenza A virus M2, which can also protect the haemagglutinin (HA) glycoprotein (Steinhauer et al., 1991) from reduced pH during secretion. Accordingly, p7 and M2 proton...
channel activity appear functionally interchangeable (Bentham et al., 2013; Griffin et al., 2004; Wozniak et al., 2010).

Previous studies indicated that genotype 1a p7 channels (H77 isolate) displayed characteristics reminiscent of a non-selective membrane pore in vitro (Li et al., 2012). This contrasts both with M2 and genotype 1b p7 (Li et al., 2012; StGelais et al., 2007) (J4 isolate), which behave as acid-activated channels with unidirectional conductance. This altered behaviour for genotype 1a p7 channels led us to investigate whether virion maturation may be affected in chimaeric genotype 1a cell culture systems (H77/JFH-1). Here, we show that H77/JFH-1 particles display greater sensitivity to acidic pH compared with JFH-1, yet they still appear to undergo maturation upon release. However, introduction of p7 sequences isolated from genotype 1a infected patients into the H77/JFH-1 backbone can significantly affect the acid stability of secreted particles, suggesting that p7 may exert a direct influence over the pH maturation process.

Results

H77/JFH-1 particles display enhanced acid sensitivity compared with JFH-1

We investigated whether particles comprising genotype 1a structural proteins (H77/JFH-1 chimaera) differed in their acid sensitivity compared with genotype 2a JFH-1 particles. Virions from intracellular and secreted compartments were transiently exposed to either neutral or reduced pH, then neutralised prior to determining residual infectivity (Fig. 1a). Infectivity of intracellular H77/JFH-1 particles was significantly diminished by relatively mild pH reductions (e.g. from pH 7.0 down to pH 6.0), whereas JFH-1 intracellular infectivity was stable until more stringent pH conditions were applied (Fig. 1a, left panel). Furthermore, as seen previously (Wozniak et al., 2010), secreted JFH-1 infectivity remained largely unaffected by transient pH reductions with ~80% of infectivity remaining at pH 4.0, indicative of virion maturation having occurred (Fig. 1a, middle panel). By contrast, secreted H77/JFH-1 infectivity was again significantly reduced at mildly acidic pH. However, comparison of the ratio of residual infectivity in both secreted and intracellular compartments revealed that a degree of pH maturation had still occurred for H77/JFH-1, despite the overall increase in acid sensitivity (Fig. 1a, right panel). Importantly, these characteristics were not due to the overall integrity of H77/JFH-1 particles, as they retained near-identical thermal stability compared with JFH-1 (Fig. 1b).

JFH-1 p7 forms an M2-like channel in vitro

We tested whether JFH-1 p7 displayed in vitro channel characteristics similar to either H77 p7, or to M2 genotype 1b p7. Previous studies have shown that liposome dye release assays robustly discriminate between M2-like channels (e.g. J4 p7) activated by reduced external pH (pH_{ext}) relative to the liposome interior, and pore-like channels (e.g. H77 p7), which are activated by shifting electrochemical gradients in either direction (Li et al., 2012; StGelais et al., 2007). As a positive control, peptides representing the conductance domain of pandemic H1N1 "swine" influenza A virus M2 were assessed for activation upon reduced pH_{ext}. In agreement with its well characterised gating mechanism (Pinto et al., 1997), M2 channels increased in activity upon reduced pH_{ext}, confirming that liposome dye release assay could reliably recapitulate channel gating behaviours observed in other systems (Fig. 1c). Comparison of JFH-1 p7 with the H77 protein, which behaved in a pore-like fashion as reported previously (Li et al., 2012), revealed JFH-1 p7 activity to be strongly activated by reduced pH_{ext}, indeed to an even greater extent than the M2 positive control (Fig. 1c).

Characterising the effects of genotype 1a patient isolate p7 sequences in the H77/JFH-1 background

As H77/JFH-1 and JFH-1 differed both with respect to p7 channel gating behaviour and virion acid stability, we hypothesised that altering p7 channel gating in an H77/JFH-1 context might have consequences for both the production of infectious H77/JFH-1 particles, as well as their pH maturation. Whilst multiple mutations have been identified that affect p7 function, these often have complex phenotypes which, combined with a lack of information regarding p7 gating mechanisms, makes them an unreliable means of manipulating channel behaviour. However, we previously identified a series of genotype 1a p7 sequences from patient isolates (Fig. 2a) that displayed variable activity and gating patterns in vitro (Li et al., 2012), providing a means to alter p7 behaviour in the H77/JFH-1 backbone using isogenic proteins that are functional in an in vivo context.

Comparison of infectious titres generated by H77/JFH-1 and JFH-1 over time revealed a similar level of infectivity, although this was reduced at late times (72 h post-transfection) in the H77/JFH-1 secreted compartment (Fig. 2b). Introduction of patient derived p7 sequences had variable effects on infectivity, although both pore-like (M2_1; S2_167; S3_175; S3_180) and M2-like (M1_4; S1_1) variants (Li et al., 2012) resulted in detectable titres (Fig. 2b). Two variants displayed either a mild (M2_1) or severe (S1_1) reduction of infectivity in both intracellular and secreted compartments. This appeared independent of their displaying pore- or M2-like activity respectively as another M2-like variant (M1_4) produced infectious virus to the same degree as parental H77/JFH-1. The S3_180 variant showed only a very mild particle infectivity defect at later times, which was unexpected as the variant contained a mutation at one of the highly conserved basic residues in the p7 loop region. The S2_167 variant was the only one found to cause an increase in secreted infectivity, although this effect was only ~3-fold. Analysis of viral protein production revealed no apparent disruption to polyprotein processing, although the abundance of variant p7 proteins varied (Fig. 2c). However, reduced/increased p7 abundance did not correlate with particle infectivity, as described recently for JFH-1 p7 carrying alanine substitutions (Bentham et al., 2013). As differences in infectious particle production did not seemingly depend on whether p7 sequences displayed either pore-like or M2-like behaviour, three were taken forward for further characterisation, namely: M1_4 and S1_1 (M2-like) and S2_167 (pore-like).

Sensitivity of patient variant sequences to small molecule p7 channel inhibitors

Genotype 1a H77 p7 displays a high level of resistance to adamantane p7 inhibitors (Griffin et al., 2008; Steinmann et al., 2007) which bind to a peripheral binding site on the membrane exposed face of the channel complex (Foster et al., 2013; Foster et al., 2011; OuYang et al., 2013), and likely stabilise the closed form of the channel complex. As H77 displays extensive sequence similarity to sensitive GT1b channels, we hypothesised that natural variation associated with switching from pore- to M2-like behaviour might influence susceptibility to this class of antivirals. Secreted infectivity was therefore assessed at 72 h post transfection in the presence of the adamantane drug, rimantadine, or the alkylated imino-sugar NN-DNJ, which acts via a different mechanism disrupting the formation of oligomeric complexes (Foster et al., 2011) (Fig. 3). Parental H77/JFH-1, JFH-1, and H77/JFH-1 harbouring variant p7 sequences were all susceptible
to NN-DNJ. By contrast, whilst H77/JFH-1, M1_4 and S2_167 remained insensitive to increasing concentrations of rimantadine, the S1_1 variant displayed sensitivity comparable with JFH-1, which is considered to be a rimantadine sensitive genotype (Foster et al., 2011; Gottwein et al., 2011).

**pH maturation of secreted genotype 1a particles is influenced by patient-derived p7 variants**

Finally, we characterised the acid sensitivity of three H77/JFH-1 clones carrying patient variant p7 sequences. Intracellular infectivity from all three patient p7 variants retained acid stability similar to prototypic H77/JFH-1, although S2_167 displayed intermediate sensitivity at pH 6.2 (Fig. 4, top panels). By contrast, analysis of secreted infectivity revealed significant differences between particles produced by H77/JFH-1 harbouring patient p7 variants and the parental clone. In all three cases, sensitivity to mild pH reductions (pH 6.0, 5.0) mirrored that of JFH-1 particles rather than H77/JFH-1 (Fig. 4, bottom panels) and this trend continued in for the M2-like variants (M1_4 and S1_1) at the lowest pH (pH 4.0). S2_167 variant particles displayed an intermediate sensitivity to pH 4.0, significantly different from either JFH-1 or H77/JFH-1 parental clones. Comparison of the ratios of intracellular and secreted infectivity revealed that the maturation of viruses carrying the S1_1 and S2_167 p7 variants was more efficient. As this ratiometric shift was a result primarily of changes in the pH sensitivity of secreted, but not intracellular particles, we conclude that p7 exerts a direct influence over the particle pH maturation process.

**Discussion**

This work demonstrates that HCV particles comprising genotype 1a (H77) structural proteins differ from those assembled by the parental genotype 2a JFH-1 clone with respect to acid sensitivity, yet their ability to switch from an acid-labile, to a more acid-stable phenotype upon release from the infected cell appears intact. This process, referred to as “pH-maturation” is specific to the effects of reduced pH rather than the overall integrity of particles, based on similar thermal stability. Furthermore, the effects of introducing patient isolate p7 sequences supports that acid stability and pH maturation are influenced by
both canonical structural proteins as well as the p7 sequence encoded by the virus.

The mechanisms underpinning the acid sensitivity of intracellular HCV particles are likely specific to the envelope glycoproteins, as these are known to undergo fusogenic change in response to low pH (Haid et al., 2009; Lavillette et al., 2006). Analogous to certain strains of influenza A virus, exposure to reduced pH as secretory vesicles become acidified via the action of vATPase would be expected to induce premature membrane fusion and so prevent efficient secretion of infectious particles. One potential role for p7 channel complexes within the membranes of secretory vesicles is therefore to prevent the build-up of proton gradients (Wozniak et al., 2010). The majority of intracellular infectious HCV virions reside within the ER in HuH7 cells (Gastaminza et al., 2008) so they are not

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**Fig. 2.** Characterisation of H77/JFH-1 carrying patient variant p7 sequences. Full length constructs were derived containing genotype 1a patient p7 sequences. Growth characteristics and protein expression were assessed following electroporation of HuH7 cells. (A) Sequences of patient isolates compared to parental H77 and JFH-1 p7 sequences. Bold indicates mutations present within patient variants compared with H77 and major, non-synonymous changes are shown on the right hand side. (B) Time course of secreted (top panels) and intracellular infectivity determined by focus forming assay. Controls comprised wild type JFH-1, H77/JFH-1 and JFH-1 carrying the GDD → GND mutation in the NS5B active site (“GND”). Results are the average of three experimental repeats with error bars representing standard error. (C) HCV protein expression was determined within cell lysates, a representative series of immunoblots is shown comparing patient isolates with controls.
exposed to reduced pH, making the dependence upon p7 proton channel function likely to be a late stage of infectious particle production. Accordingly, small molecule p7 inhibitors only prevent the accumulation of secreted infectivity and do not affect intracellular levels (Foster et al., 2013; Foster et al., 2011), presumably as titres are largely conferred by the ER-resident pool. Presumably, intracellular particles exposed to reduced pH in the absence of p7 function are degraded, likely via a proteasome independent mechanism (Gastaminza et al., 2008), which could be linked to activation of the unfolded protein response by HCV glycoproteins (Mohl et al., 2012). However, p7 also plays additional roles at early points during particle production that are likely independent of channel function (Boson et al., 2011; Gentzsch et al., 2013; Jirasko et al., 2008; Jirasko et al., 2010; Ma et al., 2011; Popescu et al., 2011; Stapleford and Lindenbach, 2011; Tedbury et al., 2011). The reduction in intracellular and secreted infectivity observed for H77/JFH-1 carrying the M2_1 and S1_1 p7 variants could therefore arise due to defects in one or both of these functions.

It is tempting to speculate that envelope glycoprotein and p7 sequences may co-evolve such that pore-like channels accompany more acid-labile glycoproteins and vice versa. This appears to hold true for H77, where the pore-like activity of its p7 protein would instantly dissipate the accumulation of protons. Similarly, the more robust JFH-1 particles are associated with an acid-activated, M2-like channel, which presumably allows for a degree of vesicle acidification to occur prior to activation. Thus, incompatible combinations of p7 and envelope proteins, i.e. acid labile glycoproteins and an M2-like p7, might be expected to significantly reduce infectious titres. However, whilst this hypothesis held for the S1_1 M2-like variant p7 in the H77 background which reduced titres, it was not borne out by M1_4, which is also M2-like in vitro. Thus, it is possible that S1_1 polymorphisms also affect p7 functions in addition to its channel activity in the H77/JFH-1 context. Nevertheless, it would be of significant interest to investigate potential genetic linkages between p7 and envelope sequences in patient cohorts.

The mechanisms underpinning the pH-maturation from acid-labile intracellular to stable secreted virions are unclear. Interestingly, secreted JFH-1 virions are largely resistant to transient pH reductions yet prolonged exposure to acidic conditions at 37 °C results in their inactivation (Tscherne et al., 2006). This suggests that energy is required to overcome a feature of secreted particles that otherwise prevents protons from permanently destroying infectivity. The effects of reduced pH on HCV glycoproteins were recently shown to be mediated via conserved histidine residues within E2 (Boo et al., 2012), suggesting that conformational changes resulting from side chain ionisation is responsible. pH maturation may therefore involve either structural rearrangements of the virion itself, or interactions with factors that prevent the acidification of the particle. Either of these possibilities may account for the shift in particle density observed between intracellular and secreted particles (Gastaminza et al., 2006), with lipoprotein interactions providing an obvious candidate. Interestingly, the acid sensitivity of JFH-1 particles in the present study differed to that observed previously (Wozniak et al., 2010), with lesser sensitivity to mild pH changes. Our method herein utilised virions concentrated and partially-purified through a sucrose cushion and resuspended in PBS, rather than harvested directly in complete medium. We hypothesise that (lipo)-proteins or other serum/cellular components co-purified and concentrated through the sucrose cushion may affect the acid sensitivity of purified particles. However, we have not performed a direct comparison as the buffering of media and PBS is also different, the latter requiring mixing with less acidic HEPES/MES buffers to reach a given pH (see Materials and methods and (Wozniak et al., 2010)).

What is not clear is how altering the p7 sequence might influence the stability of secreted virions. It is possible that p7 variants may differentially mediate the trafficking of particles through cellular compartments where pH-maturation may, or may not take place. Alternatively, p7-protein interactions may alter the recruitment of various chaperones, glycosidases or other modifiers of protein structure that might mediate the transition from pH-immature to mature particles during egress. However, both of these possibilities are related to pH solely affecting the glycoprotein components of virions, yet evidence exists to suggest that acidification may be required to mediate stages of productive virus entry in addition to glycoprotein fusion. Specifically, a protonation-mimetic mutant of a conserved E2 histidine involved in pH response (H445R) important for the entry process, was unable to overcome bafilomycin A (bafA) blockade of virus entry (Boo et al., 2012). In another study, citrate was only able to promote the infection of bafA treated cells following prolonged incubation at 37 °C, suggesting that processes additional to glycoprotein fusogenic changes are involved (Tscherne et al., 2006).
This study ruled out a role for endosomal cathepsins, implicating additional features of the virion particle. One possibility is that the nucleocapsid core may require acidification in order to promote efficient uncoating of the RNA, which may be mediated by a stoichiometrically small number of p7 channel complexes present within the virion membrane. However, recent studies of epitope-tagged p7 provided no evidence of the protein being present within the virion (Vieyres et al., 2013), although it is not clear whether glycoproteins might obscure antibody access to underlying channel complexes. Another study found no defects in specific infectivity to be present in low titre particles generated by a J6/JFH-1 chimera carrying a mutation of the p7 dibasic loop region. However, such mutations may disrupt p7 membrane insertion rather than channel activity (Bentham et al., 2013; Perez-Berna et al., 2008; StGelais et al., 2009), making it possible that functional channel complexes existed within mutant particles. Furthermore, as both p7 inhibitor studies (Griffin et al., 2008) and the uptake of HCV-like particles by hepatocytes (Saunier et al., 2003) functionally support a role for p7 during entry, more detailed analysis will be necessary to determine conclusively whether p7 channel complexes exist within particles and regulate their acid sensitivity.

Taken together, our studies show that acidic pH plays a vital role in the egress and stability of infectious HCV particles. This appears likely to vary both between genotypes as well as at the level of individual virus variants within the p7 protein.

Materials and methods

Plasmid constructs

Constructs for glutathione-S-transferase (GST) fusions of GT1a H77 and patient p7 sequences were described (Li et al., 2012). Full length constructs were based on GT2a JFH-1 (Wakita et al., 2005), including the GT1a chimera, pHJ3-5 (Yi et al., 2007), encoding core
to NS2 of HCV H77. Patient p7 sequences were amplified from cDNA using specific primers, and introduced into pHJ3-5 digested with MauBl (Fermentas) and AfEl (New England Biolabs). Details and primer sequences are available upon request.

Analysis of recombinant p7 channel activity

FLAG-tagged p7 was expressed, cleaved from GST, and purified by rHPLC as described (Clarke et al., 2006; Foster et al., 2011; StGelais et al., 2007). M2 conductance domain (aa 18–60) peptides of pandemic “swine” influenza A virus H1N1 (A/pH1N1), incorporating an N31S mutation, were obtained from Peptides International Inc., (Louisville, KY, USA). Sequence: RSCDSDDLVIASSGIGLHLLWITD-RLFCKYTRFFKLYGK-NH2. Liposome dye release assays for pH dependent channel activity were conducted as described (Li et al., 2012; StGelais et al., 2007; Wetherill et al., 2012).

HCV culture

Huh7 cells were cultured, transfected and treated with Riman- tadine (Chembridge) and NN-DNJ (Toronto Biochemicals) as described (Griffin et al., 2008). Time course experiments divided single electroporations (8 x 10^6 cells) amongst three T25 flasks (Corning) in 5 ml of HEPES-buffered (pH 7.4) DMEM, with cells/media harvested every 24 h. Infectivity was determined by focus forming assays (Griffin et al., 2008).

Virion pH sensitivity

Particle acid sensitivity was assessed using a method adapted from Wozniak et al. (2010). HEPES/MES buffers (20 mM HEPES, 20 mM MES, 133.5 mM NaCl, 2 mM CaCl2, 4 mM KCl, 1.2 mM MgSO4, 11 mM glucose) were prepared to pH 3.3, pH 3.9, pH 6.0 and pH 7.4, to yield final pH values of 4.0, 5.2, 6.2 and 7.4 when mixed 1:1 (v/v) with PBS (pH 7.4). Clarified supernatants were harvested and concentrated through a PBS/20% w/v sucrose cushion at 150,000 x g for 4 h in a Sorvall AH650 rotor. Intracellular virus was prepared by freeze-thaw lysis (x 3) of harvested cells in PBS, followed by removal of cell debris by centrifugation at 1000 x g for 5 min at room temperature in a microfuge. Purified virus stocks were mixed 1:1 with HEPES/MES buffers as above, then incubated for 10 min at 37 °C. Acidic pH was neutralised by an equivalent volume of 1 M NaOH. Residual infectivity was determined by focus forming assay.

Virion temperature sensitivity

Clarified supernatants were harvested 48 h post-transfection, aliquotted, and then placed in a water bath at 54 °C. Triplicate samples were removed at each time point and placed immediately on ice prior to determining residual infectivity by focus forming assay.

Protein analysis

Total cell lysates in enriched broth culture (EBC) lysis buffer containing 0.5% NP40 and 0.1% SDS were generated from T25 flasks as described (Griffin et al., 2008). Immunoblots were performed using mouse monoclonal anti-E2 antibody (AP33), rabbit anti-core polyclonal serum (R4128), rabbit anti-NS2 polyclonal serum, sheep anti-NS5A polyclonal serum, mouse monoclonal anti-glyceraldehyde phosphate dehydrogenase (GAPDH; GC1, Abcam) and rabbit anti-p7 polyclonal serum 2716 (Foster et al., 2011; Griffin et al., 2008; Luik et al., 2009; StGelais et al., 2009) purified and concentrated on a protein G column. 2716 is specific to the JFH-1 p7 N-terminus and is weakly cross-reactive with GT1a.

Author contributions

Performed experiments: EA, RT
Analysed data: EA, MH, SG
Planned and conceived experiments: SG, MH, DC, HL
Reviewed the paper: EA, MH, DG, HL
Wrote the paper: SG

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