Genetic and functional heterogeneity of the hepatitis C virus p7 ion channel during natural chronic infection

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Abstract

The present study describes natural genetic heterogeneity of hepatitis C virus (HCV) p7 protein, the ion channel that plays a critical role in assembly and release of HCV, within 299 variants isolated from serum specimens of 27 chronically infected patients, 12 of whom with human immunodeficiency virus (HIV) co-infection. Liver fibrosis stage was inversely correlated with p7 synonymous substitutions (dS) (p = 0.033), and indices of p7 genetic diversity were significantly higher in HIV-negative subjects compared to HIV-positive subjects (dS, p = 0.005; non-synonymous substitutions (dN), p = 0.002; dN/dS ratio, p = 0.024; amino acid distances, p = 0.007). Six p7 genes with naturally occurring unique amino acid variations were selected for in vitro study. The variants demonstrated diversified functional heterogeneity in vitro, with one variant from a subject with severe liver disease displaying hyperactive ion channel function, as well as other variants presenting altered pH-activated channel gating activities.

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Introduction

Chronic infection with hepatitis C virus (HCV) represents a major and growing global health concern, and is now the leading cause of liver cirrhosis, hepatocellular carcinoma, and death due to liver disease in the United States and other developed nations. Although 20–30% of HCV-infected patients spontaneously clear the virus, the majority of infections progress to the chronic phase, characterized by continuous, high level viremia, and a high degree of viral genetic heterogeneity. The long term clinical course of chronic HCV infection is highly variable: only about 20% of such cases develop into progressive liver disease, typically over a 2–3 decade period of infection (McMahon et al., 2010; Seef et al., 2000). The leading pathogenesis model assumes that liver damage is largely mediated by cytotoxic immune responses, and other adverse consequences of chronic inflammation, including generation of toxic free radicals, such as reactive oxygen species (Mengshol et al., 2007). However, several lines of evidence suggest that viral factors may also play a role in hepatitis C disease, the most striking being selection of cytopathic HCV infectious clones in vitro (Mishima et al., 2010), and identification of HCV-associated cytopathic effects in immune deficient, chimeric mice in vivo (Joyce et al., 2009). During human infection, studies have revealed highly significant differences in HCV genome evolution between mild and severe disease subjects, with viral sequence homogenization, and accumulation of synonymous mutations (dS) within HCV genomes more extensive during severe disease (Li et al., 2008; Sullivan et al., 2007). Our recent study of 54 near full-length HCV genomes circulating in chronically infected humans over time revealed significant fixation of amino acid sequences during severe disease in four HCV genes: p7, NS2, NS3 and NS5B (Li et al., 2011). Whereas high genetic heterogeneity of HCV likely contributes to viral persistence via immune escape mechanisms, the functional consequences of HCV genome fixation during natural, in vivo infection of humans have not been previously characterized.

The genome of HCV is a 9.6 kb positive-sense single strand RNA that encodes 5′ and 3′ untranslated regions (UTRs), plus a single polyprotein of approximately 3011 amino acids in length. Following cap-independent translation, the polyprotein is cleaved by both host and viral proteases to yield 10 functional mature proteins, including 3 putative structural proteins (core, envelope 1 (E1), and envelope 2 (E2)), an ion channel protein (p7), and 6 non-structural proteins

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The demographic and clinical characteristics of the 27 patients recruited from our cross-sectional study at the Harborview Medical Center site, Seattle, Washington, are listed in Table 1, according to HIV infection status. All 27 patients were infected with HCV genotype 1a. The HIV negative and HIV positive groups were similar in age (mean 44.3 vs. 44.0 years, respectively; \( p = 0.93 \)) as well as duration of HCV infection (mean 21.6 years vs. 17.0 years, respectively; \( p = 0.25 \)). Furthermore, there were no significant differences in either mean viral load, mean AST value in serum, or mean ALT value in serum between HIV positive and negative groups (Table 1). The status of liver disease was evaluated by liver biopsy at the time of patient recruitment, within 30 days of serum procurement for p7 sequencing studies; no difference was found between the HIV negative and HIV positive groups with respect to inflammation (mean grade 1.5 vs. 1.9, respectively; \( p = 0.21 \)) or fibrosis stages (mean stage 1.9 vs. 2.1, respectively; \( p = 0.56 \)).

**Genetic diversity of the HCV p7 gene according to HIV status and disease severity**

A total of 299 p7 variants were sequenced from the serum samples of the 27 patients recruited in Seattle. The genetic diversity of p7 variants was compared according to HIV infection status, and data are summarized in Fig. 1 and Table 2. Synonymous substitutions (\( dS \)) were more frequent than non-synonymous substitutions (\( dN \)) in p7 genes isolated from both groups, regardless of the HIV infection status (\( dS = 0.0349 \) versus \( dN = 0.0061 \) for HIV negative patients, \( p < 0.01 \); \( dS = 0.0144 \) versus \( dN = 0.0011 \) for HIV positive patients, \( p < 0.01 \)). Both synonymous and non-synonymous substitutions were significantly higher in HIV negative versus HIV positive subjects (\( p = 0.005 \) and \( p = 0.002 \) for \( dS \) and \( dN \), respectively). Similarly, the HIV negative patients showed significantly greater genetic diversity at both nucleotide (\( p = 0.003 \)) and amino acid (\( p = 0.007 \)) levels. The \( dN/dS \) ratio, generally considered as an indicator of host immune pressure, was significantly higher in HIV negative subjects compared to HIV positive subjects (\( p = 0.024 \)).

**Results**

**Patient characteristics**

The demographic and clinical characteristics of the 27 patients are shown in Table 1, according to HIV infection status. The groups were compared using the Fisher’s exact test for the gender and race or the Student t test for the other characteristics.

### Table 1

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>HIV negative (n=15)</th>
<th>HIV positive (n=12)</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age</td>
<td>44±6</td>
<td>44±9</td>
<td>0.93</td>
</tr>
<tr>
<td>Gender</td>
<td></td>
<td></td>
<td>0.09</td>
</tr>
<tr>
<td>Male</td>
<td>9</td>
<td>11</td>
<td></td>
</tr>
<tr>
<td>Female</td>
<td>6</td>
<td>1</td>
<td>0.38</td>
</tr>
<tr>
<td>Race</td>
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<td></td>
</tr>
<tr>
<td>Caucasian</td>
<td>9</td>
<td>8</td>
<td></td>
</tr>
<tr>
<td>African American</td>
<td>1</td>
<td>3</td>
<td></td>
</tr>
<tr>
<td>Hispanic</td>
<td>3</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Native American</td>
<td>2</td>
<td>1</td>
<td></td>
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<tr>
<td>HCV duration (year)</td>
<td>22±9</td>
<td>17±10</td>
<td>0.25</td>
</tr>
<tr>
<td>AST (U/L)</td>
<td>59±60</td>
<td>121±150</td>
<td>0.16</td>
</tr>
<tr>
<td>ALT (U/L)</td>
<td>76±64</td>
<td>136±135</td>
<td>0.18</td>
</tr>
<tr>
<td>HCV RNA (log10IU/ml)</td>
<td>6.5±0.6</td>
<td>6.4±1.3</td>
<td>0.87</td>
</tr>
<tr>
<td>Inflammation grade (0–4)</td>
<td>1.5±0.7</td>
<td>1.9±0.8</td>
<td>0.21</td>
</tr>
<tr>
<td>Fibrosis stage (0–4)</td>
<td>1.9±0.9</td>
<td>2.1±1.0</td>
<td>0.56</td>
</tr>
</tbody>
</table>

**Fig. 1.** Nucleotide substitutions of p7 from HCV monoinfected and HIV/HCV coinfected subjects. The Mann-Whitney U test was used to compare the median of the two groups. A) Synonymous nucleotide substitution (\( dS \)); B) Non-synonymous nucleotide substitution (\( dN \)); C) \( dN/dS \) ratio.
Table 2
Comparison of the median genetic parameters between the HCV mono- and co-infected patients. dS, the synonymous mutations, indicates the proportion of observed synonymous mutations over the number of potential synonymous mutations of the pairwise comparison. dN, the non-synonymous mutations, indicates the proportion of observed non-synonymous mutations over the number of potential non-synonymous mutations of the pairwise comparison. p values were calculated with the Mann–Whitney test.

<table>
<thead>
<tr>
<th></th>
<th>dS</th>
<th>dN</th>
<th>dN/dS</th>
<th>Nucleotide distance</th>
<th>Amino acid distance</th>
</tr>
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<tbody>
<tr>
<td>HIV neg</td>
<td>0.0349</td>
<td>0.0061</td>
<td>0.1727</td>
<td>0.0135</td>
<td>0.0154</td>
</tr>
<tr>
<td>HIV pos</td>
<td>0.0144</td>
<td>0.0011</td>
<td>0.0870</td>
<td>0.0070</td>
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</tr>
<tr>
<td>p value</td>
<td>0.005</td>
<td>0.002</td>
<td>0.024</td>
<td>0.003</td>
<td>0.007</td>
</tr>
</tbody>
</table>

When compared according to disease status, the frequency of p7 gene synonymous substitutions was significantly higher among subjects with mild disease (fibrosis scores of 0–1) compared to subjects with severe disease (fibrosis 3–4) (p = 0.016). In concordance, synonymous substitutions were negatively correlated with fibrosis stage at statistical significance (r = −0.412, p = 0.0033) (Fig. 2A). The lowest medians of non-synonymous substitution, nucleotide distance, and amino acid distance, were found in the patients with severe disease, although the distribution of these parameters was not statistically significant (Figs. 2B, C and D). The lack of statistical significance for the latter comparisons may be due to under sampling bias.

p7 amino acid variation

The amino acid residues of the 299 sequenced variants from 27 subjects were highly conserved, with an average of 93% sequences showing identical amino acid per site. 8 residue positions were highlighted with amino acid variations in more than 20% of the total sequences, including position 7 (35% V, 33% L, 16% I, and 15% T), position 16 (77% T and 23% A), position 19 (60% V vs. 100% V), 20 (80% V vs. 52% A). When comparing among disease groups, greater than 20% difference in amino acid identity was found on 6 residue positions between the mild (fibrosis 0–1) and severe (fibrosis 3–4) disease groups, including positions 6 (60% V vs. 100% V), 7 (27% I vs. 52% L), 16 (89% T vs. 63% T), 17 (75% H vs. 100% H) and 20 (80% V vs. 52% A).

Cloning and expression of naturally occurring p7 variants

Genetic data revealed interesting, potentially significant non-synonymous changes in primary amino acid sequences of several p7 genes isolated from both mild and severe disease individual cases. Therefore, in vitro functional studies were performed. Naturally occurring HCV p7 gene variants were cloned from serum specimens of 3 subjects from the Haborview cohort study (M1, M2 and S1) and 2 additional subjects enrolled in the Alaska Native cohort study (McMahon et al., 2004) (S2 and S3). From these 5 subjects, six unique p7 variants were selected for in vitro testing of ion channel activities, with H77 (genotype 1a) and J4 (genotype 1b) p7 clones serving as prototype controls (Fig. 4 and Table 3). Variant M1_4, with mutations of S21P, P38S, and A40T, was obtained from an HIV-negative Seattle patient with severe disease (fibrosis stage 1). Variant M2_1, with a mutation of H17R, was obtained from an HIV-positive Seattle patient with mild disease (fibrosis stage 0). Variant S1_1, with a mutation of Y31H, was obtained from an HIV-negative Seattle patient with severe disease (fibrosis stage 3). Variant S2_167, with mutations of H17N and F44C, was obtained from an HIV-negative patient with severe disease (fibrosis stage 3). Variants S3_175 and S3_180 were obtained from another HIV-negative patient with severe disease (fibrosis stage 3). Both variants had T16A, while the latter one had R35G and V41T.

In Fig. 3, the frequency of most common amino acids, per site, is plotted according to HIV infection status (Fig. 3A), and liver disease severity (Fig. 3B), over the full-length p7 frame. Overall, the amino acid identity per site was similar between the HIV negative and HIV positive groups (92.8% vs. 93.1%), with position 20 showing the greatest difference (73% V vs. 51% V for HIV negative and positive groups, respectively). When comparing among disease groups, greater than 20% difference in amino acid identity was found on 6 residue positions between the mild (fibrosis 0–1) and severe (fibrosis 3–4) disease groups, including positions 6 (60% V vs. 100% V), 7 (27% I vs. 52% L), 16 (89% T vs. 63% T), 17 (75% H vs. 100% H) and 20 (80% V vs. 52% A).
We recently showed that p7 acts as a proton channel in cellular membrane compartments, inducing vesicle alkalinization to protect intracellular virions from acidic pH at an as-yet undefined stage of virion release (Wozniak et al., 2010). Accordingly, we have also shown that the genotype 1b J4 p7 protein displays increased activity at reduced pH (StGelais et al., 2007), consistent with a gating mechanism mediated by ionization of luminal amino acid residues, including His17. To investigate whether amino acid variation, particularly within luminal residues, observed in patient sequences could lead to a direct effect on p7 ion channel function, we expressed selected proteins in vitro and analyzed activity in a liposome-based fluorescent dye release assay (StGelais et al., 2007). All proteins were efficiently expressed as visualized by Coomassie-stained SDS PAGE and different sequences caused variation in electrophoretic mobility, as seen previously (Griffin et al., 2005, 2008) (Fig. 5). The liposome-based dye release assay revealed one variant, S2_167 from a patient with severe disease, with markedly enhanced channel activity compared with H77 and/or J4, indicative of a hyper-activated channel (Fig. 6). This protein contained a H17N non-synonymous variation in the lumen, which is common to many genotype 2 HCV isolates, as well as F44C within the C-terminal helix.

We next tested channel activity under conditions where the pH of the external milieu (pH_{ext}) was altered relative to the liposome interior (pH 7.0). We have previously noted a marked increase in activity for J4 p7 when pH_{ext} was reduced below pH 7.0 (StGelais et al., 2007). As seen previously, J4 channel activity was markedly increased at acidic pH (Fig. 7A), whereas the prototype genotype 1a H77 protein displayed a different pattern of activation that was not enhanced by reduced pH; indeed, activity was reduced at near-neutral values (pH 6.7) compared with either mildly acidic (pH 6.2) or alkaline (pH 7.4) conditions. The majority of the patient variants tested (M2-1, S2-167, S3_175 and S3_180) behaved essentially as H77 in the pH activation assay with slightly reduced activity at pH 6.7 compared with 7.4 and 6.2 (Figs. 7C and D). Interestingly, two patient variants behaved more akin to the genotype 1b J4 p7 compared to H77, whereby channel activity was markedly increased by reducing...
pHext to 6.7 (Fig. 7B). These hailed from both mild (M1-4) and severe (S1-1) cases. M1-4 contained non-synonymous S21P and A40T changes, of which S21P would be predicted to affect the channel lumen. Interestingly, S1-1 contained a Y31H change which is also present within the channel lumen and variation at this position has previously been shown to influence virion production in a genotype 2a background (Brohm et al., 2009).

Discussion

Recently, the evolution of HCV genetic diversities has been studied in multiple cohorts with longitudinal clinical follow-ups. It has been shown that during natural HCV infection (Li et al., 2008; Sullivan et al., 2007), during HIV coinfection (Shuhart et al., 2006), and in the setting of immune suppression after liver transplantation (Li et al., 2010; Sullivan et al., 1998), genetic homogenization of HCV quasispecies is significantly associated with progression of severe hepatitis C disease. This distinctive virological phenomenon is also observed in the near full-length HCV open reading frame, with viral protein sequences significantly conserved over time in patients with severe disease (Li et al., 2011). For the present study, the sequence variability of the ion channel protein p7 was analyzed in a sub-cohort of 27 patients with chronic HCV infection, 12 of whom were coinfected with HIV. P7 genetic diversity, as indicated by synonymous substitutions, non-synonymous substitutions, dN/dS ratio, and amino acid distances, was significantly higher in HIV negative patients compared to HIV positive ones. The severity of liver disease was evaluated for all the patients by liver biopsies. The liver fibrosis stage was inversely correlated with synonymous substitutions, which is in consistence with previous studies that genetic homogenization coincidently occurs with advanced liver disease.

The p7 protein, as a member of the viroporin family, shares sequence and functional similarity to the M2 channel protein of influenza A virus. M2 channel structure has been widely studied in strains of influenza A virus with mutational analysis of the channel gating mechanism. His37 of M2 has been proposed as a pH sensor that causes a conformational shift in the gating residue Trp41 and leads to channel opening on protonation (Tang et al., 2002). Similarly, a counterpart has been identified in p7 of HCV by sequence and structural analysis, including His17 present in the lumen of the channel (Chew et al., 2009), and Tyr31 at the end of trans-membrane domain 1 (Brohm et al., 2009), likely as the gating residues. Although mutagenesis studies with H17A in the genotype 1b J4 strain showed highly reduced activity in a liposome-based fluorescent dye-release assay (StGelais et al., 2009), the same mutation showed no inhibition of channel activity in the genotype 1a H77 strain (Chew et al., 2009), and little effect on the production of infectious virions in the genotype 2a JFH1 strain (Brohm et al., 2009). The basic residues Arg 33 and Arg 35 in the highly conserved loop region between the two trans-membrane regions have also been shown essential to the p7 channel activity. Mutations R33A R35A in the basic loop region resulted in no production of infectious virions in the full length JFH1 system, while the less geometrically extreme mutant R33Q R35Q showed a substantial reduction of infectious virions (Steinmann et al., 2007).

In the present study, a large variation of p7 sequences were observed among different patient isolates, and six variants were selected for in vitro liposome dye-release assay to analyze channel activity. As the prototype control, H77 p7 showed relatively stable channel activity to various pH, while J4 p7 showed significantly increased activity when pH decreased from 7.4 to 6.2. Given that the H17A mutation does not inhibit H77 p7 channel activity in black lipid membranes (Chew et al., 2009), His17 in H77 p7 may not be as important in channel functions as the same histidine of J4 p7. In contrast to the J4 p7, in which the protonated His17 potentially

<table>
<thead>
<tr>
<th>Variant</th>
<th>Amino acid change</th>
<th>Host characteristics</th>
<th>Fibrosis stage</th>
<th>HIV status</th>
</tr>
</thead>
<tbody>
<tr>
<td>M1_4</td>
<td>S21P, P38S, A40T</td>
<td>1</td>
<td>Negative</td>
<td></td>
</tr>
<tr>
<td>M2_1</td>
<td>H17R</td>
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<td>S1_1</td>
<td>Y31H</td>
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<tr>
<td>S2_167</td>
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<td>3</td>
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<td></td>
</tr>
<tr>
<td>S3_175</td>
<td>T16A</td>
<td>3</td>
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<td></td>
</tr>
<tr>
<td>S3_180</td>
<td>T16A, R35G, V41T</td>
<td>3</td>
<td>Negative</td>
<td></td>
</tr>
</tbody>
</table>

Table 3: Major amino acid changes of the six tested p7 variants compared to J4 and H77.

Fig. 5. In vitro expression of six p7 variants. All 6 proteins were expressed as GST fusions with a FLAG tag on the N-terminal of the p7 sequence. The GST was then cleaved off and the proteins were purified by HPLC. 5 μg of each protein was loaded and stained with Coomassie Blue in the following order: 1) H77 p7; 2) M1_4 p7; 3) M2_1 p7; 4) S2_167 p7; 5) S1_1 p7; 6) S3_175 p7; 7) S3_180 p7.

Fig. 6. Real-time dye release assay after addition of p7 proteins. A) 5 μg of each of the proteins in methanol was added to 50 μM of liposomes containing CF, and dye release measured every 30 s over a period of 30 min. Controls were liposomes alone, liposomes with methanol, liposomes with the pore-forming peptide melitin and liposomes with 0.5% Triton (melitin and triton controls omitted for scale). Each sample was carried out in duplicate and the mean plotted for each sample. B) Initial reaction rates taken from the above real-time dye release assay and shown as fluorescent units released per second (FU/s). Gradients for the first 300 s were taken by plotting the regression line for each sample, then calculating the mean and standard deviation for each protein (n = 2).
induce a conformational shift to enhance channel activity, the H77 p7 channel activity is likely to remain at a relatively high level, and the slightly decreased activity at near neutral pH is likely due to bidirectional proton gradients. Such channel activity was still observed from the proton-regulated J4 channels at pH 7.4, and from H77 channels at neutral pH, which may be due to the CF concentration gradient between the liposome interior, and the external milieu forces.

The naturally occurring p7 variants we selected for study showed a wide variation in channel activity. The most active variant, S2_167, has a H17N mutation at the inner surface of pH7 lumen. The side group of asparagine is polar rather than charged, and smaller than both arginine and histidine. It is possible that this mutation can create a constitutively open channel that is unresponsive to pH. Interestingly, the J6 p7 sequence has N17, and this confers greater virion productivity (such as seen in J4) for ions to be transported across. Introducing a P acidic pH may disrupt hydrophobic interactions that make the channel complex stable, resulting in a more “open” structure. The S3_180 variant, with mutation of one of the two highly conserved basic loop residues, R35G, still appears to be a functional channel in the in vitro dye-release assay. This observation argues against the role of these basic loop residues in channel activity, although a previous study reported that the K33A/R35A mutant exhibited drastic decrease in the assembly and release of infectious intracellular virions (Jones et al., 2007). Both S3_175 and S3_180 variants have the T16A mutation and showed broadly similar activity and pH response compared to the H77 prototype. The residue at position 16 in J4 p7 is also alanine, so it is possible that this mutation is not significant in channel activity.

Two variants, M1_4 and S1_1, showed increased channel activity at acidic pH. The pH response of M1_4 is possible due to the increased hydrophobicity within the lumen as a result of S21P. A more hydrophobic lumen may require more energy or a shift in protein conformation (such as seen in J4) for ions to be transported across. Introducing a P could also cause a bend in the helix that could alter the structure significantly. The mutation of Y31H, in the variant S1_1, potentially serves as a pH-sensor gating residue at the cytosolic end of the channel that could make it responsive to pH. Interestingly, the JFH-1 strain, which shows more pH responsiveness than J4, also contains histidine at this position, although mutation of H31 showed no significant effect on virion production (Steinmann et al., 2007). The histidine mutant M2_1 showed no pH responses, although the channel activity remained constitutively high in the tested pH range. This variant contains a unique H17R substitution, in which arginine, with a much higher pKa (12.1), replaces histidine (pKa = 6.04). Since arginine is only deprotonated at much higher pH, the channel gating effect probably occurs at a higher pH range. Further experimentation is needed to elucidate if such pH gating mechanisms exist.

It remains hotly debated whether genetic heterogeneity is naturally associated with functional heterogeneity in highly mutated RNA viruses such as HCV. A recent study on HCV NS3-NS4A proteins demonstrated that the protease activities of genotype 4a, 5a, and 6a isolates were similar to those of genotype 1b and 2a references, while a genotype 2a variant demonstrated a significantly higher helicase activity compared to other HCV genotypes (Massariol et al., 2010). Meanwhile, tumor-derived HCV core variants have been shown to significantly inhibit the tumor growth factor-beta (TGF-beta) pathway, in contrast to moderate or no effects observed with non-tumor variants or reference clones (Pavio et al., 2005). In our study, we characterized the channel activity of six p7 variants isolated from serum specimens of HCV-infected patients, and our results indicated that p7 variants with genetic variation exhibit functional variability at an extended range. As a preliminary study, the number of variants is still small to draw correlation between p7 activity and disease severity. For example, the variant M2_1 from a patient with mild disease showed constitutively high activity, with no change in response to pH, while the variant S1_1 from a patient with severe disease showed relatively low activity, with an increase in response to reduced pH. However, viral genetic homogenization during severe disease highlights the possibility of variant outgrowth in a manner related to the pathogenesis of hepatitis C disease.

In summary, the present study documents genetic and functional heterogeneity of the HCV p7 protein in nature, and sets the stage for understanding whether or not naturally occurring heterogeneity in HCV translates into differences in efficiency of virogenesis, or viral disease potential. Further experiments will be performed to characterize the channel activity of the isolated p7 variants under a wide range of pH conditions. Since liposomes are unable to tolerate pH lower than 6, p7 proteins will be expressed in Xenopus oocytes followed by patch clamping to increase the pH range that can be studied. The patient variants will be further investigated in the chimeric H77/JFH1 virus developed by S. Lemon et al. (Yi et al., 2007) for details on the effects of these mutations in viral particle release and drug susceptibility.

Materials and methods

Study subjects and specimens

Twenty-seven subjects with chronic HCV genotype 1a infection were recruited at the Harborview Medical Center at Seattle, WA. All patients
were negative for hepatitis B surface antigen, negative for recent acute infection, and without previous HCV antiviral treatment. Twelve of the patients were coinfected with HIV while nine were on highly active antiretroviral therapy (HAART) at the time of study. A written informed consent was obtained from each subject prior to study participation in accordance to the regulation of Human Subjects Committee of the University of Washington. Liver biopsies were reviewed by a single study pathologist (LVT) according to the Batts and Ludwig system with Inflammation Grade (0–4) and Fibrosis Stage (0–4) evaluations (Batts and Ludwig, 1995). Serum specimens were collected within 30 days of liver biopsy and stored at −80 °C for further virological analysis. HCV genotype was determined using restriction fragment length polymorphism (RFLP) analysis of sequences amplified from the 5′ non-coding region (Davidson et al., 1995), and confirmed by nucleotide sequencing and phylogenetic analysis (see below). HCV RNA titer was determined using the third-generation branched DNA assay (VERSANT HCV RNA 3.0, Bayer Diagnostics, Tarrytown, NY), or ultrasensitive real-time RT-PCR assay.

For in vitro studies, p7 variants were cloned from 3 subjects in the Harborview cross sectional cohort (one HIV-positive and two HIV-negative), plus additional 2 viremic subjects from the Alaska Native cohort (both HIV-negative) (McManus et al., 2004). Both Alaska Native persons consented to a long-term hepatitis C study conducted by the Liver Disease & Hepatitis Program of the Alaska Native Tribal Health Consortium in Anchorage, AK. This study was approved by the Alaska Area Institutional Review Board, Native Health boards of the Alaska Native Tribal Health Consortium, and the Southcentral Foundation. All 5 subjects were infected with HCV genotype 1a, and were naive to HCV treatment. Three subjects developed severe hepatitis C disease, as indicated by advanced bridging fibrosis on liver biopsy (S1, S2 and S3), and two had consistently mild disease (stage 1 fibrosis for subject M1, and stage 0 fibrosis for subject M2).

Cloning and sequence analysis

Total RNA was extracted from the serum samples using the QIAamp Viral RNA mini kit (Qiagen, Germantown, MD). cDNA was synthesized from each of the RNA samples using the SuperScript III RT-PCR assay.

Resultant GST-FLAGp7 was expressed, cleaved and FLAG-p7 purified by reverse phase HPLC as previously described (Clarke et al., 2006).

Liposome dye release assay and pH response test

Channel forming activity of purified FLAG-p7 proteins was assessed in a liposome-based fluorescent dye release assay and incorporating assays where external reaction buffer pH was altered as previously described (StGelais et al., 2007).

References


