The p7 viroporin of the hepatitis C virus contributes to liver inflammation by stimulating production of Interleukin-1β

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A B S T R A C T

Hepatitis C is one of the most widespread infectious diseases worldwide and hepatitis C virus (HCV)-induced chronic inflammation is highly associated with progressive liver damage. It was shown that HCV infection increases levels of pro-inflammatory cytokines via activation of NOD-like receptor (NLRP3) inflammasomes, yet the underlying mechanism is still under question. We propose modulation of intracellular pH by p7, a 63 residue ion channel produced by the hepatitis C virus as a possible pathomechanism for hepatitis C-associated inflammation. Recombinant constructs corresponding to HCV genotypes 1–4 were expressed in HEK 293 and RAW 264.7 cells and changes of intracellular pH were monitored using pH-sensitive fluorescent probes as well as production of inflammatory cytokines. Presence of p7 induced general loss of vesicular acidity as well as producing a significant increase in the levels of interleukin-1β (IL-1β). Effects showed a genotype-dependent pattern of IL-1β production, in agreement with the pH-response profile of p7 channels corresponding to hepatitis C genotypes. Lowering the pH of the extracellular medium increased activity of p7 channels as well as production of IL-1β for genotypes 1, 3, and 4, but less for genotype 2. Our data are in agreement with the hypothesis that p7 activity can trigger intracellular signaling cascades that are involved in HCV-associated cytopathity.

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1. Introduction

Hepatitis C virus (HCV) infection is the second most common cause of viral hepatitis after hepatitis B and constitutes one of the most severe blood borne infections with significant morbidity all over the globe [1]. HCV infection is a major cause of acute and chronic liver disease with >3% of the world’s population infected and >170 million chronic carriers at risk of developing liver cirrhosis and/or liver cancer [2]. Although new, highly promising treatments have recently been introduced, the search for anti-viral targets is still a major necessity, especially in countries such as Egypt, where 18% of the population is reported to be infected with HCV [3].

HCV is a small enveloped virus with a positive sense single stranded RNA genome of about 9.6 kb. According to nucleotide sequence variation, patient isolates are grouped into at least six major genotypes of HCV deviating from each in as much as 30% of the sequence [4]. The genome has a single open reading frame (ORF) encoding a large polypeptide of about 3000 amino acids and is flanked by an untranslated region (UTR) at both the 3′ end and the 5′ end [5,6]. The polypeptide is co- and post-translationally cleaved by a set of cellular and viral proteases in the Endoplasmic reticulum (ER) into 10 proteins [5], including three structural proteins: core protein and two envelope glycoproteins E1 and E2 which mediate binding to co receptors and entry into hepatocytes. There are six nonstructural proteins (NS): NS2, NS3, NS4A, NS4B, NS5A and NS5B, which generate cellular conditions necessary for viral genome replication and assembly of viral particles [7]. The small 63-residue protein p7, located at the junction of the structural and non-structural proteins, is a transmembrane protein that assembles into hexa- or heptameric intracellular ion channels [8] that are activated by low pH and proton-conducting [8–10]. P7 activity thus affects membrane permeability and pH gradients of several cellular compartments and is suggested to assist in virus assembly and release [11–13]. Involvement of p7 in HCV-induced cellular pathogenesis has also been suggested [14,15]. Indeed, chronic inflammation is a major complication associated with HCV-induced liver injury. It has been shown that HCV increases the levels of proinflammatory cytokines such as IL-1β in hepatic macrophages via activation of NOD-like receptor (NLRP3) inflammasomes [16], although the mechanism of activation of the inflammasome pathway through HCV is not yet understood. Inflammasomes are molecular complexes involved in the activation of inflammatory caspases, which lead to the processing of immature proIL-1β and proIL-18 into their mature forms [16]. To date, four inflammasomes have been identified and classified by the NLR protein that they contain [16,17].
Here, we describe expression of recombinant p7 constructs in LPS-primed Raw 264.7 cells where they cause significant increase in the levels of IL-1β in a genotype dependent pattern. IL-1β production parallels the pH-response profile of p7 channels, suggesting that p7 activity can trigger intracellular signaling cascades that are involved in HCV-associated cell injury. TNF-α production was not affected by absence or presence of p7.

2. Materials and methods

2.1. Generation of p7 expression constructs

A set of p7 constructs with and without a c-myc tag for Western Blot analysis had been prepared and described previously [10].

2.2. Cell culture and transfection

Human embryonic kidney cells (HEK 293) were cultured as described [18]. Briefly, cells were grown in 10 cm tissue culture Petri dishes in Minimum Essential Medium (MEM, Sigma, Deisenhofen, Germany) supplemented with 10% FBS (Fetal Bovine Serum, Invitrogen, Karlsruhe, Germany) and Penicillin/Streptomycin at 5% CO2 and 37 °C in a water saturated atmosphere. For experiments, cells were plated on poly-l-lysine treated glass coverslips in 6 cm dishes. Transfection in a water saturated atmosphere. Cells were grown for 2 days after transfection.

2.3. Membrane preparation and Western blot analysis

Expression of p7 constructs in HEK 293 cells had been described before [10]. To test expression in RAW 264.7 cells, these were transfected with p7 constructs as described. For crude membrane preparations, cells were harvested three days post transfection using ice-cold phosphate buffered saline (PBS, 137 mM NaCl, 2.7 mM KCl, 4.3 mM Na2HPO4, 1.4 mM KH2PO4, pH 7.4) and collected by centrifugation (10 min, 1000 rpm). All subsequent steps were carried out on ice. The cell pellet was taken up in a 20-fold volume of 10 mM K-phosphate buffer pH 7.4, supplemented with protease inhibitors (Complete EDTA-free, Roche, Mannheim, Germany, 5 mM EDTA, 5 mM EGTA), homogenized using a glass potter and centrifuged (20 min, 35,000 g). The step was repeated and the membrane pellet finally resuspended in a 5-fold volume of storage buffer (25 mM K-phosphate, pH 7.4, 200 mM KCl, plus protease inhibitors). These crude membrane preparations, containing both, outer plasma membranes as well as ER and other intracellular membranes, were subjected to SDS-PAGE and Western blotting. An alkaline phosphatase-conjugated anti-c-myc antibody (Santa Cruz, Heidelberg, Germany) was used and the blot was visualized using 0.03% nitro blue tetrazolium and 0.02% 5-bromo-4-chloro-3-indolyl-phosphate in substrate buffer (100 mM Tris-HCl, 100 mM NaCl, 5 mM MgCl2, pH 9.5).

2.4. Live cell imaging of vesicular pH

HEK 293 cells were grown on poly-l-lysine coated cover slips overnight and transfected as described above. Twenty four hours post transfection, the cells were washed in HEPES buffer (10 mM HEPES, 133.5 mM NaCl, 2.0 mM CaCl2, 4.0 mM KCl, 1.2 mM MgSO4, 1.2 mM NaH2PO4, 11 mM glucose, pH 7.4) and loaded with 2 μM pH indicator LysoSensor Green DND-189 (Thermo Fisher Life Technologies, Darmstadt, Germany) diluted in 10 mM HEPES buffer at 37 °C for 30 min. The cells were washed twice with HEPES buffer and immediately imaged at excitation of λ = 443 nm and emission at λ = 500 nm. Imaging was performed using AxioCam ERC5s imaging system (Carl Zeiss, Jena, Germany). Cell fluorescence intensity was determined by image analysis of the single wavelength images stored using Carl Zeiss Zen 2012 lite-blue edition software package. For each cell, the area of interest was delineated that included the cytosolic space of the cell and the corresponding background noise values were determined from a region on the same image that was near the cell but did not contain any cells. Thus, measurement signal and background were always taken from the same image. For p71a, a control experiment using Hoechst nuclear stains (1 μg/mL for 5 min) was performed.

2.5. Quantification of IL-1β and TNF-α production

LPS-primed RAW 264.7 cells were grown in 24 well-plates, transfected as described earlier and incubated at 5% CO2 and 37 °C overnight. Cell-free supernatants were collected 24 h post-transfection and analyzed for the presence of IL-1β and TNF-α using an enzyme-linked immunosorbent assay (ELISA) according to the manufacturer’s instructions (Life Technologies, California, USA). Standards, buffers and Streptavidin-HRP solutions were prepared as described. 50 μl of the Standard Diluent Buffer was added to the zero wells followed by addition of 50 μl of standards, samples and controls to the wells and then 100 μl of biotinylated antibody (Biotin conjugate) solution was added. Following incubation at 37°C for 4 h, 100 μl of Streptavidin–HRP working solution was added and the wells were washed 4 times before the addition of 100 μl of stabilized chromogen. The absorbance of each well was then recorded against chromogen blanks on a VICTOR3 multilabel plate reader using Wallac 1420 software package (PerkinElmer, Berlin, Germany) at λ = 450 nm. A calibration curve was constructed using a set of concentration standards. The concentration of IL-1β and TNF-α of the unknown samples and controls was determined from the standard curve. Samples producing signals higher than that of the highest standard (250 pg/ml) were diluted in Standard Diluent Buffer and reanalyzed. Each sample was run in duplicate and the assay was repeated three times. Controls included a) unprimed untransfected cells, b) unprimed p7-transfected cells, c) primed untransfected and d) primed mock cells transfected with c-myc glycine receptor-c-DNA constructs. Assays were done in presence and absence of 100 μM rimantidine (Rim). The effect of decreasing the extracellular pH to 6.8 and 6.2 (using dil. HCl) was also examined.

2.6. Statistical analysis

Statistical analysis of the data was done using Graph Pad Instat software (Version 3.05 for Microsoft Windows). One sample t-test, unpaired t-test and one-way ANOVA were used for statistical testing, p < 0.05 was considered significant. All data are given as means ± standard errors of the means, unless otherwise indicated.
3. Results
3.1. Cell culture, transfection and expression

Genotype-specific constructs corresponding to the p7 sequence of genotypes 1a, 2a, 3a and 4a (Table 1) had previously been generated and characterized [10]. To verify expression in RAW 264.7 cells, crude membrane fractions were prepared three days post transfection and p7 constructs identified by SDS-PAGE and Western Blotting. Detection with an anti-myc antibody revealed a specific band at ~12 kDa. (Fig. 1). Thus, all p7 constructs expressed well in both recombinant systems.

3.2. Live cell imaging of the vesicular pH

To investigate the hypothesis that p7 acts as a proton gating channel affecting intracellular pH, p7 constructs of different HCV genotypes (1a, 2a, 3a and 4a) were expressed in HEK 293 cells, and intracellular pH was determined using the pH-sensitive probe LysoSensor Green DND-189, which exhibits a pH-dependent shift in fluorescence upon acidification. LysoSensor Green DND-189 is a weak base that is selectively concentrated in acidic granules. The dye is strongly fluorescent under acidic conditions and almost non-fluorescent in neutral – basic environment. Expression of p7 in HEK 293 cells at near neutral pH of 7.4 significantly reduced the mean cellular fluorescence intensity for all genotypes compared to untransfected and mock controls (p < 0.001), which indicates shunting of lysosomal membranes and loss of acidity in presence of p7 protein, as we had shown previously [10]. We observed mean fluorescence intensity values of 19.9 ± 10.1, 31.4 ± 7.0, 24.8 ± 7.1, 29.1 ± 6.5, and 59.0 ± 6.4 for genotypes 1a, 2a, 3a, 4a and untransfected control, respectively (Figs. 2A, 3). The activity of p7-transfected HEK 293 cells was also compared to a mock transfection control using constructs of α1 subunits of the glycine receptor, a plasma membrane ion channel. Here, a mean fluorescence intensity of 60.2 ± 9.4 was found (p < 0.001), indicating that observed action on intracellular vesicular pH was indeed due to the activity of p7 channels. Upon comparing the activity of the different genotypes to each other, HEK 293 cells expressing genotypes 2a and 4a showed higher fluorescence, indicating a lower activity, than genotypes 1a and 3a (Fig. 2A).

As a control, we tested the live cell fluorescence of p7α constructs expressed in HEK 293 cells using both, the pH sensor dye LysoSensor Green DND-189, and Hoechst dye, staining DNA, whose signal should be independent from the treatment of the cells, as was observed (Fig. 2B). This indicated that the observed changes in LysoSensor Green DND-189 fluorescence were indeed due to p7-mediated breakdown of intracellular vesicular pH. Observed activities of genotype-specific p7 constructs were in good agreement with previous measurements using the same technique, and also with an independent assay, where p7 activity is measured by the pH-dependent transport of haemagglutinin to the cell surface [8,10].

Given that p7 acts as H+ channel in living cells, we then tested the effect of decreasing extracellular pH on channel activity. Fluorescence activity of HEK 293 cells transfected with p7 of different genotypes was examined at acidic extracellular pH of 6.8 and 6.2. The mean fluorescence intensity of the p7-transfected HEK 293 cells was significantly reduced in a genotype dependent manner compared to untransfected controls (p < 0.001). While HEK 293 cells transfected with p7 of genotypes 1a, 3a, and 4a displayed enhanced activity at acidic pH confirming western blot analysis of membrane allocation of expressed p7, genotype 2a displayed a different pattern of activity that was not responsive to changes of extracellular pH with mean fluorescence intensity of 27.9 ± 0.5 at pH 6.8 and 27.2 ± 0.2 at pH 6.2, respectively (Fig. 3). Changes in pH of the culture medium were effected by adding dilute HCl. Since we only explored the range between pH 7.4 and 6.2, the buffering capacity of the medium was not exhausted, and sufficient to show the effect of pH changes on p7 activity. pH changes in the CO2-saturated atmosphere of the incubator cannot be excluded, but the observed tendencies nevertheless were robust.

The effect of the p7 inhibitor rimantadine (Rim) was then investigated (Fig. 2A, 3). Rimantadine, which was previously reported to be inhibitor of influenza virus M2 channels [14], and of hepatitis p7 channels [10,15,16], was added at a concentration of 100 μM 10 min before cell imaging. Intracellular fluorescence activity was restored for all examined genotypes 1a, 2a, 3a and 4a compared to untreated control with mean fluorescence values of 67.7 ± 5.5, 59.9 ± 3.8, 62.9 ± 4.5 and 65.1 ± 7.2, respectively (p < 0.001 in all cases), indicating significant decrease in intra vesicular pH and restoration of vesicular acidity. Differences in fluorescence intensity between p7-transfected cells after rimantadine treatment, and untransfected or mock transfected controls were not significant (p > 0.05).

Table 1

<table>
<thead>
<tr>
<th>Genotype</th>
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<td>2</td>
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<td>3</td>
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<td>4</td>
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<tr>
<td>4a</td>
<td>ALENLYVLLASASASAG7HG1LWFLVFFCAWTVKRLPGATYSLGNGFLLLLLLALPQRAYA</td>
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Sequences were compiled after Carrere-Kremer et al. [13]. Consensus sequences in bold; 1a–4a: sequences used in this study.
Fig. 2. Live cell fluorescence imaging of HEK 293 cells expressing genotype-specific constructs of p7. (A) Cells transfected with p7 cDNA constructs for genotypes 1a, 2a, 3a and 4a, and loaded with 2 μM LysoSensor Green DND-189 at pH 7.4 are shown. Wavelengths used were λ = 443 nm (excitation) and λ = 500 nm (emission). Controls include untransfected HEK 293 cells as well as cells transfected with glycine receptor α1 subunits (mock transfection). Images were taken in the absence and presence of 100 μM rimantadine. Images show transmission and fluorescence channels. Genotypes and treatments are indicated. Scale bar is 10 μm. (B) Co-staining of HEK 293 cells expressing p71a constructs, and untransfected cells in presence and absence of rimantadine. Cells were loaded with 2 μM LysoSensor Green DND-189 and costained with Hoechst nuclear stain (1 μg/ml for 5 min). Wavelengths used were λ = 443 nm (excitation) and λ = 500 nm (emission) for LysoSensor Green DND-189 and λ = 361 nm (excitation) and λ = 497 nm (emission) for the Hoechst dye.
3.3. IL-1β production

To investigate if p7 plays a role in the activation of NLRP3 inflammasomes, p7 constructs of the 4 different genotypes (1a, 2a, 3a and 4a) were expressed in LPS-primed RAW 264.7 murine macrophages. Cell-free supernatants were collected 24 h post-transfection and analyzed for the presence of IL-1β using an enzyme-linked immunosorbent assay (ELISA). A standard curve was constructed using a
serial dilution of IL-1β standards and used to calculate IL-1β concentrations in the collected samples. IL-1β was produced by all LPS primed macrophages compared to unprimed controls, confirming the key role of LPS in providing the first signal for its production. Expression of p7 significantly increased the levels of IL-1β in primed macrophages (p < 0.001) compared to those lacking the p7 protein with mean IL-1β concentration of 288 ± 13, 188 ± 24, 317 ± 17 and 281 ± 28 pg/ml for genotypes 1a, 2a, 3a and 4a, respectively (Fig. 4A). Mock controls, transfected with α1 glycine receptor cDNA, and untransfected primed controls showed only traces of IL-1β (71 ± 4 and 78 ± 6 pg/ml). Untransfected unprimed and p7-transfected unprimed controls showed no signs of IL-1β production.

To test whether IL-1β production was indeed due to p7 activity, cells were treated with the p7 inhibitor rimantadine (Fig. 4A). IL-1β levels were significantly reduced compared to untreated controls with mean IL-1β levels of 76 ± 6, 107 ± 17, 63 ± 11, and 77 ± 9 pg/ml for genotypes 1a, 2a, 3a and 4a, respectively. This suggests that expression of p7 in living cells could indeed provide a second signal required for the activation of NLRP3, and production of IL-1β. Furthermore, we used monensin, a Na+/H+ antiporter that exports H+ and dissipates vacuolar pH, to mimic the activity of p7 (Fig. 4B). Monensin-treatment enabled IL-1β production in LPS-treated RAW 264.7 macrophages with mean IL-1β levels of 338 ± 12 pg/ml. When cells expressing p71a were additionally treated with monensin (10 μM), levels of IL-1β increased significantly to 432 ± 21 pg/ml. The results indicate that H+ transportation, as well as alteration of vesicular pH caused by p7 could trigger inflammasome activation and contribute to the production of IL-1β.

We then attempted to identify the intracellular mechanism by which p7 triggers IL-1β production. Since p7 was shown from the previous assays to be a pH gated H+ channel in living cells, the effect of decreasing the extracellular pH on IL-1β levels was examined. Lowering the pH of the extracellular medium to 6.8 and 6.2 increased the production of IL-1β, in agreement with the pH-response profile of p7 channels observed earlier (Fig. 5). The levels of IL-1β significantly increased for all the tested genotypes (p < 0.001) upon reduction of pH to 6.8 and further increased when the extracellular pH was lowered to 6.2 except for genotype 2a, which was much less responsive to pH changes with mean IL-1β levels of 211 ± 13 pg/ml for pH 6.8, and 208 ± 25 pg/ml at pH 6.2 (changes were not significant, p > 0.05, one-way ANOVA).

3.4. TNF-α production

A second approach to elucidate the role of p7 in production of IL-1β and to confirm the specificity of the ionic disturbance caused by p7 in the activation of NLRP3, was followed, namely monitoring of another inflammatory cytokine, TNF-α. LPS primed murine macrophages were transfected with p7 of genotypes 1a – 4a as before, and the level of TNF-α was assayed 24 h post transfection by ELISA. The levels of TNF-α production by all LPS primed cells was significantly higher than unprimed controls with TNF-α concentrations in the range of

![Fig. 4. P7-mediated production of IL-1β in a recombinant system. (A) LPS-primed RAW 264.7 cells were transfected with p7 cDNA constructs of different HCV genotypes and production of IL-1β was assayed in presence and absence of 100 μM rimantadine (Rim) by ELISA. Data presents average IL-1β levels from three independent experiments, each run in duplicates, (** = p < 0.001, ns = not significant, one-way ANOVA). (B) IL-1β production in LPS-primed macrophages by transfection of p71a constructs, or monensin, or both. Significance of differences of monensin or monensin/p7 treatment relative to p71a alone are given (** = p < 0.01; *** = p < 0.001, ns = not significant, one-way ANOVA).](image-url)
250 pg/ml for genotypes 1a, 2a, 3a, 4a, glycine receptor-transfected (mock) controls, and untransfected controls, compared to ~35 pg/ml for untransfected-unprimed and transfected-unprimed controls, respectively (Fig. 6). TNF-\(\alpha\) production was not affected by the presence or absence of rimantadine (Fig. 6, \(p > 0.05\)), except for p72a, where TNF-\(\alpha\) levels were reduced from 248 ± 11 to 220 ± 8 pg/ml after addition of rimantadine (\(p = 0.01\), one-way ANOVA). This small reduction of ~11% was considered functionally not relevant, compared to the very low amounts of ~35 pg/ml of TNF-\(\alpha\) found in unprimed cells (Fig. 6). Thus, priming, but not any p7 activity was required for the production of TNF-\(\alpha\).

4. Discussion

With an estimated prevalence of 3% worldwide, HCV infection represents a major health threat and a significant worldwide challenge. Currently, approximately 130 million individuals are infected with HCV, the majority of which remain undiagnosed and untreated [14]. 75–85% of HCV patients develop chronic infection which is characterized by the persistent existence of the virus in the body for more than six months [7]. More importantly, two thirds of patients diagnosed with chronic infection develop chronic liver disease that progresses to cirrhosis in 5–20% of the cases. As a consequence, hepatitis C is the leading cause of cirrhosis, end-stage liver disease, and hepatocellular carcinoma requiring liver transplantation [15]. The p7 protein of the hepatitis C virus, a small hydrophobic protein of 63 amino acids that belongs to the family of viroporins [19] has been identified as a relevant contributor to virus replication and release [20–22]. So far, a role of the p7 protein in the progression of hepatitis C has not been identified. However, disruption of viroporin function is associated with abrogation of viral infectivity; rendering them promising targets for antiviral drug development. Here, p7 protein corresponding to genotypes 1a, 2a, 3a and 4a was expressed in two different recombinant systems in order to study the channel activity and further biochemical signalling of p7 in isolation, yet in a well-defined in-vivo system.

The p7 protein has been shown to form functional channels in artificial lipid black bilayer membrane preparations and function as calcium permeant ion channel [9,23,24]. A subsequent study confirmed the ion-
Hepatic macrophages, including Kupffer cells, can internalize HCV through a process of phagocytosis, where internalized viral products could then induce innate immune signaling leading to IFN-β expression [38]. This process might also induce pro-inflammatory cytokines that serve to recruit immune cells to the site of infection, thus supporting immune-mediated liver damage characteristic of chronic hepatitis C. Kupffer cells serve a specialized role to sample the local environment via phagocytosis and selectively render inflammatory signalling upon pathogen identification, including internalized HCV RNA. Indeed, it was shown that upon HCV infection IL-1β was produced via the NLRP3 inflammasome pathway in hepatic macrophages [39].

It has been claimed that viroporins including M2 of influenza virus [40,41], and the 2B protein of encephalomyocarditis virus [42], as well as 2B of poliovirus, picornavirus and enetrovirus, are capable of inducing inflammasome activation and subsequent production of IL-1β [43]. Thus, while HCV RNA targets inflammasome “signal one” via TLR7, the transient pH-shunting activity of p7 may provide stimulus for “signal two”, that induces NLRP3 activation for IL-1β maturation and secretion [39]. Consistent with a specific activation of IL-1β, activity of p7 channels did not have any effect on the production of TNF-α. Although TNF-α is indeed activated in chronic hepatitis C infections [44], this activation appears not to be affected by vesicular pH or other p7-mediated processes.

We also examined the effect of extracellular pH on IL-1β production in primed macrophages. Lowering extracellular pH resulted in higher levels of IL-1β [45]. This is consistent with the observed increase in p7 activity with decreased extracellular pH. The activity of p7 could also be mimicked using monensin, a Na+/H+ antiporter that dissipates vacuolar pH. Monensin-treatment increased IL-1β levels in LPS-treated Raw 264.7 cells. P7 functions as pH-sensitive proton channel, where decreasing extracellular pH results in an increase in p7 activity, leading to increased ionic disturbance and higher levels of IL-1β production. Similar results were reported for the influenza viroporin M2, where H+ export from acidified Golgi apparatus due to the ion channel activity of M2 acted as second signal required for the formation of NLRP3 [40]. It was suggested that the ionic perturbation in the trans Golgi network by M2 activity could activate IL-1β production [41]. It has been shown that p7 can indeed replace the activity of M2 in a haemadsorption assay, where surface transport of HA requires pH neutralization in the trans Golgi network [8,10].

The current study analyzed the role of the p7 protein of HCV as an intracellular ion channel and suggests a role of p7 in immune response and inflammation subsequent to HCV infection and development of hepatitis C. To our knowledge, this is the first report of the expression of recombinant p7 protein in a liver macrophage cell line, confirming the previous suggestion that p7 activity can provide a second signal for IL-1β production in macrophages. These findings support the classification of p7 as an important mediator of hepatitis C infection and its progression towards inflammation and cirrhosis, marking p7 as an important target for the development of novel drugs against hepatitis C.

Conflict of interest

The authors declare no competing financial interests.

Transparency document

The Transparency document associated with this article can be found, in online version.

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