Hepatitis C virus interacts with human platelet glycoprotein VI

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Hepatitis C virus (HCV) interacts with human platelets in vivo as a potential transport of infectious virions to the target liver. The binding of native viral particles with the platelet membrane glycoprotein VI (GPVI) was analysed. A consistent interaction between HCV from plasma or after purification by two different methods and the recombinant extracellular immunoglobulin (Ig)-like domains of human GPVI (hD1D2) was observed with two independent experimental approaches: pull-down and ELISA assays. Between 2 and 7% of HCV particles were specifically bound to hD1D2. The binding was inhibited by an anti-hD1D2 in a dose-dependent manner. Human D1D2 interaction with HCV was significantly higher than the murine D1D2, supporting the specificity of the interaction and to the single human domains (D1 and D2), suggesting that both Ig-like domains of the molecule are required for efficient binding. GPVI may be a platelet surface ligand for HCV playing a role in viral transport and persistence.

INTRODUCTION

Hepatitis C virus (HCV) infection is a global health problem, being a major cause of cirrhosis and hepatocellular carcinoma worldwide. The infection can also lead to a number of serious extra-hepatic manifestations (Major et al., 2001) and the existence of extra-hepatic viral reservoirs, such as lymphocytes, as well as the importance that they might have in the pathophysiology of infection becoming increasingly recognized.

HCV infection is also associated with thrombocytopenia. Several investigators have reported HCV association with platelets (de Almeida et al., 2004; Floreani et al., 1996; Li et al., 1994; Nagamine et al., 1996; Silva et al., 1992; Takehara et al., 1994). We have previously shown the interaction of immunoglobulin (Ig) G-free and IgG-complexed HCV with platelets in chronically infected patients (Hamaia et al., 2001). Viral interaction with platelets is well recognized, as it has been described for other viruses such as herpes simplex virus (Forghani & Schmidt, 1983), Vaccinia virus (Bik et al., 1982), Human immunodeficiency virus 1 (HIV-1) (Lee et al., 1998, 1993; Olinger et al., 2000), echovirus 1 (Xing et al., 2004), hantavirus (Gavrilovskaya et al., 1998) and the flavivirus dengue type 2 (Wang et al., 1995) among others. Several mechanisms have been proposed as contributing to thrombocytopenia, but recently a positive correlation between thrombocytopenia and HCV association with platelets was described (de Almeida et al., 2004), suggesting a direct viral effect on platelet count.

Platelets do not express CD81 (Levy et al., 1998), SR-BI (Rhaïnds & Brissette, 2004), DC- and L-SIGN (van Kooyk & Geijtenbeek, 2003) or the classical LDL-R (Korporaal et al., 2004; Pedreno et al., 1992), which are putative HCV receptors (Agnello et al., 1999; Gardner et al., 2003; Lozach et al., 2003; Monazahian et al., 1999; Pileri et al., 1998; Pohlmann et al., 2003; Scarselli et al., 2002). Thus, other molecule(s) might mediate the interaction between HCV and platelets.

Preliminary work in the laboratory identified a 60 kDa platelet glycoprotein interacting with HCV from human plasma using a virus overlay method under reducing conditions (S. Hamaia & J.-P. Allain, unpublished results). The band was compatible with the apparent molecular mass of platelet glycoprotein VI (GPVI) under such conditions (Clemetson et al., 1982; Nieswandt & Watson, 2003) and was developed in parallel with a specific anti-GPVI antibody. Based on this preliminary result, an interaction between HCV and platelet GPVI was hypothesized and the presently reported research conducted.

GPVI is expressed exclusively on megakaryocytes and platelets (Jandrot-Perrus et al., 2000). It is the primary signalling receptor for platelet activation by collagen (Nieswandt & Watson, 2003). GPVI is a type-I membrane glycoprotein belonging to the Ig superfamily with two N-terminal Ig C2-like domains (D1D2) (Clemetson et al., 1999), highly conserved between human and mouse (Jandrot-Perrus et al., 2000; Smethurst et al., 2004).
To investigate the potential interaction between HCV and GPVI, advantage was taken of two simple systems developed in the laboratory to preferentially purify Ig-free HCV from human plasma (Zahn & Allain, 2005) and to express calmodulin (CaM)-tagged D1D2 in Drosophila S2 cells (Smethurst et al., 2004). The former allowed the use of native viral particles rather than viral surrogates. Although recombinant E2 proteins and HCV-like particles have been of great help for identifying HCV receptor candidates, neither of them appears to fully mimic viral particles (Clayton et al., 2002; Hamaia et al., 2001; Wellnitz et al., 2002; Wunschmann et al., 2000). The binding of HCV to GPVI was studied using a sophisticated pull-down assay based on the immobilization of CaM-tagged human D1D2 (hD1D2) and a specific viral interaction with hD1D2 was found.

METHODS

Human plasma. Plasma from a patient (EH) with X-linked agammaglobulinaemia, who was infected with HCV genotype 1a, was collected at the Bristol Blood Centre by Dr T. Wallington and was aliquoted and stored at ‒70 °C on the day of collection. EH viral load was estimated by real-time quantitative PCR (Q-PCR) as approximately 1 E+05 IU ml−1. HCV-infected plasmas (S2, S3 and S12) with normal globulinaemia were from blood donors collected at the National Blood Service in Cambridge and provided by Dr G. J. M. Alexander (Hepatology Clinic, Department of Medicine, University of Cambridge Medical School, Cambridge, UK). S2 (genotype 1a), S3 (genotype 3a) and S12 (genotype 3a) viral loads were 4 E+06 IU ml−1, 6 E+06 IU ml−1 and 1 E+06 IU ml−1, respectively.

Antibodies and peptides. A polyclonal antibody against EH HCV quasispecies was developed in rabbits and total IgG (anti-EH1) was purified as previously reported (Zahn & Allain, 2005). A polyclonal antibody to hD1D2 was produced in rabbits immunized with His-tagged hD1D2 and total IgG (anti-hD1D2) was purified by Protein-G chromatography. Horseradish peroxidase (HRP)–, BSA- and biotin-conjugated 23 mer high affinity CaM-binding peptide (N9A) were synthesized as described previously (Montigiani et al., 1996).

Purification of EH and S2 HCV from plasma proteins by heparin chromatography. EH HCV and S2 HCV were purified from plasma proteins by heparin chromatography as described previously (Zahn & Allain, 2005) with minor modifications. The 0.5 M NaCl eluted fraction from heparin columns was 8–10-fold concentrated by Q-PCR and kept at 4 °C for no more than 2 days until use.

Depletion of IgG-bound S2 HCV. IgG-free HCV was purified from 500 μl S2 plasma using a Protein-G column (Amersham Pharmacia) as described previously (Zahn & Allain, 2005). The flow-through and washing fractions were pooled (corresponding to the IgG-free plasma fraction) and concentrated using Vivaspin concentrator devices as described above. Viral genomes were extracted and quantified by Q-PCR.

Purification of CaM-tagged recombinant proteins. The cloning of both extracellular Ig-like domains (D1D2) of both h(uman) and m(urine) GPVI and the single human domains (hD1, aa 1–89 and hD2, aa 87–185) to produce C-terminally CaM-tagged recombinant proteins in Drosophila has been reported (Smethurst et al., 2004). Cells expressing CaM-tagged hD1D2, hD1, hD2, md1D2 and two single chain variable domain antibodies; B2 (directed against GPIIb-IIIa Leu 33; Griffin & Ouwehand, 1995) and OS11 (derived from the malignant B-cell clone of a patient with follicular lymphoma, negative for anti-HCV) were employed. CaM-tagged proteins were purified on N-(6-aminohexyl)-5-chloro-1-naphthalenesulfonamide (W-7) affinity columns as described previously (Neri et al., 1995), exploiting the interaction between W-7 and CaM in the presence of Ca2+ (Osawa et al., 1999). Expression of protein species of the expected molecular mass was monitored by Western blot and probed with the HRP-N9A peptide. The purity of all proteins was analysed by SDS-PAGE and the concentration was measured using the BCA assay kit (PeroBio) with BSA (PeroBio) as standard.

Pull-down method to assess HCV binding to CaM-tagged proteins immobilized on biotin-N9A streptavidin-magnetic beads. The whole procedure was performed in the presence of Ca2+ (1 mM) to allow binding between the CaM tag and the N9A peptide. CaM-tagged proteins (200 ng) were incubated with the biotinylated N9A peptide (molar ratio, 1:2) for 20 min at room temperature, followed by streptavidin-magnetic beads (Dynal) for 30 min at room temperature in 50 mM Tris pH 7.4, 150 mM NaCl (TBS) containing 4% BSA. Using a magnetic particle concentrator, the beads were dragged down and washed twice with TBS. The beads were resuspended in a maximum volume of 500 μl heparin-purified HCV, IgG-free HCV or HCV-infected plasma (approx. 5000 IU RNA) in TBS supplemented with Ca2+ and BSA, and incubated for 2 h at room temperature or 37 °C with gentle rotation. After five washes with TBS/Ca2+, HCV RNA from the bead-associated material was extracted and quantified (see below). Viral binding to each recombinant protein was compared with the unpaired Mann–Whitney test (P<0.05 was considered significant) using the SPSS 11 statistics package.

HCV RNA extraction and quantification by reverse transcription (RT)-Q-PCR. Viral RNA was extracted from 140 μl plasma, Protein-G or heparin-purified HCV with the QIAamp viral RNA mini kit (Qiagen) following manufacturer’s instructions or from the magnetic bead-associated material after binding experiments using the same kit with minor modifications. Following the last wash, 560 μl Qiagen viral lysis buffer was added to each tube and incubated for 10 min at room temperature. After pelleting the magnetic beads, supernatants were transferred to new tubes and viral RNA extraction was continued as indicated by the manufacturer. HCV RNA was quantified by real-time PCR using a PE Applied Biosystems Prism model 7700 sequence detector (Applied Biosystems) as described previously (Hamaia et al., 2001) or using the Mx4000 Multiplex Quantitative PCR system (Stratagene) as described previously (Candotti et al., 1993). The C0 value, which correlates inversely with the concentration of target RNA, was determined as the cycle number where the fluorescence emission of the reporter probe increased above a threshold level. Each sample was analysed in duplicate or triplicate and the results were averaged. In each run, 10-fold serial dilutions of a WHO international standard (HCV 96/790 I, S3onal Institute for Biological Standards and Controls, Potters Bar, UK) or an in-house reference plasma sample validated against the international standard was included, and linear regression was obtained interpolating the mean C0 value against the logarithm of the amount of template. In order to assess the significance of the quantitative binding results, the intra- and inter-assay variabilities of the Q-PCR method was calculated and it ranged from 1.7 to 0.5% C0 for values of 40 to 4000 IU HCV RNA, respectively, which encompasses the values obtained in all binding experiments.

ELISA method to assess HCV binding to CaM-tagged proteins. Polystyrene Nunc-Immuno ELISA microplates maxisorp surface (Nalge Nunc International) were coated with BSA-N9A (1 μg per well) in carbonate buffer pH 9 overnight at 4 °C and blocked.
with 5% BSA for 2 h at 37 °C. The rest of the procedure was performed in the presence of CaCl₂ (1 mM) to allow interaction between CaM and N9A. CaM-tagged hD1D2, mD1D2, hD1 or hD2 recombinant proteins (1 µg per well) were incubated for 15 min at room temperature followed by the addition of heparin-purified EH HCV (5000–8000 IU) supplemented with CaCl₂ for 2 h at 37 °C. After washing, anti-EH1 (1 µg) was incubated for 2 h at 37 °C and overnight at 4 °C, followed by an HRP-conjugated anti-rabbit IgG (1:2000). The reaction was developed with 150 µl per well of the ABTS Solution HRP substrate (Roche Diagnostics). Absorbance was measured at different times at 405 nm on an ELISA plate reader (Dynex Technologies). The omission of the heparin-purified EH HCV, the anti-EH1 or the coating done with BSA instead of BSA-N9A were used as negative controls. At each time point, absorbance of viral binding to each recombinant protein was corrected for background reading when the virus was omitted. Means of quadruplicates ± SEM were calculated.

Binding of HCV E2 to CaM-tagged proteins by ELISA. CaM-tagged proteins were coated onto ELISA plates as explained above. Mammalian recombinant E2 protein genotype 1a (kindly provided by Chiron Corporation) was incubated for 2 h at 37 °C, followed by the monoclonal anti-E2 AP33 antibody (kindly provided by A. Patel, University of Glasgow, UK) and an anti-mouse-HRP antibody. Alternatively, E2 protein was captured by the AP33 antibody coated on ELISA plates and CaM-tagged proteins were incubated for 2 h at 37 °C, followed by the N9A-HRP peptide.

Pull-down experiments of HCV E2 and hD1D2 proteins. Different amounts of recombinant E2 protein were incubated with hD1D2, mD1D2 or OS11 (molar ratio E2:CaM-protein, 1:1) for 2 h at either room temperature or 37 °C, followed by the AP33 antibody and Protein A-Sepharose. After washing, proteins were eluted in reducing electrophoresis buffer, resolved by SDS-PAGE and electroblotted. CaM-tagged proteins were developed with the N9A-HRP peptide and a HRP-chemiluminescent substrate. Alternatively, E2 incubation with CaM-tagged proteins was followed by the addition of biotinylated N9A peptide and streptavidin magnetic beads; in such case, E2 protein was developed by Western blot using the AP33 antibody.

RESULTS

Native HCV interacts with the extracellular domains of human platelet GPVI

To investigate the putative interaction between HCV and GPVI, we took advantage of a CaM-tagged recombinant form of the ectodomains of human GPVI (hD1D2). Human D1D2 has the same binding specificity as platelet GPVI, since it recognizes the GPVI-specific collagen-related peptide containing the GPVI-specific GPO sequence motif (Smethurst et al., 2004). In the first set of experiments, a CaM-tagged unrelated protein, named OS11, was used as negative control of the interaction. Highly purified CaM-hD1D2 and CaM-OS11 proteins were obtained (Fig. 1a), similarly to previously observed results for this system (N. Jennings, P. Smethurst, G. Knight, M. O’Connor, L. Joutsi-Korhonen, S. Garner, I. Harmer, R. Farndale, N. Watkins & W. Ouwehand, unpublished results). Protein species matching the expected molecular mass were detected in both cases by the CaM-binding N9A peptide (Fig. 1b). The identity of the hD1D2 protein was confirmed by Western blot analysis probed with the anti-hD1D2 (data not shown).

The interaction of HCV with hD1D2 was assessed using a modification of the system employed to study the viral interaction with recombinant CD81 (Pileri et al., 1998). CaM-hD1D2 and CaM-OS11 proteins were immobilized onto streptavidin-magnetic beads using the N9A peptide conjugated to biotin and the beads were incubated with heparin-purified EH HCV. After washing, the amount of bead-associated virus was measured by RT-Q-PCR. International units of HCV bound to the recombinant proteins were estimated by interpolation of the Ct values into standard curves run in parallel. In three independent experiments, viral binding to hD1D2 was around fourfold higher than to OS11, with values ranging from 80 to 160 IU of bound HCV RNA to hD1D2 (Fig. 2). The amount of bound virus was within the confidence limit calculated for the intra-assay variability of the Q-PCR method. The percentage of input virus bound to hD1D2 ranged from 2 to 5% but was consistently lower than 1.5% for OS11.

The specificity of the viral interaction was evaluated by constructing a dose–response curve using increasing amounts of input HCV (1000–45 000 IU of heparin-purified EH HCV RNA) and a constant amount of recombinant proteins. Although HCV binding to both hD1D2 and OS11 increased with the viral input, a two- to threefold higher binding to hD1D2 than to OS11 was observed when the viral input ranged from 2000 to 5000 IU HCV RNA, reaching saturation at around 10 000 IU HCV RNA (Fig. 3a).

The effect of a specific polyclonal antibody to hD1D2 on the binding of HCV to the recombinant protein was examined. A constant amount of CaM-hD1D2 was incubated with increasing concentrations of anti-hD1D2
before the addition of heparin-purified EH HCV. As a negative control, total IgG purified from a pre-immunization rabbit serum was included at the two highest concentrations. As shown in Fig. 3(b), the binding of hD1D2 to EH HCV was specifically inhibited by the anti-D1D2 in a dose-dependent manner up to 5 μg ml⁻¹. These results suggest a specific HCV interaction with hD1D2.

**HCV binding to human and murine GPVI ectodomains**

To investigate further the specificity of the viral interaction with hD1D2, HCV binding was next compared to the murine GPVI homologue (mD1D2) using identical experimental design. In addition to OS11, another unrelated CaM-tagged protein, named B2, was included as negative control for this set of experiments. CaM-tagged mD1D2 and B2 were purified and showed protein species of the expected molecular mass with a degree of purity similar to hD1D2 and OS11 (Fig. 1a).

In six independent experiments, HCV binding was consistently higher to hD1D2 than to the murine counterpart or the control proteins. In each experiment, the Ct value obtained for hD1D2 was around 1.5 times lower than with the rest of the proteins. As shown in Fig. 4(a), the percentage of input virus bound to hD1D2 ranged from 2.5 to 5.8% (median 5.2%), whilst for other recombinant proteins the medians for bound virus were <2%. These differences were significant in all cases (Mann–Whitney test; P=0.026 when comparing viral binding to human and mouse D1D2 and P=0.002 when comparing hD1D2 to B2). Considering that the alignment of hD1D2 and mD1D2 is ungapped with 78% identity between species (Smethurst et al., 2004), these results suggested that...
relatively subtle differences between the human and murine proteins affect the binding and support the specificity of HCV binding to hD1D2.

An alternative ligand-binding assay was developed using immunological rather than PCR detection of HCV binding to hD1D2. CaM-tagged proteins were immobilized onto ELISA plates coated with BSA-N9A peptide and incubated with heparin-purified EH HCV. After washing, the amount of bound virus was detected using a specific antibody against EH HCV (anti-EH1). Differences were detected by colorimetric development of the signal recorded over time; at each time point, absorbance related to HCV binding was corrected for background by subtracting the absorbance value when virus was omitted. The absorbance curves for the negative controls when the coating was done with BSA or when the anti-EH1 was omitted were consistently lower than the mD1D2 curve (data not shown).

ELISA and Q-PCR results consistently suggested a preferential EH viral binding to the extracellular Ig-like domains of human GPVI compared to the murine counterpart or the unrelated proteins OS11 and B2.

We investigated further the ability of HCV isolated from another, but normoglobulinaemic, plasma sample (S2) purified by heparin chromatography to interact with hD1D2. As shown in Fig. 5(a, left panel) in three independent experiments, S2 viral interaction with hD1D2 was approximately 7- and 5.5-fold higher than to the mD1D2 and OS11, respectively, with values ranging from 90 to 200 IU of HCV RNA bound to hD1D2. The percentage of input virus bound to hD1D2 was 2–4 %. Moreover, in order
to confirm binding results, a viral preparation obtained by a different method (IgG-free fraction instead of heparin purification) was used to probe the binding of HCV present in such fraction to hD1D2. Similarly, viral binding to hD1D2 was higher than to mD1D2 or OS11 (Fig. 5a, middle panel), suggesting that IgG-free HCV interacts with hD1D2.

Finally, we investigated whether HCV in plasma, without any purification procedure, also interacted with hD1D2. Using three different HCV-infected plasmas (S2, S3 and S12), we observed a two- to threefold and two- to sixfold higher viral binding to hD1D2 compared with the mD1D2 or OS11, respectively (Fig. 5a, right panel and b) with values of bound virus comparable to the ones observed when using purified HCV.

Since HCV envelope E2 protein appears to mediate viral binding to CD81 (Pileri et al., 1998) and human scavenger receptor class B type I (Scarselli et al., 2002), we designed experiments to examine whether recombinant E2 would bind to hD1D2. No significant interaction could be detected either by co-immunoprecipitation or by ELISA (data not shown).

**HCV interaction with human GPVI requires both glycoprotein extracellular domains**

The viral binding to each separate extracellular domain of human GPVI (hD1 and hD2) was analysed. The purified single domains showed species of the expected molecular mass (N. Jennings, P. Smethurst, G. Knight, M. O’Connor, L. Joutsi-Korhonen, S. Garner, I. Harmer, R. Farndale, N. Watkins & W. Ouwehand, unpublished results) and a comparable degree of purity as observed for the other CaM-tagged proteins (Fig. 1a). Human D1 has one predicted N-glycosylation site that is indeed glycosylated in insect cells as confirmed by binding to Con-A lectin (N. Jennings, P. Smethurst, G. Knight, M. O’Connor, L. Joutsi-Korhonen, S. Garner, I. Harmer, R. Farndale, N. Watkins & W. Ouwehand, unpublished results),
explaining the difference in apparent molecular mass compared with hD2.

The binding of HCV to hD1D2 was always higher than to either of the single domains. The binding was measured by two different experimental designs: pull-down with magnetic beads followed by Q-PCR detection (Fig. 4a) as well as by ELISA (Fig. 4b, right panel). With both systems, higher level of binding was obtained with hD1D2 than with individual domains (P = 0.002 Mann–Whitney test, Fig. 4a), suggesting that both domains are required for efficient binding.

DISCUSSION

Little information is available on whether platelets and megakaryocytes are a target for HCV infection or are capable of interacting with HCV. We have shown that IgG-free and IgG-complexed HCV from chronically infected patients interact with platelets in vivo (Hamaia et al., 2001). Although the physiological significance of this interaction is still unclear, a number of possibilities can be envisaged. Platelets could adsorb HCV non-specifically and direct the clearance of HCV from circulation by either phagocytosis or by removal of platelets by the reticuloendothelial system. Alternatively, the association of IgG-free HCV with platelets might be related to persistence and interpreted as a way of viral dissemination and/or shielding the virus against neutralizing antibodies. It can also be speculated that if the virus infects megakaryocytes, HCV association with platelets may just represent a viral transfer during proplatelet formation and a passive vehicle for viral dissemination, as described for hog cholera virus (Gomez-Villamandos et al., 1998). Interestingly, a role in viral transmission to PBMC has been suggested for platelets and megakaryocytes during HIV-1 infection (Chelucci et al., 1998; Lee et al., 1998; Olinger et al., 2000), and recently, HCV transmission to monocytes by human platelets has been proposed (Pugliese et al., 2004).

Thrombocytopenia is a commonly detected haematological disorder during chronic HCV infection. Several mechanisms have been proposed as contributing to thrombocytopenia: sequestration of platelets in the enlarged spleen (Sanjo et al., 2003); platelet destruction mediated by platelet-associated IgG possibly leading to the sequestration in the reticuloendothelial system and also related to hypersplenism (Nagamine et al., 1996; Pockros et al., 2002; Sanjo et al., 2003); impaired hepatic production of thrombopoietin (Ishikawa et al., 1998; Martin et al., 1997; Peck-Radosavljevic et al., 1997) and a direct viral effect since a positive correlation between thrombocytopenia and HCV association with platelets has been found (de Almeida et al., 2004).

These limited data suggest a number of physiologically relevant scenarios according to which platelet–HCV interaction might play a role in the infection. We show a preferential binding of HCV to the extracellular Ig-like domains of human GPVI detected by different approaches. First, heparin-purified EH HCV binds to hD1D2 to a higher extent than to a non-related control protein (Fig. 2). This higher level of binding was reproducible and specifically detected by measuring the amount of viral molecules bound to each recombinant protein by RT-Q-PCR. Second, the interaction between HCV and hD1D2 is inhibited by a polyclonal anti-hD1D2 in a dose-dependent manner (Fig. 3b). Third, there is a two- to sixfold higher level of viral binding to the hD1D2 when compared not only with negative controls, but also with the mD1D2 counterpart and the single human GPVI domains (Fig. 4a). Results were reproducible as independent experiments using different viral preparations, belonging to different genotypes (1 and 3), yielded similar results. Moreover, HCV binding to hD1D2 was confirmed using an alternative experimental design (Fig. 4b). The percentages of input virus bound to hD1D2 (Fig. 4a) were in the range reported for viral binding values to CD81, in which binding and detection systems were similar to those used here (Pileri et al., 1998). Finally, using HCV-infected plasmas other than EH, the binding of HCV, either from plasma or purified, was reproducibly higher to hD1D2 than to either the mD1D2 or OS11 (Fig. 5). Recombinant HCV envelope E2 protein has been extensively used as a viral surrogate to probe viral interactions with host molecules. To test whether the binding of HCV to hD1D2 was mediated by E2, the ability of recombinant E2 to interact with CaM-tagged hD1D2 immobilized onto ELISA plates or to be pulled down by hD1D2 was assessed using a recombinant E2 protein (genotype 1a) produced in mammalian cells. No difference in binding was detected between the hD1D2 and the mD1D2 or the negative control with either experimental design (data not shown). These negative data suggest either that the viral interaction with hD1D2 might rather occur through E1 or the E1–E2 heteroduplex at the viral envelope surface, or that recombinant E2 protein might not fully mimic the viral envelope major glycoprotein as has been described (Clayton et al., 2002; Hamaia et al., 2001; Wunschmann et al., 2000) and therefore the interaction is not detected in our assays. Alternatively, the observed interaction might be indirect, mediated by some component of the serum that interacts with the virus and GPVI.

The observation that HCV shows binding to human but not to murine D1D2 is interesting. The sequence difference between the two species is only 22% and most of the diversity is in the apical loops of domains 1 and 2 (Smethurst et al., 2004), suggesting that subtle differences between species are sufficient to affect viral binding. Moreover, glycans are probably not involved in the binding since both proteins have a conserved single canonical N-glycosylation site in D1 (Smethurst et al., 2004). Previous studies from our group have shown that the apical loops of domain 1 harbour the primary-binding site for collagen and collagen-related peptide (Smethurst et al., 2004). The profound difference in the overall charge of the apical face of domain 1 between mice and human makes it attractive.
to postulate that the positive charge of hD1 might play an important role in HCV binding, although not sufficient, as individual domains were not able to interact with the virus (Fig. 4). If HCV binding mimics collagen binding and leads to cross-linking of the receptor, then activation of platelets may ensue, leading to P-selectin expression and subsequent removal of platelets from the circulation by the reticuloendothelial system, resulting in thrombocytopenia.

In summary, we have shown that native HCV from plasma or purified from infected patients interacts specifically with the extracellular Ig-like domains of human platelet GPVI and the interaction appears not to be mediated by E2. HCV interaction with GPVI might explain, at least partially, the viral binding to platelets in infected patients.

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