

BRIEF ARTICLES

## Interaction of hepatitis C virus envelope glycoprotein E2 with the large extracellular loop of *tupaia* CD81

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Supported by Grants from Health Department of Hubei Province (JX2B09), NSFC (30771911), National 973 Key Program (2009CB522502), and Tongji Hospital

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Received: July 16, 2007 Revised: November 5, 2008

Accepted: November 12, 2008

Published online: January 14, 2009

may be important for the understanding of the mechanisms of binding and entry of HCV to PTHs.

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**Key words:** Hepatitis C virus E2 protein; *Tupaia*; CD81, Bind; Enzyme-linked immunosorbent assay

**Peer reviewer:** Thomas Bock, PhD, Professor, Department of Molecular Pathology, Institute of Pathology, University Hospital of Tuebingen, D-72076 Tuebingen, Germany

Tian ZF, Shen H, Fu XH, Chen YC, Blum HE, Baumert TF, Zhao XP. Interaction of hepatitis C virus envelope glycoprotein E2 with the large extracellular loop of *tupaia* CD81. *World J Gastroenterol* 2009; 15(2): 240-244 Available from: URL: <http://www.wjgnet.com/1007-9327/15/240.asp> DOI: <http://dx.doi.org/10.3748/wjg.15.240>

### Abstract

**AIM:** To further analyze the interaction of *tupaia* CD81 with hepatitis C virus (HCV) envelope protein E2.

**METHODS:** A *tupaia* CD81 large extracellular loop (CD81 LEL), which binds to HCV E2 protein, was cloned and expressed as a GST-fusion protein, and interaction of HCV E2 protein with a *tupaia* CD81 LEL was evaluated by enzyme-linked immunosorbent assay (EIA).

**RESULTS:** Although *tupaia* and human CD81 LEL differed in 6 amino acid changes, *tupaia* CD81 LEL was strongly recognized by anti-CD81 antibodies against human CD81 LEL conformation-dependent epitopes. Investigating LEL CD81-E2 interactions by EIA, we demonstrated that binding of *tupaia* CD81 LEL GST fusion protein to recombinant HCV E2 protein was markedly reduced compared to binding of human CD81 LEL GST fusion protein to recombinant HCV E2 protein.

**CONCLUSION:** These data suggest that the structural differences in-between the *tupaia* and human CD81 may alter the interaction of the large extracellular loop with HCV envelope glycoprotein E2. These findings

### INTRODUCTION

Hepatitis C virus (HCV) is a major cause for posttransfusion and community-acquired hepatitis worldwide<sup>[1-4]</sup>. The majority of HCV-infected individuals develop chronic hepatitis that may progress to liver cirrhosis and hepatocellular carcinoma (HCC)<sup>[5]</sup>. Virion contains an approximately 9.5 kb long positive-strand RNA genome encoding for a single polyprotein containing 3010-3030 amino acids<sup>[6-8]</sup>. The polyprotein is cleaved co- and post-translationally by host cellular and viral proteases into at least ten different products. The predicted structural components of the virus comprise the core and two envelope glycoproteins: E1 and E2. The E2 protein is responsible for initiating viral attachment to receptor(s) on potential host cells due to its ability to bind to human cells<sup>[6,9]</sup>. CD81 has first been identified as a HCV E2 binding molecule by expression cloning by Pileri and colleagues<sup>[10]</sup>. The E2 binding site of CD81 is located within the LEL domain<sup>[10-13]</sup>. Further studies then demonstrated a key role of CD81 as a host entry factor for infection of human hepatoma cells with recombinant HCV pseudoparticle and tissue-culture-derived HCV (HCVcc)<sup>[14-18]</sup>. Furthermore, one very recent study has demonstrated evidence that CD81 is also involved in HCV infection of primary human hepatocytes using serum-derived HCV<sup>[19]</sup>. However, there appear to be subtle differences regarding the role

of CD81 for productive infection of human hepatoma cells and human hepatocytes using serum-derived or recombinant virus<sup>[19-24]</sup>.

Recently, a novel *in vitro* cell culture model system has been established for HCV with primary tupaia hepatocytes (PTHs)<sup>[25-27]</sup>. Using this cell culture system, we found that HCV E2 protein binding to PTHs and infection of PTHs with HCV could not be blocked with soluble CD81 and anti-CD81<sup>[25]</sup>. To further investigate the role of CD81 in the infection of PTHs with HCV, we cloned a tupaia CD81 large extracellular loop (LEL) and analyzed the interaction of *tupaia* CD81 with HCV E2 protein *in vitro*. The results indicate that changes occur in 6 amino acid residues of *tupaia* CD81 LEL and the ability of *tupaia* CD81 LEL to bind to HCV E2 is significantly decreased compared with human CD81 LEL.

## MATERIALS AND METHODS

### Reagents

Recombinant HCV E2 protein, and mouse anti-HCV E2 (3E5) were generously provided by M Houghton (Chiron Corp., Emeryville, CA). Human CD81 LEL (wild type and T163A mutant) and AGM CD81 LEL protein were kindly provided by Dr. S Levy (Department of Medicine, Stanford Medical School, Palo Alto, CA)<sup>[12]</sup>. Mouse monoclonal anti-CD81 antibodies 5A6 and 1D6 have been described elsewhere<sup>[11,12,28]</sup>. Glutathione S-transferase (GST) expression vector, pGEX-2T, was purchased from Amersham Pharmacia Biotech Inc.

### Cloning of *tupaia* CD81 LEL

Total cellular RNA was isolated from PTHs with RNeasy kits (Qiagen, GmbH, Hilden, Germany) according to its manufacturer's instructions. cDNA was transcribed from cellular RNA using rTth reverse transcriptase. *Tupaia* CD81 (TupCD81) LEL was amplified by PCR using Pfu DNA polymerase (Stratagene) and human CD81-specific primers covering the LEL domain (sense: 5'-TAACGG ATCCAACAAGGACCAGATTGCCAAGGA-3', anti-sense: TAACGAATTCACAGCTTCCCGGAGAAGAG CTC-3'). The PCR products were then cloned into pCR-Blunt II-TOPO (Invitrogen, Groningen, Netherlands). An EcoR I/BamH I fragment was subcloned into pGEX-2T (Amersham Pharmacia Biotech Inc.) with the same enzyme digestion. The resulting plasmid (named p2T-TupCD81 LEL) was sequenced using CD81-specific primers. Plasmids expressing African green monkey, and human CD81 LEL (both wild type and genetically modified T163A type) were kindly provided by Dr. S Levy and have been described previously.

### Expression and purification of glutathione S-transferase (GST)-CD81 LEL fusion proteins

Expression and purification of GST-CD81 LEL fusion protein were performed as previously described<sup>[13]</sup>. Briefly, BL21 *E. coli* were transformed with plasmid DNA. Transfected *E. coli* were grown in 12 mL LB

medium containing ampicillin (50 mg/L) at 30-32°C until the optical density (OD) value of the culture medium reached 0.6-0.8 at 4600. Protein expression was then induced by adding isopropyl β-D-thiogalactoside (IPTG, 0.75 mmol/L). After cells grew for additional 3 h, they were lysed by sonication and the fusion proteins were purified using the MicroSpin GST purification module (Amersham Pharmacia Biotech). GST protein expressed in control plasmid pGEX-2T was purified in parallel. The purified proteins were analyzed using SDS-PAGE under non-reducing conditions, quantified, aliquoted, and stored at -80°C until use.

### Test of HCV E2-CD81 LEL interaction

A 96-well microtiter plate was coated with GST-CD81 fusion protein (5 mg/L) at 4°C overnight. After washing 5 times with PBS and blocking with 4% dry milk (in PBS) at room temperature for 1 h, 50 μL of recombinant HCV E2 protein (with reciprocal dilutions, starting from 2 mg/L) was allowed to bind to CD81 LEL at 4°C overnight. The bound E2 was detected using anti-E2 (3E5; diluted at 1:2000 in PBS containing 1% Tween 20 and 1% BSA) and HRP-conjugated anti-mouse IgG (diluted at 1:5000 in PBS containing 1% Tween 20 and 1% BSA). GST protein and human and AGM CD81 LEL were tested as controls. For GST test, goat anti-GST (in dilution starting at 1:1000) (Amersham Pharmacia Biotech) was added, and incubated at room temperature for 1 h. The bound anti-GST was detected using HRP-conjugated anti-goat IgG. The bound HRP-conjugated second antibodies were quantified by colorimetric reaction with an Abbott OPD reagent kit (Abbott Laboratories, North Chicago, IL), and the OD value was measured at 490 nm on a Bio-Rad plate reader (Bio-Rad, Hercules, CA).

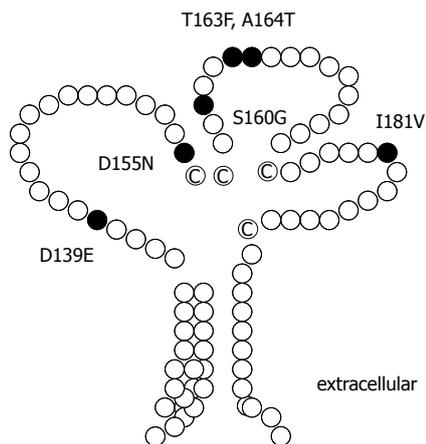
## RESULTS

### *Tupaia* CD81 LEL amino acid sequence

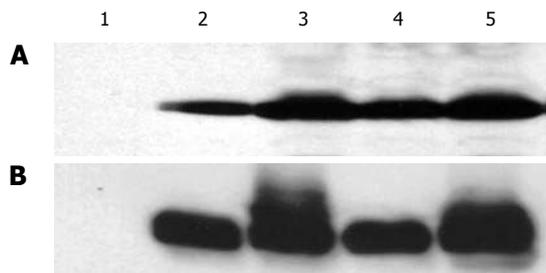
It has been demonstrated that the E2 binding site of CD81 is located within the LEL domain<sup>[10,12,13]</sup>. To study the E2-CD81 LEL interaction *in vitro*, *tupaia* CD81 LEL was cloned and sequenced. The deduced amino acid sequence of *tupaia* CD81 LEL cDNA showed mutations in 6 amino acid residues when compared with human CD81 LEL as previously showed<sup>[25]</sup>. These mutations were clustered around the E2 binding head subdomain (Figure 1), according to the 3D structure of human CD81 LEL<sup>[29]</sup>.

### Analysis of *tupaia* CD81 LEL-E2 interaction *in vitro*

To analyze the interaction between HCV glycoprotein E2 and *tupaia* CD81 LEL *in vitro*, *tupaia* CD81 LEL was expressed as a GST-fusion protein in *E. coli*. The correct expression and folding of *in vitro*-synthesized *tupaia* CD81 LEL were confirmed by analyzing the purified protein with non-reducing SDS-PAGE and immunoblot using anti-CD81 antibodies against conformation-dependent epitopes. As shown in Figure 2A and B, *tupaia* CD81 LEL was recognized by defined anti-human CD81



**Figure 1** Putative structure of *tupaia* CD81. *Tupaia* CD81 LEL cDNA was cloned and sequenced as described in Materials and Methods. Deduced amino acid sequence was compared with that of human. The *tupaia* CD81 secondary structure was drawn according to the three-dimensional structure of human CD81 LEL, and changes in six amino acids of *tupaia* CD81 LEL were illustrated by black color.

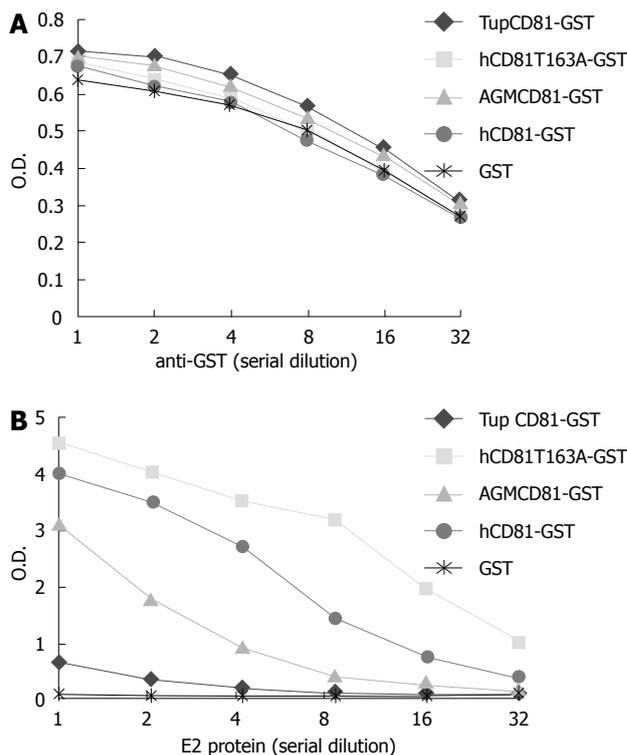


**Figure 2** Expression and purification of soluble *tupaia* CD81 LEL using mouse monoclonal anti-human CD81 5A6 (A), and 1D6 (B) antibodies. *Tupaia* CD81 LEL was expressed and purified in *E. coli* as a GST fusion protein as described in Materials and Methods. Purified proteins were subjected to SDS-PAGE, and immunoblot using mouse monoclonal anti-human CD81 5A6 and 1D6 antibodies. Tup: *tupaia*; h: human; AGM: African green monkey; hCD81 LEL T163A: human CD81 containing a mutation of T to A at amino acid residue 163; 1: GST; 2: TupCD81 LEL-GST; 3: hCD81 LELT163A-GST; 4: AGMCD81 LEL-GST; 5: hCD81 LEL-GST.

antibodies (5A6 and 1D6), similar to human or African green monkey CD81 LEL. The data suggest that folding of CD81 LEL-GST fusion protein is comparable to that of the native molecule, while slight differences might exist in the 3D structure of human and *tupaia* CD81 LEL since the staining intensity of TupCD81 was weak compared with that of human CD81 (both wild and T163A mutation type) using antibody to human CD81 conformation-dependent epitopes. Interaction of *tupaia* CD81 LEL with recombinant E2 protein was analyzed with EIA, and compared with that of human- or African green monkey-derived CD81 LEL. Binding of *tupaia* CD81 LEL to HCV E2 protein was markedly reduced compared with the native or mutant human CD81 LEL (Figure 3).

## DISCUSSION

Recombinant HCV E2 could bind to *tupaia*, but not to rat hepatocytes in a dose-dependent manner and PTHs could be infected with HCV *in vitro*<sup>[25,26]</sup>. Furthermore,



**Figure 3** Interaction between *tupaia* CD81 LEL and HCV E2 protein. Plates were coated with 100  $\mu$ L of recombinant GST or GST-CD81 LEL fusion proteins (5 mg/L), reciprocally diluted anti-GST antibody (A) (starting dilution at 1:1000) or HCV E2 protein (B) (starting concentration 2 mg/L) was added to the plates. Binding of anti-GST or HCV E2 protein was assessed as described in Materials and Methods. OD: optical density; Tup: *tupaia*; h: human; AGM: African green monkey; hCD81T163A: human CD81 containing a mutation of T to A at amino acid 165.

we demonstrated that both the binding of HCV E2 to PTHs, and infection of PTHs with HCV could not be blocked with soluble CD81 and anti-CD81 exhibiting the blocking ability of HCV E2 to bind to lymphoma cell lines, indicating that binding of E2 to PTHs and infection of *tupaia* hepatocytes with HCV might require additional or other molecules besides CD81. To further characterize the HCV E2-*tupaia* CD81 interaction, we cloned the HCV E2 binding domain of CD81, the CD81 LEL, and investigated the reaction of HCV E2 with *tupaia* CD81 *in vitro*.

CD81, a member of the super-family of tetra-spanins, comprises 4 transmembrane (TM1-4) and two extracellular loops. The HCV E2 binding domain locates in the large extracellular loop (LEL), which folds to form a mushroom-like 3D structure, and is stabilized by a number of specific interactions within the defined amino acid residues<sup>[29]</sup>. In the present study, the deduced amino acid sequence of *tupaia* CD81 LEL showed changes in only 6 amino acid residues compared to human CD81 LEL, while all the residues necessary for the 3D structure-stabilization were conserved, suggesting that soluble *tupaia* CD81 LEL folds in a manner comparable to human CD81 LEL. This hypothesis was confirmed by the cross interaction of *tupaia* CD81 LEL with anti-human CD81 antibodies against conformation-dependent epitopes (Figure 2).

It has been reported that HCV E2 binds to the

head subdomain of CD81 LEL consisting of about 60 amino acid residues<sup>[29]</sup>. Distinct amino acid mutations can affect CD81-E2 interaction. African green monkey (AGM) CD81 LEL contains only 4 amino acid residues compared with human CD81 LEL, and shows reduced E2 binding<sup>[10-12]</sup>. Reduced E2-binding of AGM CD81 is due to a mutation of phenylalanine to leucine at the amino acid residue 186 (F186L)<sup>[12]</sup>. Interestingly, mutation of threonine to alanine at the amino acid residue 163 (T163A) can enhance E2-CD81 binding<sup>[12]</sup>. In this study, all the six mutant residues of *tupaia* CD81 LEL were clustered at its head subdomain which is the binding site for HCV E2, while the phenylalanine at residue 186, and the 4 cysteine residues, which are pivotal for human CD81-HCV E2 binding, were conserved in *tupaia* CD81. When purified *tupaia* CD81 LEL was tested for its binding to recombinant HCV E2 protein, only a mild E2-CD81 interaction was observed. By contrast, human CD81 LEL (both wild-type and genetically modified CD81 containing a T163A mutation) bound firmly to E2 protein. Interestingly, threonine at residue 163 of TupCD81 LEL changed into phenylalanine. The reduced E2 binding ability of *tupaia* CD81 was not due to the amount of protein coating the plates, since the GST activity of those fusion proteins was nearly similar. From the perspective of the 3D structure of CD81 LEL, mutations at amino acid residues 155 and 181 of *tupaia* CD81 LEL may be responsible for the reduced binding in *Tupaia* CD81 to HCV E2 protein<sup>[29]</sup>. The binding of HCV E2 to AGMCD81 LEL in this study different from a previous study<sup>[12]</sup>. This discrepancy might be due to the differences in the recombinant HCV E2 proteins. E2 used in this study is C-terminally truncated at amino acid 715, whereas other investigators used E2 C-terminally truncated at amino acid 661.

Although our studies were limited to evaluate CD81 LEL-E2 interaction with EIA, and need to be confirmed in model systems expressing full-length CD81 in transfected mammalian cell lines, the differences in HCV E2 binding between *tupaia* CD81 LEL and human CD81 LEL are consistent with our previous functional data assessing E2 binding to PTHs and HCV infection of PTHs in the presence of anti-CD81 antibodies. Taken together, these results indicate that although CD81 may play a functional role as a co-factor for entry of HCV into PTHs, it is likely that other or additional molecules besides CD81 play a key role in HCV entry into PTHs. These may include other identified HCV host factors including SR-BI<sup>[27]</sup> or Claudin-1<sup>[30]</sup>. Alternatively, other not yet identified host entry factors may mediate HCV-PTH interaction.

## COMMENTS

### Background

CD81, a member of the superfamily tetraspanins, comprises four transmembrane (TM1-4) and two extracellular loops. The HCV E2 binding domain locates in the large extracellular loop (LEL). The LEL folds to form a mushroom-like 3D structure, which is stabilized by a number of specific interactions within the defined amino acids residues.

### Research frontiers

Hepatitis C virus (HCV) is a major cause for posttransfusion and community-acquired hepatitis worldwide. The majority of HCV-infected individuals develop chronic hepatitis that may progress to liver cirrhosis and hepatocellular carcinoma (HCC). The predicted structural components of HCV comprise the core and two envelope glycoproteins: E1 and E2. The E2 protein is responsible for initiating viral attachment to receptor(s) on potential host cells due to its ability to bind to human cells. HCV E2 could specifically bind to cell surface molecule CD81 expressed in lymphoma cells. Anti-HCV antibodies from chimpanzees that are protected against homologous HCV challenge by vaccination with envelope glycoproteins inhibit E2 binding to CD81, suggesting that CD81 represents a candidate receptor for HCV infection. Whether CD81-E2 interaction can mediate virion entry into host cells is unknown and has not been tested in a suitable model.

### Innovations and breakthroughs

A novel *in vitro* cell culture model system has been established for HCV with primary *tupaia* hepatocytes (PTHs). Using this cell culture system, we found that HCV E2 protein binding to PTHs and infection of PTHs with HCV could not be blocked with soluble CD81 and anti-CD81. To further investigate the role of CD81 in the infection of PTHs with HCV, we cloned a *tupaia* CD81 large extracellular loop (LEL) and analyzed the interaction of *tupaia* CD81 with HCV E2 protein *in vitro*. The results indicate that changes occur in 6 amino acid residues of *tupaia* CD81 LEL and the ability of *tupaia* CD81 LE to bind to HCV E2 is significantly decreased compared with human CD81 LEL.

### Applications

*Tupaia* CD81 has a reduced ability to bind to HCV E2 protein. HCV entry and infection of PTHs with HCV might occur through receptor(s) besides CD81.

### Peer review

The manuscript is well written, but needs clarification of the importance of the found amino acid changes with regard to the interaction of *tupaia* CD81 and HCV E2 binding.

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S- Editor Li JL L- Editor Wang XL E- Editor Zheng XM