Binding of Hepatitis C Virus E2 Glycoprotein to CD81 Does Not Correlate with Species Permissiveness to Infection

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Hepatitis C virus (HCV) glycoprotein E2 binds to human cells by interacting with the CD81 molecule, which has been proposed to be the viral receptor. A correlation between binding to CD81 and species permissiveness to HCV infection has also been reported. We have determined the sequence of CD81 from the tamarin, a primate species known to be refractory to HCV infection. Tamarin CD81 (t-CD81) differs from the human molecule at 5 amino acid positions (155, 163, 169, 180, and 196) within the large extracellular loop (LEL), where the binding site for E2 has been located. Soluble recombinant forms of human CD81 (h-CD81), t-CD81, and African green monkey CD81 (agm-CD81) LEL molecules were analyzed by enzyme-linked immunosorbent assay for binding to E2 glycoprotein. Both h-CD81 and t-CD81 molecules were able to bind E2. Competition experiments showed that the two receptors cross-compete and that the t-CD81 binds with stronger affinity than the human molecule. Recently, h-CD81 residue 186 has been characterized as the critical residue involved in the interaction with E2. Recombinant CD81 mutant proteins were expressed to test whether human and tamarin receptors interacted with E2 in a comparable manner. Mutation of residue 186 (F186L) dramatically reduced the binding capability of t-CD81, a result that has already been demonstrated for the human receptor, whereas the opposite mutation (L186F) in agm-CD81 resulted in a neat gain of binding activity. Finally, the in vitro data were confirmed by detection of E2 binding to cotton-top tamarin (Saguinus oedipus) cell line B95-8 expressing endogenous CD81. These results indicate that the binding of E2 to CD81 is not predictive of an infection-producing interaction between HCV and host cells.
MATERIALS AND METHODS

Animals and cells. A captive, outbred Saguinus oedipus tamarin (B234) was housed at the Biomedical Primate Research Center, Rijswijk, The Netherlands, and maintained under conditions that fulfilled the ethical and scientific requirements for animal use. Saguinus labiatus primary hepatocytes were kindly supplied by Ralph Laufer. S. oedipus lymphoblast cell line B98-5 was obtained from the American Type Culture Collection (ATCC) (CRL-1612). The Molt-4 (human T-cell leukemia) line was obtained from the Medical Research Council ADP Repository. The 293 (human embryonic kidney) cell line was obtained from ATCC (CRL-1573), as was EL4 (mouse lymphoma) (TIB-39).

RNA preparation. Total RNA (20 μg) from each of resuspended S. oedipus tamarin liver was used for reverse transcription using the UltraScript II RNA isolation system (Biocat), following the manufacturer’s instructions. Pellets corresponding to 5 × 106 cells of both S. labiatus primary hepatocytes and S. oedipus B98-5 cultured cells were used to prepare total RNA using the system described above.

Amplification of t-CD81 sequences. Total RNA (2.5 μg) was used as a template for first-strand cDNA synthesis in a 20-μl reaction mixture. The RNA was mixed with 10 pmol of antisense primer 98184 (5′-TCAGTACCGAAGCTGT TCCGGATG-3′) in a volume of 11 μl, denatured for 5 min at 90°C, chilled on ice, and centri fuged at 4°C. The following reagents were then added to the reaction mixture in amounts suitable to reach the indicated concentrations: 2.5% dimethyl sulfoxide, 10 U of RNasin (Promega) per ml, 1× Superscript buffer (Gibco-BRL), 10 mM dithiothreitol, and a 1.25 mM concentration of each decanuclease inhibitor (dNTP). The reaction was performed by preincubation of the mixture at 42°C for 2 min, followed by incubation with 1 μl of Superscript II reverse transcriptase (Gibco-BRL) at 42°C for 30 min. The reaction was stopped by incubation at 70°C for 5 min. PCR was performed using 2.5 μl of first-strand cDNA reaction mixture and the above primers as described above, and alkaline phosphatase was revealed by incubation at 37°C with a 1-mg/ml solution of p-nitrophenyl phosphatase in ELISA substrate buffer (10% diethanolamine buffer, 0.5 mM MgCl2, pH 9.8). Results were expressed as the difference in absorbance between the OD492 nm of the control and the OD492 nm of the automated ELISA reader (Labsystems Multiskan Bichromatic, Helsinki, Finland). Competition of the binding of t-CD81 was performed by preincubation of a nonsaturating amount of E2 protein with redundant CD81 molecules.

Cloning and expression of E2. The cDNA fragment of genotype 1a (strain H) was cloned into a pBluescript II SK+(E2) by direct cloning of PCR products. The purified PCR products were ligated into the EcoRI/XbaI sites of the vector using the Original TA cloning kit (Invitrogen). The pBluescript II SK+(E2) plasmid was constructed by cloning the 264-bp HindIII-RsaI fragment of t-CD81-ORF/pCR2.1 encoding the CD81 LEL domain (Invitrogen). This pBluescript II SK+(E2) plasmid was used to construct the 293-hCD81-ELP plasmid. The CD81 plasmid was constructed by cloning the 264-bp HindIII-RsaI fragment of t-CD81-ORF/pCR2.1 encoding the CD81 LEL domain into the filled-in EcoRI site of the pGEX-2T vector (Amersham Pharmacia Biotech). Construction of h-CD81 and h-CD81-L186F plasmids was as previously reported (9). Mutants (t-CD81-F186L and agm-CD81-L186F) were constructed as follows. Parental plasmids were digested with HindIII and ligated into the parental digested vectors.

Expression and purification of recombinant proteins. DNA of plasmids encoding CD81 LEL domains fused to glutathione-S-transferase (GST) protein was used to transform Escherichia coli DH5α competent cells, and cells were plated on Luria-Bertani (LB) agarose plates containing 100 μg of ampicillin per ml and 1% glucose. Single ampicillin-resistant colonies were inoculated in LB medium containing 100 μg of ampicillin per ml and 1% glucose and grown at 37°C with shaking. Overnight cultures were diluted 1:100 in LB-ampicillin and grown at 37°C till the optical density at 600 nm (OD600) was 0.6. Isopropyl-β-D-thiogalactopyranoside (IPTG) was added to a final concentration of 1 mM, and the culture was incubated for 3 h at room temperature. The bacterial pellet corresponding to 500 ml of induced culture was resuspended in 30 ml of phosphate-buffered saline (PBS) containing protease inhibitor cocktail (Boehringer) at 4°C. The cells were lysed by a microfluidizer (model 110-S; Microfluidics International Corp., New York, N.Y.), and the extract was centrifuged for 30 min at 38,000 × g. Glutathione-Sepharose matrix (Pharmacia) was added to the bacte- rial lysate and incubated overnight at 4°C with gentle agitation. After extensive washing with cold PBS, the matrix-bound GST fusion protein was eluted with reduced glutathione (Sigma) at a final concentration of 10 mM in 50 mM Tris HCl (pH 8.0). Proteins were supplemented with protease inhibitor cocktail tablets (1 tablet/50 ml; Boehringer Mannheim) and 0.05% NaN3, and the concentration was determined by protein assay (Bio-Rad). Finally, proteins were checked by Western blotting analysis (25), using both anti-GST polyclonal antibody (Amersham Pharmacia Biotech) and anti-CD81 mouse monoclonal antibody (13.3.22; Santa Cruz Biotechnology), and kept at −80°C in the presence of 10% glycerol.

Binding of E2 to recombinant CD81 proteins. Enzyme-linked immunosorbent assay (ELISA) plates were coated with 10 μg of recombinant GST-CD81 proteins (t-CD81, h-CD81, agm-CD81, t-CD81-F186L, and agm-CD81-L186F, with GST as the control) diluted in PBS. After an overnight incubation at 4°C, plates were washed with washing buffer (0.05% Tween 20, PBS) and nonspecific binding sites were blocked with milk buffer (5% nonfat dry milk-0.05% Tween 20-0.05% NaN3 in PBS) for 1 h at 37°C. Serial dilutions of crude cell extract containing E2 proteins were performed in milk buffer supplemented with 50 μg of GST per ml, and the dilutions were preincubated 1 h at room temperature and then added to the CD81-coated plates for an overnight incubation at 4°C. After extensive washing, anti-His tag mouse MAb (Qiagen) diluted 1/400 in 2.5% bovine serum albumin–PBS was added and incubated for 3 h at 4°C. Alkaline phosphatase-conjugated goat anti-mouse immunoglobulin G (IgG) (Sigma) diluted 1/2000 in milk buffer was used as secondary antibody. Plates were finally washed as described above, and alkaline phosphatase was revealed by incubation at 37°C with a 1-mg/ml solution of p-nitrophenyl phosphatase in ELISA substrate buffer (10% diethanolamine buffer, 0.5 mM MgCl2, pH 9.8). Results were expressed as the difference in absorbance between the OD492 nm of the control and the OD492 nm of the automated ELISA reader (Labsystems Multiskan Bichromatic, Helsinki, Finland). Competition of the binding of t-CD81 was performed by preincubation of a nonsaturating amount of E2 protein with recombinant CD81 molecules.

RESULTS

Cloning of t-CD81 sequence. To investigate the relationship between permissiveness to HCV viral infection and CD81, we
sequenced the CD81 molecule from a primate genus (Saguinus) nonsusceptible to the infection (11). RNA was prepared from the S. oedipus-derived B95-8 lymphoblast cell line, from the liver of S. oedipus, and from hepatocytes of S. labiatus tamarins. Reverse transcription-PCR amplification of the CD81 open reading frame was performed using primers complementary to the h-CD81 sequence, and the product was cloned. The sequence of all three specimens was identical, indicating that there is no variability either between the two species examined or between primary cells and the cell line B95-8. The deduced amino acid sequence differs from that of the h-CD81 sequence in five amino acid positions, 155, 163, 169, 180, and 196, all located within the LEL domain, as shown in Fig. 1.

Interaction of soluble CD81 LEL proteins with HCV E2 glycoprotein. The binding site for HCV E2 glycoprotein has been mapped to the LEL domain of the CD81 molecule (9, 19). In general, fusions between tetraspanin large extracellular domains and GST have been described as correctly folded and functionally active (4). Moreover, a recombinant bacterially expressed h-CD81 LEL fused to GST has been reported to be able to bind HCV E2 envelope protein (9). We have analyzed the binding of E2 derived from genotype 1a (H-661) to recombinant CD81 LEL-GST fusion proteins from different species. All of the recombinant CD81 proteins were produced with comparable yields. Similarly, the concentrations of purified CD81 variant proteins, as measured by their reactivity by Western blotting with anti-CD81 antibodies, were comparable (data not shown). ELISA plates were coated with various CD81 molecules, and E2 binding was measured. agm-CD81 was unable to bind E2, whereas both human and tamarin proteins bound E2 (Fig. 2A). Indeed, E2 appeared to bind with higher relative affinity to t-CD81 than to h-CD81. To evaluate this further we compared the ability of soluble h-CD81 and t-CD81 proteins to compete for E2 interaction with t-CD81 bound to a solid support. Both human and tamarin proteins inhibited the interaction; however, a 10-fold-higher concentration of h-CD81 was required for the same level of inhibition (Fig. 2B). These results support the data shown in Fig. 2A, suggesting that E2 binds with higher affinity to t-CD81.

Binding of HCV E2 to cell lines. E2 ectodomain derived from HCV genotype 1a (H-661) binds to a variety of cell lines of human origin interacting with the CD81 receptor molecule (9, 19). We analyzed the binding of E2 to the cotton-top tamarin B95-8 cell line from which the t-CD81 cDNA was cloned for expression of the protein tested in the in vitro experiments described above. The binding was performed in parallel on the human cell line Molt-4 and on the mouse cell line EL4. The presence of the CD81 molecule on the cell surface was checked by FACS analysis using a fluorescein conjugate anti-h-CD81 monoclonal antibody. The antibody was cross-reactive with the t-CD81 and stained Molt-4 and B95-8 cells in a comparable manner (data not shown). E2 bound both human and tamarin cells, as revealed by the addition of a mouse MAb against the His tag of the recombinant protein followed by an anti-mouse IgG1-phycoerythrin conjugate (Fig. 3). The E2 interaction to B95-8 occurred in a CD81-dependent manner since nonsaturating levels of E2 could be competed by preincubation of E2 with soluble recombinant t-CD81 (Fig. 4). Moreover, preincubation of cells with anti-CD81 mouse MAb completely prevents E2 binding (Fig. 5). In this experiment directly labeled anti-His MAB (Alexa 488) was used to reveal the bound antigen.

Defining the E2 binding region of t-CD81. To gain further insight into the functional equivalence of t-CD81 and h-CD81 regions interacting with E2, we constructed a mutant t-CD81, FIG. 1. Alignment of CD81 LEL amino acid sequences of h-CD81 (SWISS-PROT accession no. P18582), chimpanzee CD81 (c-CD81; GenBank accession no. AF116600), agm-CD81 (GenBank accession no. AF116599) and t-CD81 (EMBL accession no. AJ250197). *, residue 186 mutated in agm-CD81 (L186F) and in t-CD81 (F186L).

FIG. 2. (A) Binding of E2 protein to recombinant CD81 molecules. ELISA plates were coated with the CD81 recombinant molecules, and 1:3 serial dilutions of crude lysate containing E2 were tested for binding. (B) Competition of E2 binding to t-CD81. t-CD81 was used to coat an ELISA plate. Competition was performed by preincubation of E2 extract with serial dilutions of recombinant CD81 molecules. ●, t-CD81; ○, h-CD81; □, agm-CD81; △, GST (control).
introducing an amino acid substitution known to be crucial for the interaction of h-CD81 with E2 (12). The binding capability of h-CD81 is abrogated by replacing the phenylalanine at position 186 with a leucine, the corresponding residue present in agm-CD81. Hence, we mutated phenylalanine residue 186 to a leucine also in t-CD81. The mutant protein t-CD81-F186L was approximately 30-fold less active in binding E2 than the parental protein (Fig. 6). To obtain direct evidence for the requirement of a phenylalanine at position 186 for CD81-E2 interactions, the mutation L186F was introduced in agm-CD81 with the expectation that this mutation would have promoted E2 binding. Indeed, the effect of this mutation was striking, as it converted the inactive agm-CD81 to a high-affinity molecule having the ability to bind E2 with an apparent affinity threefold higher than that of the tamarin receptor (Fig. 6).

**DISCUSSION**

CD81 has been identified as a putative receptor for HCV envelope glycoprotein E2. The binding region for E2 was mapped to the LEL of the CD81 molecule (10, 19). To gain further information about the association between permissiveness to HCV infection and CD81 receptors we sequenced the CD81 gene from monkeys of the genus *Saguinus*, commonly called tamarins. Unsuccessful attempts to experimentally infect tamarins have led to the conclusion that these monkeys are resistant to HCV (11).

Cloning of the cDNA encoding CD81 from the *S. oedipus* cell line B95-8 allowed the characterization of this species’ molecule, which is very similar to the h-CD81. We confirmed that t-CD81 obtained from the B95-8 cell line corresponded exactly to the molecule present in primary cells by sequencing the cDNA directly from two independent sources of hepatocytes belonging to the species *S. oedipus* and *S. labiatus*. The t-CD81 is identical to h-CD81 in the transmembrane domains, in the cytoplasmic domains, and in the extracellular loop 1, and it has only five amino acid differences, 155, 163, 169, 180, and 196, all located within the LEL region (Fig. 1). It is interesting to note that two of the five changes are conservative (T163S and D196E). Moreover, phenylalanine 186, which has been recently shown to be crucial for the interaction with E2 (12), is present in human and tamarin receptors and it is changed to leucine in the agm-CD81.

Production of soluble CD81 fusion proteins was achieved to perform comparative analysis of the interaction of E2 with CD81 molecules from different species. E2 protein from HCV

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**FIG. 3.** FACS analysis of E2 binding to cell surface of EL4 (A), Molt-4 (B), and B95-8 (C) cell lines. Open curves, binding of E2 revealed by an MAb against the His tag; gray curves, binding of the isotype control. For panels A, B, and C, the median F.I. values were 7.8, 66.7, and 77.0, respectively.

**FIG. 4.** FACS analysis of E2 binding to B95-8 cell surface in the presence of recombinant t-CD81. Competition was performed by preincubation of subsaturating amounts of E2 with increasing concentrations of t-CD81-GST (○) or GST (△). The median F.I. for binding of E2 in the absence of the competitor was 38.8, and the median F.I. for binding of the mock antigen was 2.8.

**FIG. 5.** FACS analysis of inhibition of E2 binding to B95-8 cells by anti-CD81 MAb. B95-8 cells were incubated with the monoclonal antibody at a concentration known to saturate the cell surface before the incubation with E2. Gray curve, E2 binding (median F.I., 34.2); open curve, binding competition by anti-CD81 MAb (median F.I., 5.3). The median F.I. for binding of the mock antigen to B95-8 was 2.7.
interacts with soluble t-CD81 (Fig. 2). The comparative analysis of direct binding to E2 suggested that t-CD81 binds even better than the human receptor. This indication was confirmed by cross-competition experiments where a 10-fold-higher concentration of the human receptor than of the t-CD81 was achieved only by interacting with the almost ubiquitous CD81 molecule.

The experiments presented in this study show that t-CD81 is able to bind HCV E2 glycoprotein, possibly through the same contacts of h-CD81-E2 interaction, in spite of the fact that HCV is unable to produce an infection in these animals. Thus, binding of HCV E2 glycoprotein to CD81 does not correlate with species permissiveness to HCV infection.

We could not assess whether t-CD81 is able to bind E2 displayed on HCV virions, since attempts to reproduce experiments on binding of HCV particles to CD81, as published by Pileri et al. (19), were unsuccessful with both the h-CD81 and the t-CD81 molecules. A possible explanation of this failure might be related to the availability of a convenient source of virus. We used high-titer HCV-infected human sera (data not shown), whereas Pileri et al. used experimentally infected chimpanzee sera, possibly obtained in a very early stage of infection, preceding the occurrence of antibodies complexing the viral particles.

Whether CD81 is the key molecule for HCV attachment to cells is an as-yet-unanswered question (21). We cannot exclude the possibility that HCV could gain entry into tamarin cells via CD81 and that infection would be blocked at a subsequent stage of the replicative cycle. On the other hand, CD81 may not be the sole receptor molecule involved in the interaction of the virus with the cells. We favor the hypothesis that a molecule exclusive for permissive species may be cooperative with CD81. One possibility is that cell entry of HCV requires, as does that of human immunodeficiency virus (7, 17), a coreceptor that would strengthen the interaction with CD81 and/or determine the viral tropism for the liver, which could not be achieved only by interacting with the almost ubiquitous CD81 molecule.

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