Hepatitis C Virus Envelope Protein E2 Binds to CD81 of Tamarins

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Received June 9, 2000; returned to author for revision August 1, 2000; accepted August 31, 2000

Since recombinant envelope glycoprotein E2 of hepatitis C virus (HCV) binds to CD81 on human and chimpanzee cells, it has been suggested that CD81 may be a receptor for HCV. Humans and chimpanzees are the only species known to be susceptible to HCV infection. E2 has been reported not to bind to CD81 of the African green monkey, mouse, or rat, suggesting that binding of HCV to CD81 is species specific and may determine susceptibility to infection with HCV. We investigated the interaction between E2 of HCV and CD81 of tamarins, a group of small New World monkeys frequently used for the study of human viruses. Tamarins are not susceptible to HCV infection. Nonetheless, we found that three different forms of HCV E2 (intracellular, secreted, and cell surface-displayed) bound more efficiently to recombinant tamarin CD81 than to human CD81, as determined by ELISA and immunofluorescence. The affinity of the interaction was approximately 10-fold higher for tamarin than for human CD81. Binding of E2 to CD81 on cultured or primary tamarin cells was demonstrated by flow cytometry. In contrast to previous reports, there was also a low-affinity interaction between E2 and African green monkey CD81. Thus, the HCV E2 interaction with CD81 is not limited to humans and chimpanzees and does not predict susceptibility to HCV infection.

INTRODUCTION

Hepatitis C virus (HCV) is an important cause of chronic liver disease worldwide (Houghton, 1996). Humans and chimpanzees are the only species known to be susceptible to HCV infection. The lack of a small animal model and a reliable cell culture system has hampered HCV research (Houghton, 1996). Tamarins, a group of New World monkeys of the genus *Saguinus*, are susceptible to several human viruses and have been used in hepatitis research for decades (Deinhardt, 1978). Tamarins are most likely the natural hosts of GB viruses A and B, the viruses most closely related to HCV (Bukh and Apgar, 1997; Simons et al., 1995). Although early studies suggested that tamarins were susceptible to non-A, non-B hepatitis (reviewed in Tabor, 1989), more recent studies using HCV-specific assays indicate that tamarins are not susceptible to HCV (Garson et al., 1997).

The factors responsible for host specificity and cell tropism of HCV are largely unknown. However, it is reasonable to assume that the envelope glycoproteins are responsible for virus attachment and entry into susceptible cells. HCV is a member of the *Flaviviridae* family of viruses. It has a lipid envelope into which two envelope glycoproteins (E1 and E2) are inserted as heterodimers (reviewed in Dubuisson, 1999). The envelope glycoproteins of HCV have been expressed in mammalian cells. However, when expressed as recombinant proteins, the majority of E1 and E2 is misfolded. Furthermore, E1 and E2 are retained in the endoplasmic reticulum (ER), suggesting that HCV, like other members of the *Flaviviridae* family, buds from this compartment. Retention in the ER can be overcome by deleting the transmembrane domain of E2 (Flint et al., 1999, 2000). The composition and arrangement of carbohydrate chains on E2 of infectious HCV is not known. Thus, it is difficult to establish whether any recombinant E2 protein has the appropriate conformation for glycosylation pattern.

Two candidate cellular receptors for HCV have recently been proposed, the LDL receptor (Agnello et al., 1999) and CD81 (Pileri et al., 1998). Originally, Rosa et al. (1996) showed that recombinant E2 of HCV could bind to human or chimpanzee cells, and that the binding could be inhibited by serum from chimpanzees that were protected from HCV infection after vaccination with recombinant E1/E2 proteins. The molecule responsible for binding E2 was subsequently identified as CD81 (Pileri et al., 1998). CD81 is a member of the tetraspannin family, and thus has two extracellular loops. The binding site for
HCV E2 protein was mapped to the large extracellular loop (LEL), also designated second extracellular loop (EC2) (Flint et al., 1999; Pileri et al., 1998). Recombinant human LEL expressed in E. coli binds to HCV E2 (Flint et al., 1999).

The majority of the CD81 molecule is highly conserved among mammalian species. However, the C-terminus of the LEL contains species-specific differences. The mouse and human CD81 LEL differ from each other at 17 of the 89 amino acid positions, whereas the African green monkey and human LEL differ from each other at only four amino acid positions (Fig. 1). Of the two published chimpanzee CD81 sequences, one is identical to that of humans and one differs by a single amino acid (Flint et al., 1999; Pileri et al., 1998). HCV E2 protein has been reported to bind CD81 on human or chimpanzee cells, but not on African green monkey or rat cells (Flint et al., 1999). Furthermore, recombinant human, but not mouse, LEL could bind to HCV particles in serum (Pileri et al., 1998). These data suggested that CD81 binding correlated with susceptibility to HCV. However, the number of species studied so far is limited. To evaluate tamarins as potential hosts for HCV/GB virus B chimeric virus constructs (Bukh et al., 1999), we investigated the binding of HCV E2 to tamarin CD81.

RESULTS

Sequence of tamarin CD81 differed from that of human and African green monkey

The cDNA sequence of CD81 LEL was determined for two tamarin species, S. oedipus and S. mystax. The LEL of the two tamarin species differed at a single nucleotide position, but the deduced amino acid sequences were identical (Fig. 1). Tamarin LEL (89 amino acids) differed from human and African green monkey LEL at five and six amino acid positions, respectively, and from mouse LEL at 17 amino acid positions.

HCV E2 had higher affinity for tamarin CD81 LEL than for human CD81 LEL

An ELISA assay was developed in which purified HCV E2 protein was coated onto the solid phase. Binding of thioredoxin-LEL fusion protein (trx-LEL) was detected with a mouse monoclonal antibody (MAb) to thioredoxin and an alkaline phosphatase-labeled anti-mouse antibody. Two different forms of purified E2 were used, secreted E2\textsubscript{383–715} (Chiron) (Petracca et al., 2000), since different forms of E2 are reported to have different CD81 binding properties (Flint et al., 2000; Forns et al., 2000). Human, tamarin, and African green monkey trx-LEL each bound to both E2 preparations (Fig. 2). Tamarin LEL-binding was more efficient than human LEL-binding to intracellular E2\textsubscript{383–715} (Chiron) (Petracca et al., 2000), since different forms of E2 are reported to have different CD81 binding properties (Flint et al., 2000; Forns et al., 2000). Human, tamarin, and African green monkey trx-LEL each bound to both E2 preparations (Fig. 2). Tamarin LEL-binding was more efficient than human LEL-binding to intracellular E2\textsubscript{383–715} (Chiron) (Petracca et al., 2000), since different forms of E2 are reported to have different CD81 binding properties (Flint et al., 2000; Forns et al., 2000). Human, tamarin, and African green monkey trx-LEL each bound to both E2 preparations (Fig. 2). Tamarin LEL-binding was more efficient than human LEL-binding to intracellular E2\textsubscript{383–715} (Chiron).
both forms of E2, whereas African green monkey LEL displayed less-efficient binding. Mouse LEL or thioredoxin alone expressed from the same vector did not bind to E2. The affinities of the E2 interaction with CD81 of the three different primate species were estimated by a competitive ELISA (Rath et al., 1988), in which soluble E2 at various concentrations was added to the trx-LEL before performing the ELISA. This experiment confirmed that tamarin LEL had higher affinity than human LEL for either form of E2, and that African green monkey LEL had a low, but detectable, affinity for each form of E2 (Table 1). The intracellular E2<sub>383–715</sub> (Chiron) had higher affinity than secreted E2<sub>388–664</sub> (Abbott) for CD81 LEL of all three primate species tested.

To determine whether a particular tamarin-specific amino acid substitution accounts for the enhanced E2 affinity of tamarin CD81 LEL, we cloned and expressed recombinant trx-LEL molecules having each of four single tamarin-specific substitutions introduced into the human sequence. The fifth substitution, D196E, was not analyzed, since this is also present in the African green monkey CD81 LEL and was studied in that context (Higginbottom et al., 2000) and found not to affect E2 binding. No single substitution was able to increase the affinity of the human LEL to that of tamarin LEL (Fig. 3). The T163S substitution, both alone and in combination with N180S, enhanced E2 binding to some extent, but not to the level of the complete tamarin LEL. The other substitutions either did not affect or slightly reduced E2 binding. Thus, a combination of two to five of the substitutions must be required for the increased E2 affinity of tamarin CD81 LEL compared to that of the human molecule.

**CD81 LEL of human, tamarin, and African green monkey origin bound to HCV E2 expressed on the cell surface**

We recently characterized E2<sub>384–661</sub> expressed on the cell surface and found it to be reactive with conformation-sensitive MAb and with human CD81 LEL. Therefore, we also analyzed binding of the different primate LEL to the well-characterized and apparently well-folded E2<sub>384–661</sub> form that was transiently expressed on the surface of Huh-7 cells. Cells were not fixed and were incubated with trx-LEL and antibodies directly in the culture dish so that only surface-displayed E2 was accessible to the trx-LEL. Bound trx-LEL protein was detected with a mouse MAb to thioredoxin, followed by fluorescein isothiocyanate (FITC)-labeled anti-mouse immunoglobulin. Human or tamarin LEL bound efficiently to the cells expressing E2, as detected by both immunofluorescence

**FIG. 3.** Effect of single tamarin-specific amino acid substitutions in the human trx-LEL of CD81 on binding to secreted E2<sub>388–664</sub> (Abbott) as determined by ELISA.

**FIG. 4.** Binding of human, tamarin, African green monkey, or mouse recombinant trx-LEL to Huh-7 cells expressing E2 from the plasmid pE2surf-661, as determined by immunofluorescence microscopy. Left panel: Cells are stained before fixation so that E2 on the cell surface only is available for LEL binding. Right panel: Cells are stained after acetone fixation so that both intracellular and cell surface-displayed E2 are available for LEL binding.

<table>
<thead>
<tr>
<th>CD81 LEL</th>
<th>Secreted E2&lt;sub&gt;388–664&lt;/sub&gt; (Abbott)</th>
<th>Intracellular E2&lt;sub&gt;383–715&lt;/sub&gt; (Chiron)</th>
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<tbody>
<tr>
<td>Tamarin</td>
<td>$10^{-8}$</td>
<td>$10^{-6.5}$</td>
</tr>
<tr>
<td>Human</td>
<td>$10^{-6.5}$</td>
<td>$10^{-7.5}$</td>
</tr>
<tr>
<td>African green monkey</td>
<td>$&lt;10^{-8}$</td>
<td>$10^{-6}$</td>
</tr>
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* A 25% inhibition was obtained with $10^{-6}$ M soluble E2; a higher concentration was not tested.
microscopy (Fig. 4) and flow cytometry. Although nearly saturating concentrations of trx-LEL were used, tamarin LEL resulted in brighter fluorescence than did human LEL. By flow cytometry, cells incubated with mouse LEL were considered negative, whereas reaction with tamarin LEL yielded 35% positive cells with mean fluorescence intensity (FI) of 1202 compared to 28% positive cells with mean FI of 867 after incubation with human LEL. Incubation with African green monkey LEL resulted in 2.2% positive cells with mean FI of 218. Numbers refer to a single experiment, but similar results were obtained in a repeat experiment. Additionally, a few cells that had reacted with African green monkey LEL were identified by immunofluorescence microscopy (Fig. 4). Since it was reported that CD81 has higher affinity for intracellular E2 than for secreted E2 (Flint et al., 2000), we analyzed cells after fixation with acetone, so that intracellular E2 was also accessible. This resulted in increased fluorescence of the cells incubated with African green monkey LEL (Fig. 4). Negative control cells transfected with a plasmid expressing HCV E1 did not bind LEL of any of the four species tested (data not shown).

HCV E2 protein bound to CD81 on tamarin lymphoid cells

To verify that CD81 naturally expressed on cells gives results comparable to those obtained with recombinant LEL fusion proteins, we tested tamarin and African green monkey cell lines as well as tamarin peripheral blood mononuclear cells (PBMC) for E2 binding by flow cytometry, using published methods (Flint et al., 1999; Higginbottom et al., 2000; Rosa et al., 1996). Human PBMC and a mouse lymphoid cell line were used as positive and negative controls, respectively. The presence of CD81 on the cells was first confirmed by staining with anti-CD81 MAb JS64, although not to the same extent as for human cells using the same reagent concentrations. This was likely the result of the higher affinity of E2 for tamarin CD81, possibly combined with lower affinity of MAb JS64 for tamarin CD81. It cannot be excluded, however, that E2 binds not only to CD81 but also to additional molecules on these cells.

In addition, tamarin (S. mystax) PBMC were tested for binding of secreted E2<sub>388-664</sub> (Abbott). Results from two animals are shown in Figs. 6E and 6F. The average shift in fluorescence intensity was greater than that observed with human PBMC. The interaction was inhibited by anti-CD81 MAb JS64 (Fig. 6F) and tamarin, but not mouse, trx-LEL (data not shown).

Confirming the low-affinity interaction of African green monkey CD81 with E2 was technically more difficult, since the flow cytometry assay is less sensitive than is the ELISA or immunofluorescence assay utilizing trx-LEL. African green monkey-derived COS-7 cells were previously reported not to bind E2 (Flint et al., 1999; Higginbottom et al., 2000). In our studies, these cells displayed only minimal binding of intracellular or secreted E2 (data not shown). However, a cell line derived from primary African green monkey kidney cells (AGMK) (Potash et al., 1997) expressed a level of CD81 similar to that of COS-7 cells as determined by staining with MAb JS-81 (Fig. 5). This cell line showed weak but significant binding of intracellular E2<sub>383-715</sub> (Chiron) that was specific, since the flow cytometry assay is less sensitive than is the ELISA or immunofluorescence assay utilizing trx-LEL. African green monkey-derived COS-7 cells were previously reported not to bind E2 (Flint et al., 1999; Higginbottom et al., 2000). In our studies, these cells displayed only minimal binding of intracellular or secreted E2 (data not shown). However, a cell line derived from primary African green monkey kidney cells (AGMK) (Potash et al., 1997) expressed a level of CD81 similar to that of COS-7 cells as determined by staining with MAb JS-81 (Fig. 5). This cell line showed weak but significant binding of intracellular E2<sub>383-715</sub> (Chiron) that was specific, because binding was inhibited by anti-CD81 MAb JS64 or human, but not mouse, trx-LEL (Figs. 7A–7C). Minimal binding, if any, was seen with secreted E2<sub>388-664</sub> (Abbott), consistent with its lower affinity for CD81 (Fig. 7D).

**DISCUSSION**

Without a cell culture system it is difficult to confirm the role of the E2–CD81 interaction in virus entry into cells. The original finding that CD81 molecules of human or chimpanzee, but not of mouse, origin bind to E2 suggested that CD81 binding may determine species specificity of HCV infection (Pileri et al., 1998). African green monkey CD81, differing by only four amino acids from human CD81, was subsequently reported not to bind E2, which supported the concept that E2 binds only to CD81 of species susceptible to HCV (Flint et al., 1999; Higginbottom et al., 2000).

Our results are at variance with the concept that CD81 binding predicts susceptibility to HCV, since HCV E2 binds strongly to tamarin CD81 and tamarins are apparently not susceptible to HCV infection. A major concern about studies of this kind is how faithfully recombinant E2 protein represents E2 in the E1/E2 heterodimers of the virus particle. In fact, only one report so far has demonstrated that hepatitis C virions bind to CD81, and in that case recombinant trx-LEL, rather than native CD81, was tested (Pileri et al., 1998). We addressed this problem by using three different well-characterized preparations of E2, one secreted (Lesniewski et al., 1995), one intracellular (Petracca et al., 2000), and one displayed on the surface of cells (Forns et al., 2000). Although the affinity for CD81 differed among the E2 prep-
arations, they gave essentially the same result with regard to the species differences, i.e., E2 bound more efficiently to tamarin CD81 than to human CD81, and there was a low-affinity interaction with the African green monkey CD81.

The affinity data were collected to compare the binding affinities of CD81 from the different primates. The accuracy of the method in absolute numbers is moderate (Rath et al., 1988). However, data agreed well with previously reported affinity measurements of the E2–CD81 interaction. Petracca et al. (2000) calculated the affinity of intracellular E2_{383–715} (Chiron) for purified human LEL as $1.8 \times 10^{-8}$ M, noting that the affinity for human trx-LEL is five to 10 times lower. Rosa et al. (1996) estimated the binding affinity of secreted E2_{384–715} to human MOLT-4 cells as approximately $10^{-8}$ M. Flint et al. (2000) reported

![Graphs showing CD81 expression of various cells](image)
that intracellular E2 has higher affinity for CD81 than
does secreted E2, and suggested that variation in glyco-
sylation may account for this difference. The present
results are consistent with those of Flint et al.
(2000), since the intracellular E2 had higher affinity than se-
creted E2 for CD81 of all three primate species tested. It
should be noted, however, that the two purified E2 prep-
arations used in the present study were derived from
different HCV strains; thus, the sequence, as well as the
C-terminal truncation site, differed slightly (Lesniewski et
al., 1995; Petracca et al., 2000), which could also affect
the affinity for CD81.

Analysis of the substitutions distinguishing tamarin
CD81 from human CD81 showed that the T163S substi-
tution resulted in increased affinity for E2, whereas the
three other individual tamarin-specific substitutions did

FIG. 6. Binding of E2 to human, mouse, or tamarin cells measured by flow cytometry. Cells were incubated with secreted E2
(Abbott) [all except (D)] at 10 μg/ml followed by MAb HS3 and PE-anti-mouse IgG1 MAb (solid line), and compared with cells stained with the same antibodies but in
the absence of E2 (solid graph). Inhibition of E2 binding by preincubation of the cells with anti-CD81 MAb JS64 is indicated by dotted line. Graphs
(E) and (F) show PBMC from two different tamarins. (A) Human PBMC. (B) Mouse WEHI-231 cells. (C) Tamarin B95-8 cells. (D) Tamarin B95-8 cells
incubated with intracellular E2 (Chiron). (E) Tamarin PBMC (S. mystax 864, inhibition not tested). (F) Tamarin PBMC (S. mystax 863).
not enhance E2 binding. This is analogous to the finding that a T → A substitution present at the same position (163) in African green monkey CD81 enhances E2 binding (Higginbottom et al., 2000). Therefore, a combination involving two or more substitutions must be required to maximize the affinity of tamarin CD81 for E2.

The present demonstration that African green monkey CD81 binds E2 contrasts with previous reports, in which the absence of binding is described in great detail using both flow cytometry and ELISA (Flint et al., 1999; Higginbottom et al., 2000). The discrepancy may be explained by the fact that the previous studies were done with secreted E2 only, and that a less-sensitive ELISA assay was used. In our studies the ELISA assay was far more sensitive when wells were coated with E2 instead of the LEL fusion protein as published previously (Higginbottom et al., 2000). The flow cytometry assay is clearly less sensitive than ELISA for low-affinity interactions. By flow cytometry, we found that only intracellular E2383–715 (Chiron) but not secreted E2388–664 (Abbott) bound to African green monkey cells, which is in agreement with the previous reports.

Whether the HCV E2–CD81 interaction has any relevance for virus entry into cells is still an open question. The poor internalization of CD81 and binding of E2 to cells that do not express CD81 led Petracca et al. (2000) to suggest the presence of a coreceptor. Takikawa et al. (2000) used a cell-fusion assay and found that CD81 alone was not sufficient for fusion. Immunomodulatory effects of CD81 engagement were previously described (Flint et al., 1999) and this may well be the main function of the E2–CD81 interaction. The preferential binding of intracellular E2 to CD81 leads us to speculate that excess E2 is released from lysed cells and that this released E2 is more biologically relevant for CD81 binding than is E2 in virions. On the other hand, if CD81 actually is a receptor for HCV, the present data suggest that lack of attachment to CD81 is not the factor preventing HCV infection of tamarins and African green monkeys. The block to infection may appear at the level of coreceptor

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**FIG. 7** Binding of E2 to African green monkey kidney cells (AGMK) measured by flow cytometry. Cells were incubated with intracellular E2383–715 (Chiron) [all except (D)] at 10 μg/ml followed by MAb H53 and PE-anti-mouse IgG1 MAb (solid line), and compared with cells stained with the same antibodies in the absence of E2 (solid graph). Graphs (A)–(C) show that the interaction could be inhibited by anti-CD81 MAb JS64, and human but not mouse trx-LEL. (A) Preincubation of the cells with anti-CD81 MAB JS64 is indicated by dotted line. (B) Preincubation of the E2 protein with human trx-LEL (dotted line) (C) Preincubation of the E2 protein with mouse trx-LEL (dotted line). (D) Cells were incubated with secreted E2388–664 (Abbott).
or postentry events. In the latter case, construction of HCV/GB virus B chimeric viruses may provide a useful approach to the study of HCV in a small animal model.

Following the initial submission of this report, a study by Meola et al. (2000) was published, which also demonstrated that tamarin CD81 binds to HCV E2 with a higher affinity than does human CD81.

MATERIALS AND METHODS

HCV E2 preparations

Purified secreted E2 (a.a. 388–664) (Lesniewski et al., 1995) was kindly provided by Dr. I. K. Mushahwar (Abbott Laboratories, North Chicago, IL). Purified intracellular E2 (a.a. 383–715) (Petracca et al., 2000; Spaete et al., 1992) was kindly provided by Dr. M. Houghton (Chiron Corporation, Emeryville, CA). The E2 protein (a.a. 384–661) expressed on the surface of Huh-7 cells was described elsewhere (Forns et al., 2000). Cells were transfected with the plasmid pE2surf-661, encoding a.a. 384–661 of E2 cloned into pDisplay (Invitrogen, Carlsbad, CA), in frame between a signal sequence and the transmembrane domain of human platelet-derived growth factor receptor. All three E2 proteins were derived from HCV-H or HCV-1, which are related HCV genotype 1a strains, and thus differed slightly from each other in amino acid sequence.

Cloning, expression, and purification of CD81 LEL

The LEL of CD81 was expressed in fusion with thioredoxin, as previously described (Pileri et al., 1998). RNA was extracted from the following cells or tissues using Trizol (Gibco BRL, Gaithersburg, MD): human hepatoma cells (Huh-7), liver homogenate of a tamarin (S. oedipus), and COS-7 cells (African green monkey kidney). Mouse liver RNA was purchased from Stratagene (La Jolla, CA). Sequence encoding LEL was amplified by RT-PCR with the primers CD81-19S and CD81-693AS (Table 2). PCR products were sequenced directly. The nearly complete cDNA sequence of S. oedipus CD81 (nucleotides 40–672 of the open reading frame) was deposited in GenBank (Accession No. AF274885). For cloning, a second round of PCR was performed using primers For-hEC2 and Rev-hEC2 (Pileri et al., 1998) for amplification of DNA encoding tamarin and mouse LEL respectively. African green monkey LEL DNA was amplified using For-hEC2 and Rev-tEC2.

TABLE 2

<table>
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<tr>
<th>Primer</th>
<th>Sequence</th>
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<tr>
<td>CD81-19S</td>
<td>ACCAAGTGCATCAAGTACCTG</td>
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<tr>
<td>CD81-693AS</td>
<td>CCGGATGCCAACAGCAAGGCAC</td>
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<tr>
<td>For-tEC2</td>
<td>GGGCGGGTGATCCCGGGGGTGAGTCGAGTTGTCACAAGAGACC</td>
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<tr>
<td>Rev-tEC2</td>
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<td>For-mEC2</td>
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<tr>
<td>Rev-mEC2</td>
<td>CCCGAGCTTCACAGCTCCCAGGAAGAGCTCAGTG</td>
</tr>
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</table>

Note. For-tEC2, Rev-tEC2, For-mEC2, and Rev-mEC2 are modifications of the primers For-hEC2, and Rev-hEC2 (Pileri et al., 1998) for amplification of DNA encoding tamarin and mouse LEL respectively. African green monkey LEL DNA was amplified using For-hEC2 and Rev-tEC2.
was used for all proteins shown in Fig. 3. After induction as above, cells were spun down and dissolved in 20 ml ice-cold 20 mM Tris–HCl, pH 8, 2.5 mM EDTA, 20% sucrose; incubated for 10 min; pelleted; and dissolved in 20 ml ice-cold 20 mM Tris–HCl, pH 8, 2.5 mM EDTA, after which they were again incubated for 10 min and pelleted. The supernatant was filtered, precipitated with 30% ammonium sulfate, centrifuged at 16,000 g, and the protein pellet was dissolved in PBS.

Binding of CD81 LEL to HCV E2 analyzed by ELISA

Binding of recombinant trx-LEL to purified E2 was studied by ELISA, and the affinity of the interaction was estimated by inhibition with various concentrations of soluble E2 protein (Rath et al., 1988). A microtiter plate (Costar 3690; Costar, Cambridge, MA) was coated with purified recombinant E2[388–664] (Abbott Laboratories) or E2[383–715] (Chiron) at 2 μg/ml overnight at 4°C, and subsequently blocked with 5% nonfat dry milk for 1 h at room temperature. The different trx-LEL preparations were added at twofold dilutions and incubated for 2 h at room temperature. Bound trx-LEL was detected by incubating with a mouse MAAb to thioredoxin (Invitrogen) for 1 h at room temperature followed by alkaline phosphatase-conjugated anti-mouse IgG antiserum (Pierce, Rockford, IL) for 1 h at room temperature, and substrate for 30 min. For affinity determination, samples were tested at a dilution giving approximately 50% saturation in the above-mentioned ELISA. Diluted thioredoxin–LEL preparations with or without added soluble E2 (at 10-fold dilutions, maximum concentration 60 μg/ml) were added to the wells, and the ELISA was performed as described above. The reduction of optical density in the presence of soluble E2 and, subsequently, the concentration needed for a 50% reduction were calculated.

Binding of CD81 LEL to E2-expressing cells analyzed by immunofluorescence microscopy and flow cytometry

Huh-7 cells cultured on chamber slides or in six-well plates were transfected with the plasmid pE2surf-661 or pE1surf-347 (Forns et al., 2000) using Superfect Reagent (Qiagen, Valencia, CA). Plasmid pE2surf-661 encodes a.a. 384–661 of E2 cloned into pDisplay (Invitrogen). Plasmid pE1surf-347 encodes HCV envelope protein E1 (a.a. 192–347) in the same vector and was used as a negative control. Cells were analyzed 48 h after transfection. For surface staining, cells were washed with wash buffer (WB; PBS, 1% fetal calf serum, 0.05% sodium azide) and stained directly. For intracellular staining, cells were fixed and permeabilized with acetone before staining. Recombinant trx-LEL preparations of the different species (or only thioredoxin expressed from the same vector and purified in the same way) were added at 2 μg/ml. Slides were incubated for 1 h at room temperature, washed with WB, and incubated with a mouse MAAb to thioredoxin (Invitrogen) for 1 h, followed by a FITC-conjugated antibody to mouse IgG for 1 h. After washing, unfixed cells were fixed with acetone, mounted, and examined by fluorescence microscopy. For flow cytometry, stained, unfixed cells were scraped off the six-well plate and analyzed in a FACScan instrument (Becton-Dickinson, San Jose, CA). Data were processed using Cellquest software (Becton-Dickinson).

Binding of E2 to CD81-expressing cells analyzed by flow cytometry

The following cells were tested for E2 binding: tamarin cell line B95-8 (Epstein–Barr virus-transformed B lymphocytes of S. oedipus, ATCC CRL-1612; ATCC, Manassas, VA), mouse B-cell lymphoma line WEHI-231 (ATCC CRL-1702), African green monkey kidney cell line COS-7, African green monkey kidney cell line AGMK (Potash et al., 1997), and human and tamarin PBMC. Cells grown in suspension were harvested by centrifugation and washed once in WB before staining. COS-7 and AGMK cells were scraped off the flask to avoid trypsin, or stained attached to a six-well culture plate using the same reagent concentrations as above, and scrapped off the plate before flow cytometry analysis. Human and tamarin PBMC were isolated from 3 to 10 ml of fresh heparinized blood using Ficoll–Paque Plus (Amersham Pharmacia Biotech, Piscataway, NJ). For tamarin cells, additional lysis of remaining erythrocytes with ammonium chloride lysis buffer for 3 min was necessary. Detection of CD81 on the cells was done using standard flow cytometry techniques and the anti-human CD81 MAbs 1.3.3.22 (Santa Cruz Biotechnology) or JS-81 (Pharmingen, San Diego, CA), or hamster anti-mouse CD81 MAAb 2F7 (Pharmingen).

For the E2-binding assay, approximately 1 × 10⁶ cells were incubated for 1 h at room temperature with purified E2 [secreted E2[388–664] (Abbott) or intracellular E2[383–715] (Chiron) at 10 μg/ml in 100 μl WB]; negative control cells were incubated in WB only. Cells were washed twice by centrifugation in WB and incubated with anti-E2 monoclonal antibody H53 (Cocquerel et al., 1998) (provided by Dr. J. Dubuisson) at 5 μg/ml for 1 h at room temperature, followed by two washes and incubation with a phycoerythrin-labeled rat MAAb to mouse IgG1 (Becton Dickinson Immunochemistry Systems, San Jose, CA). The use of a monoclonal anti-IgG1 secondary antibody enabled confirmation of the E2–CD81 binding specificity by blocking with anti-CD81 MAAb JS64 (Coulter Cytometry, Miami, FL) that belongs to subclass IgG2a. Cells were finally washed twice and resuspended in wash buffer. The difference in fluorescence intensity between cells incubated with or without E2 was the readout, and this was previously shown to represent E2 binding (Flint et al., 1999; Rosa et al., 1996). Confirmation of E2 binding spec-
HCV E2 BINDS TO TAMARIN CD81


Acknowledgments

We thank Mr. David Stephany and Dr. Kevin Holmes, Flow Cytometry Unit, NIAID, National Institutes of Health, for technical support and helpful discussions. We also thank Drs. Issa K. Mushahwar and Michael Houghton for providing purified E2 protein, Dr. J. Dubuisson for providing monoclonal antibodies, and Dr. J. Cohen for providing the B95-8 cell line. This study was supported in part by National Institutes of Health Grant RO1-AI38368 and the Swedish Foundation for International Cooperation in Research and Higher Education (STINT).

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