The envelope glycoprotein E2 of hepatitis C virus (HCV) is the target of neutralizing antibodies and is presently being evaluated as an HCV vaccine candidate. HCV binds to human cells through the interaction of E2 with the tetraspanin CD81, a putative viral receptor component. We have analyzed four different E2 proteins from 1a and 1b viral isolates for their ability to bind to recombinant CD81 in vitro and to the native receptor displayed on the surface of Molt-4 cells. A substantial difference in binding efficiency between these E2 variants was observed, with proteins derived from 1b subtypes showing significantly lower binding than the 1a protein. To elucidate the mechanism of E2-CD81 interaction and to identify critical regions responsible for the different binding efficiencies of the E2 variants, several mutants were generated in E2 protein regions predicted by computer modeling to be exposed on the protein surface. Functional analysis of these E2 derivatives revealed that at least two distinct domains are responsible for interaction with CD81. A first segment centered around amino acid residues 613 to 618 is essential for recognition, while a second element including the two hypervariable regions (HVRs) modulates E2 receptor binding. Binding inhibition experiments with anti-HVR monoclonal antibodies confirmed this mapping and supported the hypothesis that a complex interplay between the two HVRs of E2 is responsible for modulating receptor binding, possibly through intramolecular interactions. Finally, E2 proteins from different isolates displayed a profile of binding to human hepatic cells different from that observed on Molt-4 cells or isolated recombinant CD81, indicating that additional factors are involved in viral recognition by target liver cells.
HVR1, rather than being a truly variable segment, might actually adopt one out of a range of closely related conformations that is compatible with recognition of the virus’ cellular receptor.

CD81 was recently reported to bind HCV through interaction with E2 and was hypothesized to act as a viral receptor component (32). Consistent with this view, high titers of antibodies neutralizing the binding of E2 to CD81 (NOB antibodies) have been shown to correlate with protection against HCV infection in chimpanzees (32). It was also reported that CD81 engagement by HCV may lead to autoimmune or immune evasion (6, 39, 41). CD81 is a member of the tetraspanin family of membrane proteins, characterized by four transmembrane domains, a short intracellular region, and two extracellular loops. This cell surface protein is widely expressed and frequently found in association with other membrane-exposed loops. This cell surface protein is widely expressed and frequently found in association with other membrane-exposed loops.

Production and characterization of E2 proteins. E2 plasmids were transfected into 293 cells by using a calcium phosphate transfection kit (catalog no. 2-463335; 5-3 Prime). At 48 h after transfection, cell supernatant and crude cell extract were prepared as described below. Supernatants were cleared by centrifugation at 3,000 × g for 30 min at 4°C. They were then concentrated 20 times with Centricon Plus-80 filters (catalog no. U652SL38; Amicon), supplemented with 10% glycerol, 25 mM HEPES, and 1°C. The supernatant was collected, filtered, and put over subconfluent HepG2 cells for 12 h. The medium was then replaced with fresh medium, and after 36 h cells were harvested and stained with fluorescence isothiocyanate (FITC)-conjugated anti-hCD81 MAb (Santa Cruz Biotechnology). The FITC-positive cells were analyzed and sorted with a FACS Vantage cell sorter (Becton Dickinson). The HepG2-R2 subclone was isolated by limiting dilution of hCD81-positive cells.

Materials and Methods

Construction of E2 plasmids. E2 proteins from genotypes 1a (strain H) and 1b (strains BK, N2, and J) were cloned in plasmid V1JnsTPA (8) as described subsequently found in association with other membrane-exposed loops. This cell surface protein is widely expressed and frequently found in association with other membrane-exposed loops.

Concentration, as were the HuH7 and HepG2 human hepatoma cell lines. The HepG2-R2 subclone was obtained by infection of HepG2 cells with a recombinant retrovirus containing the human CD81 (hCD81) gene. The hCD81 gene was amplified by PCR and directly cloned into the pLlB retroviral vector (Clontech), giving rise to plasmid pLlB-hCD81. To produce the recombinant retroviral particles, pLlB-hCD81 was transfected into the FT67 amphotropic packaging cell line (37). At 48 h after transfection, the supernatant was collected, filtered, and put over subconfluent HepG2 cells for 12 h. The medium was then replaced with fresh medium, and after 36 h cell supernatant was concentrated by centrifugation at 3,000 × g for 30 min at 4°C. They were then concentrated 20 times with Centricon Plus-80 filters (catalog no. U652SL38; Amicon), supplemented with 10% glycerol, 25 mM HEPES, and 1°C. The supernatant was collected, filtered, and put over subconfluent HepG2 cells for 12 h. The medium was then replaced with fresh medium, and after 36 h cells were harvested and stained with fluorescence isothiocyanate (FITC)-conjugated anti-hCD81 MAB (Santa Cruz Biotechnology). The FITC-positive cells were analyzed and sorted with a FACS Vantage cell sorter (Becton Dickinson). The HepG2-R2 subclone was isolated by limiting dilution of hCD81-positive cells.

Cell lines. The Molt-4 cell line (human T-cell leukemia) and the 293 (human embryonic kidney) cell line were obtained from the American Type Culture Collection, as were the HuH7 and HepG2 human hepatoma cell lines. The HepG2-R2 subclone was obtained by infection of HepG2 cells with a recombinant retrovirus containing the human CD81 (hCD81) gene. The hCD81 gene was amplified by PCR and directly cloned into the pLlB retroviral vector (Clontech), giving rise to plasmid pLlB-hCD81. To produce the recombinant retroviral particles, pLlB-hCD81 was transfected into the FT67 amphotropic packaging cell line (37). At 48 h after transfection, the supernatant was collected, filtered, and put over subconfluent HepG2 cells for 12 h. The medium was then replaced with fresh medium, and after 36 h cells were harvested and stained with fluorescence isothiocyanate (FITC)-conjugated anti-hCD81 MAB (Santa Cruz Biotechnology). The FITC-positive cells were analyzed and sorted with a FACS Vantage cell sorter (Becton Dickinson). The HepG2-R2 subclone was isolated by limiting dilution of hCD81-positive cells.

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Pull-down experiments. Cells were harvested, washed in PBS, allowed to bind for RT 1 h with E2 concentrated supernatant, washed twice with PBS, and
lysed in PBS–1% Triton in the presence of protease inhibitor cocktail (Boehringer Mannheim) for 20 min at 37°C. Lysates corresponding to 10⁶ cells were loaded onto a sodium dodecyl sulfate (SDS)–10% polyacrylamide gel to perform Western blotting for the detection of cell-bound E2. For detection of recombinant E2 protein of genotype 1a, the rat monoclonal antibody 6-1/a (13) was used diluted 1:50 in Tris-buffered saline–Triton X-100–5% nonfat dry milk, followed by incubation with anti-rat horseradish peroxidase (Dako) conjugate diluted 1:2,000. The chemiluminescent substrate Super Signal West Pico (Pierce) was used for detection, and immuno-reactive proteins were visualized by exposure on X-ray film (Kodak Biomax ML).

**Binding of E2 to recombinant GST-hCD81.** The hCD81-LEL was expressed as a fusion protein with the C terminus of glutathione S-transferase (GST-hCD81) and purified as previously described (27). The fusion protein was derived from a best-frame downstream of the TPA to enforce secretion of the mature and extend this observation, we generated expression plasmids encoding these and two additional E2 proteins of subtype 1b isolates (BK and J). The recombinant proteins were expressed as C-terminal truncations ending at position 661 for the H, BK, and J strains and at position 662 for the N2 strain variant, which contains an extra amino acid in HVR2 (Fig. 1). This choice was made on the basis of previous observations suggesting that this particular form of the E2 ectodomain has a higher tendency to fold in a correct conformation (28). In all cases the E2-coding sequence was cloned in frame downstream of the TPA to enforce secretion of the expression products, and a six-histidine tag was engineered at the C terminus to detect the various proteins with a single reagent (Fig. 2).

Expression of recombinant proteins in transiently transfected 293 cells was assayed by GNI capture ELISA of whole-cell extracts and crude cell culture supernatants. All four proteins displayed similar expression levels, and significant amounts of recombinant products were secreted in the medium (between 30 and 50% of the total expression product [data not shown]). H and N2 strain E2 recombinants from both intracellular and secreted fractions reacted with conformation-sensitive MAbs (H33 and 166, respectively) in ELISA, confirming that a detectable proportion of these expression products is folded (data not shown). Recognition by MAb H3 is of particular relevance, since this antibody is specific for a conformation- and time-dependent epitope on non-disulfide-bridged E2 in complex with E1, which is believed to represent native prebudding forms of the HCV envelope (7, 30). We could not perform similar experiments with the BK and J proteins, since both H33 and 166 MAbs are strain specific (7, 44).

Consistent with the above observations, a significant amount of monomeric protein was detected in both the intracellular and the secreted fractions of all four expression products by non-denaturing SDS-polyacrylamide gel electrophoresis (SDS-PAGE) (see Fig. 4A and data not shown). In general, the percentage of monomer in relation to the total amount of expression product was quite consistent between the four proteins and in different preparations of the same E2 variant (about 50% [data not shown]).

Only monomeric E2 is expected to fold in an active conformation and to bind CD81 (12). We confirmed this finding by pull-down experiments with CHO cells transfected with an expression vector encoding full-length hCD81. Mock-transfected CHO cells were used as negative control for this experiment. Soluble E2 secreted into the cell culture medium from transiently transfected 293 cells was incubated with the CD81-transfected CHO cells, and the bound material was then separated from the bulk supernatant by low-speed centrifugation and analyzed by nonreducing SDS-PAGE and Western blotting with the anti-E2 MAb 6/1a (12). As shown by the migra-
tion pattern of the recovered E2H, a significant enrichment of the monomeric form was obtained upon binding to native CD81 displayed on the surface of transfected cells, while no species reacting with the anti-E2 MAb was precipitated by mock-transfected cells (Fig. 3A, lanes 1, 5, and 6). We then performed pull-down experiments with Molt-4 cells, which display large amounts of CD81 expressed by the endogenous gene, and found essentially equivalent results (Fig. 3A, lane 2).

Detection of specific and saturable binding of E2 to Molt-4 cells by FACS was previously demonstrated (17, 35). We also determined the time and temperature dependence of this interaction. Binding of E2H to Molt4 cells reaches equilibrium after 40 min at RT. The kinetics of this interaction does not improve at 37°C, while E2H binds with a very slow kinetics at 4°C (Fig. 3C). To show that binding of E2 to Molt-4 cells is mediated by CD81, we performed competition assays with an anti-CD81 MAb (MAb 1.3.3.22). E2H recognition by Molt-4 cells was abolished by the anti-CD81 MAb in a dose-dependent manner but was not affected by an unrelated MAb (see Fig. 8B and data not shown). Finally, cell surface binding of E2H was efficiently competed out by an excess of purified GST-hCD81, with a 50% inhibitory concentration of about 1.5 μM (Fig. 3B). These results confirmed that Molt-4 cells specifically recognize E2 via CD81.

**CD81 binding of E2 is strain specific.** Based on the above observations, prior to testing the four E2 natural variants in CD81 binding assays, we determined the amount of monomeric protein present in either the intracellular or the secreted fraction of each expression product. This was done by running serial dilutions of all E2 preparations on nonreducing SDS-PAGE and by comparing the amount of monomeric forms after Western blotting and quantification by densitometric scanning of the autoradiographs. The E2H protein was used as a reference, and the normalized values of the other three proteins were expressed as relative monomer content. Only small variations in the contents of monomeric E2 were detected among different variants or in various preparations of the same protein (within a twofold difference) (Fig. 4A).

**FIG. 1.** Sequence alignment of the H, BK, N2, and J strain E2 ectodomain sequences used in the binding assays. The two HVRs (HVR1 and HVR2) are indicated.
probably due to the presence of modified glycans which reduce E2 recognition by CD81 (12). However, the relative binding potencies exhibited by the secreted forms of the four variants perfectly matched the reactivity pattern of the intracellular fractions (data not shown).

The four E2 variants were also tested for their ability to bind native CD81 displayed on the surface of Molt-4 cells by FACS analysis. Binding assays were performed by incubating serial dilutions of secreted E2 from expression products after normalizing them for the amount of monomeric species (Fig. 4B). In this case, E2N2 successfully bound Molt-4 cells more efficiently than the 1b variants (Fig. 4C and D). E2N2 displayed a relative binding efficiency on Molt-4 cells higher than that measured by ELISA on purified GST-hCD81 (a fourfold reduction compared to E2N2); however, the overall binding profile for the four E2 isolates was unchanged (E2N2 > E2N2 > E2N2 > E2N2). Thus, the hierarchy of binding efficiency to CD81 displayed by E2 proteins from different viral isolates does not depend on their glycosylation state or on the CD81 expression system and reflects instead an intrinsic property of the E2 proteins.

Deletion of HVR1 leads to increased CD81 binding of E2.

The different CD81 binding efficiencies displayed by the four E2 variants suggested that segments containing variations in amino acid sequence between the four strains should be responsible for the observed pattern. We therefore generated expression plasmids encoding HVR1 deletion mutants of E2H and E2N2 by directly fusing the TPA leader sequence to residue Ile411 (Fig. 2). The ability of pV1jnstpaHVR1E2 plasmids to drive expression of the encoded mutant proteins was assayed by transient transfection of 293 cells. GNI capture ELISA using whole-cell extract and culture medium from transfected cells indicated that significant amounts of the two mutants were expressed and secreted (reference 45 and data not shown). HVR1 deletion did not affect the aggregation state of the recombinant proteins, since similar amounts of monomeric species were detected for the complete ectodomain (wild type [wt]) and the mutant proteins by nonreducing SDS-PAGE and Western blot analysis. Furthermore, both ΔHVR1E2H and ΔHVR1E2N2 mutants were recognized by the conformation-sensitive MAb s H33 and 166, respectively, with an efficiency comparable to that observed for the wt proteins (data not shown). Taken together, these data indicate that the folding of HVR1 deletion recombinants is very similar to that of the wt ectodomain.

Cell culture supernatants from 293 transfected cells were again analyzed for the amount of monomeric products, and serial dilutions of ΔHVR1E2 mutant preparations were tested for binding to Molt-4 cells by FACS. Both mutants showed increased binding activity on Molt-4 cells with respect to the parental proteins (Fig. 5), which was higher for the HVR1 deletion protein from the N2 strain (threelfold) than for the corresponding H strain mutant (twofold). Similar results were obtained when binding experiments were performed with bacterially expressed GST-hCD81 (three- and twofold increases for the N2 and H mutants, respectively) (Fig. 5).

It is possible that HVR1 exerts a negative effect on E2-CD81 binding by masking the E2 region responsible for CD81 recognition. In agreement with this hypothesis, an anti-HVR1 MAb (MAb 9/27) (12) directed against the C-terminal half of the HVR (residues 395 to 408) was able to inhibit E2H binding to CD81 with a 50% inhibitory concentration of about 10 mM (data not shown).

The HVR1 and HVR2 regions of the N2 strain negatively modulate E2-CD81 interaction in a cooperative fashion. The role of the HVRs was further investigated by generating swapping mutants between the H and N2 isolates. No change in the efficiency of binding to GST-hCD81 or Molt-4 cells was observed by replacing only the HVR1 region of E2H with the corresponding sequence of the N2 strain (mutant HVR1N2E2H) (Fig. 6A). Similarly, replacement of only the E2H HVR2 region with that of the 1b isolate did not affect CD81 recognition (HVR2N2E2H mutant). However, a double E2H mutant containing both HVRs from the N2 isolate resulted in a fourfold reduction in CD81 binding (mutant HVR1N2HVR2N2E2H) (Fig. 6A).
A different phenotype was displayed by the inverse mutation: replacement of both the HVR1 and HVR2 regions of the N2 protein with the corresponding HVRs of the H variant did not inhibit CD81 binding but rather improved receptor recognition (mutant HVR1\(\text{HVR2}\)HVR2\(\text{HVR1}\)N2) (Fig. 6B). An even better binding was displayed when the HVR1 region was deleted from the latter derivative, consistent with the hypothesis of a generally negative effect mediated by the HVR1 region (mutant \(\text{HVR1}\)HVR2\(\text{HVR1}\)N2) (Fig. 6B).

Thus, it appears that in both the 1a and 1b proteins HVR1 negatively affects E2-CD81 interaction but that the concomitant presence of the two HVRs from the N2 isolate is required to achieve maximal inhibition.

Identification of an E2 sequence required for CD81 recognition. Our results are consistent with the hypothesis that the two HVRs can modulate access to a primary CD81 binding site. To identify this element, we took advantage of an E2 model recently generated by a combination of secondary-structure prediction and fold recognition methods (44). Based on this model, three protein segments were postulated to represent putative CD81 binding sites: (i) a region spanning residues 474 to 494 and including HVR2, (ii) the fragment between positions 522 and 551, and (iii) a short stretch of eight amino acids from residue 612 to 620. The last fragment appears to be spatially close to HVR2, and it is highly conserved across HCV isolates (data not shown). Therefore, we generated a substitution mutant with mutations of the six residues from position 613 to 618 (YRLWHY to SAASAS). The resulting protein variant (E2\(\text{mut613-618}\)) showed an expression profile and monomer content similar to those of the wt protein but was no longer able to bind hCD81 either as a recombinant GST fusion or on the surface of Molt-4 cells (Fig. 6A).

The profile of E2 binding to human hepatic cells is different from that observed on Molt-4 cells. Since HCV is a hepatotropic virus, we extended our studies to human cells of hepatic origin. Human hepatoma HuH7 cells display a highly differen-
tiated phenotype and are the only cell line shown to support replication of subgenomic HCV replicons to date (3). They also express CD81 and, therefore, were chosen for E2 binding assays. Pull-down experiments confirmed that only the monomeric fraction of E2 can bind to HuH7 cells (Fig. 3A, lane 3). The same E2 preparations from different isolates previously tested for their content in monomeric form and tested in a dose-response FACS-based experiment for binding to Molt 4 cells. ΔMFI represents the MFI with the background fluorescence subtracted. The data show the results of a representative experiment performed in triplicate. (C) FACS data, plotted as histograms of fluorescence intensity against relative cell number, for the binding on Molt-4 cells of a normalized dose of E2 monomer from each preparation of E2 variants. E2H (dotted line), E2N2 (thin line), E2BK (dashed line), and E2J (thick line) histograms are indicated by arrows; the grey histogram represents the fluorescence intensity measured with a supernatant from mock-transfected cells. (D) Binding values for the different E2 variants are reported as percentage of E2H reactivity calculated as described in Materials and Methods. Binding to the bacterially expressed CD81 (GST-hCD81) was measured by ELISA, and the background signal observed on GST carrier protein was subtracted. Average values from two replicates were determined. Binding to Molt-4 cells was measured by FACS. The background signal measured in control reactions using equal amounts of cell culture supernatant from mock-transfected cells was subtracted from the MFI for each reaction.

FIG. 4. E2 binding to CD81 is strain specific. (A) Western blot analysis of serial dilutions of the secreted E2 proteins expressed from plasmids encoding E2 from different viral isolates (E2H, E2BK, E2N2, and E2J). In nonreducing SDS-PAGE, E2 aggregates are the slow-migrating species, while the faster-migrating band corresponds to the monomer and is heterogeneous in size due to glycosylation. MW, molecular mass markers. (B) Secreted E2 proteins were normalized for their content in monomeric form and tested in a dose-response FACS-based experiment for binding to Molt 4 cells. MW, molecular mass markers.

CD81-transformed HepG2 cells and selected a population of cells with high levels of CD81 display by FACS. Several individual clones were isolated by limiting dilution and characterized for CD81 expression and E2 binding. A clone (HepG2-R2) with a CD81 surface display comparable to that measured on Molt-4 cells was chosen for further studies (Fig. 7C). Once again, only monomeric E2 was able to bind HepG2-R2 cells in pull-down experiments (Fig. 3A, lane 4). The amount of E2 recovered after binding to HepG2-R2 cells was higher than that observed with Molt-4 or HuH7 cells. This may reflect an intrinsically higher binding efficiency of HepG2-R2 due to E2 recognition of another E2 receptor present on HepG2 cells (the scavenger receptor type B class I) which is able to interact with soluble E2 in a CD81-independent manner (Fig. 7D) (36).
the E2H mut613-618 derivative to HepG2-R2 cells (Fig. 8A). abolished CD81 binding, did not completely impair binding of hepatic cells, since mutation of residues 613 to 618, which involved in CD81 recognition must contribute to the binding to sequence variability.

Furthermore, E2 interaction surface distinct from that involved in CD81 recognition must contribute to the binding to hepatic cells, since mutation of residues 613 to 618, which abolished CD81 binding, did not completely impair binding of the E2H mut613-618 derivative to HepG2-R2 cells (Fig. 8A). Consistently with this hypothesis, recognition of E2H with Molt-4 cells (MAb 1.3.3.22) (Fig. 8A). To prove that such CD81-independent binding is not a specific property of the mutant protein but also contributes to the recognition of wt E2H by hepatic cells, we performed binding inhibition experiments with the anti-CD81 MAB 1.3.3.22 on HepG2-R2 cells. These results confirmed that HepG2-R2 can recognize E2H via at least two independent molecules, since only 40% of the binding was eliminated by preincubating cells with saturating amounts of MAb 1.3.3.22, while no change was observed with an unrelated isotypic MAB as a negative control (Fig. 8B and data not shown). In contrast, E2 recognition by Molt-4 cells was completely abolished by using the same amount of MAb 1.3.3.22 (Fig. 8B).

**DISCUSSION**

A major stumbling block in understanding the HCV infection mechanism is the lack of an efficient cell culture system to study viral attachment and entry. An alternative approach toward this end is the identification and characterization of interactions between viral and host cell components. The recent discovery that the human cell surface CD81 molecule is a putative HCV receptor component and devising of in vitro and ex vivo assays to measure interaction between CD81 and recombinant forms of the HCV E2 glycoprotein constitute a step forward in resolving this issue. Information on the E2 determinants responsible for CD81 interaction is of paramount importance in deciphering the rules that regulate viral attachment to target cells and elaborating effective strategies for prevention and therapy. As a matter of fact, antibodies capable of blocking HCV E2 interaction with CD81 (NOB antibodies) are elicited during natural or experimental infection as well as by vaccination with a recombinant E1/E2 complex (18, 32). However, NOB antibodies are difficult to induce, and the only immunogens that have been shown to elicit such a response are recombinant E1/E2 complexes produced in mammalian cells or plasmid DNA encoding E2 (14, 32). Production of pure, correctly folded E1/E2 heterodimers in sufficient amounts to immunize large numbers of individuals presents a difficult task, and while DNA vaccination is effective in small mammals, it resulted in significant paucity of immunization in larger animals or humans. Thus, the design of novel immunogens able to induce a neutralizing response is highly desirable but requires additional knowledge on the structural and functional properties of the HCV envelope proteins and the specific sites for interaction with CD81 or novel receptor components. This situation is rendered even more complicated by the fact that HCV is not a single virus but is a complex mixture of variants with significant sequence heterogeneity in protein regions deemed important for virus infection.

We previously approached the problem of identifying putative E2 regions for CD81 interaction by generating a theoretical model of its tertiary structure (44). This exercise led to three distinct protein fragments being identified as potentially important for interaction with CD81. In the present work we have generated a set of E2 substitution mutants with mutations in two of these regions and tested them for their ability to recognize isolated hCD81 as a recombinant GST fusion expressed in E. coli or in its native form as displayed on the surface of Molt-4 cells.

For our binding assays, we chose to express E2 as C-terminal truncations in mammalian cells, since soluble and correctly folded E2 can be obtained by deleting the transmembrane
HVR1 HHVR2 HE2N2 and common 1a and 1b subtypes (strains H, BK, N2, and J) and we chose four different HCV isolates belonging to the most proteins, to increase the general relevance of our observations, preparations normalized for their content in monomeric form. Therefore, to allow for quantitative measurements of binding, pull-down experiments using paired CHO cell lines with or confirmed this observation by reported that the only form capable of binding to CD81 is folded E2 (12, 28). We confirmed this observation by pull-down experiments using paired CHO cell lines with or without hCD81, Molt-4 cells, or hepatic cells and subsequent analysis of the cell-bound fraction by nonreducing SDS-PAGE. Therefore, to allow for quantitative measurements of binding, all assays throughout this work were performed on protein preparations normalized for their content in monomeric form.

Since the E2 glycoprotein is one of the most variable HCV proteins, to increase the general relevance of our observations, we chose four different HCV isolates belonging to the most common 1a and 1b subtypes (strains H, BK, N2, and J) and examined their ability to interact with CD81. In line with our earlier observations (44), all recombinant E2 proteins from the latter three isolates were markedly less efficient than the H strain E2 in binding to bacterially expressed CD81 and to the native protein displayed on Molt-4 cells. This finding led us to hypothesize that protein fragments corresponding to less conserved E2 segments might contribute to CD81 recognition and could be responsible for the different binding levels of the four E2 variants. We focused our attention on the E2 HVRs for three reasons. First, they display the most variable sequences in the whole protein. Second, MAbs against either of the two HVRs can inhibit E2 binding to CD81, and virus recognition by Molt-4 cells is affected by anti-HVR1 MAbs (25, 35, 44). Finally, HVR2 lies in a protein segment predicted to be exposed on the surface of the virus (44).

We discovered that HVR1 deletion mutants from both the H and N2 strains increased binding efficiency. This finding together with the capability of anti-HVR1 MAbs to inhibit CD81 recognition suggested that HVR1 is not directly involved in receptor interaction but can negatively affect binding, either by steric hindrance or by blocking E2 in an unfavorable conformation. Replacement of both E2H HVRs with the corresponding sequences from the E2N2 variant generated a poor CD81 binder, whose efficiency of recognition by Molt-4 cells was comparable to that of the E2N2 protein. However, neither of the two E2N2 HVRs was able by itself to confer this phenotype. Thus, it appears that, at least in our experimental system, a particular combination of HVR1 and HVR2, such as that of the N2 strain, is required to achieve maximum inhibition. In line with this observation, in the E2N2 protein a double substitution mutant with the HVRs of the H strain protein led to an increase in binding. It should be noted that also in this chimeric protein, deletion of HVR1 improved binding, further confirming this protein region as a negative modulator.

One hypothesis to explain these results is that intramolecular interactions between the two HVRs occurs, and due to an intrinsic flexibility, the E2 protein shifts from an open to a closed conformation, with the former being more competent for CD81 recognition. Like many other viral envelope proteins, E2 also probably undergoes conformational changes during cell entry, envelope assembly, and disassembly. These changes could be blocked either by antibody binding or by intramolecular interaction between the two HVRs. According to this hypothesis, recent data indicate that the conformation of E2 changes upon binding to CD81, irrespective of the molecular context: as a soluble ectodomain, as a full-length E1/E2 complex, or as virus-like particles (16, 29). Furthermore, binding of nonneutralizing Fab to E2 can cause conformational changes that result in reduced susceptibility to Fab-mediated NOB activity (4).

According to the previously reported E2 model, the HVR2 is located close to aa 613 to 618 (44), one of the regions proposed to be involved in the interaction with CD81. Replacement of this segment with an unrelated sequence (E2H mut613-618) led to a complete loss of CD81 interaction, while nonetheless maintaining the capacity to bind to HepG2-R2 cells. Within a second E2 region predicted to contribute to CD81 recognition (aa 522 to 551) (44), the protein segment from residue 524 to 531 has been identified as another potential site of receptor interaction (29). This study also detected a
further element, residues 412 to 423, where antibody binding inhibits E2-CD81 interaction (29). The E2 protein was modeled on the structure of tick-borne encephalitis virus E envelope protein (34) on the basis of the combined results of secondary-structure prediction and threading and mapping methods (44). This model was subsequently indirectly validated by elucidating the structure of the E1 fusion glycoprotein of Semliki Forest virus (SFV) (23), since that structure was found to be remarkably similar to that of the tick-borne encephalitis virus E protein. In the HCV E2 model, HVR1 and the region spanning residues 522 to 551 are located on the opposite side of an elongated molecule with respect to the HVR2 and the region from aa 613 to 618. They could form a...
The mode of interaction of E2 with human hepatoma cells must be significantly different from that occurring on Molt-4 cells, since the profile of binding of E2 from the H, BK, N2, and J strains to these cell lines is remarkably different. This is unlikely to be due to differential glycosylation. In fact, hCD81 has only one potential Asn glycosylation motif; however, it resides at the very end of the intracellular C-terminal end of the protein outside of the LEL domain. Since no alternative CD81 isoform has been described to date, we do not believe that the different patterns of recognition for Molt-4 and liver-derived cell lines would result from variations in the coding sequence.

A more plausible explanation for this phenomenon could be the presence on the surface of hepatic cells of another molecule, distinct from CD81, which is capable of interacting with HCV E2. In line with this premise, binding of E2H to HepG2-R2 cells was reduced, but not completely eliminated, by a blocking anti-CD81 MAb, while binding inhibition experiments with the same MAb on Molt-4 confirmed that E2 recognition by these cells is solely due to the surface-displayed tetraspanin. Additional evidence supporting the existence of a putative HCV coreceptor is provided by the E2H mut613-618 derivative, which is unable to bind Molt-4 cells or isolated recombinant CD81 but still interacts with hepatoma cells, albeit less efficiently than the wt protein. The ability of this mutant to interact with HepG2-R2 cells was not affected by the blocking anti-CD81 MAb, confirming that E2 elements located outside the CD81 interaction surface contribute to recognition of hepatic cells, which appears to be, at least in part, CD81 independent. Finally, soluble E2 bound to HepG2 cells in spite of the fact that these cells do not display detectable amounts of CD81. During revision of this paper we were able to identify the scavenger receptor type B class I as the molecule displayed on the surface of hepatic cell lines which is able to interact with soluble E2 independently from CD81 (36). Thus, it appears that in HepG2-R2 cells as well as in liver cells that express both CD81 and scavenger receptor type B class I, binding of E2 is not restricted to a particular variant, while differential binding of E2 variants mediated by CD81 is apparently a lymphocyte-specific phenomenon and hence may have little relevance to the process of liver cell infection. However, the engagement of CD81 by E2 on the surface of T or NK cells has been recently shown to provide different signals which potentially lead to autoimmunity or immune evasion, respectively (6, 39, 41). Thus, even if no direct correlation between E2-CD81 binding efficiency and the outcome of the disease is presently available, our findings provide important information for a better understanding of the role played by HCV attachment to nonhepatic cells in the modulation of innate or adaptive immune responses.

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