Mapping of a Conformational Epitope Shared between E1 and E2 on the Serum-derived Human Hepatitis C Virus Envelope*

Monoclonal antibody D32.10 produced by immunizing mice with a hepatitis C virus (HCV)-enriched pellet obtained from plasmapheresis of a chronically HCV-infected patient binds HCV particles derived from serum of different HCV1a- and HCV1b-infected patients. Moreover, this monoclonal has been shown to recognize both HCV envelope proteins E1 and E2. In an attempt to provide novel insight into the membrane topology of HCV envelope glycoproteins E1 and E2, we localized the epitope recognized by D32.10 on the E1 and/or E2 sequence using Ph.D.-12\textsuperscript{TM} phage display peptide library technology. Mimotopes selected from the phage display dodecapeptide library by D32.10 shared partial similarities with \textsuperscript{H}RHWTQGNC\textsuperscript{C} of the HCV E1 glycoprotein and with both 61\textsuperscript{Y}RLWHYPC\textsuperscript{C}1\textsuperscript{E} and 480PDQRPY-CWHYPKPC\textsuperscript{C} of the HCV E2 glycoprotein. Immunoreactivity of D32.10 with overlapping peptides corresponding to these three HCV regions confirmed these localizations and suggested that the three regions identified are likely closely juxtaposed on the surface of serum-derived particles as predicted by the secondary model structure of HCV E2 derived from the tick-borne encephalitis virus E protein. This assertion was supported by the detection of specific antibodies directed against these three E1E2 regions in sera from HCV-infected patients.

Infection with hepatitis C virus (HCV)\textsuperscript{1} represents an important public health problem worldwide because it is a major cause of chronic hepatitis, cirrhosis, and hepatocellular carcinoma (1). HCV is an enveloped positive single-stranded RNA virus that is a member of the Flaviviridae family (2). It is now classified within a third genus, designated Hepacivirus (3). Its genomic organization consists of one large translational open reading frame encoding a polyprotein of ∼3000 amino acids, which is bracketed by 5'- and 3'-noncoding regions (2). The HCV polyprotein is processed into functional proteins by host and viral proteases (4). The three structural proteins, viz. core and envelope glycoproteins E1 and E2, are located within the N terminus of the polyprotein, whereas the nonstructural proteins reside within the C-terminal part (5). Glycoproteins E1 and E2 are believed to be type 1 integral transmembrane proteins, with C-terminal hydrophobic anchor domains. Until now, structure-function analyses of HCV gene products have been carried out using artificial cell expression systems in which E1 and E2 form two kinds of complexes in vitro (6–9): high molecular mass aggregates that contain intermolecular covalent bonds and native complexes in which E1 and E2 associate by noncovalent interactions. The contribution of either complex to the structure of the proteins on viral particles is unknown; however, the latest complexes are thought to correspond to the native association of E1 and E2. The lack of an efficient tissue culture system for propagating the virus and low levels of HCV particles in plasma samples are the main factors responsible for slow progress in this research area.

In an attempt to obtain information on the exact nature of the interaction between E1 and E2, we recently produced murine monoclonal antibodies directed against E1E2 complexes expressed on native HCV particles isolated from serum of chronically infected patients.\textsuperscript{2} In this study, the new monoclonal antibody (mAb) D32.10, which specifically recognizes serum-derived HCV particles and both HCV glycoproteins E1 and E2, was obtained. The fine epitope localization of mAb D32.10 was established using phage display peptide library technology. Intriguingly, its epitope is located in the E1E2 association site (WHY), encompassing CD81-binding region-1, as depicted on the predicted HCV E1E2 heterodimeric model proposed by Yagnik et al. (10) on the basis of tick-borne encephalitis virus. More exciting, such E1 and E2 sequences, which are recognized by our mAb D32.10, are also reactive with specific antibodies present in human sera from HCV-infected patients.

EXPERIMENTAL PROCEDURES

Antibody Production and Characterization—mAb D32.10 was generated as previously described.\textsuperscript{3} Briefly, viral material was obtained in large amounts from plasmapheresis of a chronically HCV-infected patient (HCV-L, genotype 1b).

An HCV-enriched pellet was prepared by successive ultracentrifugations. The final pellet (concentrated 240-fold) contained ∼10\textsuperscript{10} copies of HCV RNA/mg of protein as determined by Amplicor\textsuperscript{TM} HCV Monitor\textsuperscript{TM} (Roche Diagnostics, Meylan, France). BALB/c mice were inoculated with 100 µg, i.e. 10\textsuperscript{6} copies of HCV RNA (two injections), of this viral preparation and boosted with 50 µg (two injections) before fusion with X63 myeloma cells (11). Hybridoma culture supernatants were screened for the presence of HCV-specific antibodies by indirect enzyme immunoassay (ELISA) and for HCV polypeptide specificity by immunoblotting using the immunogen as an antigenic probe (12).

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\textsuperscript{2}Petit, M.-A., Jolivet-Reynaud, C., Peronnet, E., Michal, Y., Dubuisson, J., and Trepo, C., 11th International Symposium on Viral Hepatitis and Liver Disease Proceedings, April 6–9, 2003, Sydney, Australia.

\textsuperscript{3}The abbreviations used are: HCV, hepatitis C virus; mAb, monoclonal antibody; EIA, enzyme immunoassay; BSA, bovine serum albumin; ELISA, enzyme linked-immunoassay assay; Endo H, endoglycosidase H; TBS, Tris-buffered saline; PBS, phosphate-buffered saline.
reactivity recognized by mAb D32.10 was also analyzed by ELISA of each fraction recovered after centrifugation in a sucrose density gradient of the HCV-enriched pellet.

**HCV Indirect EIA**—96-Well polystyrene plates (Falcon from BD Biosciences, Le Pont de Claix, France) were coated with four different HCV preparations (1 mg/ml protein) diluted from 10⁻¹ to 10⁻⁸ (corresponding to 100 μg/ml to 1 ng/ml). The plates were incubated overnight at 4 °C and then saturated with 20 μl Tris-HCl (pH 7.5) and 100 mM NaCl containing 5% (v/v) bovine serum albumin (BSA) (Tris/NaCl/BSA buffer). mAb D32.10 diluted in a mixture of Tris/NaCl/BSA buffer and 50% normal human serum at a concentration of 5 μg/ml was added to each well and incubated for 2 h at 37 °C. The bound antibody was detected with a horseradish peroxidase-conjugated Fab(′)₂ fragment of anti-mouse IgG antibodies (diluted 1:1000; Dako Corp.) as the secondary antibody. Protein bands were visualized by enhanced chemiluminescence (ECL® system, Amersham Biosciences). Glycosidase digestion was performed as previously described by Sato et al. (14) on circulating HCV virions. The HCV-enriched pellet (HCV-L, 4 μg) was treated with 5, 10, or 20 milliunits/ml peptide N-glycosidase A (Roche Applied Science) in 100 mM citrate/phosphate buffer (pH 6.0) for 18 h at 37 °C. Deglycosylation of purified HCV particles was also performed by overnight incubation at 37 °C in 50 mM sodium acetate buffer (pH 5.5) containing endoglycosidase H (Endo H, 5 milliunits/ml; Roche Applied Science), 20 mM dithiothreitol, and 0.1% Triton X-100. The experiment was performed under the same conditions as described for the peptide N-glycosidase A or Endo H digestion, except the enzyme was omitted. Samples were then treated with electrophoresis sample buffer containing reducing agent and analyzed by SDS-PAGE.

**Dodecapeptide Library Screening**—The Ph.D.-12™ phage display peptide library kit was obtained from New England Biolabs, Inc. This is a combinatorial peptide 12-mer fused to the minor coat protein (pIII) of M13 phage. The displayed peptide 12-mers are expressed at the N terminus of pIII. The library consists of ~1.9 × 10¹⁰ electrooptered sequences, amplified once to yield ~20 copies of each sequence in 10 μl of the supplied phage. Three biopannings were performed according to the manufacturer’s recommendations, with an overnight culture of *E. coli* ER2537 (17) with an Applied Biosystems DNA sequencer Version 4.5 software (Eastman Kodak Co.). Basically, the regions of interest were considered as positive when superior to the cutoff, corresponding to the mean of negative controls multiplied by 2.1.

**Western Immunoblot Experiments**—The untreated HCV-enriched pellet (HCV-L) was used as an antigenic probe (12, 13) at concentrations from 0.1 to 1 mg/ml. The antigen was subjected to SDS-PAGE on 12.5% gels under reducing or nonreducing conditions (2% SDS ≤ 5% β-mercaptoethanol). After protein transfer onto polyvinylidene difluoride membranes, immunoblotting was performed using mAb D32.10 (2-5 μg/ml, diluted in 50% normal human serum) as the primary antibody, IgG bound was then detected by incubation with a peroxidase-conjugated Fab(′)₂ fragment of anti-mouse immunoglobulins (diluted 1:10,000; Dako Corp.) as the secondary antibody. Protein bands were visualized by enhanced chemiluminescence (ECL® system, Amersham Biosciences). Glycosidase digestion was performed as previously described by Sato et al. (14) on circulating HCV virions. The HCV-enriched pellet (HCV-L, 4 μg) was treated with 5, 10, or 20 milliunits/ml peptide N-glycosidase A (Roche Applied Science) in 100 mM citrate/phosphate buffer (pH 6.0) for 18 h at 37 °C. Deglycosylation of purified HCV particles was also performed by overnight incubation at 37 °C in 50 mM sodium acetate buffer (pH 5.5) containing endoglycosidase H (Endo H, 5 milliunits/ml; Roche Applied Science), 20 mM dithiothreitol, and 0.1% Triton X-100. The amino acid sequences of peptides were compared with the HCV E1 and E2 protein sequences using MacVector 6.0 (Oxford Molecular). Tentative searches for best local identities (18) were carried out using the supplied phage. Three biopannings were performed according to the manufacturer’s recommendations, with an overnight culture of *E. coli* ER2537 (17) with an Applied Biosystems DNA sequencer Version 4.5 software (Eastman Kodak Co.). Basically, the regions of highest similarity were detected with the LFASTA program, which tentatively searches for best local identities (18).

**Peptide Synthesis on N-terminal Sequencing**—The simultaneous synthesis of different peptide sequences was performed on a dodecapeptide library using *P. fluorescens* (N-[9-fluorenylmethoxycarbonyl) amino acid chemistry (19). Each peptide was generated in nanomolar quantities suitable for immunological detection. Antibody reactivity with membrane-bound peptides was analyzed by an indirect colorimetric immunosay as described previously (20). Spots corresponding to peptides with antibody reactivity produced a positive blue signal, which was quantitated by visualization and expressed as relative intensity on a scale ranging from 0 to 5.

**ELISA with Synthetic Peptides and Human Sera**—Wells were coated overnight at 4 °C with 100 μl of streptavidin at a final concentration of 10 μg/ml in 0.1 M carbonate buffer (pH 9.6) and blocked for 1 h at 37 °C with phosphate-buffered saline (PBS) containing 10% goat serum. The plates were washed again with PBS/Tween, 100 μl of serum diluted 1:50 in PBS/Tween containing 10% goat serum were added and incubated for 2 h at 37 °C. The plates were washed again with PBS/Tween, the peroxidase-conjugated goat anti-human IgG (Jackson ImmunoResearch Laboratories, Inc.) at a final concentration of 1:1000 was added and incubated overnight at 4 °C. The plates were washed in PBS/Tween and incubated with TBS/Tween. The amino acid sequences of peptides were compared with the HCV E1 and E2 protein sequences using MacVector 6.0 (Oxford Molecular). Tentative searches for best local identities (18) were carried out using the supplied phage. Three biopannings were performed according to the manufacturer’s recommendations, with an overnight culture of *E. coli* ER2537 (17) with an Applied Biosystems DNA sequencer Version 4.5 software (Eastman Kodak Co.). Basically, the regions of highest similarity were detected with the LFASTA program, which tentatively searches for best local identities (18).

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**RESULTS**

mAb D32.10 Recognizes Native Serum-derived HCV1a and HCV1b Particles—Four different HCV RNA-positive enriched pellet preparations (HCV1, HCV2, HCV3, and HCV4) were obtained as previously described (2) from three different patients (L., F., and Fan.) and tested by EIA for their recognition by mAb D32.10. As shown in Fig. 1, mAb D32.10 detected up to 10 ng of protein/ml of each preparation. The detection was linear between 10 μg and 100 ng or 1 μg and 10 ng/ml protein. HCV1 (the immunogen, genotype 1b) and HCV2 were two preparations derived from the same patient (L.). HCV3 (F.) and HCV4 (Fan.) were obtained from two other patients with severe cutaneous vasculitis, cryoglobulinemia, and chronic hepatitis C requiring plasma exchanges. The patient Fan. (HCV4) was initially found to have two distinct genotypes in serum, 1a and 2a (21). Our results show that mAb D32.10. is able to recognize determinants not restricted to genotype 1b of the immunogen.

mAb D32.10 Reacts with Large E1E2 Complexes and E1 and
related products were not identified after the treatment. The sensitivity of E1E2 complexes expressed on natural HCV particles to Endo H digestion was investigated. As shown in Fig. 2C, only a diffuse shift in molecular mass was observed for both E2 (from 68 to 42 kDa) and E1 (from 34 to 24 kDa) proteins, suggesting that E1 and E2 on serum-derived native HCV particles possess mainly a complex Endo H-resistant glycosylation.

**Epitope Mapping of mAb D32.10 Using Phage Display**—To further characterize the epitope recognized by mAb D32.10, the antibody was used to screen a phage display dodecapeptide library. After three rounds of selection, 4% the phage input was found in the eluate, indicating amplification of specifically bound phage. Thus, 88 clones were randomly isolated; their DNAs were sequenced; and the amino acid sequences of inserts were deduced. Forty-eight different sequences were obtained, and some of them were found in several examples. However, when tested in an ELISA for their immunoreactivity with mAb D32.10, none of them gave a positive signal, indicating that the binding affinity was too low to be detectable. The 48 clone sequences were compared with the sequences of HCV E1 and E2. Five and three sequences presented similarities to residues of E1 located in regions 292–305 and 347–356, respectively (Table I) whereas seven, four, and two sequences shared some similarities with residues of E2 located in regions 481–501, 610–631, and 685–698, respectively (Table II). Moreover, as shown in Table III, three of these different motifs shared similarities with both E1-(292–305) and E2-(482–499) or E2-(612–626), and two of them shared similarities with both E1-(347–356) and E2-(482–499) or E2-(612–626).

**Immunoreactivity of Selected Phage-displayed E1 and E2 Sequences with mAb D32.10**—To evaluate the significance of these different localizations on both E1 and E2 sequences, regions 291–315 and 347–356 of E1 and regions 473–498, 607–627, and 686–697 of E2 were reproduced by the Spotscan approach as overlapping synthetic pentadecapeptides offset by one and tested for their immunoreactivity with mAb D32.10. A strong positive signal was obtained with peptides corresponding to E1-(292–306) (Table IV, part A), whereas E1-(347–356) was not recognized by mAb D32.10 (data not shown). As shown in Tables V (part A) and VI (part A), peptides corresponding to E2-(482–499) and E2-(612–626), respectively, were also immunoreactive with mAb D32.10, and no signal was detected with E2-(686–697) (data not shown). The immunoreactive E1 and E2 regions were reproduced as overlapping octapeptides. Indeed, the immunoreactivity of E1-(292–306) (pentadecapeptide TFSPRRHWTTGQCN) could by restricted to 297RHWT–501, whereas E2-(482–499) or E2-(612–626) (pentadecapeptide LVDYPYRLWHYPCT) to 613YRLWHYPCT–921 (Table VI, part B). However, as shown in Table V (part B), overlapping octapeptides corresponding to E2-(480–494) (pentadecapeptide PDQRPYCWHPKPC) gave two non-overlapping zones of weaker immunoreactivity: 575PDQRPY686 and 485WHYPKPC, indicating that the recognition of each octapeptide by mAb D32.10 is partial. Indeed, it is difficult to discriminate between E2-(480–494) and E2-(613–621) because these two regions contain the same motif (WHYP) reported by Yagnik et al. (10) to be involved in the heterodimerization of E1E2.

**Determination of Critical Residues of the mAb D32.10 Epitope by Alanine Replacement Analysis**—The contribution to antibody binding of the conserved residues between the phage-displayed peptides and the E1 and E2 proteins was assessed by preparing a series of alanine analogs of sequences 292–306, 480–494, and 608–622. A residue was defined as critical to binding if its replacement with alanine induced a decrease of at least 50% in the signal of the corresponding peptide compared.

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**Fig. 1.** mAb D32.10 reactivity with HCV particles in EIA. The binding of mAb D32.10 to HCV particles of genotype 1b (HCV1, HCV2, or HCV3) or 1a (HCV4) purified from three different chronically infected patients was measured by indirect EIA. Plates were coated with the individual HCV-enriched pellets used at a concentration of 1 mg/ml and diluted to 10−1 (100 μg/ml) to 10−6 (1 ng/ml). Purified mAb D32.10 IgGs were used at a concentration of 5 μg/ml and diluted in Tris/NaCl/BSA containing 50% normal human serum. The antibody bound to antigen-coated wells was then reacted with horseradish peroxidase-conjugated anti-mouse immunoglobulins as the secondary antibody; plates were revealed using o-phenylenediamine and H2O2; and the absorbance was read at 450 nm. The cutoff value corresponds to the mean of three negative controls multiplied by 2.1.

**E2 Glycoproteins**—Using the immunogen (HCV1) as an antigenic probe, the HCV polypeptide specificity of mAb D32.10 was tested by immunoblot analysis under reducing and nonreducing conditions (Fig. 2A). When two concentrations of the same sample (2.5 and 5 μg) (lanes 2 and 3, respectively) were analyzed under reducing conditions (2% SDS + 5% β-mercaptoethanol), mAb D32.10 recognized major bands at 60–68 kDa and another band at 31 kDa, corresponding to E2 and E1, respectively. mAb D32.10 also recognized disulfide-linked complexes recovered in the upper part of the gel (>200 kDa) when the HCV1 preparation was treated with 2% SDS only. These high molecular mass bands (lane 1) could correspond to hetero-oligomeric E1E2 complexes.2

Asparagine-linked complex-type sugar chains have been shown to be present on the surface of native virions of HCV (14); thus, the ability of mAb D32.10 to recognize HCV-specific proteins after treatment of the HCV1 preparation with peptide N-glycosidase A at different concentrations (20, 10, and 5 milliunits/ml) was examined. As shown in Fig. 2B (lanes 1–3, respectively), mAb D32.10 reacted with all of the deglycosylation products of E1 (28, 25, 23, and 21 kDa), but especially with the 25-kDa (twice glycosylated) and 21-kDa (non-glycosylated) species, which accumulated at the highest concentration of the enzyme. Although E1-related products could be clearly detected by mAb D32.10 after deglycosylation, neofomed E2-
with the unmodified peptide. As shown in Fig. 3, for the three peptides, the only critical residues were Cys 306, Cys 494, and Cys 620, respectively, whereas the replacement of Cys 304 and Cys 486 with alanine in peptides 292–306 and 480–494, respectively, had no effect. Regarding the contribution to antibody binding of the conserved residues between the phage-displayed peptides and the E1 and E2 proteins, the absence of an effect of the corresponding alanine analogs on the respective immunoreactivities of the three peptides is in agreement with the fact that the peptides displayed on phage did not give a detectable milliunits/lane 2 (lane 2). The proteins were subjected to SDS-PAGE; and after transfer, immunoblotting was performed using mAb D32.10 diluted in 50% normal human serum to a final concentration of 3 g/ml. Antibody binding was detected with horseradish peroxidase-conjugated anti-mouse immunoglobulins as the secondary antibody. Bands were visualized using the ECL system. The molecular masses of markers (lanes M) and HCV proteins are indicated in kilodaltons on the left and right, respectively. Asterisks indicate deglycosylated forms of E1 and E2.

Fig. 2. mAb D32.10 reactivity with HCV proteins in Western blotting. A, serum-purified HCV particles (HCV1, genotype 1b) were used as antigenic probes to determine the polypeptide specificity of mAb D32.10 under nonreducing (lane 1) and reducing (lanes 2 and 3) conditions. B, the effect of peptide N-glycosidase A (PNGase A) at 20 (lane 1), 10 (lane 2), 5 (lane 3), and 0 (lane 4) milliunit/ml on the binding of mAb D32.10 to HCV envelope proteins E1 and E2 was studied. C, the HCV-enriched pellet was untreated (lane 1) or was treated with Endo H (5 milliunits/µl) (lane 2). The proteins were subjected to SDS-PAGE; and after transfer, immunoblotting was performed using mAb D32.10 diluted in 50% normal human serum to a final concentration of 3 µg/ml. Antibody binding was detected with horseradish peroxidase-conjugated anti-mouse immunoglobulins as the secondary antibody. Bands were visualized using the ECL system. The molecular masses of markers (lanes M) and HCV proteins are indicated in kilodaltons on the left and right, respectively. Asterisks indicate deglycosylated forms of E1 and E2.
signal by themselves because they did not contain any Cys residue. Indeed, according to the manufacturer, the low frequency of Cys in the Ph.D.-12™ library (0.4% instead of 5–10% for the other amino acids) does not favor the selection of peptides with Cys. However, as this library provides an increased repertoire of overlapping peptides, it allows the target to select sequences with multiple weak contacts instead of a few strong interactions. In conclusion, these results suggest that, in sequences with multiple weak contacts instead of a few strong interactions, the localization of the mAb D32.10 epitope, we assessed the availability of these sequences on the viral particle surface as well as their potential immunogenicity during in vivo HCV infection by testing these peptides with 44 sera from HCV-infected patients and 11 sera from healthy individuals. Using a cutoff of recognition calculated for each peptide (mean of the values obtained with HCV-negative sera + 3 S.D.), positive responses were obtained with 6 out of 44 HCV-positive sera against E1-(292–306) (Fig. 4A), 6 out of 44 against HCV-positive sera E2-(480–494) (Fig. 4B), and 16 out of 44 HCV-positive sera against E2-(608–622) (Fig. 4C). Sera A7, A14, A21, A33, A39, and A40 gave a positive signal with the three peptides, whereas E2-(608–622) was also recognized by 10 more sera. This indicates that these E1E2 sequences or, more likely, the corresponding epitope recognized by mAb D32.10 was able to induce an immune response in some HCV patients during infection.

**DISCUSSION**

Until now, the low levels of HCV particles present in patient plasma and the lack of an efficient cell culture system for HCV propagation had precluded a direct analysis of the virion envelope glycoproteins. The envelope glycoproteins have been shown to assemble into a functional subunit of HCV virions (9, 22). However, anti-HCV monoclonal antibodies (A4, A11, and H2) obtained from E1E2 heterodimers produced in such a heterologous system failed to bind to HCV RNA-containing particles from serum (7). This suggests that the

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**TABLE III**

| Amino acid similarities between phage clones and both E1 and E2 sequences |
|---|
| Amino acids in boldface correspond to similarities to E1, and underlined amino acids to similarities to E2. |
| E1-(292–305), E2-(482–499/612–626) |
| Clone 1 | SP | RH | Y3 | ELQ |
| Clone 4 | SP | RH | W | VP |
| Clone 11 | TS | QP | RH | GQPAT |
| E1-(347–356), E2-(482–499/612–626) |
| Clone 6 | W | EM | PR | AT |
| Clone 8 | W | EM | PR | AT |

**TABLE IV**

**Immunoreactivity of overlapping peptides offset by one spanning residues 291–315 of the HCV E1 glycoprotein**

| Peptides | E1-(291–315) sequence | Spot intensity |
|---|---|---|
| A. Pentadecapeptides | FTFSPPRHWTQGCN | 2 |
| | TFSPPRHWTQGCN | 4 |
| | SPRRHWTQGCNC | 1.5 |
| | PRRHWTQGCNSIPY | 1 |
| | RHWTQGNCNSIYPG | 0.5 |
| | HWTTQGNCNISYPGH | 0 |
| | WTQGCNCNSIFYPGH | 0 |
| | TQGCNCHIFYPGHTG | 0 |
| B. Octapeptides | TFSPRHWH | 0 |
| | FSPRHHWT | 0 |
| | SRRHWT | 0 |
| | PRRHTQ | 0 |
| | RRHTQG | 0 |
| | HWTTQGC | 3 |
| | WTQGCN | 3 |
| | TQGCNC | 4 |

**TABLE V**

**Immunoreactivity of overlapping peptides offset by one spanning residues 473–498 of the HCV E2 glycoprotein**

| Peptides | E2-(473–498) sequence | Spot intensity |
|---|---|---|
| A. Pentadecapeptides | SYANGSGPDQRPYCWW | 0 |
| | YANGSGPDQRPYCWW | 0 |
| | ANSGSGPDQRPYCWW | 0 |
| | NGSQDPQRCWYHP | 0 |
| | GSDQRPQCHYHPPPK | 0 |
| | GPDQRPCHYHPPPK | 0 |
| | PDQRPYCWHPPKPC | 3 |
| | DQRPYCWHPPKPCG | 0.5 |
| | QRPYCWHPPKPCG | 1 |
| | PYCWHPKPCGIV | 1 |
| | PYCWHPKPCGIV | 0.5 |
| B. Octapeptides | SQPDQRPY | 0 |
| | GPDQRC | 2 |
| | PDQRPYC | 0 |
| | DQRPYCWH | 0 |
| | QRPYCWH | 0 |
| | RPYCWHYP | 0 |
| | PCHYWPFP | 0 |
| | YCWHPKP | 0 |
| | CHEWPKPC | 2 |
| | WHPKPCG | 1 |
| | YPPKPCG | 1 |
| | PPKPCGIV | 0 |

**TABLE VI**

**Immunoreactivity of overlapping peptides offset by one spanning residues 607–627 of the HCV E2 glycoprotein**

| Peptides | E2-(607–627) sequence | Spot intensity |
|---|---|---|
| A. Pentadecapeptides | CLVDYYPRLWHYPC | 3 |
| | LDYPYPLRLWHYPC | 4 |
| | VDPYRLWHYPTCTIN | 2 |
| | DYPYRLWHYPTCTIN | 1 |
| | YRPYRLWHYPTCTINYT | 0 |
| | YRPYRLWHYPTCTIN | 0 |
| | YRLWHYPTCTIN | 0 |
| | LVDYPYR | 0 |
| | VDYPYR | 0 |
| | DYPYR | 0 |
| | YR | 0 |
| | LRLWHYPC | 4 |
| | RLWHYPCT | 3.5 |
| | LWHPCT | 1 |
| | WHYPC | 0 |
produced monoclonal antibodies by immunization of mice with glycoproteins, E1 and E2, is not yet known. Therefore, we exact nature of the interaction between the two HCV envelope 44390 diluted to a final concentration of 20

reactivities of the control and substituted peptides with mAb D32.10 by alanine scanning. Spot intensities correspond to the respective

strongly reacted with a doublet at 60

probes in Western blotting, we determined that mAb D32.10

specificity for HCV particles from different origins (subtypes 1a

determination of E1 and E2 regions. Region 292–306 of E1 and regions 480–494 and 608–622 of E2 as pentadecapeptides interacted with mAb D32.10 in ELISA. Using overlapping octapeptides, we determined that 297-RHWTQG-CN306 of the HCV E1 protein and both 613-YRALLYWHYPCG621 and 486-PDQRPYCWHPKP3C494 of the HCV E2 protein were reactive with mAb D32.10. The two regions identified in E2 contained the same motif (WHY) reported by Yagnik et al. (10) as likely to be involved in the heterodimerization of E1E2. Indeed, it is difficult to discriminate between these two regions. Because two non-overlapping zones (479-GPDQRPYC866 and 487-WHYPKPC494) separately bound to mAb D32.10, this suggests that mAb D32.10 specifically recognized each octapeptide and thus the complete sequence (amino acids 480–494). The three identified regions contain cysteine (C) residues, which could be involved in intramolecular and/or intermolecular disulfide bridges. Indeed, Cys306, Cys494, and Cys620, located in the C-terminal part of each peptide, were shown by alanine substitution to be the critical residues for strong reactivity with mAb D32.10. Interestingly, Cys620 from one monomer has been proposed as the most likely candidate for covalent interaction in E2 homodimerization (10). In addition, E2(476–494), containing Cys486 and Cys494 and including the WHY motif, might be important for heterodimeric association between E1 and E2 (10). Finally, E1(297–306), reacting with mAb D32.10, contains two amino acids (NC) in the C-terminal part belonging to the fourth core glycosylation site, Asn305-Cys306-Ser307, which is involved in the coprecipitation of E1 and E2 and would be the less efficiently glycosylated (only 66% of E1) in vitro (26). On the other hand, it has been demonstrated that treatment of HCV particles recovered from the circulation of infected humans with peptide N-glycosidase A results in a significant decrease in their ability to bind to lectins RCA1 (Ricinus communis) agglutinin-I and WGA (Tricicum vulgaris) wheat germ agglutinin (14). Our data show that treatment of the HCV1 preparation with peptide N-glycosidase A led to deglycosylation of E1 and that mAb D32.10 recognized the deglycosylated forms, especially the 25-kDa species. This suggests that site 4 is very likely less efficiently glycosylated in vivo than in vitro (26, 27) and probably plays an important role in the protein folding and antigenicity of native HCV envelope complexes. Surprisingly, deglycosylation of serum-derived HCV particles with Endo H allowed us to only partially remove high mannosetype oligosaccharides (even at the highest concentrations under denaturing conditions) (data not shown), suggesting the presence of a mixture of both Endo H-resistant complex glycans and Endo H-sensitive forms. Thus, our data show that the E1 and E2 proteins in the natural HCV particles contain complex glycans that are susceptible to cleavage by peptide N-glycosidase A, but resistant to Endo H, indicating that circulating HCV particles pass through the Golgi during cellular transport.

![Fig. 3. Analysis of critical residues of the mAb D32.10 epitope by alanine scanning. Spot intensities correspond to the respective reactivities of the control and substituted peptides with mAb D32.10 diluted to a final concentration of 20 μg/ml.](image-url)
Together, these results indicate that the three regions identified in E1 and E2 by mAb D32.10 are closely juxtaposed on the surface of serum-derived HCV particles. Secondary structure prediction of the ectodomain of HCV E2 using only HCV sequences suggested an overall low secondary structure content (≈37%) of predominantly β-strands. It is noteworthy that Flavivirus envelope glycoprotein E from tick-borne encephalitis virus shows functional similarity to HCV E2 (28), and these proteins are similar from the point of view of the parameters in these fold recognition methods (10). Tick-borne encephalitis virus envelope glycoprotein E could thus be considered as a good candidate for model building of HCV E2. In the current

![Graphs A, B, and C showing human anti-HCV antibody binding to selected E1 and E2 sequences.](image-url)
model of HCV E2 (strain H), the 661-amino-acid truncated E2 protein is sufficient to bind CD81 (29), to be exported (30), and to heterodimerize with E1 (31). Therefore, it could be assumed that amino acids 384–661 may represent the structural core of a functional E2 protein. All of these considerations and the puzzle specificity of our anti-E1E2 mAb D32.10 support the model proposed for the quaternary structure of envelope glycoproteins E1 and E2 by Yagnik et al. (10). Our results suggest that the mAb D32.10 epitope encompasses the E1E2 association site (amino acids 487–489, WHY), hypervariable region 2 (amino acids 474–482), and CD81-binding region-1 (amino acids 474–494) as well as residues 612–620, involved in CD81 binding and/or dimerization of E2. This implies that all of these regions would share the same site on the viral surface, in accordance with the formation of a head-to-tail E2 homodimeric pair, covalently linked, of heterodimers with E1 (10). Because such a model of HCV E2 was based on the tick-borne encephalitis virus glycoprotein E structure, it would be reasonable to assume that its physical relationship to the viral membrane could be also similar.3 The presence in sera from HCV-infected patients of specific antibodies that were also able to react simultaneously with the three regions of E1 and E2 recognized by mAb D32.10 strongly supports their juxtaposition on the surface of circulating enveloped HCV particles and their immunogenicity in mice as well as in humans.

When baculovirus-derived HCV-like particles were used as a capture antigen in ELISA (32), no binding of mAb D32.10 was observed (data not shown), suggesting that envelope domains comprising amino acids 297–306 (E1), amino acids 480–490 (E2), and amino acids 613–621 (E2) are not accessible to this monoclonal antibody on the surface of HCV-like particles synthesized in insect cells. This finding may indicate that such recombinant particles (33) express envelope proteins in a conformation presenting antigenic properties that are distinct from those of the HCV particles circulating in the sera of chronically infected patients. The different patterns of envelope expression either in insect cells (two glycosylated forms of E1) or in mammalian cells (a single glycosylated protein) involve significant differences in glycosylation, which could probably explain the differences in the particles’ morphology and antigenicity.

The HCV envelope glycoprotein complex is likely a key antigenic structure for an effective vaccine against the virus. Knowledge of its exact structure on the surface of native HCV particles will therefore present a significant step forward. A monoclonal antibody such as D32.10 that recognizes potential E2E2 and E1E2 association sites and the CD81-binding site could then become a valuable tool for studying HCV neutralization mechanisms and for identifying complete enveloped infectious HCV virions needed for further structural analysis of the native HCV envelope E1E2 complexes.

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REFERENCES

1. Saito, I., Miyamura, T., Ohbayashi, A., Harada, H., Katayama, T., Kikuchi, S., Watanabe, Y., Koi, S., Onji, M., Ohta, Y., Choo, Q. L., Houghton, M., and Kuo, G. (1990) Proc. Natl. Acad. Sci. U. S. A. 87, 6547–6549
2. Choo, Q. L., Richman, K. H., Han, J. H., Berger, K., Lee, C., Dong, C., and Kuo, C. (1989) Proc. Natl. Acad. Sci. U. S. A. 86, 3451–3455
3. Bukh, J., and Apparg, C. L. (1997) Virology 229, 429–436
4. Rice, C. M. (1996) in Fields Virology (Fields, B. N., Knipe, D. M., and Howley, P. M., eds) pp. 931–959, Lippincott-Raven, Philadelphia
5. Clarke, B. (1997) J. Gen. Virol. 78, 2397–2410
6. Coquerel, L., Meunier, J. C., Piliez, A., Wychowski, C., and Dubuisson, J. (1998) J. Virol. 72, 2183–2191
7. Delsereznyder, V., Piliez, A., Wychowski, C., Blight, K., Xu, J., Hahn, Y. S., Rice, C. M., and Dubuisson, J. (1997) J. Virol. 71, 697–704
8. Dubuisson, J. (2000) Curr. Top. Microbiol. Immunol. 242, 135–148
9. Dubuisson, J., Hau, H. H., Cheung, R. C., Greenberg, H. B., Russell, D. G., and Rice, C. M. (1994) J. Virol. 68, 6147–6160
10. Yagnik, A. T., Lahm, A., Meola, A., Roccasecca, R. M., Ercole, A., Nicosia, A., and Tramontano, A. (2000) Proteins 40, 355–366
11. Buttin, G., LeQueru, G., Phale, L., Lin, E. C., Medrano, L., and Casenave, P. A. (1978) Curr. Top. Microbiol. Immunol. 81, 27–36
12. Petit, M.-A., Capel, F., Rissot, M. M., Daugas, C., and Pilot, J. (1987) J. Gen. Virol. 68, 2759–2770
13. Petit, M.-A., Dubanchet, S., and Capel, F. (1989) Mol. Immunol. 26, 531–537
14. Sato, K., Okamoto, H., Aiha, S., Hoshi, T., Tanaka, T., and Mishiro, S. (1993) J. Virol. 67, 4777–4781
15. Scott, J. K., and Smith, G. P. (1990) Science 245, 386–390
16. Sambrook, J., Fritsch, E. F., and Maniatis, T. (1982) Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
17. Sanger, F., Nicklen, S., and Coulson, A. R. (1977) Proc. Natl. Acad. Sci. U. S. A. 74, 5463–5467
18. Pearson, W. R., and Lipman, D. J. (1988) Proc. Natl. Acad. Sci. U. S. A. 85, 2444–2448
19. Franc K. D. R. (1998) Tetrahedron 44, 6031–6040
20. Jolivet-Renaud, C., Dalbon, P., Viola, F., Yvon, S., Paranos-Baccala, G., Piga, N., Bridon, L., Trabaud, M. A., Battaill, N., Shibai, G., and Joivet, M. (1998) J. Med. Virol. 56, 390–399
21. Trepo, C., Berthillon, P., and Vitiolaki, L. (1998) Ann. Oncol. 9, 469–470
22. Ralston, R., Thudium, K., Berger, K., Kuo, C., Gervase, B., Hall, J., Selby, M., Kuo, G., Houghton, M., and Choo, Q. L. (1993) J. Virol. 67, 6753–6761
23. Okamoto, H., Kurai, K., Okada, R., Yamamoto, K., Liu, H., Tanaka, T., Fukuda, S., Tuda, F., and Mishiro, S. (1992) Virology 198, 331–341
24. Hikikata, M., Kato, N., Otsuzyuma, Y., Nakagawa, M., and Shimotohno, K. (1991) Proc. Natl. Acad. Sci. U. S. A. 88, 5547–5551
25. Sparte, R. R., Alexander, D., Rugreden, M. E., Choo, Q. L., Berger, K., Crawford, K., Kuo, C., Leng, S., Lee, C., Ralston, R., Thudium, K., Thung, J., W., Kuo, G., and Houghton, M. (1992) Virology 188, 819–830
26. Meunier, J. C., Fourriaul, A., Choukh, A., Cahour, A., Coquerel, L., Dubuisson, J., and Wychowski, C. (1999) J. Gen. Virol. 80, 877–886
27. Dubuisson, J., Duve, S., Meunier, J. C., Op De Breech, A., Cacan, R., Wychowski, C., and Coquerel, L. (2000) J. Biol. Chem. 275, 30605–30609
28. Roy, F. A., Heinz, F. X., Mandl, C., Kunz, C., and Harrison, S. C. (1995) Nature 375, 291–296
29. Flint, M., Maiten, C., Louns-Price, L. D., Shotton, C., Dubuisson, J., Monk, P., Higginbottom, A., Levy, S., and McKeating, J. A. (1999) J. Virol. 73, 6235–6244
30. Selby, M. J., Glazer, E., Masiarz, F., and Houghton, M. (1994) Virology 204, 114–122
31. Patel, J., Patel, A. H., and McLachlan, J. (1999) J. Gen. Virol. 80, 1681–1690
32. Baumert, T. F., Wellnitz, S., Aono, S., Aono, S., H, Herion, D., Tilman Gerlach, J., Pape, G. R., L., Liu, J., Hofmagne, J. H., Blum, H. E., and Lang, T. J. (2000) Hepatology 32, 610–617
33. Wellnitz, S., Klimps, B., Barth, H., Itu, S., Depla, E., Dubuisson, J., Blum, H. E., and Baumert, T. F. (2002) J. Virol. 76, 1181–1185

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