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Characterization of Functional Hepatitis C Virus Envelope Glycoproteins

Anne Op De Beeck,[1]† Cécile Voisset,[1]† Birke Bartosch,[2] Yann Ciczora,[1] Laurence Cocquerel,[1] Zhenyong Keck,[3] Steven Foung,[3] François-Loïc Cosset,[2] and Jean Dubuisson[1]*

CNRS-UPR2511, Institut de Biologie de Lille and Institut Pasteur de Lille, Lille,[1] and Laboratoire de Vectorologie Rétrovirale et Thérapie Génique, INSERM U412, IFR 74, Ecole Normale Supérieure de Lyon, Lyon,[2] France, and Department of Pathology, Stanford University, Stanford, California[3]

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Hepatitis C virus (HCV) encodes two envelope glycoproteins, E1 and E2, that assemble as a noncovalent heterodimer which is mainly retained in the endoplasmic reticulum. Because assembly into particles and secretion from the cell lead to structural changes in viral envelope proteins, characterization of the proteins associated with the virion is necessary in order to better understand how they mature to be functional in virus entry. There is currently no efficient and reliable cell culture system to amplify HCV, and the envelope glycoproteins associated with the virion have therefore not been characterized yet. Recently, infectious pseudotype particles that are assembled by displaying unmodified HCV envelope glycoproteins on retroviral core particles have been successfully generated. Because HCV pseudotype particles contain fully functional envelope glycoproteins, these envelope proteins, or at least a fraction of them, should be in a mature conformation similar to that on the native HCV particles. In this study, we used conformation-dependent monoclonal antibodies to characterize the envelope glycoproteins associated with HCV pseudotype particles. We showed that the functional unit is a noncovalent E1E2 heterodimer containing complex or hybrid type glycans. We did not observe any evidence of maturation by a cellular endoprotease during the transport of these envelope glycoproteins through the secretory pathway. These envelope glycoproteins were recognized by a panel of conformation-dependent monoclonal antibodies as well as by CD81, a molecule involved in HCV entry. The functional envelope glycoproteins associated with HCV pseudotype particles were also shown to be sensitive to low-pH treatment. Such conformational changes are likely necessary to initiate fusion.

Hepatitis C virus (HCV) is a major cause of chronic hepatitis, liver cirrhosis, and hepatocellular carcinoma worldwide (39). This small enveloped positive-strand RNA virus has been classified within its own genus, *Hepacivirus*, within the *Flaviviridae* family, which also comprises the *Flavivirus* and *Pestivirus* genera (54). Its genome encodes a single polyprotein precursor of just over 3,000 amino acid residues. This polyprotein precursor is co- and posttranslationally processed by cellular and viral proteases to yield at least 10 polypeptides (36, 41). The two viral envelope glycoproteins, E1 and E2, are released from the HCV polyprotein precursor after cleavage by a host signal peptidase(s) (reviewed in reference 20).

No efficient and reliable cell culture system is available to amplify HCV (36). The current knowledge on the characterization of HCV envelope glycoproteins is based on cell culture transient-expression assays. HCV glycoproteins are type I transmembrane proteins with a large N-terminal ectodomain and a C-terminal hydrophobic anchor. During their synthesis, the ectodomains of HCV glycoproteins are targeted to the endoplasmic reticulum (ER) lumen, where they are modified by N-linked glycosylation. E1 and E2 possess up to 6 and 11 potential glycosylation sites, respectively (27). HCV envelope glycoproteins have been shown to assemble into oligomeric complexes. They can form a heterodimer of E1 and E2 stabilized by noncovalent interactions as well as heterogeneous disulfide-linked aggregates (19). Extensive characterization of the noncovalent heterodimer strongly suggests that this oligomer is the prebudding form of the functional complex, which will probably subsequently play an active role in the process of entry into host cells (15).

Immunolocalization studies and glycan analyses have shown that the noncovalent E1E2 heterodimer is located in the ER (15, 22). In addition, the transmembrane domains of E1 and E2 have been shown to play a major role in ER retention of the E1E2 complex (10, 12, 14). Several attempts have been made to mutate the transmembrane domains of HCV envelope glycoproteins in order to readdress them to the plasma membrane with the objective of making pseudotyped viruses or developing a cell-cell fusion assay (6, 26, 40, 43, 51, 57, 59). However, the transmembrane domains of HCV envelope glycoproteins also play a role in heterodimerization (46), and this function is lost when these transmembrane domains are replaced by those of other proteins (12, 40). In addition, such mutations also abolish the entry functions of HCV envelope glycoproteins (31).

Recently, infectious pseudotype particles that are assembled by displaying unmodified HCV envelope glycoproteins on retroviral core particles have been successfully generated (4, 31). The data that have been accumulated on these pseudotype particles strongly suggest that they mimic the early steps of HCV infection. Indeed, they exhibit a preferential tropism for hepatic cells, and they are specifically neutralized by anti-E2

* Corresponding author. Mailing address: Unité Hépatite C, CNRS-UPR2511, Institut de Biologie de Lille, 1 rue Calmette, BP 447, 59021 Lille cedex, France. Phone: (33) 3 20 87 11 60. Fax: (33) 3 20 87 12 01. E-mail: jean.dubuisson@ibl.fr.

† A.O.D.B. and C.V. contributed equally to the results of this study.

monoclonal antibodies (MAbs) as well as sera of HCV-infected patients. These HCV pseudotype particles (HCVpp) therefore represent the best tool currently available to study functional HCV envelope glycoproteins. In this report, we characterized HCVpp-associated envelope proteins by use of conformation-dependent MAbs. We showed that the functional unit is a noncovalent E1E2 heterodimer containing complex or hybrid type glycans. We did not observe any evidence of a maturation by cellular endoprotease cleavage during their transport through the secretory pathway. In addition, conformational changes in HCV envelope glycoproteins were observed after low-pH treatment.

MATERIALS AND METHODS

Cell culture. Huh-7 human hepatocellular carcinoma cells (45) and 293T human embryo kidney cells (293tsA1609neo) obtained from the American Type Culture Collection (Manassas, Va.) were grown in Dulbecco's modified essential medium (Invitrogen) supplemented with 10% fetal bovine serum.

Production of HCVpp and infection assays. Production of HCVpp and infection assays have been described previously (4). In this work, we used the cytomegalovirus (CMV)-Gag-Pol murine leukemia virus (MLV) packaging construct, containing the MLV *gag* and *pol* genes and the MLV-green fluorescent protein (GFP) (4) or MLV-Luc (C. Voisset and J. Dubuisson, unpublished data) plasmid, encoding an MLV-based transfer vector containing a CMV-GFP or CMV-Luc internal transcriptional unit. Due to better sensitivity, the MLV-Luc plasmid was used for the neutralization assays. The HCV sequence used in this work is derived from the H strain (genotype 1a) (23). In some experiments, modified HCV envelope proteins were used. Plasmids pTM1/E1†HA and pTM1/E2†HA (13) were used to construct phCMV-E1-HA and phCMV-E2-HA, respectively. In these plasmids, the last residue (Ala) at the C terminus of E1 and E2 has been replaced by an Arg to avoid signal sequence cleavage. In several experiments, 293T cells were metabolically labeled from 16 to 40 h posttransfection with ^{35}S-Protein Labeling Mix (Amersham Biosciences) as described previously (19).

Antibodies. The MAbs used in this work have been described previously: A4 (19); H2 (15); H14 (8); H31, H33, H35, H44, H47, H48, H50, H52, H53, H54, H57, H60, and H61 (24, 25, 48); 1/39, 3/11, 6/16, 6/53, and 9/86a (25); CBH2, CBH4B, CBH4D, CBH4G, CBH5, CBH7, CBH11, and CBH17 (28). The rat anti-hemagglutinin (anti-HA) MAb (3F10) was purchased from Roche.

Immunoprecipitation and endoglycosidase digestions. Metabolically labeled 293T cells and HCVpp obtained from cell culture supernatants were lysed with 0.5% Igepal CA-630 in TBS (50 mM Tris-HCl [pH 7.5]–150 mM NaCl). Immunoprecipitations were carried out as described previously (21). In some experiments, HCVpp were acidified with 150 mM morpholineethanesulfonic acid (MES) at 37°C for 20 min at pH 5.5. The pH was back-neutralized with 150 mM triethanolamine (pH 7.5) before immunoprecipitation. For endoglycosidase digestion, immunoprecipitated proteins were eluted from protein A-Sepharose in 30 μl of dissociation buffer (0.5% sodium dodecyl sulfate [SDS] and 1% 2-mercaptoethanol) by boiling for 10 min. The protein samples were then divided into three equal portions: one for digestion with endo-β-*N*-acetylglucosaminidase H (endo H), one for digestion with peptide:*N*-glycosidase F (PNGase F), and one for an undigested control. Digestions were carried out for 1 h at 37°C in the buffer provided by the manufacturer (New England Biolabs). Digested samples were mixed with an equal volume of 2× Laemmli sample buffer and analyzed by SDS-polyacrylamide gel electrophoresis (PAGE).

Western blotting. Proteins bound to nitrocellulose membranes were revealed by enhanced chemiluminescence detection as recommended by the manufacturer (NEN). Briefly, after separation by SDS-PAGE under reducing conditions, proteins were transferred to nitrocellulose membranes by using a Trans-Blot apparatus (Bio-Rad) and revealed with specific MAbs (A4, 3/11, H52, or 9/27) followed by rabbit anti-mouse (A4 and H52) or anti-rat (3/11 and 9/27) immunoglobulin conjugated to peroxidase (dilution, 1/1,000; Dako).

CD81 pull-down assay. Recombinant fusion proteins containing the large extracellular loop of human or murine CD81 fused to glutathione *S*-transferase were preadsorbed onto glutathione-Sepharose 4B beads according to the manufacturer's recommendations (Pharmacia Biotech, Uppsala, Sweden). Pull-down experiments were performed as described previously (11). Precipitates were separated by SDS-PAGE followed by Western blotting with an anti-E1 (A4) or anti-E2 (3/11) MAb.

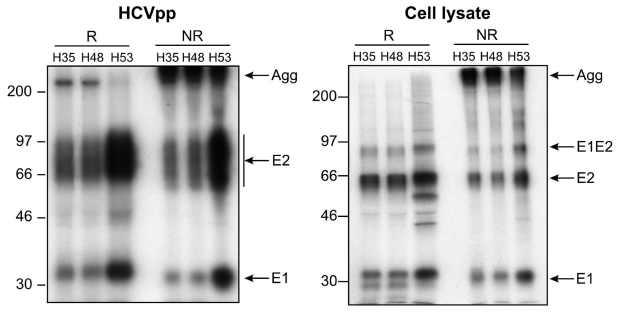

FIG. 1. Noncovalent E1E2 heterodimers are incorporated into HCVpp. At 16 h posttransfection, 293T cells transfected to produce HCVpp were metabolically labeled for 24 h. The supernatant (HCVpp) and cell lysate were immunoprecipitated with anti-E2 MAbs (H35, H48, and H53). The immunoprecipitates were analyzed by SDS-PAGE under reducing (R) or nonreducing (NR) conditions. Sizes of protein molecular mass markers are given on the left (in kilodaltons). HCV envelope proteins E1 and E2 as well as aggregates (Agg) and SDS-resistant E1E2 complexes are indicated on the right.

RESULTS

Noncovalent E1E2 heterodimers are associated with HCVpp. Extensive characterization of HCV envelope glycoproteins has shown that these proteins assemble as a noncovalent heterodimer which is most likely the prebudding form of the functional complex (15). Because assembly into particles and secretion from the cells lead to structural changes in viral envelope proteins, we were interested in analyzing the modifications associated with HCV envelope glycoproteins incorporated into HCVpp. Although we cannot exclude some heterogeneity in HCV proteins incorporated into HCVpp, we focused our work on proteins isolated with the help of conformation-dependent MAbs, some of which have been shown to have a neutralizing activity against HCVpp.

Conformation-dependent anti-E2 MAbs (H35, H48, and H53) were used to characterize HCV envelope glycoproteins incorporated into HCVpp. MAbs H35 and H48 have been shown to have a neutralizing activity when incubated with HCVpp (4), indicating that these antibodies recognize functional HCV envelope glycoproteins. As shown in Fig. 1 (HCVpp), two bands corresponding to E1 and E2 were detected after immunoprecipitation with MAbs H35, H48, and H53. Since these MAbs recognize an epitope present on E2, these results indicate that hetero-oligomers of HCV envelope glycoproteins are incorporated into HCVpp (Fig. 1). Similar results were obtained when infectious HCVpp were purified on a sucrose density gradient (data not shown). It is worth noting that HCVpp-associated E2 had a slower and more diffuse migration pattern than the cell-associated form. This is likely due to modifications of the glycans by Golgi enzymes (see below). When analyzed under nonreducing conditions, the same bands corresponding to E1 and E2 were still observed, indicating that HCV envelope glycoproteins formed noncovalent complexes involving E1 and E2. As observed before (42), some aggregates were observed on the top of the gel, but they represented a minor fraction of HCV envelope glycoproteins. Indeed, the intensities of the bands corresponding to E1 and E2 were very similar when analyzed under reducing and nonreducing conditions. Although the majority of E1 and E2 en-

velope glycoproteins associated with HCVpp form noncovalent heterodimers, we cannot exclude the possibility that the disulfide-linked E1E2 complexes play a role in HCV entry. When analyzed under reducing conditions, two glycoforms of E1 were immunoprecipitated from the cell lysate (Fig. 1, Cell lysate), as previously observed (18). Interestingly, only the fully glycosylated form of E1 was associated with HCVpp (Fig. 1, HCVpp). HCV glycoprotein complexes containing the lower-glycosylation form of E1 are therefore not incorporated into HCVpp. This finding is in agreement with the observation that this lower glycosylated form of E1 was associated with aggregates in the cell lysate, as it was detected only under reducing conditions (Fig. 1, Cell lysate).

Altogether, these results indicate that HCV envelope glycoproteins are associated with HCVpp as a noncovalent heterodimer.

Maturation of the glycans associated with HCV envelope glycoproteins. HCV envelope glycoproteins are modified by N-linked glycosylation. Analyses of the glycans bound to the intracellular HCV envelope glycoprotein heterodimer have indicated that only high-mannose type oligosaccharides are associated with these proteins (15, 22). The absence of modification of the glycans associated with HCV envelope glycoproteins indicates that they are not modified by Golgi enzymes, which is in agreement with their localization in the ER or an ER-like compartment.

To determine whether the glycans associated with HCV envelope glycoproteins expressed in 293T cell are modified by Golgi enzymes, their resistance to endo H treatment was determined. Indeed, endo H removes the chitobiose core of high-mannose oligosaccharides and some hybrid forms of N-linked sugars but not the complex forms (53). Hence, resistance to digestion with endo H is indicative that glycoproteins have moved from the ER to at least the medial and *trans*-Golgi regions, where complex sugars are formed. PNGase F treatment, which removes all types of N-linked glycans, was used as a control of deglycosylation. Since H35 and H48 had a lower relative affinity, we used MAb H53 to analyze the glycans associated with E1 and E2. Although H53 is not neutralizing, the proteins precipitated by this antibody have a pattern similar to those precipitated by MAbs H35 and H48 (Fig. 1). As shown in Fig. 2A (Cell lysate), cell-associated HCV envelope glycoproteins remained endo H sensitive in a steady-state labeling experiment, suggesting that the bulk of E1 and E2 expressed in 293T cells is located in an early compartment of the secretory pathway, as previously shown in other cell lines (15). As previously observed (19), the PNGase F-treated E2 migrated slightly faster than the endo H-treated protein, and the PNGase F-treated E1 had a slightly slower migration profile than the endo H-treated form (Fig. 2A, Cell lysate). In the case of E2, this could be explained by the presence of a residual *N*-acetylglucosamine at each glycosylation position after endo H treatment. The slightly slower migration of PNGase F-treated E1 is likely due to abnormal behavior of this protein after modification of its hydrophilicity as observed in C-terminally truncated forms (44).

To determine whether the glycans associated with HCV envelope glycoproteins incorporated into HCVpp are resistant to endo H treatment, metabolically labeled HCV envelope glycoproteins associated with HCVpp were immunoprecipitated with MAb H53 and either left untreated or treated with endo H or PNGase F. As shown in Fig. 2A, HCVpp-associated E2 treated with endo H had a migration profile similar to that of the untreated protein. An additional faint band of 46 kDa was also detected, indicating some heterogeneity in the sensitivity to endo H. Together, these data indicate that most E2 glycans are endo H resistant. Interestingly, most HCVpp-associated E1 treated with endo H migrated faster than the untreated protein but more slowly than the deglycosylated form from the cell lysate (Fig. 2A; compare E1** and E1*). This indicates that at least one E1 glycan can be resistant to endo H treatment, while the others are sensitive and therefore likely of the high-mannose type.

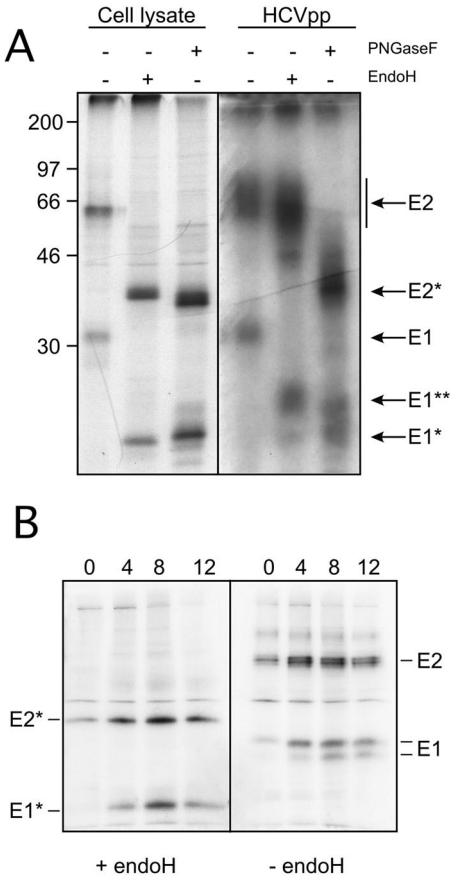

FIG. 2. Analyses of the glycans associated with HCV envelope proteins. (A) The glycans associated with HCV envelope glycoproteins are modified by Golgi enzymes. 293T cells transfected to produce HCVpp were labeled for 24 h. Supernatants (HCVpp) and cell lysates were immunoprecipitated with the anti-E2 MAb H53. The immunoprecipitates were either left untreated or treated with endo H or PNGase F and analyzed by SDS-PAGE. Deglycosylated forms of E1 and E2 are indicated by an asterisk. The endo H-resistant form of E1 is indicated by two asterisks. Sizes of protein molecular mass markers are given on the left (in kilodaltons). (B) The bulk of HCV envelope glycoproteins expressed in 293T cells is not modified by Golgi enzymes. 293T cells transfected for 24 h were pulse-labeled for 30 min and chased for different times as indicated (in hours). Cell lysates were immunoprecipitated with the anti-E2 MAb H53. The immunoprecipitates were either left untreated or treated with endo H and analyzed by SDS-PAGE. HCV envelope proteins E1 and E2 are indicated on the right. Deglycosylated forms of E1 and E2 are indicated by an asterisk.

PNGase F removes all types of N-linked glycans. However, after PNGase F treatment, two fast-migrating bands were detected for HCVpp-associated E1 (Fig. 2A). One migrated at the size of the fully deglycosylated protein, and the other had a slightly slower mobility, suggesting that this form still contained at least one glycan. Such a partial resistance to PNGase F treatment has already been observed for truncated forms of HCV glycoprotein E2 (J. Dubuisson, unpublished data) as well as for other proteins (30). Whether the PNGase F-resistant glycan of E1 is the same as the endo H-resistant glycan remains to be determined.

When HCV envelope proteins are overexpressed in 293T cells to produce HCVpp, a fraction of these proteins accumulate at the plasma membrane, the site where these particles have been suggested to bud (4, 16, 31). This suggests that HCV envelope glycoproteins that accumulate at the plasma membrane have followed the secretory pathway; thus, their glycans should be modified by Golgi enzymes. Because no endo H-resistant form of HCV envelope glycoproteins was detected in steady-state labeling, the sensitivity of HCV envelope glycoproteins to digestion with endo H was analyzed in a pulse-chase experiment, which is a more sensitive approach. As shown in Fig. 2B, HCV envelope proteins remained endo H sensitive, and even after 12 h of chase, no endo H-resistant form of E1 and/or E2 was clearly detected, suggesting that the fraction that migrates to the cell surface must be very small. This is in agreement with the presence of ER retention signals in HCV envelope glycoproteins (10, 12). Detection of E1 and/or E2 at the surfaces of 293T cells by immunofluorescence (4, 16, 31) is therefore likely due to small amounts of E1E2 proteins escaping the ER retention machinery.

Altogether, these results indicate that HCV envelope glycoproteins incorporated into HCVpp issue from a very small population of proteins escaping the ER retention machinery and are thus modified by Golgi enzymes and contain complex or hybrid type glycans.

Maturation of HCV envelope glycoproteins. Most viral envelope proteins are matured by cellular endoprotease cleavage during their transport through the secretory pathway (32). It was therefore interesting to determine whether such a maturation also occurred for HCV envelope glycoproteins. As shown in Fig. 1, SDS-PAGE analyses showed no evidence of a protease cleavage in E1 or E2. HCVpp-associated E2 migrated more slowly than the cell-associated form, and the migration profiles of E1 were very similar for the HCVpp- and cell-associated forms (Fig. 1). In addition, after deglycosylation by PNGase F treatment, neither E1 nor E2 from HCVpp migrated faster than the deglycosylated cell-associated forms (Fig. 2A). However, we could not exclude a cleavage that would not be detected by SDS-PAGE. Indeed, some C-terminally truncated forms of E1 have been shown to migrate more slowly than expected due to abnormal behavior in SDS-PAGE (44). Therefore, we could not totally rule out a cleavage very close to the N or C terminus of E1 or E2. To exclude an N-terminal cleavage, HCV envelope glycoproteins associated with HCVpp were analyzed by Western blotting with antibodies recognizing an epitope located at the N terminus of E1 or E2. If a cleavage has occurred, the peptide should be separated from the rest of the protein when analyzed by SDS-PAGE, and such antibodies should not recognize the bulk of the protein in Western blotting. We used MAbs A4 and 9/27, which are directed against epitopes located at the N termini of E1 (amino acids 197 to 207) and E2 (amino acids 396 to 407), respectively. As shown in Fig. 3, an E2 protein of the appropriate size was detected by Western blotting with MAb 9/27, indicating that the N terminus of E2 had not been cleaved. As a control to confirm the absence of a partial cleavage, we used an antibody recognizing an epitope located elsewhere on E2 (MAb 3/11) and compared the amounts of protein recognized by the two antibodies. As shown in Fig. 3, there was no evidence of any relative decrease in the amount of E2 recognized by MAb 9/27, suggesting that no partial cleavage had occurred. Although 12 residues separate the N terminus of E2 from the epitope recognized by MAb 9/27, a cleavage within this 12-amino-acid hypervariable segment is unlikely. As shown in Fig. 3, an E1 protein of the appropriate size was also detected by Western blotting with MAb A4, indicating that the N terminus of E1 had not been cleaved. In the absence of an antibody recognizing an epitope located elsewhere on E1, we compared the E1/E2 ratios between cell lysates and purified pseudotyped particles to detect a partial cleavage. Similar ratios were observed (data not shown), suggesting that no partial cleavage had occurred at the N terminus of E1.

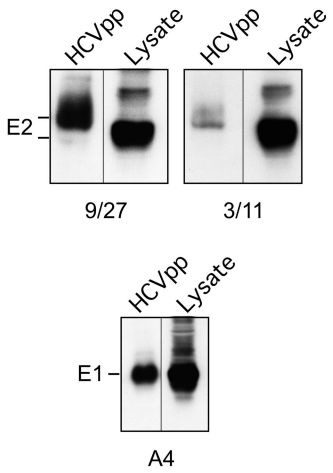

FIG. 3. Detection of the N termini of HCV envelope glycoproteins. HCVpp purified on a 20-to-60% sucrose gradient and lysates from cells expressing E1E2 were analyzed by Western blotting with MAbs recognizing an epitope located at the N terminus of E1 (MAb A4, recognizing amino acids 197 to 207) or E2 (MAb 9/27, recognizing amino acids 396 to 407). The anti-E2 MAb 3/11 was used as a control.

To exclude a C-terminal cleavage, HCV envelope glycoproteins were tagged with an HA epitope at their C termini, and HCV envelope glycoproteins associated with HCVpp were analyzed by immunoprecipitation with MAb H53 followed by Western blotting with the anti-HA antibody. Western blot analyses with anti-E1 and anti-E2 MAbs indicated that E1†HA and E2†HA were efficiently incorporated into pseudoparticles when these proteins were coexpressed with their wild-type partners (Fig. 4A). Similar results were obtained when HCVpp were purified on a sucrose density gradient (data not shown). The presence of the tag reduced the infectivity of the pseudoparticles to approximately 35% in the case of E2 and 4% in the case of E1 (Fig. 4B). Since the HA tag does not alter E1E2

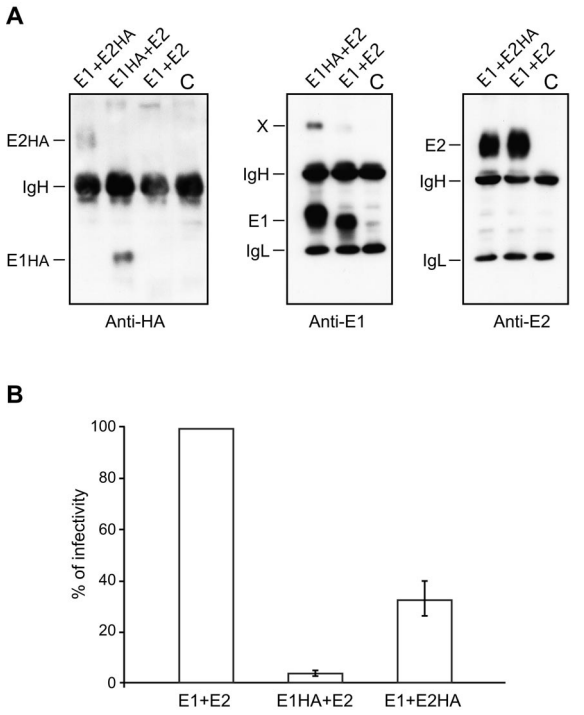

FIG. 4. Detection of the C termini of HCV envelope glycoproteins. (A) The envelope glycoproteins associated with HCVpp generated with either E1 plus E2, E1†HA plus E2, or E1 plus E2†HA or without envelope glycoproteins (control [C]) were analyzed by immunoprecipitation with MAb H53 followed by Western blotting with the anti-HA MAb 3F10, the anti-E1 MAb A4, or the anti-E2 MAb H52. Immunoglobulin light (IgL) and heavy (IgH) chains and SDS-resistant E1 oligomers (X) are indicated. (B) Infectivity of HCVpp containing HCV envelope proteins tagged at their C termini with an HA tag was evaluated by using the luciferase reporter gene. The infectivity of HCVpp containing E1†HA plus E2 or E1 plus E2†HA was compared to that of HCVpp containing wild-type HCV envelope glycoproteins (E1 plus E2).

assembly (13), it likely affects the function of the transmembrane domains of these proteins during the fusion process, as shown for some mutations introduced into the transmembrane domain of the G protein of vesicular stomatitis virus (VSV) (9). As shown in Fig. 4A, E1†HA and E2†HA were detected by Western blotting with the anti-HA antibody, indicating that these proteins had not been cleaved in their C-terminal regions.

Altogether, these results indicate that there is no evidence that HCV envelope glycoproteins incorporated into HCVpp are cleaved by a cellular endoprotease during their transport through the secretory pathway. However, we cannot totally exclude cleavage of a small proportion of HCV envelope proteins associated with HCVpp.

Epitope exposure on mature E1E2 glycoproteins. MAbs are potentially very useful tools for structure-function studies of HCV envelope proteins and for defining B-cell epitopes that might have some relevance for therapeutic and/or vaccine development. Large panels of MAbs recognizing HCV envelope glycoproteins have been generated. Since there is no efficient and reliable cell culture system to amplify HCV, these MAbs have been screened against recombinant HCV envelope glycoproteins for their selection, and most of them have also been generated by immunizing mice or rats with the same recombinant proteins. The relevance of these tools has therefore not been confirmed on proteins that are functional for virus entry. HCVpp make it possible for the first time to reanalyze such antibodies in the context of potentially fully functional HCV envelope glycoproteins.

The binding of a panel of anti-E2 MAbs that have been generated in several laboratories, including ours (8, 15, 24, 25, 28, 48), to HCV glycoproteins associated with HCVpp was analyzed by immunoprecipitation. Among these MAbs, 3/11, H35, and H48 have been shown to neutralize approximately 70% of HCVpp infectivity, and H54 and H60 have been shown to neutralize approximately 40% of HCVpp infectivity (4, 31). As shown in Fig. 5, all the conformation-dependent MAbs described previously (H2, H31, H33, H35, H44, H48, H50, H53, H54, H57, H60, H61, and 9/86a) recognized HCV envelope glycoproteins associated with HCVpp. MAb H14, which specifically recognizes aggregates of HCV envelope glycoproteins (8), did not immunoprecipitate HCVpp-associated proteins. Interestingly, a series of human MAbs (CBH2, CBH4B, CBH4D, CBH5, CBH7, and CBH17) also recognized HCV glycoproteins associated with HCVpp. It is worth noting that all the conformation-independent MAbs (H47, H52, 1/39, 3/11, 6/16, 6/53, and CBH17) recognized the envelope glycoproteins associated with HCVpp less efficiently than the majority of the conformation-dependent MAbs (H2, H31, H33, H35, H44, H48, H50, H53, H54, H57, H60, H61, CBH2, CBH4B, CBH4D, CBH5, and CBH7). This suggests that conformation-sensitive epitopes are better exposed on functional HCV envelope glycoproteins.

Since human MAbs have been obtained from B cells of HCV-infected patients, they represent antibodies that have been generated against native antigens in the course of a normal HCV infection. In the absence of an efficient cell culture system to amplify HCV, these antibodies therefore represent valuable tools for the characterization of HCV envelope glycoproteins. To further characterize these antibodies, we analyzed their capacities to neutralize HCVpp. As shown in Fig. 6, two antibodies (CBH5 and CBH7) significantly reduced the infectivity of HCVpp. The incompleteness of the neutralization is likely due to the heterogeneity of E2 glycosylation. Indeed, due to the high level of glycosylation of E2 (27), some modified glycans might potentially partially mask epitopes. However, the observation that CBH5 and CBH7 have a neutralizing activity provides additional support for the observation that HCVpp mimic the early steps of infection of native HCV.

Enveloped viruses enter cells through two main pathways: direct fusion at the plasma membrane and receptor-mediated endocytosis. In the latter case, the fusion of the viral envelope protein(s) is triggered by low pH within the endosome. It has been shown that the infectivity of HCVpp is pH dependent (5, 31). This suggests that changes in the conformation of HCV envelope proteins occur at low pH. We were thus interested in seeing whether such changes would be detectable with our MAbs. HCV envelope glycoproteins associated with HCVpp were exposed to a low pH before immunoprecipitation with our panel of anti-E2 MAbs. As shown in Fig. 5, some changes were observed. For most antibodies, there was a slight reduction in the signal of E2 after low-pH treatment. Interestingly,

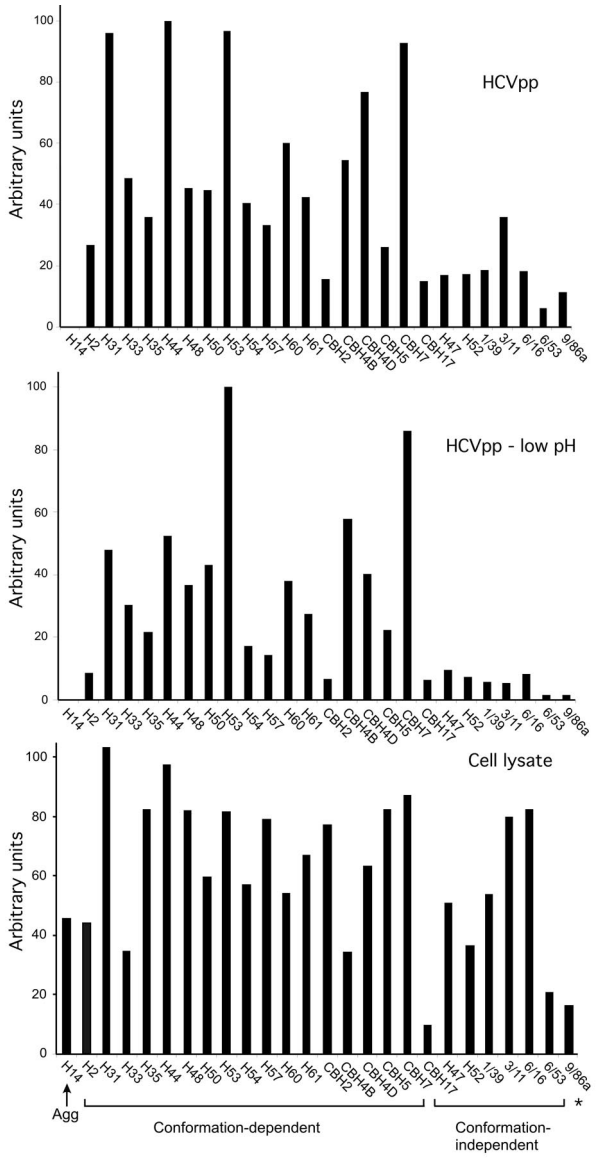

FIG. 5. Epitope exposure on HCV glycoproteins associated with HCVpp. 293T cells transfected to produce HCVpp were labeled for 24 h. Supernatants (HCVpp) and cell lysates were immunoprecipitated with anti-E2 MAbs. In some experiments, supernatants were treated with MES (pH 5.5) for 20 min at 37°C (HCVpp–low pH) and pH was neutralized before immunoprecipitation. The intensities of immunoprecipitated E2 were determined by phosphorimaging. MAbs are grouped as conformation dependent or conformation independent. The epitopes of the following antibodies have been identified: 6/16 (amino acids 384 to 395), 9/86a (amino acids 384 to 407), 3/11 (amino acids 412 to 423), 1/39 (amino acids 432 to 443), H47 (amino acids 448 to 463), 6/53 (amino acids 544 to 551), and H52 (amino acids 644 to 655). Although it recognizes HVR1, MAb 9/86a (asterisked at the bottom) is conformation dependent. H14 (Agg) recognizes aggregates of HCV envelope glycoproteins.

the signal was almost totally abolished for a series of conformation-independent MAbs (1/39, 3/11, 6/16, 6/53, and 9/86a). In addition to changes in the recognition of E2 epitopes, the low-pH treatment also induced some dissociation of E1E2 complex. As shown for MAb H50 as an example, the amount of E1 that coprecipitated with E2 was reduced after low-pH treatment (Fig. 7). The intensities of the bands were measured with a PhosphorImager, and the quantitative analysis of the bands indicated that approximately 75% of E1E2 complexes were dissociated. Together, these data indicate that conformational changes occur in E1E2 at low pH.

E1E2 heterodimer associated with HCVpp interacts with CD81. Human CD81 has been repeatedly shown to interact with recombinant soluble E2, E1E2 complex, HCV-like particles, and HCV particles from infectious plasma (7, 17, 25, 28, 34, 47, 50, 55, 60, 63). For the characterization of functional HCV envelope glycoproteins, it is therefore important to analyze their interaction with CD81. A CD81 pull-down assay was performed for this purpose. As shown in Fig. 8, both E1 and E2 from HCVpp were pulled down with human CD81 but not with the murine protein. These data confirm that the E1E2 heterodimer associated with HCVpp interacts with human CD81, which is in agreement with CD81 playing a role in HCV entry (4, 31).

DISCUSSION

HCV envelope glycoproteins in their mature form, as they are present at the surface of the particle, have never been characterized. Because HCVpp contain fully functional envelope glycoproteins, these envelope proteins, or at least a fraction of them, are supposed to be in a mature conformation similar to that present on native HCV particles. HCVpp therefore represent the best tool currently available to characterize functional HCV envelope glycoproteins. Here, we characterized HCVpp-associated envelope proteins with conformation-

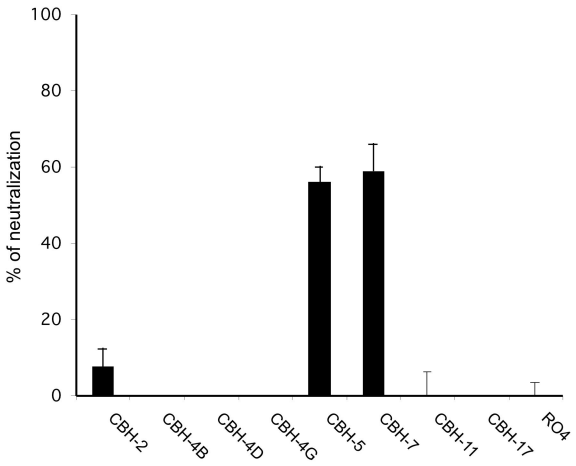

FIG. 6. Neutralization of HCVpp by human MAbs. HCVpp were preincubated, before infection of Huh-7 cells, with saturating concentrations (20 μg/ml)of human anti-E2 MAbs. The saturating concentrations were determined by analyzing the extent of neutralization of HCVpp with several concentrations of several neutralizing antibodies (data not shown). A negative-control experiment using a nonspecific human MAb (RO4) was performed. Control pseudotype particles produced in the absence of envelope proteins were used to establish the background level, which was always below 1% (data not shown). Pseudotype particles bearing VSV-G protein were used in a control neutralization experiment. No neutralization response was observed with these control particles (data not shown). Results, determined by measuring the luciferase activity, are expressed as percentages of neutralization.

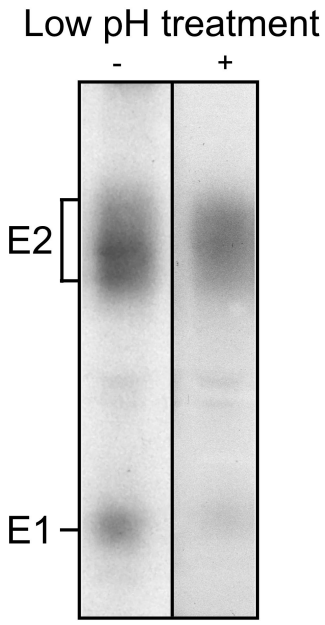

FIG. 7. Low-pH treatment dissociates E1E2 complexes. 293T cells transfected to produce HCVpp were labeled for 24 h. Supernatants, either left untreated or exposed to a low pH, were immunoprecipitated with the anti-E2 MAb H50. Immunoprecipitates were analyzed by SDS-PAGE.

dependent MAbs, some of which had been shown to have neutralizing activity against HCVpp. We showed that these HCV envelope glycoproteins associated with HCVpp formed a noncovalent E1E2 heterodimer containing complex or hybrid type glycans. In contrast to what has been observed for most viral envelope proteins, we did not detect any cleavage by a cellular endoprotease during their transport through the secretory pathway. In addition, HCV envelope glycoproteins were recognized by a large panel of MAbs and were shown to interact with CD81 and to be sensitive to low-pH treatment.

Noncovalent E1E2 heterodimers are associated with HCVpp. From sedimentation analyses, there is currently no evidence that larger oligomers of E1E2 are formed (A. Op De Beeck, unpublished data). Interestingly, some of the MAbs used to characterize the E1E2 complex show neutralizing activity (4; this work), indicating that they recognize fully functional envelope glycoproteins. Earlier studies of HCV envelope glycoproteins analyzed in transient expression systems have shown that these proteins can form noncovalent E1E2 heterodimers as well as heterogeneous disulfide-linked aggregates (19, 52). Extensive characterization of the noncovalent heterodimer with conformation-dependent MAbs has strongly suggested that this oligomer is most likely the prebudding form of the functional complex (15). We now show that the mature HCV glycoproteins form a complex showing some similarities to the prebudding E1E2 heterodimer. Very recently, a MAb produced by immunizing mice with an HCV antigen obtained from a chronically infected plasma has been shown to recognize disulfide-bound complexes that might potentially be formed of E1 and E2 (49). However, there is no evidence that the HCV envelope glycoproteins recognized by this MAb are associated with infectious particles, because there was no purification of viral particles either to generate the antibody or to analyze its reactivity. There is, therefore, no clear evidence that this MAb recognizes envelope glycoproteins that are functional in HCV entry.

HCV envelope glycoproteins E1 and E2 possess up to 6 and 11 potential glycosylation sites, respectively (27). It has been confirmed experimentally by site-directed mutagenesis that four of the five putative glycosylation sites of E1 of genotype 1a can be occupied by N-glycans (42). The glycosylation sites occupied in E2 have not been determined yet. However, expression of HCV glycoprotein E2 followed by deglycosylation indicates that a large number of glycosylation sites are occupied. Analyses of the glycans bound to the intracellular HCV envelope glycoprotein heterodimer have indicated that high-mannose type oligosaccharides are associated with these proteins (15, 22). A fraction of HCV envelope glycoproteins overexpressed in 293T cells has been shown to accumulate at the plasma membrane, where they are supposed to be incorporated into HCVpp (4, 16, 31). However, our results suggest that expression of E1 and E2 on the cell surface is likely due to the accumulation of small amounts of E1E2 proteins escaping the ER retention machinery. The envelope glycoproteins associated with HCVpp contain complex type glycans. These proteins have therefore been transported through the secretory pathway, and some of the N-linked glycans associated with them have been modified by Golgi enzymes. This is in agreement with lectin binding analyses of HCV particles isolated from infected patients which suggest that the envelope glycoproteins of HCV might contain complex type glycans (56). Although the glycans associated with E2 proteins incorporated into HCVpp are modified by Golgi enzymes, most E1 glycans remain high-mannose type glycans. Indeed, endo H treatment experiments suggest that E1 might have a single glycan of the

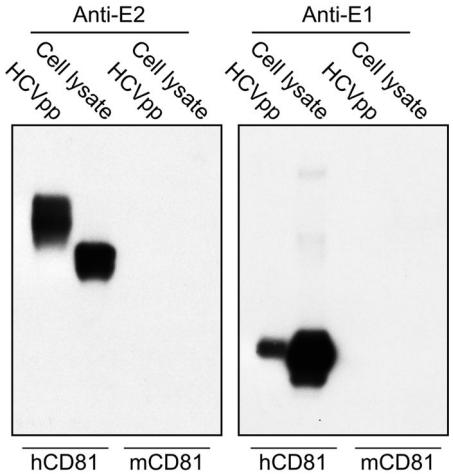

FIG. 8. E1E2 heterodimers associated with HCVpp interact with CD81. Supernatants (HCVpp) and cell lysates of 293T cells transfected to produce HCVpp were pulled down with a recombinant fusion protein containing the large extracellular loop of human CD81 (hCD81) or murine CD81 (mCD81) fused to glutathione *S*-transferase preadsorbed onto glutathione-Sepharose 4B beads. Precipitates were separated by SDS-PAGE followed by Western blot analysis with an anti-E1 (A4) or anti-E2 (3/11) MAb.

complex type. This conclusion contrasts with the previous observation that a C-terminally truncated form of E1 had all its glycans resistant to endo H treatment after secretion (44). These conflicting observations suggest that the presence of E2 masks the access of the Golgi enzymes to most E1 glycans. Alternatively, the truncated form of E1 might be more accessible to Golgi enzymes due to its misfolding. It has also been shown previously that different glycoforms of intracellular E1 can be observed (18). Here, we show that only the fully glycosylated form of E1 is incorporated into HCVpp.

The surface proteins of many enveloped viruses are initially synthesized as inactive precursors, and proteolytic cleavage is often required for maturation and full functional activity. In several virus families, this processing step is carried out by cellular proprotein convertases (32). In the case of the flaviviruses, the envelope contains two proteins, E and M. The latter is synthesized as a precursor called prM (29). Newly synthesized E and prM proteins associate to form heterodimers (2, 62) that are incorporated into immature virions by budding into the ER lumen (38). The particles are then transported through the secretory pathway, and shortly before release from the cell, they are converted to the active form by cleavage of prM by a cellular furin protease in the *trans*-Golgi network (58). Heterodimeric interactions between prM and E are important for proper folding of E (3, 33, 37) and probably also for protection of the immature virion against acid inactivation during transport through acidic vesicles (29). Such a maturation of the envelope glycoprotein complex is not observed for HCV. Whether modification of the glycans associated with HCV envelope proteins is responsible for the maturation of the fusion-competent E1E2 complex remains to be determined.

HCV envelope glycoproteins associated with HCVpp are sensitive to low-pH treatment. This finding is in agreement with the observation that HCVpp cell entry is pH dependent (5, 31). Conformational changes in viral fusion proteins are necessary for exposure of the fusion peptide, which interacts with the target membrane and thus initiates fusion. Interestingly, low-pH treatment induces dissociation of the E1E2 heterodimer. This is likely necessary to induce homo-oligomerization of the active form of the fusion protein, as shown for alphavirus envelope glycoproteins (61). There is, however, some controversy about the identity of the HCV fusion protein. It was first proposed that E1 might be a good candidate, because sequence analyses suggest that the ectodomain of E1 might contain a putative fusion peptide (26). However, potential structural homology with other fusion proteins from the same family, as well as with other type II fusion proteins, suggests that E2 should be the fusion protein (35, 64). Mutagenesis studies of the putative fusion peptides of the envelope glycoproteins associated with HCVpp, as described for the flavivirus envelope protein E (1), will be helpful for further characterization of the HCV fusion protein.

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REFERENCES

1. **Allison, S. L., J. Schalich, K. Stiasny, C. W. Mandl, and F. X. Heinz.** 2001. Mutational evidence for an internal fusion peptide in flavivirus envelope protein E. J. Virol. **75:**4268–4275.
2. **Allison, S. L., J. Schalich, K. Stiasny, C. W. Mandl, C. Kunz, and F. X. Heinz.** 1995. Oligomeric rearrangement of tick-borne encephalitis virus envelope proteins induced by an acidic pH. J. Virol. **69:**695–700.
3. **Allison, S. L., K. Stadler, C. W. Mandl, C. Kunz, and F. X. Heinz.** 1995. Synthesis and secretion of recombinant tick-borne encephalitis virus protein E in soluble and particulate form. J. Virol. **69:**5816–5820.
4. **Bartosch, B., J. Dubuisson, and F. L. Cosset.** 2003. Infectious hepatitis C pseudo-particles containing functional E1E2 envelope protein complexes. J. Exp. Med. **197:**633–642.
5. **Bartosch, B., A. Vitelli, C. Granier, C. Goujon, J. Dubuisson, S. Pascale, E. Scarselli, R. Cortese, A. Nicosia, and F. L. Cosset.** 2003. Cell entry of hepatitis C virus requires a set of co-receptors that include the CD81 tetraspanin and the SR-B1 scavenger receptor. J. Biol. Chem. **278:**41624–41630.
6. **Buonocore, L., K. J. Blight, C. M. Rice, and J. K. Rose.** 2002. Characterization of vesicular stomatitis virus recombinants that express and incorporate high levels of hepatitis C virus glycoproteins. J. Virol. **76:**6865–6872.
7. **Chan-Fook, C., W. R. Jiang, B. E. Clarke, N. Zitzmann, C. Maidens, J. A. McKeating, and I. M. Jones.** 2000. Hepatitis C virus glycoprotein E2 binding to CD81: the role of E1E2 cleavage and protein glycosylation in bioactivity. Virology **273:**60–66.
8. **Choukhi, A., A. Pillez, H. Drobecq, C. Sergheraert, C. Wychowski, and J. Dubuisson.** 1999. Characterization of aggregates of hepatitis C virus glycoproteins. J. Gen. Virol. **80:**3099–3107.
9. **Cleverley, D. Z., and J. Lenard.** 1998. The transmembrane domain in viral fusion: essential role for a conserved glycine residue in vesicular stomatitis virus G protein. Proc. Natl. Acad. Sci. USA **95:**3425–3430.
10. **Cocquerel, L., S. Duvet, J.-C. Meunier, A. Pillez, R. Cacan, C. Wychowski, and J. Dubuisson.** 1999. The transmembrane domain of hepatitis C virus glycoprotein E1 is a signal for static retention in the endoplasmic reticulum. J. Virol. **73:**2641–2649.
11. **Cocquerel, L., C.-C. Kuo, J. Dubuisson, and S. Levy.** 2003. CD81-dependent binding of hepatitis C virus E1E2 heterodimers. J. Virol. **77:**10677–10683.
12. **Cocquerel, L., J.-C. Meunier, A. Pillez, C. Wychowski, and J. Dubuisson.** 1998. A retention signal necessary and sufficient for endoplasmic reticulum localization maps to the transmembrane domain of hepatitis C virus glycoprotein E2. J. Virol. **72:**2183–2191.
13. **Cocquerel, L., A. Op de Beeck, M. Lambot, J. Roussel, D. Delgrange, A. Pillez, C. Wychowski, F. Penin, and J. Dubuisson.** 2002. Topologic changes in the transmembrane domains of hepatitis C virus envelope glycoproteins. EMBO J. **21:**2893–2902.
14. **Cocquerel, L., C. Wychowski, F. Minner, F. Penin, and J. Dubuisson.** 2000. Charged residues in the transmembrane domains of hepatitis C virus glycoproteins play a key role in the processing, subcellular localization and assembly of these envelope proteins. J. Virol. **74:**3623–3633.
15. **Deleersnyder, V., A. Pillez, C. Wychowski, K. Blight, J. Xu, Y. S. Hahn, C. M. Rice, and J. Dubuisson.** 1997. Formation of native hepatitis C virus glycoprotein complexes. J. Virol. **71:**697–704.
16. **Drummer, H. E., A. Maerz, and P. Poumbourios.** 2003. Cell surface expression of functional hepatitis C virus E1 and E2 glycoproteins. FEBS Lett. **546:**385–390.
17. **Drummer, H. E., K. A. Wilson, and P. Poumbourios.** 2002. Identification of the hepatitis C virus E2 glycoprotein binding site on the large extracellular loop of CD81. J. Virol. **76:**11143–11147.
18. **Dubuisson, J., S. Duvet, J. C. Meunier, A. Op De Beeck, R. Cacan, C. Wychowski, and L. Cocquerel.** 2000. Glycosylation of the hepatitis C virus envelope protein E1 is dependent on the presence of a downstream sequence on the viral polyprotein. J. Biol. Chem. **275:**30605–30609.
19. **Dubuisson, J., H. H. Hsu, R. C. Cheung, H. B. Greenberg, D. G. Russell, and C. M. Rice.** 1994. Formation and intracellular localization of hepatitis C virus envelope glycoprotein complexes expressed by recombinant vaccinia and Sindbis viruses. J. Virol. **68:**6147–6160.
20. **Dubuisson, J., F. Penin, and D. Moradpour.** 2002. Interaction of hepatitis C virus proteins with host cell membranes and lipids. Trends Cell Biol. **12:**517–523.
21. **Dubuisson, J., and C. M. Rice.** 1996. Hepatitis C virus glycoprotein folding: disulfide bond formation and association with calnexin. J. Virol. **70:**778–786.
22. **Duvet, S., L. Cocquerel, A. Pillez, R. Cacan, A. Verbert, D. Moradpour, C. Wychowski, and J. Dubuisson.** 1998. Hepatitis C virus glycoprotein complex localization in the endoplasmic reticulum involves a determinant for retention and not retrieval. J. Biol. Chem. **273:**32088–32095.
23. **Feinstone, S., H. J. Alter, H. P. Dienes, Y. Shimizu, H. Popper, D. Blackmore, D. Sly, W. T. London, and R. H. Purcell.** 1981. Non-A, non-B hepatitis in chimpanzees and marmosets. J. Infect. Dis. **144:**588–598.
24. **Flint, M., J. Dubuisson, C. Maidens, R. Harrop, G. R. Guile, P. Borrow, and**

J. A. McKeating. 2000. Functional characterization of intracellular and secreted forms of a truncated hepatitis C virus E2 glycoprotein. J. Virol. **74:**702–709.
25. **Flint, M., C. Maidens, L. D. Loomis-Price, C. Shotton, J. Dubuisson, P. Monk, A. Higginbottom, S. Levy, and J. A. McKeating.** 1999. Characterization of hepatitis C virus E2 glycoprotein interaction with a putative cellular receptor, CD81. J. Virol. **73:**6235–6244.
26. **Flint, M., J. M. Thomas, C. M. Maidens, C. Shotton, S. Levy, W. S. Barclay, and J. A. McKeating.** 1999. Functional analysis of cell surface-expressed hepatitis C virus E2 glycoprotein. J. Virol. **73:**6782–6790.
27. **Goffard, A., and J. Dubuisson.** 2003. Glycosylation of hepatitis C virus envelope proteins. Biochimie **85:**295–301.
28. **Hadlock, K. G., R. E. Lanford, S. Perkins, J. Rowe, Q. Yang, S. Levy, P. Pileri, S. Abrignani, and S. K. Foung.** 2000. Human monoclonal antibodies that inhibit binding of hepatitis C virus E2 protein to CD81 and recognize conserved conformational epitopes. J. Virol. **74:**10407–10416.
29. **Heinz, F. X., and S. L. Allison.** 2000. Structures and mechanisms in flavivirus fusion. Adv. Virus Res. **55:**231–269.
30. **Hirani, S., R. J. Bernasconi, and J. R. Rasmussen.** 1987. Use of *N*-glycanase to release asparagine-linked oligosaccharides for structural analysis. Anal. Biochem. **162:**485–492.
31. **Hsu, M., J. Zhang, M. Flint, C. Logvinoff, C. Cheng-Mayer, C. M. Rice, and J. A. McKeating.** 2003. Hepatitis C virus glycoproteins mediate pH-dependent cell entry of pseudotyped retroviral particles. Proc. Natl. Acad. Sci. USA **100:**7271–7276.
32. **Klenk, H. D., and W. Garten.** 1994. Activation cleavage of viral spike proteins by host proteases, p. 241–280. *In* E. Wimmer (ed.), Cellular receptors for animal viruses. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
33. **Konishi, E., and P. W. Mason.** 1993. Proper maturation of the Japanese encephalitis virus envelope glycoprotein requires cosynthesis with the premembrane protein. J. Virol. **67:**1672–1675.
34. **Lambot, M., S. Fretier, A. Op De Beeck, B. Quatannens, S. Lestavel, V. Clavey, and J. Dubuisson.** 2002. Reconstitution of hepatitis C virus envelope glycoproteins into liposomes as a surrogate model to study virus attachment. J. Biol. Chem. **277:**20625–20630.
35. **Lescar, J., A. Roussel, M. W. Wien, J. Navaza, S. D. Fuller, G. Wengler, and F. A. Rey.** 2001. The fusion glycoprotein shell of Semliki Forest virus: an icosahedral assembly primed for fusogenic activation at endosomal pH. Cell **105:**137–148.
36. **Lindenbach, B. D., and C. M. Rice.** 2001. *Flaviviridae*: the viruses and their replication, p. 991–1042. *In* D. M. Knipe, P. M. Howley, D. E. Griffin, R. A. Lamb, M. A. Martin, B. Roizman, and S. E. Straus (ed.), Fields virology, 4th ed. Lippincott Williams & Wilkins, Philadelphia, Pa.
37. **Lorenz, I. C., S. L. Allison, F. X. Heinz, and A. Helenius.** 2002. Folding and dimerization of tick-borne encephalitis virus envelope proteins prM and E in the endoplasmic reticulum. J. Virol. **76:**5480–5491.
38. **Mackenzie, J. M., and E. G. Westaway.** 2001. Assembly and maturation of the flavivirus Kunjin virus appear to occur in the rough endoplasmic reticulum and along the secretory pathway, respectively. J. Virol. **75:**10787–10799.
39. **Major, M. E., B. Rehermann, and S. M. Feinstone.** 2001. Hepatitis C viruses, p. 1127–1162. *In* D. M. Knipe, P. M. Howley, D. E. Griffin, R. A. Lamb, M. A. Martin, B. Roizman, and S. E. Straus (ed.), Fields virology, 4th ed. Lippincott Williams & Wilkins, Philadelphia, Pa.
40. **Matsuura, Y., H. Tani, K. Suzuki, T. Kimura-Someya, R. Suzuki, H. Aizaki, K. Ishii, K. Moriishi, C. S. Robison, M. A. Whitt, and T. Miyamura.** 2001. Characterization of pseudotype VSV possessing HCV envelope proteins. Virology **286:**263–275.
41. **McLauchlan, J., M. K. Lemberg, R. G. Hope, and B. Martoglio.** 2002. Intramembrane proteolysis promotes trafficking of hepatitis C virus core protein to lipid droplets. EMBO J. **21:**3980–3988.
42. **Meunier, J.-C., A. Fournillier, A. Choukhi, A. Cahour, L. Cocquerel, J. Dubuisson, and C. Wychowski.** 1999. Analysis of the glycosylation sites of hepatitis C virus (HCV) glycoprotein E1 and the influence of E1 glycans on the formation of the HCV glycoprotein complex. J. Gen. Virol. **80:**887–896.
43. **Meyer, K., A. Basu, C. T. Przysiecki, L. M. Lagging, A. M. Di Bisceglie, A. J. Conley, and R. Ray.** 2002. Complement-mediated enhancement of antibody function for neutralization of pseudotype virus containing hepatitis C virus E2 chimeric glycoprotein. J. Virol. **76:**2150–2158.
44. **Michalak, J.-P., C. Wychowski, A. Choukhi, J.-C. Meunier, S. Ung, C. M. Rice, and J. Dubuisson.** 1997. Characterization of truncated forms of hepatitis C virus glycoproteins. J. Gen. Virol. **78:**2299–2306.
45. **Nakabayashi, H., K. Taketa, K. Miyano, T. Yamane, and J. Sato.** 1982. Growth of human hepatoma cell lines with differentiated functions in chemically defined medium. Cancer Res. **42:**3858–3863.
46. **Op De Beeck, A., R. Montserret, S. Duvet, L. Cocquerel, R. Cacan, B. Barberot, M. Le Maire, F. Penin, and J. Dubuisson.** 2000. Role of the transmembrane domains of hepatitis C virus envelope proteins E1 and E2 in the assembly of the noncovalent E1E2 heterodimer. J. Biol. Chem. **275:**31428–31437.
47. **Owsianka, A., R. F. Clayton, L. D. Loomis-Price, J. A. McKeating, and A. H. Patel.** 2001. Functional analysis of hepatitis C virus E2 glycoproteins and virus-like particles reveals structural dissimilarities between different forms of E2. J. Gen. Virol. **82:**1877–1883.
48. **Patel, A. H., J. Wood, F. Penin, J. Dubuisson, and J. A. McKeating.** 2000. Construction and characterization of chimeric hepatitis C virus E2 glycoproteins: analysis of regions critical for glycoprotein aggregation and CD81 binding. J. Gen. Virol. **81:**2873–2883.
49. **Petit, M. A., C. Jolivet-Reynaud, E. Peronnet, Y. Michal, and C. Trepo.** 2003. Mapping of a conformational epitope shared between E1 and E2 on the serum-derived human hepatitis C virus envelope. J. Biol. Chem. **278:**44385–44392.
50. **Pileri, P., Y. Uematsu, S. Campagnoli, G. Galli, F. Falugi, R. Petracca, A. J. Weiner, M. Houghton, D. Rosa, G. Grandi, and S. Abrignani.** 1998. Binding of hepatitis C virus to CD81. Science **282:**938–941.
51. **Pohlmann, S., J. Zhang, F. Baribaud, Z. Chen, G. J. Leslie, G. Lin, A. Granelli-Piperno, R. W. Doms, C. M. Rice, and J. A. McKeating.** 2003. Hepatitis C virus glycoproteins interact with DC-SIGN and DC-SIGNR. J. Virol. **77:**4070–4080.
52. **Ralston, R., K. Thudium, K. Berger, C. Kuo, B. Gervase, J. Hall, M. Selby, G. Kuo, M. Houghton, and Q.-L. Choo.** 1993. Characterization of hepatitis C virus envelope glycoprotein complexes expressed by recombinant vaccinia viruses. J. Virol. **67:**6753–6761.
53. **Robbins, P. W., R. B. Timble, D. F. Wirth, C. Hering, F. Maley, G. F. Maley, R. Das, B. W. Gibson, N. Royal, and K. Biemann.** 1984. Primary structure of the *Streptomyces* enzyme endo-β-*N*-acetylglucosaminidase H. J. Biol. Chem. **259:**7577–7583.
54. **Robertson, B., G. Myers, C. Howard, T. Brettin, J. Bukh, B. Gaschen, T. Gojobori, G. Maertens, M. Mizokami, O. Nainan, S. Netesov, K. Nishioka, T. Shini, P. Simmonds, D. Smith, L. Stuyver, and A. Weiner.** 1998. Classification, nomenclature, and database development for hepatitis C virus (HCV) and related viruses: proposals for standardization. International Committee on Virus Taxonomy. Arch. Virol. **143:**2493–2503.
55. **Roccasecca, R., H. Ansuini, A. Vitelli, A. Meola, E. Scarselli, S. Acali, M. Pezzanera, B. B. Ercole, J. McKeating, A. Yagnik, A. Lahm, A. Tramontano, R. Cortese, and A. Nicosia.** 2003. Binding of the hepatitis C virus E2 glycoprotein to CD81 is strain specific and is modulated by a complex interplay between hypervariable regions 1 and 2. J. Virol. **77:**1856–1867.
56. **Sato, K., H. Okamoto, S. Aihara, Y. Hoshi, T. Tanaka, and S. Mishiro.** 1993. Demonstration of sugar moiety on the surface of hepatitis C virions recovered from the circulation of infected humans. Virology **196:**354–357.
57. **Siler, C. A., J. P. McGettigan, B. Dietzschold, S. K. Herrine, J. Dubuisson, R. J. Pomerantz, and M. J. Schnell.** 2002. Live and killed rhabdovirus-based vectors as potential hepatitis C vaccines. Virology **292:**24–34.
58. **Stadler, K., S. L. Allison, J. Schalich, and F. X. Heinz.** 1997. Proteolytic activation of tick-borne encephalitis virus by furin. J. Virol. **71:**8475–8481.
59. **Takikawa, S., K. Ishii, H. Aizaki, T. Suzuki, H. Asakura, Y. Matsuura, and T. Miyamura.** 2000. Cell fusion activity of hepatitis C virus envelope proteins. J. Virol. **74:**5066–5074.
60. **Triyatni, M., B. Saunier, P. Maruvada, A. R. Davis, L. Ulianich, T. Heller, A. Patel, L. D. Kohn, and T. J. Liang.** 2002. Interaction of hepatitis C virus-like particles and cells: a model system for studying viral binding and entry. J. Virol. **76:**9335–9344.
61. **Wahlberg, J. M., and H. Garoff.** 1992. Membrane fusion process of Semliki Forest virus. I. Low pH-induced rearrangement in spike protein quaternary structure precedes virus penetration into cells. J. Cell Biol. **116:**339–348.
62. **Wengler, G., and G. Wengler.** 1989. Cell-associated West Nile flavivirus is covered with E+pre-M protein heterodimers which are destroyed and reorganized by proteolytic cleavage during virus release. J. Virol. **63:**2521–2526.
63. **Wunschmann, S., J. D. Medh, D. Klinzmann, W. N. Schmidt, and J. T. Stapleton.** 2000. Characterization of hepatitis C virus (HCV) and HCV E2 interactions with CD81 and the low-density lipoprotein receptor. J. Virol. **74:**10055–10062.
64. **Yagnik, A. T., A. Lahm, A. Meola, R. M. Roccasecca, B. B. Ercole, A. Nicosia, and A. Tramontano.** 2000. A model for the hepatitis C virus envelope glycoprotein E2. Proteins **40:**355–366.