

## Formation of Native Hepatitis C Virus Glycoprotein Complexes

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**The hepatitis C virus (HCV) glycoproteins (E1 and E2) interact to form a heterodimeric complex, which has been proposed as a functional subunit of the HCV virion envelope. As examined in cell culture transient-expression assays, the formation of properly folded, noncovalently associated E1E2 complexes is a slow and inefficient process. Due to lack of appropriate immunological reagents, it has been difficult to distinguish between glycoprotein molecules that undergo productive folding and assembly from those which follow a nonproductive pathway leading to misfolding and aggregation. Here we report the isolation and characterization of a conformation-sensitive E2-reactive monoclonal antibody (H2). The H2 monoclonal antibody selectively recognizes slowly maturing E1E2 heterodimers which are noncovalently linked, protease resistant, and no longer associated with the endoplasmic reticulum chaperone calnexin. This complex probably represents the native prebudding form of the HCV glycoprotein heterodimer. Besides providing a novel reagent for basic studies on HCV virion assembly and entry, this monoclonal antibody should be useful for optimizing production and isolation of native HCV glycoprotein complexes for serodiagnostic and vaccine applications.**

Hepatitis C virus (HCV) is the major causative agent of non-A, non-B hepatitis. HCV, which is classified in the *Flaviviridae* family (9), is an enveloped virus with a positive-stranded RNA genome of approximately 9,500 nucleotides. Its genome contains highly conserved 5' (15) and 3' (23, 39) noncoding regions. The 5' noncoding region is followed by a long open reading frame of 9,030 to 9,099 nucleotides that is translated into a single polyprotein of 3,010 to 3,033 amino acids (28). This polyprotein is processed by a combination of host and viral proteinases to produce at least 10 distinct products: NH<sub>2</sub>-C-E1-E2-p7-NS2-NS3-NS4A-NS4B-NS5A-NS5B-COOH (reviewed in reference 35). Polyprotein cleavages in the structural region (C/E1, E1/E2, and E2/p7 and p7/NS2) are catalyzed by a host signal peptidase localized in the endoplasmic reticulum (ER) (17, 25). Cleavage at the NS2/NS3 site is mediated by an HCV-encoded proteinase which encompasses the NS2 region and the NS3 serine proteinase (12, 18). Cleavages at the C/E1, E1/E2 and NS2/NS3 sites are cotranslational, whereas those at the E2/p7 and p7/NS2 occur posttranslationally and generate two precursors for E2: E2-NS2 and E2-p7 (13, 25, 30, 36).

The low levels of HCV particles in patient samples and lack of a cell culture system supporting efficient HCV replication or particle assembly have hampered the characterization of virion glycoprotein complexes. Our current understanding of HCV biogenesis is based on cell culture transient-expression assays with viral and nonviral expression vectors. These studies have shown that the HCV E1 and E2 glycoproteins interact to form complexes, which have been proposed as functional subunits of the HCV virus particle (5, 13, 24, 34). In the presence of nonionic detergents, two forms of E1E2 complexes are detected: an E1E2 heterodimer stabilized by noncovalent inter-

actions and heterogeneous disulfide-linked aggregates, which most probably represent misfolded complexes. Kinetics studies indicate that glycoprotein folding and the formation of noncovalently associated E1E2 complexes are slow and inefficient (5, 6). Folding and heterodimer formation occur in association with the ER chaperone calnexin, and assembled glycoprotein complexes appear to be retained in a pre-*trans*-Golgi compartment (5, 6).

To date, glycoprotein-specific antibodies used for these studies appear to recognize conformation-independent epitopes or a mixture of conformation-dependent and -independent epitopes. For this reason, it has been difficult to distinguish between the productive folding pathway, leading to the formation of mature complexes, and the nonproductive pathway, leading to aggregation and misfolding (5, 6). In this study, we produced an E2-reactive monoclonal antibody (MAB) which specifically reacts with noncovalently associated HCV glycoproteins but not large aggregates. Extensive characterization of the complex recognized by this MAB suggests that it may represent the prebudding form of the HCV virion glycoprotein heterodimer.

### MATERIALS AND METHODS

**Plasmids.** Plasmids for expression of HCV-H strain cDNA segments were constructed as follows. pTM3/HCV1-745 (C-E1-E2) was assembled by ligation of three fragments, one derived from pTM3/HCV1C-966 (*NcoI-StuI* [26]) and two derived from pTM3/HCV364-1207.A746.X (*NcoI-HindIII*, *AscI-StuI* [26]). Prior to ligation, the *HindIII* site was filled in with T4 DNA polymerase. The construction of pTM3/HCV1-809 (C-E1-E2-p7) involved several steps including the introduction of tandem TAA and TAG termination codons immediately following the C terminus of p7 (residue 809) by PCR. The region amplified by PCR was checked by sequence analysis. pSINrep5/HCV171-383 (E1), which contains an N-terminal signal sequence (residues 171 to 191) preceding E1 (residues 192 to 383), was constructed by ligating appropriate fragments from pSINrep5/HCVsigE1-myc (*XhoI-SsrI* [27]), and pSINrep5 (*XbaI-SsrI* [3]). pSINrep5/HCV370-745 (E2) was assembled by ligation of appropriate fragments from pTM3/HCV364-1207.A746.X (*AscI-HindIII*) and pSINrep5/HCV370-809 (*AscI-StuI* [42]). Prior to ligation the *HindIII* site was filled in with T4 DNA polymerase.

**Cells and viruses.** The CV-1, HepG2, and BHK-21 cell lines were obtained from the American Type Culture Collection, Rockville, Md. CV-1 and HepG2 cells were grown in Dulbecco's modified essential medium supplemented with 10% fetal bovine serum. BHK-21 cell monolayers were grown in minimal essen-

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tial medium supplemented with 5% fetal bovine serum and 10% tryptose phosphate broth.

Stocks of vTF7-3, a vaccinia virus recombinant expressing the T7 DNA-dependent RNA polymerase (10), and vaccinia virus-HCV recombinants (vHCV1-745, vHCV1-809, vHCV1-1488, and vHCV1-3011) containing sequences of the HCV-H strain (13, 25; also see above) were grown in CV-1 monolayers and partially purified (22), and titers of infectious progeny were determined by plaque assay on CV-1 cells.

Recombinant Sindbis viruses expressing the E1 (SINrep5/HCV171-383) or E2 (SINrep5/HCV370-745) glycoprotein with the respective signal sequences were generated in BHK-21 cells as described previously (3).

**Antibodies.** Anti-E1 A4 and anti-E2 A11 MAbs have been described previously (5). To produce other anti-HCV glycoprotein MAbs, HepG2 cells coinfecting with vTF7-3 and vHCV1-1488 were lysed in 0.5% Nonidet P-40 (NP-40) in Tris-buffered saline (TBS; 20 mM Tris-Cl [pH 7.4], 137 mM NaCl, 2mM EDTA). HCV glycoprotein complexes were sedimented through a sucrose gradient as described below. Fractions containing E1E2 heterodimers (5) were pooled, and HCV glycoprotein complexes were purified by immunoaffinity on protein A-Sepharose (Pharmacia-LKB) with anti-E1 MAb A4. HCV-glycoprotein complexes bound to the immunobeads were injected into BALB/c mice to produce HCV-specific, antibody-secreting hybridomas as described previously (16). Screening was performed in 96-well plates containing HepG2 cells that had been coinfecting with vTF7-3 and vHCV1-1488, fixed with paraformaldehyde, and permeabilized with Triton X-100 as described previously (5). Concentrated MAbs were produced *in vitro* by using a MiniPerm apparatus (Heraeus) as recommended by the manufacturer. The Myc1-9E10.2 hybridoma cell line (MAb c-myc) was obtained from the American Type Culture Collection (ATCC CRL-1729). Anti-calnexin MAb AF8 was kindly provided by M. B. Brenner (21), a rabbit antiserum to protein disulfide isomerase (PDI) was purchased from Stress-Gen.

**Metabolic labeling and immunoprecipitation.** Cells were infected and metabolically labeled with  $^{35}\text{S}$ -Translabel (ICN) as previously described (5, 6). These were lysed with 0.5% NP-40 in 10 mM Tris-Cl (pH 7.5)-150 mM NaCl-2 mM EDTA. Iodoacetamide (20 mM) was included in the lysis buffer for experiments in which disulfide bond formation was assayed. Immunoprecipitations were carried out as described previously (6). For quantitative experiments, gels were analyzed with a PhosphorImager (Molecular Dynamics).

**Sedimentation through sucrose gradients.** Labeled cell lysates were layered on a 10-ml gradient of 5 to 20% sucrose in TBS containing 0.1% NP-40. Following centrifugation at 4°C for 24 h at 36,000 rpm in a Beckman SW41 rotor, 11 fractions were collected from the bottom of the gradient and analyzed by immunoprecipitation as described above. Molecular mass markers (Combithek, calibration protein I; Boehringer-Mannheim) were sedimented in a parallel sucrose gradient.

**Western blotting.** Proteins bound to nitrocellulose membranes (Hybond-ECL; Amersham) were analyzed by enhanced chemiluminescence detection (Amersham) as recommended by the manufacturer. Briefly, after separation by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), proteins were transferred to nitrocellulose membranes by using a Trans-Blot apparatus (Bio-Rad) and revealed with specific MAbs (diluted 1/2,000) followed by goat anti-mouse immunoglobulin conjugated to horseradish peroxidase (diluted 1/1,000; DAKO).

**Immunolabeling.** HepG2 cells coinfecting with vTF7-3 and vaccinia virus-HCV recombinants were fixed 15 h postinfection in picric acid-formaldehyde, embedded in Tissue Tek (Miles Laboratories), and snap frozen in liquid nitrogen and 2- to 3- $\mu\text{m}$  sections were cut with a cryostat (33). Double-immunofluorescent staining was performed by overnight incubation at 4°C with rabbit antiserum to PDI (diluted 1/200) and MAb H2 (diluted 1/400), followed by incubation for 1 h at room temperature with donkey anti-mouse (fluorescein isothiocyanate) and anti-rabbit (tetramethylrhodamine-5-isothiocyanate) immunoglobulins (diluted 1/100; Jackson Immunoresearch). For immunoelectron microscopy, HepG2 cells coinfecting with vTF7-3 and vaccinia virus-HCV recombinants were fixed for 30 min at 4°C with 4% paraformaldehyde and permeabilized in phosphate-buffered saline (PBS) containing 0.025% Triton X-100. Preembedding immunoperoxidase staining was performed by incubation for 4 h at 4°C with MAb H2 (diluted 1/2,000) followed by incubation for 1 h at 4°C with peroxidase-conjugated rabbit anti-mouse immunoglobulins (DAKO; diluted 1/200) and revealed with 3,3'-diaminobenzidine. The cells were then fixed in 1% osmium tetroxide and prepared for electron microscopy as described previously (33).

**Serum immunoprecipitation and quantitative-competitive reverse transcription-PCR.** The H77 patient serum, containing HCV-H subtype 1a, has been previously described (37) and was used in these experiments. Initially, HCV-H H77 serum was diluted to  $10^5$  RNA molecules/ml in 0.2% bovine serum albumin (BSA) in PBS. The diluted virus (300  $\mu\text{l}$ ) was preincubated with 3  $\mu\text{g}$  of MAb c-myc for 2 h at 4°C with continuous rocking. Nonspecific antibody complexes were pelleted at  $500 \times g$  for 10 s after incubation for 1 h at 4°C with 30  $\mu\text{l}$  of protein A-agarose (Sigma). A 100- $\mu\text{l}$  sample of the resultant supernatant was incubated with 3  $\mu\text{g}$  of either MAb c-myc or MAb H2 for 2 h at 4°C with rocking and then incubated with protein A-agarose and pelleted as above. The pellet was washed extensively in 0.2% BSA in PBS prior to the addition of 100  $\mu\text{l}$  of 0.2% BSA in PBS containing 20  $\mu\text{g}$  of tRNA, 1% SDS, 50 mM EDTA, and 50  $\mu\text{g}$  of proteinase K per ml. Following incubation at 37°C for 1 h, the RNA was

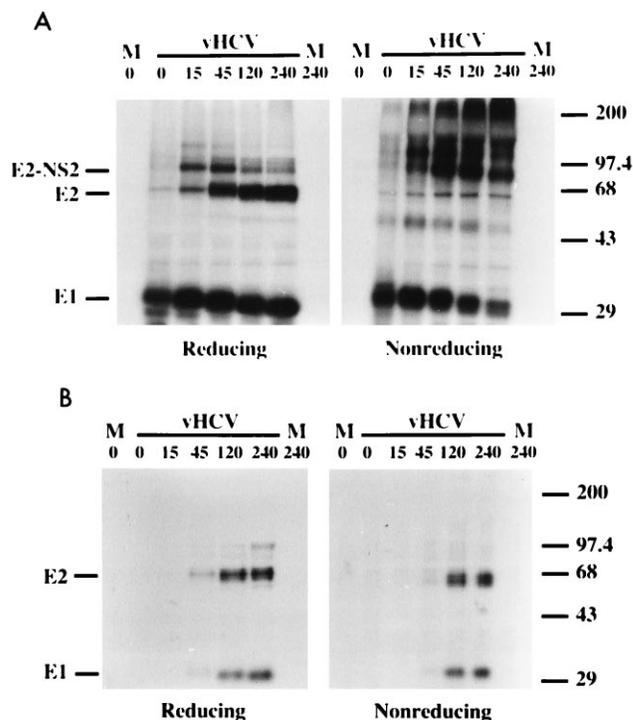


FIG. 1. Kinetics of E1 and E2 glycoprotein precipitation by MAb A4 (A) and H2 (B). HepG2 cells infected with vTF7-3 alone (lanes M) or coinfecting with vTF7-3 plus vHCV1-1488 (lanes vHCV) were pulse-labeled for 5 min and chased for the indicated times (in minutes). After immunoprecipitation, samples were separated by SDS-PAGE (10% polyacrylamide) under reducing or nonreducing conditions. HCV-specific proteins are indicated on the left. Sizes (in kilodaltons) of protein molecular mass markers are indicated on the right.

extracted with phenol-chloroform and ethanol precipitated and the final RNA pellet was resuspended in 10  $\mu\text{l}$  of  $\text{H}_2\text{O}$ . RNA was also extracted from the supernatant fraction.

RNA was analyzed by quantitative-competitive reverse transcription (RT)-PCR with primers designed to amplify a region of the HCV RNA genome upstream of the poly(U)-polypyrimidine tract (23). Briefly, 1  $\mu\text{l}$  of RNA was mixed with 0 to  $10^4$  molecules of competitor RNA, containing a 29-bp deletion (23), and cDNA was synthesized at 43°C for 1 h in a 5- $\mu\text{l}$  reaction mixture containing 50 mM Tris-Cl (pH 8.3), 75 mM KCl, 3 mM  $\text{MgCl}_2$ , 10 mM dithiothreitol, 0.5 mM deoxynucleoside triphosphates, 5 pmol of primer (no. 288), 4 U of RNasin, and 20 U of Superscript II (Gibco/BRL). Samples were then heated to 96°C for 10 min prior to the addition of 45  $\mu\text{l}$  of a PCR mix containing 25 pmol of the desired primers (no. 228 and 288), 50 mM Tris-Cl (pH 9.2), 2.5 mM  $\text{MgCl}_2$ , 16 mM  $(\text{NH}_4)_2\text{SO}_4$ , 150  $\mu\text{g}$  of BSA per ml, and 0.2  $\mu\text{l}$  of KlenTaqLA (from Wayne Barnes, Washington University). After 35 cycles of amplification (15 s at 95°C, 30 s at 60°C, and 15 s at 72°C), a nested PCR step (20 cycles) was performed under the same conditions with 25 pmol of an internal primer pair (no. 289 and 290) and 1  $\mu\text{l}$  of the first amplification product.

## RESULTS

**Identification of a MAb which selectively recognizes noncovalently associated HCV glycoproteins.** To isolate MAbs which might selectively recognize properly folded and assembled E1E2 complexes, E1E2 heterodimers were purified by sedimentation in a sucrose gradient to remove most of the aggregates and injected into BALB/c mice for MAb production. In pulse-chase experiments, one MAb (H2) showed a different pattern of immunoprecipitation compared to previously described MAbs (Fig. 1) (5). The HCV glycoproteins precipitated by MAb H2 were not detected until 45 min of chase, and their intensity increased during the chase (Fig. 1B). The delay in detection of proteins precipitated by MAb H2 suggested that the formation of the epitope recognized by MAb H2

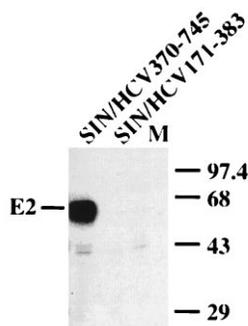


FIG. 2. Identification of the glycoprotein recognized by MAb H2. BHK-21 cells infected with SINrep5/HCV171-383 (E1) or SINrep5/HCV370-745 (E2) or mock infected (M) were labeled from 4 to 8 h postinfection. After immunoprecipitation by MAb H2, samples were separated by SDS-PAGE (10% polyacrylamide). Sizes (in kilodaltons) of protein molecular mass markers are indicated on the right.

depends on the folding of E1 and/or E2. For MAb H2, the pattern was similar under nonreducing and reducing conditions (Fig. 1B). In contrast, under nonreducing conditions, disulfide-linked complexes and/or aggregates were detected in the upper part of the gel for MAb A4 (Fig. 1A). When HCV glycoproteins were expressed separately, MAb H2 was shown to recognize E2 but not E1 (Fig. 2) or the E2 precursor, E2-NS2 (Fig. 1). However, the kinetics of precipitation of E1 and E2 by MAb H2 were very similar when these proteins were coexpressed (Fig. 1B; see below). This indicates that even if MAb H2 is E2 specific, it precipitates mostly E1E2 complexes when the two proteins are coexpressed. MAb H2 did not recognize denatured E2 by Western blot analysis (data not shown). Collectively, these results indicate that MAb H2 recognizes an epitope present on E2 and specifically precipitates noncovalently associated HCV glycoproteins which may represent the native complex.

**Properties of HCV glycoprotein complexes precipitated by MAb H2.** We previously showed that HCV glycoproteins associate rapidly with calnexin but dissociate slowly (6). Calnexin is a lectin-like chaperone of the ER which associates transiently with numerous newly synthesized polypeptides and folding intermediates of secretory glycoproteins during their

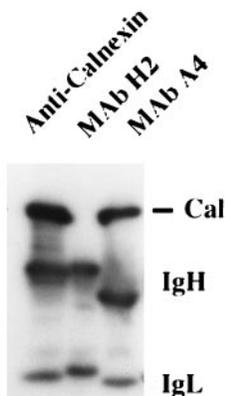


FIG. 3. Association of HCV glycoproteins with calnexin. HepG2 cells coinfecting with vTF7-3 and vHCV1-1488 were lysed with 1% digitonin in TBS and used for immunoprecipitation with anti-HCV MAbs (A4 and H2) or anti-calnexin MAb. Immunoprecipitates were revealed by Western blotting with anti-calnexin MAb. Cal, calnexin; IgH and IgL, immunoglobulin heavy and light chains, respectively.

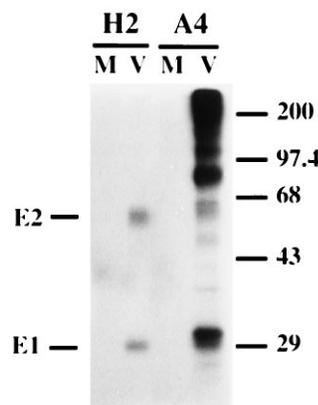


FIG. 4. Oxidation of E1 glycoprotein. HepG2 cells infected with vTF7-3 alone (lanes M) or coinfecting with vTF7-3 and vHCV1-1488 (lanes V) were pulse-labeled for 5 min and chased for 4 h. The E1 glycoprotein was immunoprecipitated with anti-E1 MAb A4 or coprecipitated with anti-E2 MAb H2. Immunoprecipitates were analyzed under nonreducing conditions by SDS-PAGE (10% polyacrylamide). The sizes (in kilodaltons) of protein molecular mass markers are indicated on the right.

maturation until they are properly folded (1, 14). As shown in Fig. 3, MAb H2 did not coprecipitate calnexin, suggesting that these HCV glycoproteins have dissociated from calnexin and are properly folded. Recently, we showed that the disulfide bond formation is slow for E1 (6). Under nonreducing conditions, the anti-E1 MAb A4 precipitates two forms of E1, which correspond to its reduced and oxidized forms; only the oxidized form of E1 was immunoprecipitated by MAb H2 (Fig. 4). The mobility of the latter was not altered by *in vivo* dithiothreitol treatment (data not shown), indicating that E1 coprecipitated by MAb H2 has acquired a compact configuration and is properly folded (2). The oxidized form of E2 is not shown in Fig. 4 since deglycosylation of E2 is necessary to detect an increase in mobility under nonreducing conditions (6). Precipitation by MAb H2 confirmed that deglycosylated E2 was in its oxidized form (data not shown).

Since acquisition of protease resistance often correlates with folding and assembly of oligomers (4), we tested the sensitivity of HCV glycoproteins to protease digestion. As shown in Fig. 5, the HCV glycoprotein complexes precipitated by MAb H2 were not sensitive to V8 protease treatment whereas those precipitated by MAb A4 were extensively degraded. At a con-

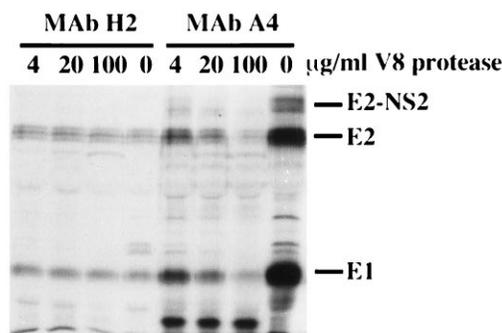


FIG. 5. Sensitivity of HCV glycoproteins to V8 protease treatment. HepG2 cells coinfecting with vTF7-3 and vHCV1-1488 were pulse-labeled for 5 min and chased for 4 h. Cell lysates were then treated with the indicated concentration of V8 protease for 5 min at 37°C and cooled on ice. After immunoprecipitation with MAb A4 or H2, the samples were separated by SDS-PAGE (10% polyacrylamide).

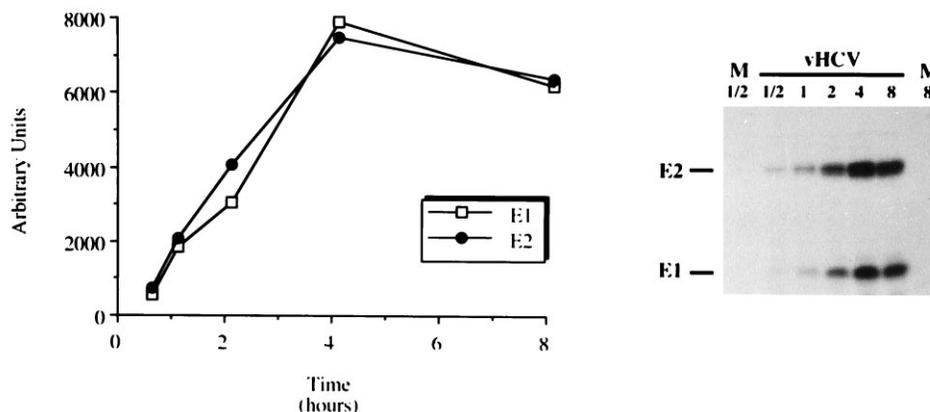


FIG. 6. Kinetics of folding of E2 and assembly of the complex. HepG2 cells infected with vTF7-3 alone (lanes M) or coinfecting with vTF7-3 and vHCV1-1488 (lanes vHCV) were pulse-labeled for 5 min and chased for the indicated times (in hours). After immunoprecipitation, the samples were separated by SDS-PAGE (10% polyacrylamide) and the amounts of radioactivity associated with E1 and E2 bands were quantitated with a PhosphorImager.

centration of 100  $\mu\text{g/ml}$ , only a small fraction ( $\approx 5\%$ ) of the glycoproteins were protease resistant and precipitable by MAb A4. The amount of these resistant forms was essentially identical to the amount precipitated with MAb H2 with or without prior protease digestion. These results indicate that most of the HCV glycoproteins are misfolded and protease sensitive even after 4 h of chase. MAb H2 selectively recognizes glycoprotein complexes which are dissociated from calnexin and protease resistant, properties which are characteristic of properly folded and assembled glycoprotein oligomers.

**Assembly kinetics and oligomeric state of HCV glycoprotein complexes.** Using MAb H2, we reexamined the kinetics of HCV oligomer formation. As shown in Fig. 6, the level of MAb H2-reactive E2 became detectable by 1 h and peaked by 4 h of chase. The levels of coprecipitated E1 paralleled those of E2, and the E1/E2 ratio was similar at each time point. These results suggest that the appearance of MAb H2 reactivity coincides with the delayed formation of properly folded and assembled E1E2 oligomers. Similar results were obtained with vaccinia virus recombinants expressing the entire HCV polyprotein or truncated forms terminating at C terminus of E2 or p7 (data not shown), indicating that sequences downstream of E2 do not markedly influence the assembly of glycoprotein oligomers, as assayed with MAb H2.

The formation and size distribution of the oligomers recognized by MAb H2 were examined by conducting pulse-chase experiments followed by separation of nonionic detergent-solubilized complexes by sedimentation through sucrose gradients. E1 and E2 precipitated by MAb H2 sedimented between molecular mass standards of 68 and 158 kDa (Fig. 7), suggestive of a heterodimeric E1E2 complex as previously proposed (5). No change in the sedimentation behavior of this complex was observed after different times of chase (1, 2, and 4 h [data not shown]). The distribution of MAb H2-reactive E1E2 complexes was narrow compared with the broad distribution observed for complexes precipitated by MAb A4. This probably reflects greater homogeneity in properly folded and assembled E1E2 complexes compared to the size heterogeneity of those recognized by MAb A4, which include misfolded complexes.

**Subcellular localization of MAb H2-reactive E1E2 complexes.** As a potential indicator of intracellular trafficking of the MAb H2-reactive E1E2 complex, we examined the acquisition of endo- $\beta$ -N-acetylglucosaminidase H (endo H) resistance in pulse-chase experiments. No endo H-resistant forms were detected (Fig. 8) even after 8 h of chase (data not shown),

which suggests that this complex might not be transported through the *trans* Golgi apparatus. Similar results were obtained with the vaccinia virus-HCV expression constructs described above (data not shown). Lack of recognition was not due to masking of the MAb H2 epitope, since this MAb can precipitate a secreted form of E2 which has acquired complex glycans as evidenced by resistance to endo H (29). E2-p7, which is readily resolved from E2 only after deglycosylation (13, 25), was also precipitated by MAb H2 (Fig. 8). This suggests that two mature complexes exist: E1E2 and E1E2-p7.

Immunostaining experiments indicated that the intracellular mature complexes colocalize with PDI, a protein which resides in the ER (Fig. 9). Immunoelectron microscopy confirmed the ER localization of the HCV glycoproteins recognized by MAb H2, and no cell surface labeling was observed (Fig. 10). In

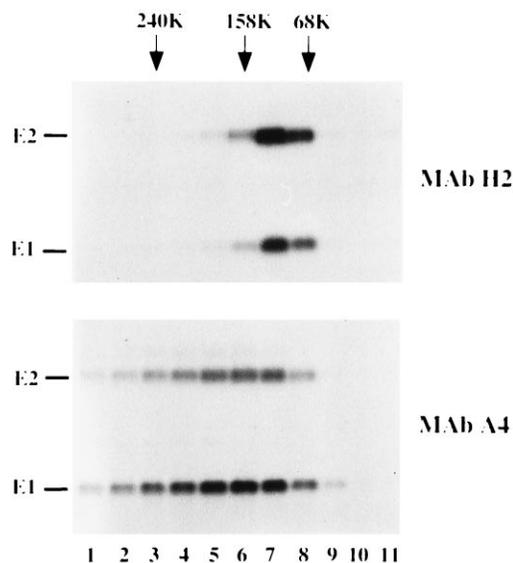


FIG. 7. Characterization of the oligomers. HepG2 cells coinfecting with vTF7-3 and vHCV1-1488 were pulse-labeled for 5 min and chased for 4 h. Cells were lysed with a buffer containing NP-40 and sedimented in sucrose gradients (5 to 20%) as described in Materials and Methods. Immunoprecipitations with MAb A4 or H2 were performed on each fraction, and the samples were separated by SDS-PAGE (10% polyacrylamide). Molecular mass markers sedimented in a parallel sucrose gradient are indicated at the top.

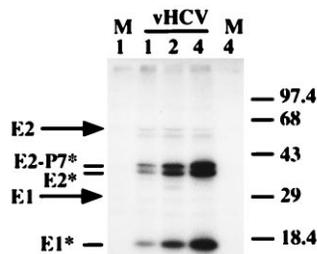


FIG. 8. Sensitivity of HCV glycoproteins to endo H treatment. HepG2 cells infected with vTF7-3 alone (lanes M) or coinfecting with vTF7-3 and vHCV1-1488 (lanes vHCV) were pulse-labeled for 5 min and chased for the indicated times (in hours). Cell lysates were immunoprecipitated with MAb H2 and then treated with endo H as recommended by the manufacturer. Samples were separated by SDS-PAGE (13% polyacrylamide). Deglycosylated proteins are indicated by asterisks. Arrows indicate the expected migration of nondeglycosylated E1 and E2. Sizes (in kilodaltons) of protein molecular mass markers are indicated on the right. The predicted protein sequences of E1 and E2 contain 5 and 11 N-linked potential glycosylation sites, respectively.

addition, secretion of HCV glycoproteins from HepG2 cells was not detected with either MAb H2 or other glycoprotein-specific MAbs (data not shown). These results indicate that both properly folded and misfolded E1E2 (and E1E2-p7) complexes are retained in the ER.

#### Recognition of MAb H2 to HCV RNA containing particles.

An immunoprecipitation-based assay was developed to determine the capacity of MAb H2 to recognize HCV RNA-containing particles (HRCP) in a well-characterized serum sample containing HCV-H subtype 1a. This patient serum has been shown previously to have a high specific infectivity of  $10^6$  to  $10^7$  chimpanzee infectious doses/ml and  $10^7$  to  $10^8$  HCV RNA molecules/ml. Since a significant fraction of the HRCP must be infectious, this sample was assayed for binding to the MAb H2. Following immunoprecipitation with MAb H2 or a c-myc MAb as a nonspecific control, RNA was isolated from the supernatant (unbound fraction) and pellet (bound fraction) and analyzed by competitive RT-PCR. As shown in Fig. 11, MAb H2 and the negative control, MAb c-myc, consistently bound 10 to 50 HRCP. On the other hand,  $\sim 1,000$  HRCP remained in the unbound fraction, suggesting that the MAb H2 is unable to recognize and bind the majority of HRCP (see Discus-

sion). Furthermore, two additional HCV glycoprotein-specific MAbs, A11 (E2) and A4 (E1), were tested in this immunoprecipitation assay and were also unable to bind to a larger fraction of the HRCP in the H77 serum sample (data not shown). These results were confirmed by a solid-phase immunocapture assay essentially described by Shindo et al. (38).

## DISCUSSION

This report describes the production and characterization of a conformation-sensitive E2-specific MAb which appears to selectively recognize noncovalently associated E1E2 heterodimers which have been released from calnexin. These complexes, which appear to be retained in the ER, may represent the prebudding form of the HCV virion glycoproteins.

The appearance of the E2-specific H2 epitope was slow and coincided with the release of E1E2 oligomers from calnexin. This suggests that formation of the conformation-sensitive H2 epitope represents a late folding step in oligomer maturation or that the H2 epitope is present earlier but masked by interaction of the complex with calnexin. Previous kinetic studies on intramolecular disulfide bond formation indicated that folding of E1 was slow ( $t_{1/2} \approx 1$  h) whereas it occurred rapidly for E2 and was complete upon cleavage of the E2-NS2 precursor (6). Thus, it seems likely that some other step in E2 maturation limits production of the H2 epitope. Proline isomerization is important for protein folding because prolines are usually free to isomerize when polypeptide chains are in the unfolded state but are constrained in properly folded chains (31). Proteins with peptidylprolyl *cis-trans* isomerase activity catalyze slow isomerization of X-P peptide bonds (11). Due to the large number of prolines in E2 (24 residues for genotype 1a), proline isomerization may be rate limiting for E2 folding and production of the H2 epitope.

Interestingly, both E2 and unprocessed E2-p7 were present in E1E2 complexes recognized by MAb H2. It is not known whether one or both of these E2 forms is present in mature HCV particles. In the case of the pestivirus classical swine fever virus, processing in this region of the polyprotein is similar but E2-p7 is either not present in mature virus or present at levels too low to be detected (7).

One consistent and striking observation is the inefficient production of properly assembled E1E2 oligomers, as assayed

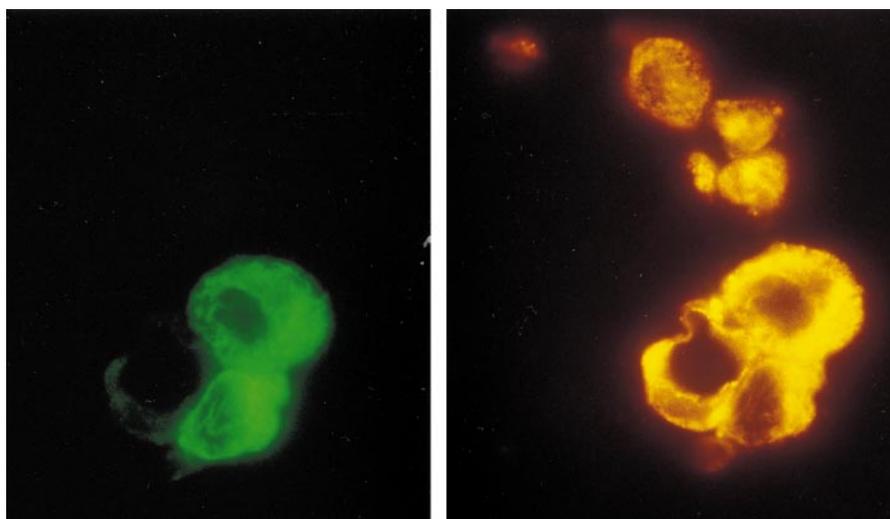


FIG. 9. Colocalization of HCV glycoproteins (revealed by MAb H2) and PDI by immunofluorescence as described in Materials and Methods.

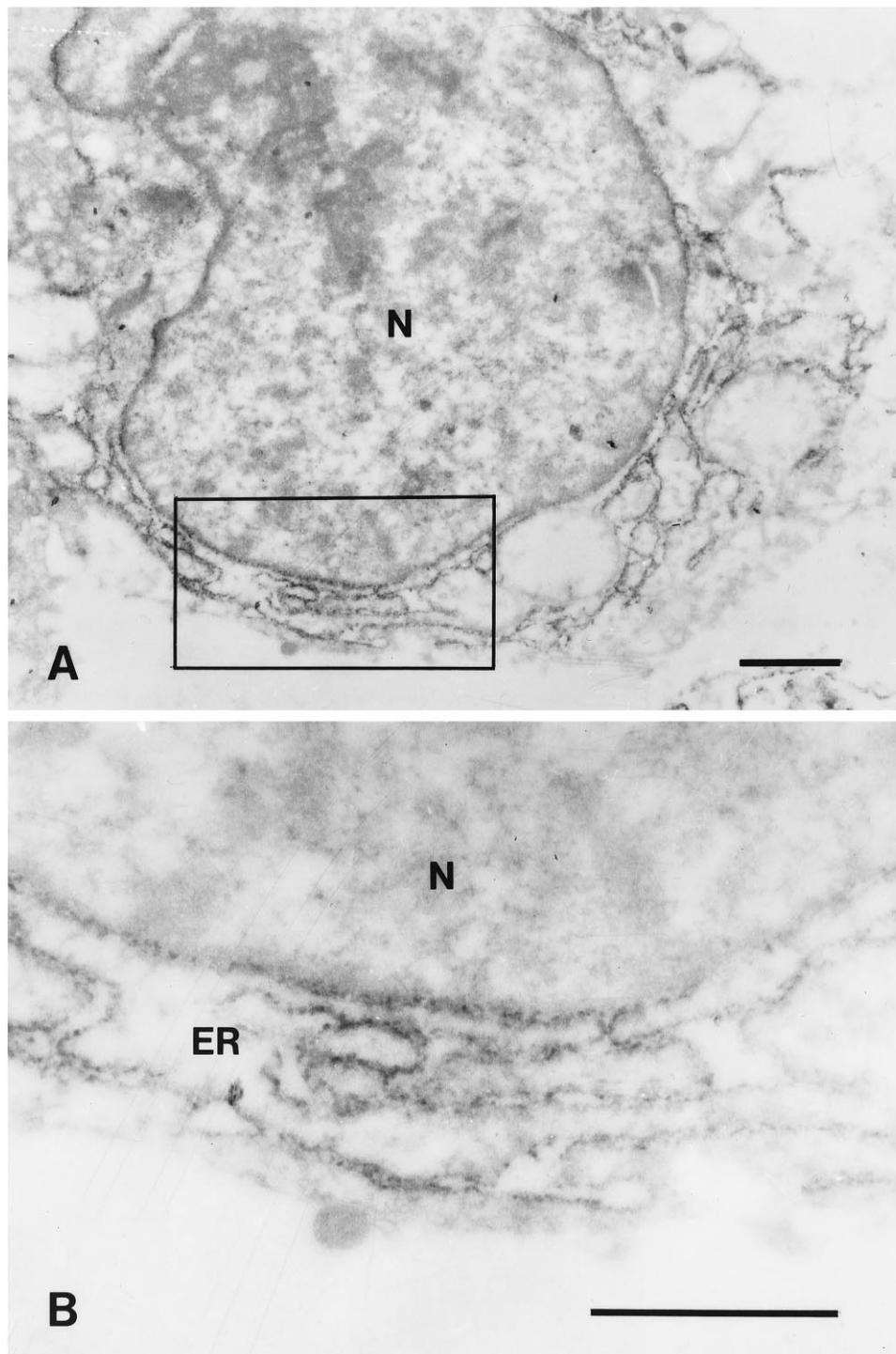


FIG. 10. Localization of HCV glycoproteins (revealed by MAh H2) by immunoelectron microscopy, using a preembedding immunoperoxidase technique as described in Materials and Methods. 3,3'-Diaminobenzidine granules were detected in the ER and in membranes in close proximity to the nucleus (N). Panel B is an enlargement of the rectangle in panel A. Bars, 1  $\mu$ m.

by H2 reactivity, protease resistance, or sedimentation on sucrose gradients. Slow folding of HCV glycoproteins may increase the fraction of these proteins shunted into competing nonproductive pathways, such as aggregation and aberrant disulfide bond formation (8). It is unclear if this tendency toward aggregation is due to abnormally high-level production driven

by the vaccinia virus and Sindbis virus expression systems. Slow folding kinetics and a high concentration of nascent, unfolded polypeptides could enhance aggregation. Alternatively, these observations may also apply at the presumed lower levels of expression in authentic HCV-infected cells. In the context of virus replication, inefficient folding of the HCV glycoproteins

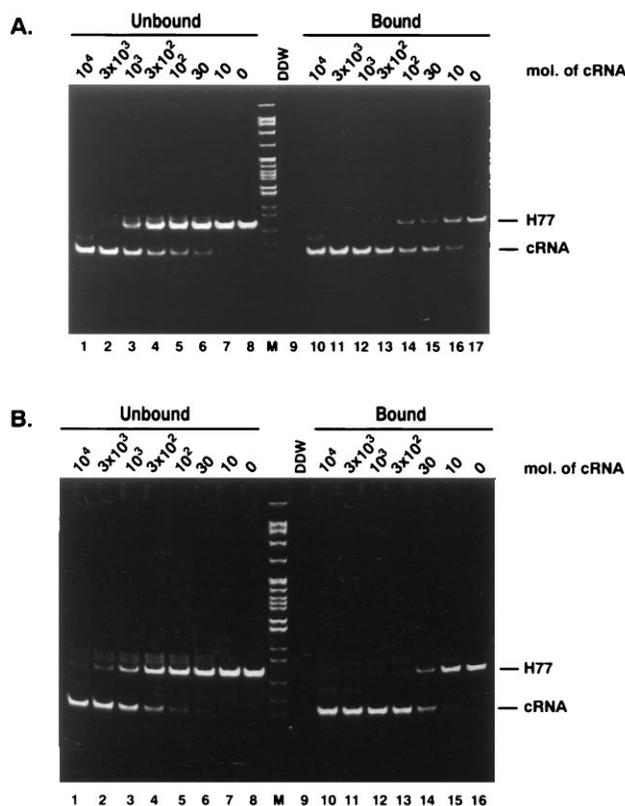


FIG. 11. Quantitation of the number of H77 viral particles bound to MAb H2 (A) or MAb c-myc (B) after immunoprecipitation. RNA was extracted from the unbound and bound fractions, and 3- or 10-fold dilutions of competitor RNA (cRNA) were added as indicated and subjected to RT followed by PCR and nested PCR as described in Materials and Methods. Products were separated by electrophoresis on a 6% polyacrylamide gel, and the positions of the product corresponding to the genomic RNA (H77) and the cRNA are indicated on the right.

could downregulate particle formation and virus replication to minimize exposure of viral antigens to the immune system and/or reduce pathogenicity. The erythropoietin receptor is an interesting example of a protein where poor folding appears to downregulate cell surface expression by arresting the protein in the ER and targeting it for degradation (20). The introduction of a single mutation in the erythropoietin receptor enhanced its folding and increased its cell surface expression. However, neither the murine nor the human erythropoietin receptor has this mutation, raising the possibility that poor folding has been selected during evolution to limit transport of the protein to the cell surface.

The ER localization and lack of complex glycans suggest that the H2-reactive E1E2 complex does not leave the ER or at least is not translocated beyond the *cis* Golgi, even after dissociation from calnexin. As we proposed previously (5), HCV glycoprotein complexes may contain a retention signal to allow budding into an intracellular subcellular compartment. As mentioned above, efficient particle formation has not been observed in transient-expression assays, suggesting that essential viral or host factors are missing or blocked. Thus, the H2-reactive E1E2 complexes most probably represent a pre-budding form of the glycoprotein oligomer. For the flavivirus, virions appear in intracellular vesicles (probably modified ER) and are released from cells via the exocytosis pathway (reviewed in reference 32). Complex glycans are acquired in the

*trans* Golgi (for some flaviviruses), and the virion glycoprotein precursor prM is cleaved just prior to the release of infectious virus. It has now become clear that there is no single "mature" conformation or quaternary structure for viral glycoproteins since oligomeric structures change at different stages during assembly, release, and entry. Similarly, the conformation of the prebudding HCV glycoprotein complex may differ from the structure after incorporation into virions or transit through the secretory pathway.

Since the *in vitro* data suggest that MAb H2 recognizes a native complex, it was surprising that MAb H2 failed to bind to a significant fraction of HRCF in a genotype 1a serum sample of high specific infectivity. The lack of recognition by MAb H2 could be due to two possibilities. First, the H2 epitope may be present and accessible on infectious virus, but if this fraction is small relative to the total HRCF, it may be undetectable above background binding. Alternatively, the H2 epitope may be absent from the surface of infectious HRCF. Given the conditions used to assay H2 reactivity in our cell culture assays, mild detergent treatment may be required to disrupt the viral lipid envelope and expose the H2 epitope. On the other hand, it is well documented that circulating virus is associated with either immunoglobulin (19) or very low density lipoprotein (VLDL) (40, 41). The HRCF in the H77 serum sample appear to be of low density and largely free of bound antibody (19). Hence, it is possible that bound VLDL mask the H2 epitope even if it is present on the surface of HRCF. In this case, association of HRCF and VLDL, either at the time of virus assembly or after release, could provide yet another mechanism for HCV to avoid immune recognition.

In the absence of a tractable cell culture replication system, it has been difficult to study the formation of HCV particles and their interaction with susceptible host cells. The MAb described in this report, which recognizes a complex which is a good candidate for the prebudding form of the E1E2 oligomer, should be useful for further studies of HCV glycoprotein biogenesis, folding, and assembly, as well as for characterization of virion structure. In addition, this MAb provides a tool for isolating native complexes for binding studies aimed at elucidating the HCV receptor(s). Besides these basic yet largely unexplored areas, the H2 MAb can be used to optimize production and isolation of properly folded complexes for use in diagnostic assays and as possible subunit vaccines.

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