EXPRESSION AND STRUCTURAL PROPERTIES OF A CHIMERIC PROTEIN BASED ON THE ECTODOMAINS OF E1 AND E2 HEPATITIS C VIRUS ENVELOPE GLYCOPROTEINS

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Abstract

Hepatitis C virus encodes two enveloped glycoproteins, E1 and E2, which are involved in viral attachment and entry into target cells. We have obtained in insect cells infected by recombinant baculovirus a chimeric secreted recombinant protein, $E1_{341}E2_{661,}$ containing the ectodomains of E1 and E2. The described procedure allows the purification of approximately 2 mg of protein from 1 L of culture media. Sedimentation velocity experiments and SDS-PAGE in the absence of reducing agents indicate that the protein has a high tendency to self-associate, the dimer being the main species observed. All the oligomeric forms observed maintain a conformation which is recognized by the conformation-dependent monoclonal antibody H53 directed against the E2 ectodomain. The spectroscopic properties of $E1_{341}E2_{661}$ are those of a three-dimensionally structured protein. Moreover, the chimeric protein is able to bind to human antibodies present in HCV-positive human sera. Accordingly, this chimeric soluble polypeptide chain may be a valuable tool to study the structure-function relationship of HCV envelope proteins.

Keywords: Hepatitis C Virus, envelope protein, E1, E2, baculovirus, glycosylation

Introduction

Hepatitis C virus (HCV) is a major cause of chronic hepatitis, liver cirrhosis, and hepatocellular carcinoma worldwide [1]. This enveloped and positive-stranded RNA virus belongs to the *Hepacivirus* genus of the *Flaviviridae* family [2]. There is no vaccine for HCV, and current antiviral therapies are based on the use of polyethylene glycol-modified interferon in combination with ribavirin. However, these treatments are expensive, relatively toxic, and effective in only half of the treated patients [3].

Structural and non-structural viral proteins are produced by cleavage of a polyprotein precursor by both host cell signal peptidases and viral proteinases [4, 5]. The envelope glycoproteins, E1 and E2 are classified as type I integral transmembrane proteins with a N-terminal ectodomain and a C-terminal hydrophobic anchor domain. During their synthesis, the ectodomains of HCV envelope glycoproteins are targeted to the endoplasmic reticulum lumen, where they are highly modified by N-linked glycosylation [6]. *In vitro* expression studies have shown that both glycoproteins associate to form a stable heterodimer, which accumulate in the endoplasmic reticulum, the proposed site for HCV assembly and budding [7].

This heterodimer is thought to be the functional complex at the surface of the virus [7], being involved in the virus entry into the cell. E2 seems to be the protein responsible for the interaction with cell receptors that include the CD81 tetraspanin, the scavenger receptor BI (SR-BI) and Claudin-1, a tight junction protein that recently has been proposed as co-receptor in a late step for HCV entry [8-10]. Also, E2 elicits production of neutralizing antibodies against the virus, and is involved in viral morphogenesis [9, 11]. Antibodies specific for epitopes within one of the hypervariable regions of E2 have been reported to inhibit binding of E2 to cells and

to block HCV infectivity *in vitro* and *in vivo* [12-14]. The role of E1 (residues 192 to 383) in HCV infection remains unclear; however, several antibodies directed against E1 were able to neutralize cell entry, presumably at a stage distinct from receptor binding [15-17]. E1 and E2 are major candidates for anti-HCV vaccine because they may harbour neutralizing antibody epitopes [18 , 19]; the envelope proteins may also work as a vaccine for chronically infected individuals who have a low immune response to E1 and E2 [20].

Because of the difficulties in propagating HCV in cell culture, many aspects of HCV life cycle remain unclear. A major advance in the investigation of HCV entry was the development of pseudoparticles (HCVpp), consisting of native HCV envelope glycoproteins E1 and E2 assembled into retroviral core particles [21, 22]. This system is potentially powerful to identify and characterize molecules that block HCV entry. Furthermore, data obtained with HCVpp can also now be confirmed with the help of the recently developed cell culture system that allows efficient amplification of HCV [23, 24].

Knowledge of the three-dimensional structure of HCV envelope proteins E1 and E2 will be of great value in the quest for a vaccine, in explaining existing data and in designing novel experiments. Despite that E1 and E2 have been expressed in several prokaryotic [25] or eukaryotic [26-29] cell lines, few data concerning the structure of isolated proteins have been obtained. Although the E2 ectodomain has been characterized as an independent folding unit [30], several studies indicate that the folding of E1 depends on the presence of E2 protein, and thus it should be characterized once the E1E2 complex has fully folded [31]. However, the only available structural data of the E1E2 complex are based on the use of conformation dependent monoclonal antibodies using the complete envelope glycoproteins

associated with partially purified HCVpp [21, 32]. Moreover, the reconstitution of "native" E1E2 heterodimers in liposomes has been reported [33]. However, only 1-5 μg of pure protein for every 10^7 cells could be obtained, which does not seem enough to achieve a full biochemical characterization of this protein in solution. In order to circumvent all these problems, we have designed a chimeric protein containing the E1 and the E2 ectodomains connected by a small hydrophilic peptide ($E1_{341}E2_{661}$). In this work, we described the production and characterization of $E1_{341}E2_{661}$ using the baculovirus/insect cell system. Only the chimeric polypeptide which is secreted soluble to the cell supernatant was purified to homogeneity. Approximately 2 mg of protein from 1 L of culture media can be purified. The protein has a high tendency to self-associate, the dimer being the main species observed. The spectroscopic properties are those of a folded polypeptide chain. Moreover, the chimeric protein is able to bind to human antibodies present in HCV-positive human sera.

Materials and methods

Plasmids construction

The cDNAs encoding E1 (residues 192-341) and E2 (residues 380-661) ectodomains were obtained by RT-PCR from the viral RNA of a strain 1HCV-PT, genotype 1a. The primers used for E1 were: 5′- ggg gaa ttc atg **cat cac cat cac cat cac** TAC CAA GTG CGC AAC TCC ACG – 3′ (forward) and 5′- ggg GAT CCG GAG CAG CTG AGC – 3′ (reverse). For E2 the primers used were: 5′- gcc atg GGC GTC GAC CCG GAA ACC CAC – 3′ (forward) and 5′- g ggc ggc cgc tta **gtg atg gtg atg gtg atg** aga tct CTC GGA CCT GTC CCT GTC - 3′ (reverse). Bases in uppercase letters indicate sequences of E1 and E2. Bases in bold lowercase letters denote codons for 6xHis tags added to the amino terminus of E1 and to the carboxy terminus of E2 ectodomains. Two restrictions sites (underlined), *Eco*RI and *Bam*HI for E1, *Nco*I and *Not*I for E2, were created at the 5′ and 3′ ends of both ectodomain genes. In order to connect E1 and E2 ectodomains by a flexible FLAG peptide, the E2 gene was subcloned into the pET30a plasmid (Novagen) digested with *Nco*I and *Not*I. The pET30a vector contains the sequence encoding the enterokinase recognition site, which is part of the FLAG tag, upstream of the cloning site. The resulting plasmid, pET30-E2 was used as template for a third PCR reaction using the same E2 reverse primer and the following forward primer: 5′ - c ggg atc cca **gac tac aag gac gac gac gac aag** – 3′; this oligonucleotide introduces the complete sequence codifying the FLAG peptide (in bold lowercase letters) as well as a *Bam*HI restriction site (underlined). Finally both amplified ectodomain cDNAs were digested with their corresponding restriction endonucleases (*Eco*RI and *Bam*HI for E1, *Bam*HI and *Not*I for FLAG-E2) and cloned into the *Eco*RI/*Not*I digested pAcGP67A baculovirus

transfer vector (Pharmingen) in a three fragment ligation to create pAcGP67A-$E1_{341}E2_{661}$.

Insect cell culture and transfections

The insect cell line *Spodoptera frugiperda* (*Sf9*) was cultured in Insect X-Press serum-free media (BioWhittaker) at 27 ºC. *Sf9* cells were cotransfected with Baculogold™ DNA (Pharmingen) and the recombinant transfer vector pAcGP67A-$E1_{341}E2_{661}$ as indicated by the manufacturer. Baculogold™ DNA is a modified wild baculovirus DNA which contains a lethal deletion and cannot develop into a viable virus by itself. Recombination between the flanking regions of the polyhedrin gene from the transfer vector and modified wild-type baculovirus DNA therefore results in 100% recombinant baculovirus DNA. Several rounds of culture amplified the recombinant virus, and a high titer virus stock solution was harvested. To express the protein on a larger scale, High Five™ insect cells (Invitrogen) were grown in Insect X-Press serum-free media prior to infection with high titer virus (>10^8 pfu/ml) at a multiplicity of infection of 5-10.

Purification of $E1_{341}E2_{661}$

Typically, 500 ml of recombinant baculovirus-infected insect cell cultures were harvested approximately 120 h postinfection and the cells pelleted by centrifugation at 5000 g for 10 min. The supernatant was dialyzed against 20 mM Tris-HCl pH 7, 50 mM NaCl and loaded onto a Ni^{2+}-Nitrilotriacetic acid agarose (Ni-NTA-agarose) column (Qiagen) which had been previously equilibrated with the same buffer. About 3 ml of gel were used per liter of culture supernatant. The flow rate was adjusted to 0.5 ml/min. Once the protein solution had entered the column, it was washed with dialysis buffer containing 10 mM imidazole and later 30 mM

imidazole. The recombinant $E1_{341}E2_{661}$ protein was eluted with 200 mM imidazole in dialysis buffer. The presence of $E1_{341}E2_{661}$ was monitored by SDS-PAGE throughout the purification.

Protein analysis

Protein concentrations were determined spectrophotometrically from the absorbance at 280 nm and the extinction coefficient calculated from the amino acid analysis. The absorption spectra were recorded on a Beckman DU-640 spectrophotometer. The amino acid analysis of hydrolyzed aliquots was performed on a Beckman 6300 automatic analyzer. Approximately 20 μg of purified protein were hydrolyzed with 5.9 N tridistilled HCl at 110 ºC for 24 h. Norleucine was used as an internal standard. Automated Edman protein degradation of $E1_{341}E2_{661}$ was performed using an Applied (model 494) gas-phase sequencer.

SDS-PAGE

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed according to Laemmli using 15% polyacrylamide gels [34]. Samples were subjected to gel electrophoresis under either nonreducing or reducing conditions (with 5% (v/v) β-mercaptoethanol) and the proteins were stained with Coomassie brilliant blue R-250. The molecular mass of the protein bands was estimated by comparison with protein markers of known molecular mass (Prestained SDS-PAGE Standards, Bio-Rad).

Western blotting

After SDS-PAGE, proteins were transferred to nitrocellulose membranes (Hybond-ECL; Amersham) in 48 mM Tris-HCl, pH 9.0, containing 39 mM glycine, 0.0375% SDS and 20% (v/v) methanol, for 1 h at 1 mA/cm^2, by using a V20-SDB apparatus (Scie-Plas). To detect $E1_{341}E2_{661}$, membranes were incubated with a HRP-conjugated monoclonal anti-His (Sigma) or anti-FLAG (Sigma) at a 1:3000 dilution. The peroxidase reaction was developed with 3,3'-diaminobenzidine tetrahydrochloride/H_2O_2. For the detection of the recombinant protein with the mouse anti-E1 (USBiologicals) and rabbit anti-$E2_{661}$ antibodies, nitrocellulose membrane was incubated with these antibodies diluted 1:1000 with 0.1% Tween 20 in PBS. Following overnight incubation at 4 ºC, the membrane was washed extensively with PBS containing 0.1% Tween 20. The membrane was then incubated with a goat anti-mouse or goat anti-rabbit antibody diluted 1:3000 for 2 hours. After membrane washing, the immunoblots were developed as described above. When the membranes were incubated with individual sera from HCV-positive human patients, $E1_{341}E2_{661}$ was detected via Enhanced Chemiluminiscence (ECL). After blotting, the membrane was incubated with the human sera diluted 1:20, washed and incubated with HRP-conjugated anti-human IgG (Fc) (Sigma) diluted at 1:1000. $E1_{341}E2_{661}$ was detected by incubating the membranes with ECL reagents (Amersham Life Sciences) and exposure to photographic film. The volumes of the bands were estimated by densitometry using UVIBand V97 (UVItec)

Polyclonal antibody against recombinant protein, $E2_{661}$ [30], was prepared by immunizing New Zealand white rabbits over a 6-week period by weekly injection of the protein (100 μg) in complete Freund´s adjuvant.

Protein Deglycosylation

Protein samples were digested with N-glycosidase F (PNGase F, Roche). Digestion was carried out for 16 h at 37 ºC in 20 mM sodium phosphate, pH 7.0, 50 mM EDTA, and 1% (p/v) octylglucoside. Digested samples were mixed with 3X Laemmli sample buffer and analyzed by SDS-PAGE. The proteins were stained with Coomassie brilliant blue R 250. The proteins were also transferred to nitrocellulose membranes that were subsequently incubated with the lectin concanavalin A conjugated to biotin. The glycoproteins were detected using HRP-streptavidin at a 1:1000 dilution.

Circular Dichroism

CD measurements were carried out on a Jasco spectropolarimeter, model J-715. All the measurements were conducted at 25 ºC with cells thermostated with a Neslab RTE-111 water bath. Far-UV CD spectrum was measured at a protein concentration of 0.15 mg/ml using protein dialyzed against 20 mM Tris-HCl pH 7, 50 mM NaCl. The pathlength was 1 mm. Five scans were averaged for each measurements and the contribution of the buffer was always subtracted. The spectra were calculated by using 110 as the mean residue molecular mass and the results are expressed in terms of residue molar ellipticity in $deg \cdot cm^2 \cdot dmol^{-1}$. The secondary structure of the protein was evaluated by computer fit of the dichroism spectra according to Convex Constraint Analysis (CCA) [35]. This method relies on an algorithm that calculates the contribution of the secondary structure elements that give rise to the original spectral curve without referring to spectra from model systems. The secondary structure was also predicted by the GOR IV method [36].

Fluorescence spectroscopy

Emission spectra were obtained at 25 ºC using an SLM AMINCO 8000C spectrofluorimeter, fitted with a 450-W xenon arc. Excitation and emission slit widths were set at 4 nm. The protein concentration was 0.05 mg/ml and a 0.4 x 1 cm cuvette was used. Buffer was 20 mM Tris-HCl pH 7, 50 mM NaCl. Excitation was performed at 275 or 295 nm, and the emission spectra were recorded over the range 285-450 nm. The contribution of the buffer was always subtracted. The tyrosine contribution to the emission spectra was calculated by subtracting the emission spectrum measured at λ_{exc} = 295 nm multiplied by a factor from that measured at λ_{exc} = 275 nm. The factor was obtained from the ratio between the fluorescence intensities measured with λ_{exc} = 275 and λ_{exc} = 295 nm at wavelengths above 380 nm, where there is no tyrosine contribution.

Analytical ultracentrifugation

The sedimentation velocity experiments were carried out on a Beckman Optima XL-A analytical ultracentrifuge equipped with UV-VIS optics detection system, using an An60Ti rotor and 12 mm double-sector centerpieces. The experiments were done with a protein concentration of 5 μM. They were carried out at 20 ºC. The buffer employed was 20 mM Tris-HCl pH 7, 50 mM NaCl. The sedimentation coefficient distributions were calculated by modelling on the sedimentation velocity data using the *c(s)* method [37], as implemented in the SEDFIT program, from which the corresponding sedimentation coefficients (s-values) were obtained.

Enzyme Linked Immunosorbent Assay (ELISA)

96 wells microtitre plates (Costar 3690) were coated overnight at 4 ºC with 100 ng/well of purified recombinant $E1_{341}E2_{661}$ diluted to 1 μg/ml in 0.05 M carbonate-bicarbonate buffer, pH 9.6. Unbound antigen was washed out, and the wells were blocked with 3% non-fat dry milk in PBS for 60 min at room temperature. After washing the wells were incubated at 37ºC for 2 h with HCV-positive and negative human sera. Seven positive and seven negative sera were used at a dilution of 1:200. The plates were then washed three times with PBS/0.05% Tween 20 and incubated at 37ºC for 1 h with HRP-conjugated anti-human IgG (Fc) diluted at 1:10.000. Bound antibodies were detected by adding 100 mM sodium citrate, pH 5.0, 4% Methanol buffer containing H_2O_2 and the substrate *o*-phenylenediamine dihydrochloride (Merck). The optical density at 492 nm was measured using an ELISA Expert 96 microplate reader (ASYS Hitech). The absorbance values obtained with a preimmune serum were subtracted. ELISA inhibition assays were performed as described in [38]. After coating with 100 μl of antigen (1μg/mL), the plates were incubated with HCV infected patients sera (diluted 1:20) previously mixed with different dilutions of the inhibitor (rabbit anti-$E2_{661}$ serum or monoclonal anti-E1) (10^{-6}-10^{-1}). Binding of human IgG was detected as described above. The inhibition data were normalized to inhibition of binding by pre-immune serum. Sera from infected and control patients were provided by Dr. Fernando Vivanco (Fundación Jiménez Díaz, Madrid, Spain).

Immunoprecipitation

A 50 μl aliquot of rabbit anti-mouse immunoglobulin G bound to Sepharose beads (Pharmacia-LKB) was incubated with either 2 μl of anti-E2 monoclonal antibody H53, 2 μl of anti-E1 monoclonal antibody (USBiological) or 2 μl of rabbit

serum for 1 h at 4 ºC in 10 mM Tris-Cl, pH 7.5, containing 0.2% NP-40, 150 mM NaCl and 2 mM EDTA (TBS-NP-40). The MAb H53 is conformation-dependent and was a generous gift of Dr. Jean Dubuisson. Beads were then incubated with 2 μg of purified $E1_{341}E2_{661}$ for 1 h at 4ºC. Between each step, the beads were washed twice with TBS–NP-40. After the last step, they were washed three times with this buffer and once with distilled water. The precipitates were then boiled for 5 min in SDS-PAGE sample buffer and analyzed on a 12% polyacrylamide gel. After electrophoresis and transfer to nitrocellulose membranes, protein $E1_{341}E2_{661}$ was detected by incubating the membranes with a polyclonal goat anti-E2 antibody (USBiological) at a 1:500 dilution followed with a rabbit anti-goat antibody conjugated to HRP diluted at 1:3000. The peroxidase reaction was developed with 3,3'-diaminobenzidine tetrahydrochloride/H_2O_2.

Results

Expression and purification of $E1_{341}E2_{661}$

Recombinant $E1_{341}E2_{661}$ has 466 amino acids, 150 corresponding to positions 192 to 341 of E1, 278 corresponding to positions 384 to 661 of E2 and the rest being due to the cloning strategy, the FLAG sequence between E1 and E2 and the His tags used to purify the recombinant protein (Fig. 1).

The chimeric protein $E1_{341}E2_{661}$ was expressed in High Five™ insect cells transfected with the pAcGP67A-$E1_{341}E2_{661}$ plasmid along with wild-type viral DNA. In a homologous recombination event, the $E1_{341}E2_{661}$ gene was inserted into the viral genome. The protein was expressed by infecting a new batch of insect cells using amplified recombinant virus. Cells were grown and protocols were carried out as described in Materials and methods. The analysis of the different fractions with a HRP conjugate anti-His antibody showed that the protein was produced soluble both intracellularly and in the extracellular medium (Fig. 2A, lanes 1, 2). Moreover, after cell lysis by sonication, it was observed that the protein was also able to form insoluble inclusion bodies (Fig. 2A, lane 3). Only the protein which was expressed in a soluble form and secreted to the extracellular medium was subsequently purified using affinity chromatography on a Ni-nitrilotriacetic acid-agarose column (Fig. 2B). The protein that elutes with 200 mM imidazole was determined to be > 95% pure by densitometry of stained SDS gels. Following this procedure, approximately 2 mg of $E1_{341}E2_{661}$ protein were obtained from 1 L of culture media. The purified protein was recognized by the monoclonal anti-FLAG as well as by a monoclonal anti-E1 antibody and the polyclonal anti-$E2_{661}$ antibody (Fig. 2C).

Biochemical characterization of $E1_{341}E2_{661}$

The amino acid composition of the recombinant protein determined by amino acid analysis was coincident with that deduced from the cDNA sequence (data not shown). The absorption spectrum was characteristic of a soluble protein with a maximum at 280 nm and a shoulder at 290 nm. The extinction coefficient calculated from the spectrum and using the protein concentration calculated from the amino acid analysis was 110342 $M^{-1}cm^{-1}$. Moreover, the Edman degradation of the purified protein confirmed the sequence of the first six amino-terminal amino acids of $E1_{341}E2_{661}$: ADPGYL. This result showed that the signal peptide gp67 had been correctly cleaved by cellular proteases, giving rise to the secretion of the processed protein.

SDS-PAGE of the recombinant protein in the presence of reducing agents showed a single band with a molecular mass of 64 kDa (Fig. 3A, lane 2) while the theoretical mass based on the amino acid sequence is 51.5 kDa. Then, $E1_{341}E2_{661}$ may be glycosylated in the 5/6 and 11 potential glycosylation sites of E1 and E2, respectively [6], most of which are well-conserved [6, 39]. In fact, when the recombinant protein was treated with PNGase F, which releases asparagine-linked (N-linked) oligosacharides from glycoproteins, the molecular mass of $E1_{341}E2_{661}$ decreased to 55 kDa (Fig. 3A, lane 3). Nevertheless, carbohydrates were still detected with concanavaline A in the recombinant protein treated with PNGase F (Fig. 3B, lane 2), indicating either that $E1_{341}E2_{661}$ contain N-glycosidic bonds which are not accessible to PNGase F or the existence of O-glycosidic bonds.

We have used sedimentation velocity to determine the oligomeric nature of $E1_{341}E2_{661}$ (Fig. 4). The calculated molecular mass of the most abundant species was 150 kDa with a sedimentation coefficient of 7s. Considering that the expected

molecular mass of the $E1_{341}E2_{661}$ monomer determined by SDS-PAGE is 64 kDa, the main form observed by ultracentrifugation is compatible with a dimer (Fig. 4, peak b). However, the recombinant protein was also present as monomer (Fig. 4, peak a) and higher order oligomers formed by more than two units of monomers, such us trimers (peak c), tetramers (peak d) and other higher forms (peaks e, f and others not shown) (Fig. 4). Based on the area under each peak the following proportion of each form was estimated: 13% monomer, 31 % dimer, 17% trimer and 13% tetramer. The analysis of the recombinant protein by SDS-PAGE in the absence of reducing agents also indicated the oligomeric nature of the purified protein (Fig. 4, inset). Under these conditions only monomers, dimers and trimers were observed while a high percentage of the protein does not enter into the gel.

Spectroscopic analysis of $E1_{341}E2_{661}$

The spectroscopic characterization of $E1_{341}E2_{661}$ was carried out by means of circular dichroism and fluorescence spectroscopies. The far-UV CD spectrum of $E1_{341}E2_{661}$ showed a minimum at 208 nm and a shoulder at 223 nm (Fig. 5). Deconvolution of this spectrum using the program Convex Constraint Analysis (CCA) [35] yielded the following percentages of secondary structure elements: 13% α-helix, 48% β-sheet, and 39% non-ordered structure. The predictive GOR IV method [36], which is based upon the propensity of each amino acid to adopt a particular secondary structure, yielded similar results: 8% α-helix, 32% β-sheet and 60% non-ordered structure.

The fluorescence emission spectrum of $E1_{341}E2_{661}$ is depicted in Figure 6. Upon excitation at both 275 and 295 nm, the recombinant protein exhibited a maximum at 331 nm. The shape of the spectrum indicates that the fluorescence of

this protein is highly dominated by tryptophan residues. In fact, the difference between the fluorescence spectra obtained upon excitation at 275 and 295 nm, the latter being normalized, indicates that the contribution of tyrosine residues to the recombinant protein fluorescence upon excitation at 275 nm was very low, approximately 5%. The position of the maximum indicates that the tryptophan residues occupy a relatively low hydrophobic environment. When the protein is treated with 8 M urea, the fluorescence intensity increased by 15% and the maximum is red shifted to 345 nm (Fig. 6).

Antigenic characterization of $E1_{341}E2_{661}$

A panel of seven HCV-positive and seven HCV-negative human sera was used to assess the antigenic properties of $E1_{341}E2_{661}$. The recombinant protein was able to bind under native conditions to antibodies present in all seven positive sera tested as assessed by ELISA (Fig. 7, sera 1-7). The values of the absorbance at 492 nm were much higher than those obtained with the negative controls (Fig. 7, serum 8; only one negative serum is depicted). Moreover, the difference in the values of the absorbance is consistent with the HCV titer as determined by COBAS TaqMan HCV test [40] (data not shown). On the other hand, the same sera were used in immunoblotting experiments after protein separation by SDS-PAGE (Fig. 7, inset). The data were quantified by densitometry. The volumes were normalized with respect to that obtained with serum 3 which gave the highest value (Figure 7, inset). There was no correlation between these values and those obtained by ELISA. Thus, sera 1, 4 and 7 which are among the ones that gave the highest absorbance values in ELISA were the ones which yielded the lowest volume values (approximately 30% of that of serum 3 which, on the other hand, yielded one of the lowest values in ELISA). This is

indicative of the existence of both continuous and discontinuous epitopes, the latter being the ones which are lost under denaturing conditions.

The conformation of the chimeric protein was examined by immunoprecipitation with the monoclonal anti-E2 antibody H53, and with a monoclonal antibody anti-E1. The $E1_{341}E2_{661}$ protein was efficiently immunoprecipitated by both antibodies. Since H53 is a conformation-dependent monoclonal antibody, it can be assumed that the E2 moiety of the chimeric recombinant protein possesses a native-like conformation. When the immunoprecipitated protein was analyzed by SDS-PAGE in the absence of reducing agents, the same pattern of bands shown in the inset of Figure 4, lane 2 was observed. In consequence it can be stated that all the oligomeric forms were able to react with the monoclonal antibody. The recombinant protein was able to bind to Huh7 cells and when the binding was performed in the presence of anti-CD81 it was drastically reduced (data not shown).

In order to distinguish the presence of anti-E1 and anti-E2 antibodies in the sera of infected individuals, an inhibition experiment using a polyclonal anti-$E2_{661}$ antibody was carried out (Fig. 8). The presence of an excess of rabbit anti-E2 antibodies would prevent the binding to $E1_{341}E2_{661}$ of the anti-E2 antibodies present in the HCV-positive sera. Several dilutions of the polyclonal anti-E2 were assayed. Taking sera 5 and 7 as examples, the binding of IgGs to $E1_{341}E2_{661}$ was practically abolished by the anti-$E2_{661}$ antibody from a dilution of 10^{-3}, although their patterns of inhibition are markedly different (Fig. 8A). To assure maximum inhibition, the rest of the sera were mixed with a 10^{-1} dilution of the anti-$E2_{661}$ antibody. At this value, inhibition studies showed that the polyclonal anti-$E2_{661}$ antibody blocked the binding of the IgGs from the seven HCV-positive sera to different levels (Fig. 8B), the

average being of inhibition 72 % (Fig. 8, horizontal line). To assess the presence of both anti-E1 and anti-E2 IgGs in the serum of the infected individuals, the same experiment was carried out in the presence of an excess of monoclonal anti-E1 and using sera 3 and 5 as examples. When these sera were incubated with a 10^{-1} dilution of monoclonal anti-E1, an 80% inhibition was observed for serum 3 while only a 15% inhibition was observed for serum 5. Thus, serum 3 would contain mainly antibodies which bind to E1 while those antibodies present in serum 5 would bind to E2. Consequently, and although the number of sera may not be statistically significant, the response to each envelope protein would depend on the infected individual.

Discussion

Hepatitis C virus encodes two enveloped glycoproteins, E1 and E2, which are involved in viral attachment and entry into target cells as well as in the fusion of viral and cellular membranes [41-44]. Most of the knowledge concerning the structural properties of these proteins is based on transient expression experiments. Their implication in the infection mechanism has also been investigated using different surrogate systems such as HCV-like particles, HCV pseudotype retroviral particles or replication-competent recombinant vesicular stomatitis virus encoding HCV envelope proteins [21, 45, 46].

However, the reports about the properties of isolated proteins are scarce. Previous attempts have been made to obtain the ectodomains of the proteins E2 and E1 separately. Expression in *Escherichia coli* led to the production of inclusion bodies which can only be solubilized with chaotropic agents. In the case of E2 the non-glycosylated recombinant protein thus obtained was able to interact with the virus receptor CD81 and it was recognized by a number of well-characterized anti-E2 antibodies in a similar way to that of native glycosylated forms [25, 47]. The structural proteins of HCV have also been produced in mammalian expression system [48, 49], yeast cells and recombinant baculovirus-infected insect cells [25, 29, 30, 50, 51]. In all cases, the majority of the recombinant proteins exhibit a molecular mass much higher than that expected because of the hyperglycosylation of the protein. In the case of $E1_{341}$, this protein was recently obtained from yeast and mammalian cells as a cysteine-blocked monomer only in presence of a detergent or reconstituted as 100 nm-particles when the detergent was eliminated [29].

Another strategy towards the understanding of the structure-function relationship of HCV envelope proteins is to obtain a tandem chimeric protein based

on HCV E1 and E2 envelope glycoproteins. In this report we have achieved the expression and purification of a chimeric recombinant protein, $E1_{341}E2_{661}$, containing the ectodomains of E1 and E2 linked by a hydrophilic and flexible FLAG region. To overcome the hyperglycosylation problems encountered in yeast, we have expressed $E1_{341}E2_{661}$ using a baculovirus expression system. Among others, it has the advantage of producing the protein in large amounts, and also the system leads to post-translational modifications which are similar to those observed in mammalian cells. Thus, by using High Five™ insect cells we have obtained the $E1_{341}E2_{661}$ protein secreted to the extracellular medium. After purification by affinity chromatography on Ni-NTA-agarose, we obtained, approximately, 2 mg of $E1_{341}E2_{661}$/liter of media. As evidenced below, this protein possesses all the features of a native soluble protein.

We have also expressed the same sequence both in *E. coli* and yeast cells (data not shown). The protein was produced in bacteria in a really high yield as an insoluble 50 kDa polypeptide but all attempts to solubilize it failed. In this respect, although it has been recently described the purification and application of bacterially expressed chimeric protein E1E2 [52], after purification in the presence of 6 M urea, the protein had to be dialyzed in the presence of both 0.1% Triton X-100 and 0.2% BSA which precludes any subsequent structural study. By using the yeast *Pichia pastoris* a highly glycosylated and insoluble non secreted protein was obtained. Taken together, these facts indicate that the proper glycosylation is an indispensable factor for the global folding of this structural protein. In fact, it has been reported that the glycans of HCV envelope glycoproteins play a major role in protein folding and/or in HCV entry [53-55].

The $E1_{341}E2_{661}$ protein used throughout this study was secreted to the cellular medium by virus-infected insect cells. However, it was also produced intracellularly in both soluble and insoluble forms which were more heterogeneous in size than the secreted one. This may be indicative that only the properly folded and processed polypeptide chains are able to enter the secretion pathway. On the other hand, the E1 and E2 ectodomains are separated by a hydrophilic sequence which contains the FLAG sequence with the enterokinase cleavage sequence which could be used to obtain both domains in a separate and soluble form. Nevertheless, all attempts to cleave that peptide bond failed.

The deconvolution of the circular dichroism far-UV spectrum of the recombinant $E1_{341}E2_{661}$ protein showed that β-sheet is the major ordered secondary structure element. The percentages of secondary structure deduced from the CD spectrum are coincident with those deduced from the amino acid sequence. Moreover, the fluorescence emission maximum corresponding to the tryptophan residues of the protein is centered at 331 nm, indicating that these residues are located in a moderately hydrophobic environment. Upon denaturation with urea, this maximum is red-shifted to 345 nm which is the value described for Trp residues in aqueous solution. Besides, the quantum yield of Trp residues undergoes a considerable increase which would indicate that the Trp fluorescence is quenched by nearby residues in the native conformation but not in the open and denatured conformation observed in the presence of urea. In consequence, the spectroscopic properties of the secreted protein are those of a three-dimensionally structured protein.

We have also evaluated the ability of $E1_{341}E2_{661}$ to bind to human antibodies with a panel of HCV-positive human sera by both ELISA and immunoblotting.

Under the conditions employed in ELISA assays the recombinant protein maintains its native conformation and the IgGs recognize both lineal and discontinous epitopes. However, in presence of SDS and β-mercaptoethanol, conditions employed in immunoblotting, the protein is completely unfolded and only the lineal epitopes are those which are recognized by IgGs. $E1_{341}E2_{661}$ was able to bind to the antibodies present in all HCV-positive sera tested. Reduction and denaturation of the recombinant protein diminished the binding to some of the sera, although to a different extent, indicating the presence of both continuous and discontinuous epitopes that need the correct network of disulfide bridges to maintain its conformation.

E1 and E2 HCV envelope glycoproteins have been shown to form noncovalent heterodimers as well as heterogeneous disulfide-linked aggregates [56, 57]. Characterization of the noncovalent heterodimer with conformation dependent monoclonal antibodies has suggested that this oligomer is likely the prebudding form of the functional complex [58]. Moreover, E1 and E2 glycoproteins interact to constitute oligomeric complexes which are the functional subunits of the HCV virion [56]. On the other hand, it has been suggested that the disulfide-linked aggregates are a consequence of an inefficient folding of HCV envelope proteins [59]. We have used analytical ultracentrifugation experiments to study the oligomeric nature of $E1_{341}E2_{661}$. The obtained results are indicative of a high tendency to self-associate, property that has already been reported [60]. The dimer is the most abundant species present in solution under the conditions employed, although higher order oligomers are also present. These $E1_{341}E2_{661}$ homodimers have to be maintained by hydrophobic interactions which are disrupted by SDS when the protein is analyzed in SDS-PAGE in the absence of reducing agents. However, disulfide bridges must be

involved as well in the formation of the dimer and the other oligomeric species which are also observed under these conditions. On the other hand, all the oligomeric species observed for the recombinant chimeric protein maintain a correctly folded conformation as assessed with the conformation-dependent monoclonal antibody H53 directed against the E2 ectodomain, and are not just mere aggregates with unfolded conformation. Another proof of the correct conformation of the chimeric protein is the fact that it was also able to bind to human hepatome Huh7 cells through CD81 which has been described as a cellular receptor.

Although there are several lines of evidence that indicate that E1 and E2 have separable functional properties, to have both ectodomains in a single polypeptide chain would have advantages over the separated domains. For instance, it has been described that both envelope proteins are required for maximal infection by HCV [61, 62]. Besides, Michalak and coworkers have shown that the folding of E1 is helped by the coexpression of E2 [28]. Moreover, two earlier studies indicate that HCV envelope glycoproteins cooperate for the formation of a functional complex and that both glycoproteins have to be co-expressed to analyze their functional properties [63, 64]. Taking into account the results described in this manuscript, it seems safe to state that $E1_{341}E2_{661}$ recombinant protein is properly folded and, at least in the E2 moiety, presents antigenic properties similar to those in the HCV virion. This chimeric soluble polypeptide chain formed by E1 and E2 ectodomains may be a valuable tool to study the structure-function relationship of HCV envelope proteins, comparable to other surrogate systems such as HCV-like particles [45], HCVpp [21] or E1E2-liposomes [33] as well as in the development of future vaccines.

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Figure captions

Fig. 1. Amino acid sequence of $E1_{341}E2_{661}$. Amino acids shown in bold are those which belong to E1 and E2 while those underlined are introduced by cloning. Positions 1 to 16 of the recombinant protein are due to cloning and to the 6xHis used to purify it. DYKDDDDK is the FLAG sequence which contains the sequence DDDDK which is recognized by enterokinase. Residues 175-176, AM , are introduced by the cloning procedure. Positions 177 to 180 of the recombinant protein correspond to residues 380 to 383 of E1. The sequence RSHHHHHH was used to increase the possibility to purify the protein by affinity chromatography.

Fig. 2. Analysis of the expression of $E1_{341}E2_{661}$ by High Five™ cells. (A) Western blot analysis of the extracellular (lane 1) and the intracellular soluble (lane 2) and insoluble (lane 3) $E1_{341}E2_{661}$ produced by baculovirus infected insect cells. After transferring to nitrocellulose membranes, proteins were detected with a peroxidase-conjugated monoclonal anti-His antibody as described in the Materials and methods section. (B) SDS-PAGE of purified $E1_{341}E2_{661}$ recombinant protein (lane 2). The samples were previously reduced with 5% (v/v) β-mercaptoethanol and boiled for 5 min. The gel was stained with Coomassie Brilliant blue R-250. Protein size markers (lane 1). (C) Western blot analysis of purified $E1_{341}E2_{661}$ using three different antibodies: a monoclonal antibody raised against FLAG region, a monoclonal against E1 glycoprotein, and a rabbit anti-$E2_{661}$ antibody.

Fig. 3. Analysis of the deglycosylation of $E1_{341}E2_{661}$ by PNGase F. (A) SDS-PAGE stained with Coomassie blue. (1) Protein size markers; (2) Purified $E1_{341}E2_{661}$; (3)

$E1_{341}E2_{661}$ treated with PNGase F; (B) SDS-PAGE stained with concanavalin A. (1) Purified $E1_{341}E2_{661}$; (2) $E1_{341}E2_{661}$ treated with PNGase F. Digestion with PNGase F was carried out for 16 h at 37 ºC in 20 mM sodium phosphate, pH 7.0, 50 mM EDTA, and 1% (v/v) octylglucoside. After transferring, the nitrocellulose membranes were incubated with biotinylated concanavalin A and the proteins were detected using peroxidase-conjugated streptavidin as described in the Materials and methods section.

Fig. 4. Sedimentation velocity analysis of $E1_{341}E2_{661}$. The results are shown as the sedimentation coefficient distribution *c(s)*. The experiment was carried out at 5 μM native $E1_{341}E2_{661}$. (a) monomer, (b) dimer, (c) trimer, (d) tetramer. (Inset) SDS-PAGE in the absence of reducing agents. (1) Protein size markers, (2) Purified $E1_{341}E2_{661}$. The positions of (a) monomer, (b) dimer, (c) trimer are marked. The gel was stained with Coomassie Brilliant blue R-250.

Fig. 5. Far-UV circular dichroism spectrum of $E1_{341}E2_{661}$. The spectrum was recorded between 190 and 250 nm with a protein concentration of 0.15 mg/ml in a cylindrical cuvette of 0.1 cm pathlength. The buffer was Tris-HCl 20 mM, pH 7.0, 50 mM NaCl. The spectrum was recorded five times, averaged and corrected for buffer contributions. Data were collected at 25 ºC and are expressed as residue molar ellipticity.

Fig. 6. Fluorescence emission spectra of $E1_{341}E2_{661}$. The excitation wavelength was 275 nm (—) and 295 nm (---). The emission spectra were recorded between 300 and 450 nm. The spectrum obtained after excitation at 295 nm was normalized at

wavelengths above 380 nm. The contribution of Tyr residues (•••) to the emission spectrum was calculated as described in the Materials and methods section. Protein concentration was 0.05 mg/ml. The buffer was Tris-HCL 20 mM, pH 7, NaCl 50 mM. The spectrum in the presence of 8 M urea was also recorded (– •• –). Spectra were collected at 25 ºC. The contribution of the buffer was always subtracted.

Fig. 7. ELISA with HCV-positive human sera. Microtitre wells were coated overnight at 4 ºC with $E1_{341}E2_{661}$ recombinant protein at 100 ng/well. Seven HCV-positive human sera (1-7) and one HCV-negative human sera (8) were used at a dilution of 1:20. Bound antibodies were detected with peroxidase conjugated anti-human IgG (Fc) diluted at 1:10000 as described in the Materials and methods section. The absorbance values were normalized to that of serum 3 which was taken as 1.0. The results shown are the mean ± standard deviation of three different experiments. (Inset) Immunodetection of $E2_{661}$ with HCV-positive human sera. Purified $E1_{341}E2_{661}$ was denatured with SDS and β-mercaptoethanol. After SDS-PAGE, the proteins were transferred to nitrocellulose membranes which were incubated with individual sera from HCV-positive patients at a dilution of 1:20. Finally, the membranes were incubated with peroxidase-conjugate goat anti-human IgG diluted at 1:10000 and $E2_{661}$ was detected with ECL detection reagents. The volumes of the bands, which were quantified by densitometry, are shown above each band. The data shown are representative of those obtained in three different experiments.

Fig. 8. Inhibition studies of the binding of $E1_{341}E2_{661}$ to HCV-positive sera. (A) Inhibition studies of the binding of the IgGs from sera 5 (– –●– –) and 7 (——○——)

to the $E1_{341}E2_{661}$ protein at different dilutions of rabbit anti-$E2_{661}$ antibody. (B) A polyclonal rabbit anti-$E2_{661}$ antibody at 10^{-1} dilution was used to block the binding of the recombinant $E1_{341}E2_{661}$ to IgGs, from 7 HCV-positive sera. The results shown are the mean ± standard deviation of three different experiments. The horizontal line shows the average of the inhibition of all 7 HCV infected patients sera.

Figure 1

1 ADPGYLLEFM HHHHHHYQVR NSTGLYHVTN DCPNSSIVYE AADAILHTPG

51 CVPCVHEGNA SRCWVALTPT VATRDGKLPT TQLRRHIDLL VGSATLCSAL

101 YVGDLCGSVF LVGQLFTFSP RRHWTTQDCN CSIYPGHITG HRMAWDMMMN

151 WSPTAALVVA QLLRIPDYKD DDDKAMGVDP ETHVTGGTAA QTTAGLVSLL

201 SPGAKQDIQL INTNGSWHIN STALNCNDSL YTGWLAGLFY HHKFNSSGCP

251 ERFASCRPLT DFAQGWGPIS HANGSGPDQR PYCWHYPPKP CGIVPAKSVC

301 GPVYCFTPSP VVVGTTDRSG APTYSWGAND TDVFVLNNTR PPLGNWFGCT

351 WMNSTGFTKV CGAPPCVIGG VGNNTLHCPT DCFRKHPEAT YSRCGSGPWI

401 TPRCLVNYPY RLWHYPCTIN YTIFKVRMYV GGVEHRLEAA CNWTRGERCN

451 LEDRDRSERS HHHHHH

Figure 2

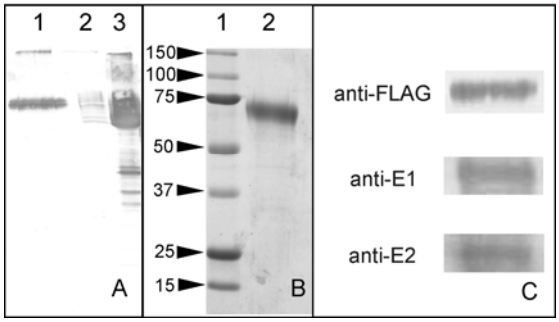

Figure 3

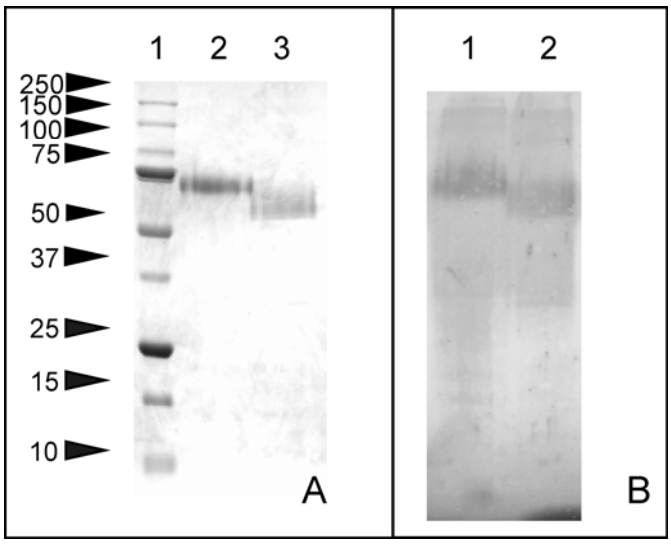

Figure 4

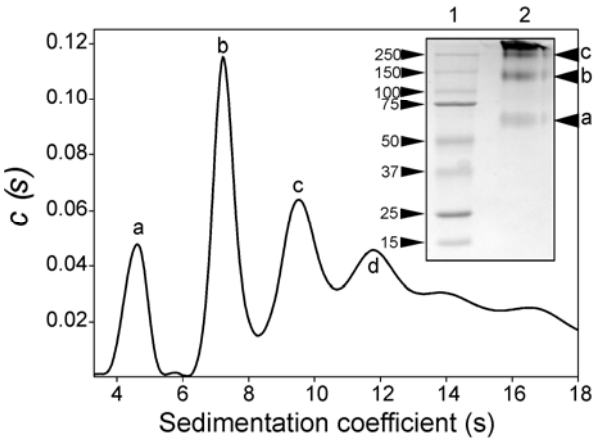

Figure 5

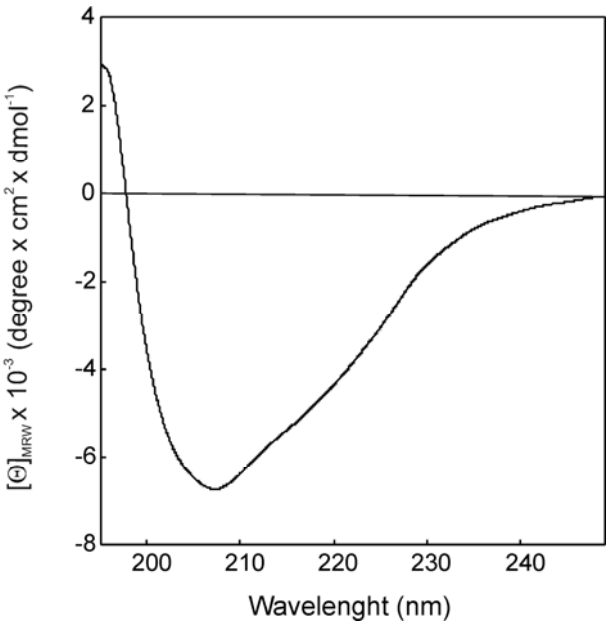

Figure 6

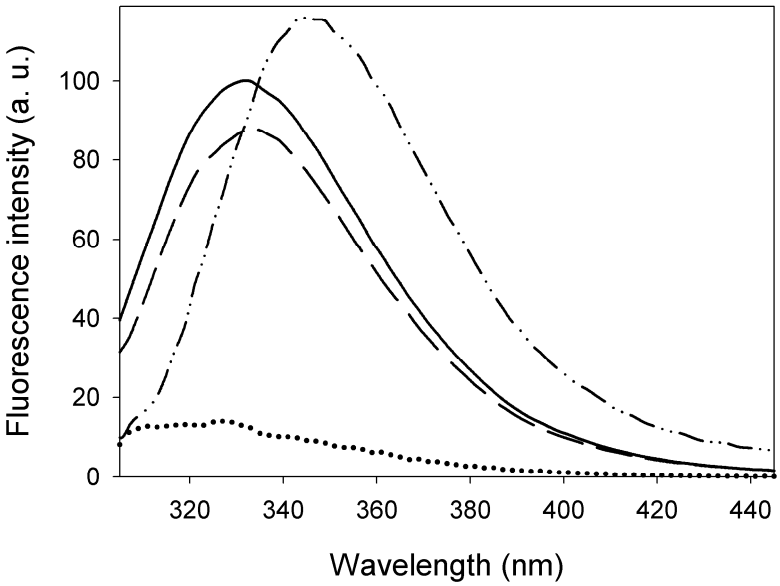

Figure 7

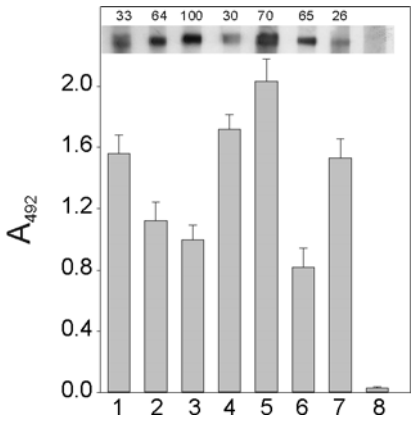

Figure 8