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Characterisation of bacterially expressed structural protein E2 of hepatitis C virus

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Abstract

The E2 glycoprotein is a structural component of the hepatitis C virus (HCV) virion. It interacts with putative cellular receptors, elicits production of neutralising antibodies against the virus, and is involved in viral morphogenesis. The protein is considered as a major candidate for anti-HCV vaccine. Despite this, relatively little is known about this protein. Previous studies have focused on the antigenic and functional analysis of the glycosylated forms. This report describes expression of the ectodomain of E2 (recE2) in *Escherichia coli* cells, its purification, and initial characterisation of its structural and functional properties. It is demonstrated that the purified protein forms small soluble aggregates, which retain functional characteristics of its native counterpart, i.e., it interacts with a putative cellular receptor, CD81, and is recognised by both conformation-dependent and -independent anti-E2 monoclonal antibodies.

Keywords: HCV; Hepatitis C virus; E2; Structural glycoprotein; Virus–receptor interaction; CD81

Hepatitis C virus (HCV) is an enveloped virus containing a positive-strand RNA genome. It encodes two glycoproteins, E1 and E2, which are components of the virus envelope [1]. The E2 glycoprotein extends from amino acid (aa) 384 to 746 of the viral polyprotein and it carries two hypervariable regions. It is thought to be a type I integral trans-membrane protein, with an N-terminal ectodomain and a C-terminal hydrophobic anchor domain [2]. It interacts with E1 and is accumulated within the endoplasmic reticulum (ER), the proposed site for HCV assembly and budding [3–5]. Truncated, soluble E2 devoid of the transmembrane domain (TMD) is secreted from the cells. Both truncated E2 and virus-like particles (VLP) containing E2 interact with the large extracellular loop (LEL) of the human cell surface protein CD81, suggesting that CD81 a receptor for HCV [6–8]. With regard to E2 glycosylation, it has been demonstrated that enzymatically de-glycosylated form of E2 binds CD81 with efficiency similar to that of core-glycosylated form, while highly glycosylated protein has substantially reduced affinity to CD81 [9]. Purification of glycosylated, soluble E2 lacking its TMD expressed in mammalian cells has been previously reported [9]. However, only low levels of expression in animal cell cultures have been achieved. Moreover, the purified product is highly heterogeneous mainly due to differential glycosylation patterns, even when the protein is isolated from single cellular compartment. All these contribute to the fact that structural analysis of this key viral protein has not yet been possible. Against this background, we set out to characterise a functional recombinant E2 (recE2) expressed in bacterial cells.

Methods

Plasmid constructs expressing E2 fragments

6× His tagged E2

The sequences encoding a C-terminally 6× His tagged ectodomain (aa 385–663) were cloned into two *Escherichia*

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coli expression vectors, pBAD18 (Invitrogen) and modified pET11c (Novagen), as follows. The E2 sequence was amplified by PCR using an infectious HCV cDNA clone of genotype 1b strain ([10], kindly provided by J. Bukh) as a template. Amplification of the E2 fragment was performed with the following primers.

Forward primer—5′-TATAGGGGTATTAATA***CA TATG***ACCCACACGACGGGGAGG-3′. It contains an initiator ATG incorporated into a *Nde*I site (shown in italics) followed by nucleotides representing the 5′ end of E2. The reverse primer for pBAD18 vector was 5′-GC*TCTAGA*TTAGTGATGGTGATG GTGATG**GCTGAGTTCTGACCTATCC**-3′ carrying 19 bases complementary to the E2 (in boldface type), followed by sequences encoding 6× His residues, TAA stop codon, and a *Xba*I recognition site (shown in italics). The reverse primer for pET11c vector was similar to the one above, except it carried a *Kpn*I restriction site.

Trx–E2 fusion

The sequence encoding $E2_{661}$ fragment was fused to the 3′ end of His-patched thioredoxin (Trx) through a linker by PCR with appropriate primers. The final PCR fragment was digested with *Nde*I and *Hin*dIII and cloned into pGEMEX-1 vector (Promega).

Expression of E2 fragments

The E2 gene constructs were expressed in *E. coli* BL21 (DE3), Rosetta-gami DE3, or Rosetta-gami pLysS cells (Novagen) following induction with varying final concentration (0.02–1 mM) of IPTG. Temperature of incubation also varied from 15 to 37 °C. Cells were grown in Luria broth to a density of 0.4 A_{600} prior to adding IPTG and then grown further to a density of about 1.0 A_{600}.

Purification of E2 on Ni–NTA

The induced cells were lysed with lysozyme in the presence of 1 mM AEBSF (4-(2-aminoethyl)benzenesulphonyl fluoride, Melford Lab.). Triton X-100 was then added to 1% final concentration and the lysate was centrifuged at 13,000 rpm for 15 min. The pellet was resuspended in guanidine buffer (PBS, 6 M guanidine, 5 mM imidazole, 1 mM AEBSF, 1% Triton X-100, and 1 mM DTT), then the suspension was centrifuged at 13,000 rpm for 30 min, and the supernatant was applied to Ni–NTA equilibrated in guanidine buffer. E2 was allowed to bind to the resin for at least 1 h, then the unbound material was washed three times in PBS, 10 mM imidazole, allowing 10 min during each wash for renaturation, followed by elution in PBS containing 200 mM imidazole. Only freshly purified E2 was used for further applications.

Purification of GST–hCD81 on glutathione–agarose

Human CD81 LEL fused to glutathione-*S*-transferase (GST–hCD81) was purified from the plasmid pGEX-EC2 as previously described [8].

Interaction of E2 with GST–hCD81 bound to glutathione–agarose

Freshly prepared E2 was added to GST–hCD81 bound to glutathione–agarose. GST–hCD81 + E2 complexes were allowed to form for at least 1 h, the unbound material was washed three times in PBS, and then the complexes were eluted with glutathione elution buffer.

Sucrose gradient centrifugation

0.5 ml freshly purified E2 or E2 + GST–hCD81 complexes were layered onto a 12 ml of 20–60% sucrose gradient and centrifuged at 30,000 rpm for 3 h (in case of E2), or overnight (E2 + GST–hCD81 complexes). One millilitre fractions were collected from the bottom of the tubes and the pelleted material was resuspended in remaining buffer (approximately in 200 μl). The fractions were analysed by Western immunoblotting using anti-E2 and anti-GST antibodies.

ELISA

For ELISAs, plates were coated with freshly prepared E2 in PBS, 200 mM imidazole. E2 was usually titrated 1:2 in each row. After overnight incubation at 4 °C, plates were washed three times with PBS, blocked for 2 h with 2% skimmed milk in PBS, and again washed three times with PBS. After that, test proteins (GST–hCD81 or antibodies) in PBST (PBS, 0.05% Tween 20) were added and incubated for at least 2 h. Then the plates were washed three times with PBST. For GST–hCD81, polyclonal anti-GST antibodies were added, and incubation and washing procedures were repeated. The plates were then incubated with the appropriate secondary antibodies conjugated with HRP and developed using tetramethyl benzidine (TMB) substrate. The reaction was quenched by adding 50 μl of 0.5 M H_2SO_4 to each well, and the absorption was read at 450 nm. In case of GST–hCD81 + E2 interaction, the plates were coated overnight with purified GST–hCD81 in glutathione elution buffer, and the next day freshly prepared E2 was titrated in place of test protein. E2 was detected using an anti-E2 MAb AP33 [11].

Immunoblotting

Proteins were electrophoretically transferred to Hybond ECL membranes (Amersham) [12]. Membranes were incubated with appropriately diluted anti-E2 MAb

AP33 or anti-GST polyclonal antiserum. The immunoreactive proteins were detected using goat anti-mouse or anti-rabbit immunoglobulin G–horseradish peroxidase conjugate and enhanced chemiluminescence reagents (Amersham).

Results and discussion

Expression of recombinant E2

The major goal of this study was to express, isolate, and characterise recombinant non-glycosylated E2 protein (recE2) produced in *E. coli* cells. Previous attempts to bacterially express and isolate E2 fused to a protein moiety, GST, have shown that the resultant protein was insoluble [13]. In this study, we generated a construct carrying a truncated E2 gene from which the C-terminal TMD was deleted. This construct is essentially similar to the one expressed previously in animal cell cultures and characterised in some detail [8,11,14]. A 6× His tag was introduced at the C-terminus of the protein to facilitate purification of the product. We chose to express recE2 in *E. coli* cytoplasm in order to get it in a high yield sufficient for further analysis.

In an attempt to find optimal conditions for recE2 expression, with special emphasis on level of soluble E2, different backbone vectors were used initially, namely pET11c, pBAD18, and a plasmid carrying a gene encoding His-patched thioredoxin. A fusion to the 3′ end of His-patched thioredoxin was made as it had been shown that in some cases this increases solubility of a target polypeptide. We checked different host cell backgrounds, including BL21 (DE3) and Rosetta-gami strains (Novagen), which allow proteins to form S–S bonds in the cytoplasm. In most cases, a wide temperature range (+15–+37 °C) was tried for the expression. Unfortunately, in all these cases the polypeptide was found predominantly in inclusion bodies (data not shown). We therefore decided to attempt purification of the protein from the inclusion bodies generated in BL21(DE3) cells carrying a pET11c-based construct. This system was found to give the best yield of the protein (5–10 mg/L of the culture).

Complete solubilisation of the inclusion bodies was achieved in 6 M guanidine–hydrochloride supplemented with 1% Triton X-100 and a reducing agent, DTT (dithiothreitol). The overall scheme for the isolation of recE2 was as follows. The dissolved protein was adsorbed onto Ni–NTA resin. Then, the immobilised protein was allowed to refold in native buffer without reducing agents to facilitate formation of S–S bonds. The protein was eluted in a buffer containing 200 mM imidazole. A variety of factors such as pH, temperature, buffer composition, detergents, etc., were checked out to find conditions for getting better elution of recE2 in a proper form.

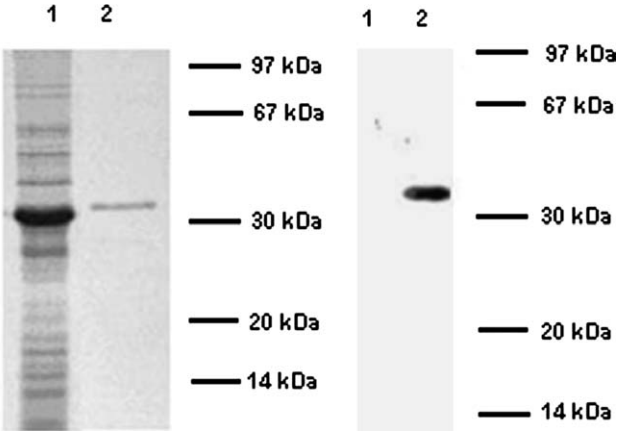

Fig. 1. Electrophoretic analysis of the recE2. Left: recE2 found in inclusion bodies (lane 1) and following purification (lane 2) was subjected to SDS–PAGE and the proteins were detected by Coomassie brilliant blue staining. Right: lysates of the parent *E. coli* cells (lane 1) or those expressing recE2 (lane 2) were subjected to Western immunoblotting using an anti-E2 MAb AP33 and the bound antibodies were detected as described in Methods. MW markers are indicated on the right of each panel.

We found that under many conditions most of the immobilised recE2 remained bound to the resin and could be eluted only by SDS. Efficient elution was achieved by incubation of the protein immobilised on the Ni-resin with elution buffer for about an hour at room temperature. Fig. 1, left shows an electrophoretic analysis of purified recE2, the identity of which is confirmed by Western immunoblotting using an anti-E2 MAb (Fig. 1, right).

Structural state of purified recE2

To ascertain its physical form (monomer, oligomer or higher order structures), the eluted recE2 was subjected to sucrose gradient centrifugation. As a control, apoferritin, a globular protein with molecular weight 443 kDa, was used in parallel. Following centrifugation, fractions were collected from the bottom of the gradient and analysed by Western blotting using an anti-E2 MAb AP33. The migration of apoferritin in gradient was determined by its colour. As shown in Fig. 2, soluble recE2 was found in fractions 1–2 as compared to apoferritin in fractions 3 and 4 (not shown). This indicated that most of the recE2 had a sedimentation velocity slower than that of apoferritin. This means that recE2, which has molecular weight of 30 kDa, forms small aggregates consisting of no more than 10–15 E2 monomers. The rest of recE2 formed heavy aggregates found on the bottom of centrifuge tube after sedimentation. It should be noted that for better visualisation of material in the pellet fivefold more material was loaded on the gel in Fig. 2 for the electrophoretic analysis compared to that from the other fractions. Similar centrifugation of recE2 that had been stored or incubated at 4 °C even for several hours

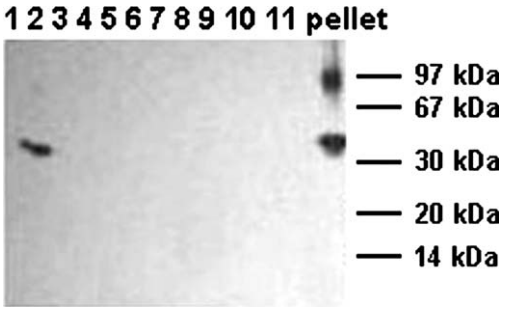

Fig. 2. Analysis of the freshly purified recE2 by sedimentation velocity centrifugation in sucrose gradient. The recE2 from individual fractions was subjected to SDS–PAGE followed by Western immunoblotting with anti-E2 AP33 MAb.

resulted in higher order structures which sedimented to the bottom of the tube (data not shown). Thus, recE2 appeared to initially form small soluble aggregates, which upon further storage were converted to higher order forms. For this reason, only freshly made recE2 immediately after elution from the Ni-resin was used in all further experiments.

Interaction of recE2 with CD81

The E2 glycoprotein has been shown to interact specifically with the large extracellular loop of human CD81, its putative cellular receptor [6–8]. In the absence of a suitable in vitro cell culture system for efficient infection of target cells with HCV, the E2–CD81 interaction has been used as a criterion for determining functionality of different recombinant E2 forms [6,15]. To test whether the bacterially expressed recE2 generated in this study is indeed functional, we performed several independent assays to determine its ability to interact with human CD81. In the first assay, the freshly produced recE2 was incubated with the GST–hCD81 and then the complex was subjected to sucrose gradient centrifugation. Aliquots of the fractionated gradient were then analysed by Western immunoblotting. Consistent with data shown in Fig. 2, the bulk of the recE2 was found in the first three fractions (Fig. 3A). Western

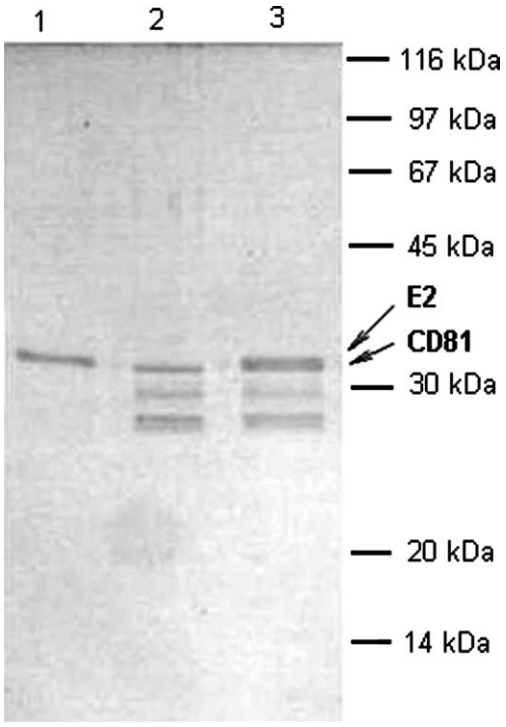

Fig. 4. Interaction between recE2 and GST–hCD81 as identified by the pull-down assay. Freshly prepared recE2 was added to GST–hCD81 bound to glutathione–agarose beads, unbound material was washed out, bound complexes were eluted by reduced glutathione and analysed by SDS–PAGE. Lane 1, sample of recE2; lane 2 GST–hCD81 eluted from glutathione–agarose in the absence of recE2; and lane 3, complexes eluted from glutathione–agarose after incubation with recE2.

immunoblotting with anti-CD81 antibody demonstrated that the same fractions also contained the GST–hCD81 fusion protein (Fig. 3B). This is in contrast to the control experiment in which GST–hCD81 was mostly found in fraction 1 when it alone was subjected to sucrose gradient centrifugation (data not shown). Interestingly, CD81 was also found to co-migrate with the higher order recE2 aggregates at the bottom of the tube (Figs. 3A and B).

Next, we incubated GST–hCD81 immobilised on glutathione–agarose beads with freshly prepared recE2. As a control, recE2 was incubated with the beads only. The

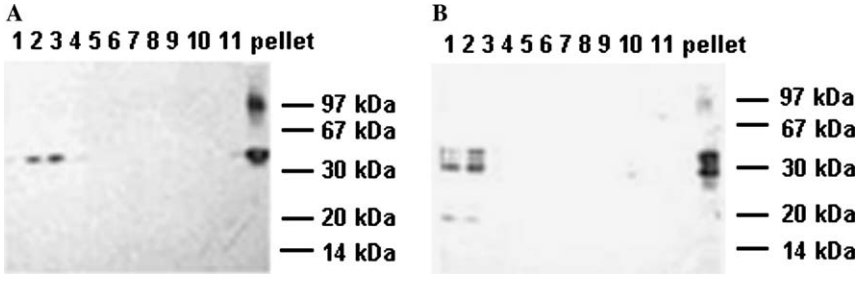

Fig. 3. Interaction of recE2 with GST–hCD81 as analysed by sedimentation velocity centrifugation in sucrose gradient. The recE2 bound to the Ni-resin was allowed to form complexes with GST–hCD81, and the bound complex was eluted with imidazole. The sample was then subjected to centrifugation in sucrose gradient, and individual fractions were analysed by SDS–PAGE followed by Western immunoblotting using an anti-E2 MAb AP33 (A) or anti-GST antiserum (B).

unbound material was washed out and the bound complex was analysed by SDS–PAGE. As shown in Fig. 4, recE2 was specifically 'pulled-down' by GST–hCD81 immobilised on glutathione–agarose beads (lane 3), but not by the beads only (data not shown).

To further validate this interaction, we performed an ELISA-based assay where different amounts of the recE2 were added to GST–hCD81 coated wells, and the bound protein was detected using an anti-E2 MAb AP33. The recE2 was able to bind CD81 in a dose-dependent fashion (Fig. 5A). This is consistent with the previously reported data with mammalian cell-expressed glycosylated E2 [8]. Similar results were obtained in a reciprocal assay where GST–hCD81 was captured by the recE2 coated to ELISA dish wells (Fig. 5B). Again, this interaction was dependent on the concentration of recE2. Taken together, our data strongly indicate that the *E. coli*-expressed recE2 is able to bind human CD81 in a specific manner.

Interaction of recE2 with anti-E2 monoclonal antibodies

We next evaluated the properties of the recE2 for its ability to bind a selected panel of anti-E2 MAbs. All antibodies were raised against glycosylated E2 produced in mammalian cells and their corresponding epitopes have been mapped and characterised [8,11]. These antibodies recognise epitopes spanning most part of E2 ectodomain (Fig. 6). Overall, all antibodies reacted with the recE2 in a concentration-dependent manner, albeit with varying affinity (Fig. 7). Although for obvious reasons it is inappropriate to compare the relative affinity of these MAbs to glycosylated E2 reported previously and bacterially expressed recE2 described here, the general pattern

Fig. 6. Epitopes on E2 recognised by anti-E2 antibodies used in this study.

of antibody reactivity to both types of proteins was very similar. Interestingly, MAbs AP436 and ALP42 recognising aa 464–475 and 524–535, respectively, were previously shown to interact with truncated (but not the full-length) E2 only (see in [11]). Furthermore, a conformation-dependent MAb H53 that has been reported to interact with the functional, but not inactive, form of E2 [16], also reacted albeit weakly with the bacterially expressed E2. Together, these data suggest at least some conformational similarities between the glycosylated and non-glycosylated forms of E2.

In conclusion, this study shows that non-glycosylated recE2 produced in *E. coli* forms aggregates under all the conditions for the polypeptide expression and purification tested so far. Initially, small aggregates are formed, which then tend to form much larger structures upon storage. The protein carries 18 Cys residues, which presumably are involved in S–S bond formation. The purification scheme of recE2 described here is expected to allow it to form disulphide bonds while bound to the resin in a monomeric form. [In most cases, aggregation is a consequence of rather non-specific hydrophobic interactions among the polypeptide molecules.] The E2 is largely a hydrophobic protein—such proteins even when folded properly, tend to aggregate in hydrophilic environment. We are currently developing conditions to stabilise E2 in bulk solution. It is possible that the glyco-

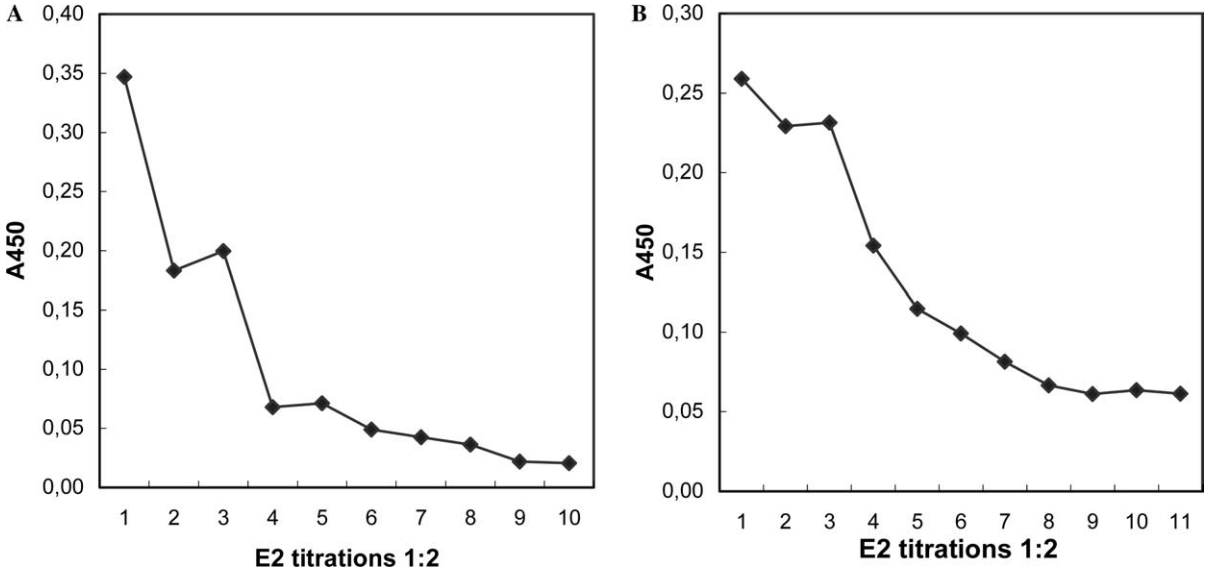

Fig. 5. Interaction between recE2 and hCD81 probed by ELISA-based assay. (A) A series of twofold dilutions of recE2 titrated on the plate coated with GST–hCD81 and the bound protein was detected with anti-E2 AP33 MAb. (B) GST–hCD81 added to the plate coated with different dilutions of recE2. The bound GST–hCD81 was detected with anti-GST MAb.

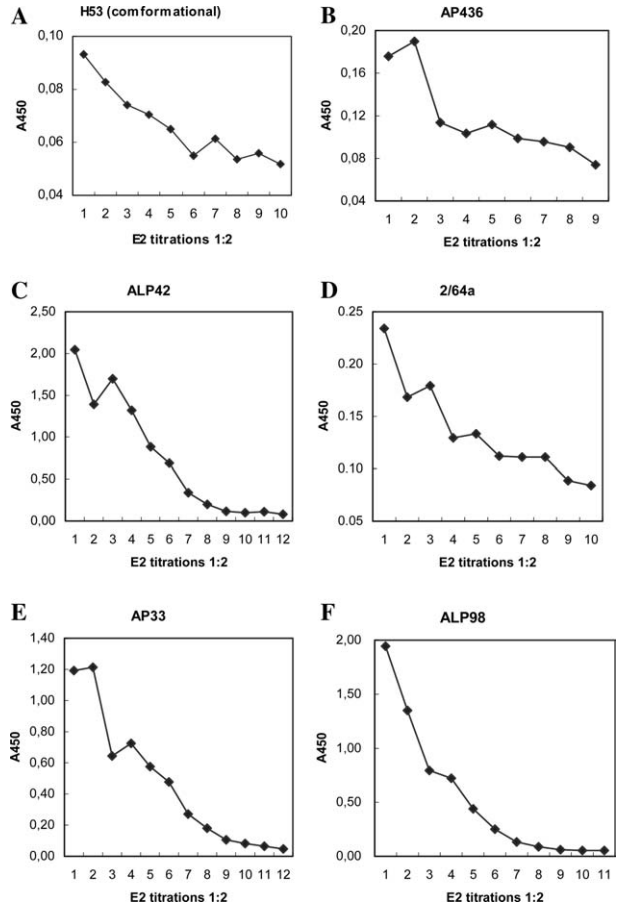

Fig. 7. Recognition of recE2 by different antibodies in ELISA.

side moiety plays a role in solubility of native E2 glycoprotein. As it has been mentioned already, even initial structural characterisation of glycosylated E2 has not yet been reported and, consequently, there are no data to compare it with the form obtained in this study. It should be noted that viral proteins quite often form aggregates or particles upon their expression in recombinant systems, yet in some cases these forms retain full functionality of the parent protein. For example, HBsAg antigen of hepatitis B virus expressed in yeast is produced in a particulate form [17]. This form of HBsAg has been used for its structure–function analysis and anti-HBV vaccine development [18]. Several lines of evidence indicate that the recombinant HCV E2 aggregates generated in this study are functional. Indeed, they interact with the virus receptor, CD81. In addition, they are recognised by a number of well-characterised anti-E2 antibodies in a fashion similar to that of the native glycosylated protein. Moreover, the conformation-dependent antibody H53 was also able to recognise non-glycosylated recE2 in its aggregate form. The reactivity of E2 with this antibody has been used as a criterion for functionality of the native glycoprotein [16]. Together, the antigenic and CD81-binding properties of the recE2 indicate conformational similarities with that of its native glycosylated counterpart. Overall, our results demonstrate that the non-glycosylated recE2 retains functionality of the native E2 glycoprotein, can be purified in adequate amounts and, therefore, can be used as a surrogate model of native E2.

Acknowledgments

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