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Characterization of secreted and intracellular forms of a truncated hepatitis C virus E2 protein expressed by a recombinant herpes simplex virus

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A replication-defective herpes simplex virus type 1 (HSV-1) recombinant lacking the glycoprotein H (gH)-encoding gene and expressing a truncated form of the hepatitis C (HCV) E2 glycoprotein (E2-661) was constructed and characterized. We show here that cells infected with the HSV/HCV recombinant virus efficiently express the HCV E2-661 protein. Most importantly, cellular and secreted E2-661 protein were both readily detected by the E2-conformational mAb H53 and despite the high expression levels, only limited amounts of misfolded aggregates were detected in either the cellular or secreted fractions. Furthermore, cell-associated and secreted E2-661 protein bound to the major extracellular loop (MEL) of CD81 in a concentration-dependent manner and both were highly reactive with sera from HCV-infected patients. Finally, BALB/c mice immunized intraperitoneally with the recombinant HSV/HCV virus induced high levels of anti-E2 antibodies. Analysis of the induced immunoglobulin G (IgG) isotypes showed high levels of IgG2a while the levels of the IgG1 isotype were significantly lower, suggesting a Th1-type of response. We conclude that the HSV-1 recombinant virus represents a promising tool for production of non-aggregated, immunologically active forms of the E2-661 protein and might have potential applications in vaccine development.

INTRODUCTION

Hepatitis C virus (HCV), a positive-strand RNA virus, is regarded as the major causative agent of non-A, non-B hepatitis, which can lead to cirrhosis, liver failure and hepatocellular carcinoma (Cohen, 1999; Hoofnagle, 1997; Reed & Rice, 2000; Saito *et al.*, 1990). Currently, there are more than 170 million people infected with HCV worldwide (Lauer & Walker, 2001). The only available therapy is interferon-α in combination with ribavirin. This treatment is expensive and shows only moderate response rates (Shad & McHutchison, 2001). Consequently, the development of diagnostic tools, improved therapies and both therapeutic and prophylactic vaccines against HCV are high-priority goals.

The HCV genome encodes two putative envelope glycoproteins, E1 and E2 (Reed & Rice, 2000). Both glycoproteins are thought to be type I transmembrane proteins that accumulate in the endoplasmic reticulum (ER) and interact non-covalently to form a heterodimer (Duvet *et al.*, 1998). Glycoprotein folding and formation of non-covalently associated E1E2 complexes are slow and inefficient processes that occur in association with the ER-resident chaperone calnexin (Choukhi *et al.*, 1998). The folding of E2 is independent of E1 but E2 is required for the proper folding of E1, suggesting that E2 acts as a chaperone for the folding of E1 (Cocquerel *et al.*, 1998). The E2 protein is (depending on the genotype) a 363–370 amino acid (aa) glycoprotein (58–74 kDa) with 20 conserved cysteines and 9 potential *N*-glycosylation sites (Depraetere & Leroux-Roels, 1999). At its carboxyl terminus, E2 has a hydrophobic anchor (aa 718–746) that retains the native E2 glycoprotein in the ER (Duvet *et al.*, 1998). Truncated E2-proteins

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lacking this region are soluble and secreted upon expression in mammalian cells (Michalak *et al.*, 1997; Mizushima *et al.*, 1994).

Owing to the lack of an *in vitro* system to propagate the virus and thus of sufficient quantities of HCV virions, truncated soluble mimics of E2 have been analysed for their potential use in the study of virus–host interactions and/or vaccine development (Depraetere & Leroux-Roels, 1999). In particular, a secreted form of E2 from HCV strain H truncated at aa 661 (E2-661) has been shown previously to react with a panel of conformation-sensitive antibodies, indicating that E2-661 is folded in a conformation comparable to the E2 in the native E1E2 complex (Deleersnyder *et al.*, 1997; Flint *et al.*, 2000). Most importantly, E2-661 was found to bind to CD81, a host cell-surface protein suggested to play a role in the virus attachment/entry process (Pileri *et al.*, 1998). Thus, truncated forms of the E2 protein may be useful reagents for *in vitro* immunobiological studies, vaccine development and generation of HCV diagnostics. Furthermore, recombinant chimeric E2 proteins have been generated through the replacement of the ER anchor with the transmembrane domain of other proteins, such as from vesicular stomatitis virus (Buonocore *et al.*, 2002), influenza A virus haemagglutinin (Flint *et al.*, 1999), CD4 (Cocquerel *et al.*, 1998; Siler *et al.*, 2002) or platelet-derived growth factor receptor (Forns *et al.*, 1999) in order to express the truncated from of E2 on the cell surface.

To optimally produce high quantities of correctly folded and glycosylated antigens, a large number of systems have been exploited for the expression of HCV glycoproteins (Depraetere & Leroux-Roels, 1999; Op De Beeck *et al.*, 2001). Prokaryotic and yeast expression systems lead to high-level expression of non-glycosylated proteins (Lok *et al.*, 1993; Mita *et al.*, 1992; Yokosuka *et al.*, 1993), and approaches using baculovirus-infected insect cells produce large quantities of partially glycosylated antigens (Lanford *et al.*, 1993; Matsuura *et al.*, 1992). Expression in eukaryotic systems has been based on transient or stable transfections of cells with DNA vectors (Chien *et al.*, 1993; Zaaijer *et al.*, 1994) or infections with recombinant viruses (Buonocore *et al.*, 2002; Siler, 2002; Dubuisson *et al.*, 1994; Fournillier-Jacob *et al.*, 1996). Eukaryotic expression systems are believed to yield native, properly glycosylated antigens, but only recombinant viruses combine high quantity with satisfactory quality. Another limitation of the eukaryotic expression systems is their tendency to generate disulfide-linked E2 aggregates, which are most likely non-functional dead-end products, yet may play a role in viral escape mechanisms (Choukhi *et al.*, 1999; Dubuisson, 1998). This occurs primarily after DNA transfection (Flint *et al.*, 2000) but also, to a variable degree, in certain viral expression systems (Michalak *et al.*, 1997).

In this study we evaluate, for the first time, the potential use of a recombinant herpes simplex virus type 1 (HSV-1)-based vector for expression of a truncated form of the HCV E2 from genotype 1a. HSV-1 is a large double-stranded neurotropic DNA virus and is a common human pathogen (Roizman & Knipe, 2001; Whitley & Roizman, 2001). Nevertheless, recent extensive studies have shown that the new generation of defective HSV-1 recombinants (both replication-defective or replication-competent attenuated viruses) are suitable expression vectors with potential applications in gene therapy or vaccine development (Brockman & Knipe, 2002; Da Costa *et al.*, 2001; Latchman, 2001). Here, we used a replication-defective HSV-1 recombinant which lacks the UL22 gene encoding the essential glycoprotein H (gH) (Forrester *et al.*, 1992). Complementing CR1 cells expressing gH are required for the propagation of the recombinant virus as gH is required for HSV entry into the target cells. Viruses produced from the complementing cell line can infect normal cells, but the virus is restricted to a single round of replication since the resulting virus progeny lacks gH and is therefore non-infectious. The gH-deleted HSV-1 recombinant, also known as a disabled infectious single cycle (DISC) herpes simplex virus, is a well-characterized viral vector system with extensive pre-clinical safety data (Loudon *et al.*, 2001). In addition, by virtue of its capacity for a single round of replication in infected cells, the DISC-HSV virus is predicted to support efficient expression of a foreign gene.

In this study we present evidence indicating that the DISC-HSV recombinant virus efficiently expresses non-aggregated, immunobiologically active intracellular and secreted forms of the E2-661 protein and induce a specific Th1-type antibody response against HCV E2 protein in a murine system.

METHODS

Cell culture. Vero, HepG2 and HEK 293 cell lines were obtained from the ATCC. CR1 cells, which are Vero cells stably transfected with the HSV UL22 gene (encoding the gH protein), were obtained from Xenova. All cell lines were grown in Dulbecco's modified essential medium (DMEM; Seromed) supplemented with 10 % foetal bovine serum (FBS; Seromed), glutamine (2 mM) and penicillin/streptomycin (5 IU ml^{-1}, 50 mg ml^{-1} respectively; Sigma).

Antibodies. MAbs H33, H47 and H53 were kindly supplied by Jean Dubuisson (Institute Pasteur, Lille, France) (Cocquerel *et al.*, 1998; Deleersnyder *et al.*, 1997). Hybridoma cell lines producing rat anti-E2 mAbs 3/11, 6/53 and 1/39 were kindly provided by Jane McKeating (University of Reading, Reading, UK). Hybridoma supernatants were used without further purification.

Generation and propagation of DISC-HCV recombinant viruses. DISC-HSV-1 strain 17 was kindly supplied by Xenova. Plasmid pIMJ28 (kindly supplied by Xenova) is a shuttle vector designed to contain two expression cassettes. The first expression cassette contains an open reading frame (ORF) encoding the green fluorescent protein (GFP) under the control of the Rous sarcoma virus promoter. GFP serves as a marker gene and is used for the selection and purification of recombinant viruses. The second cassette consists of an immediate early cytomegalovirus (*ie*CMV) promoter followed by a multiple cloning site in which the gene of interest is inserted, a poly(A) tail and stop codons in all reading frames.

E2 sequences were amplified from a vector containing the full-length cDNA sequence of the HCV H strain (genotype 1a), kindly provided by Genevieve Inchauspe (INSERM, Lyon, France).

For DISC-gEE2^{661}, an ORF consisting of the HSV gE leader sequence (HSV nt 141183–141309) serving as a signal sequence and aa 384–661 of HCV was subcloned in-frame into pIMJ28. All constructs were confirmed by sequencing and a sequence change at aa 370 leading to a substitution of glutamic acid for lysine was corrected. A DISC-GFP, which encodes the GFP under the control of the *ie*CMV promoter, was constructed as a mock-infection control.

DISC-HCV recombinants were constructed using the *Pac*I-based ligation technique as previously described by Xenova (Loudon *et al.*, 2001). The ligation mix was transfected into gH-expressing CR1 cells using Lipofectamine (Life Technologies). Recombinant GFP fluorescent viruses were purified using limiting dilution assays and single plaques were screened by PCR using E2-specific primers and verified by Southern blots (Sambrook, 1989). DISC-HCV recombinants were maintained and titrated in CR1 cells.

Standard infection, metabolic labelling and immunoprecipitation. Confluent monolayers of Vero or CR1 cells were grown in six-well plates (Corning Costar) or 25 cm^2 flasks and were inoculated with 5 p.f.u. in 199V medium containing 1 % FBS (Life Technologies). After 2 h adsorption at room temperature, the medium was replaced with fresh 199V medium containing 1 % FBS and infection allowed to proceed.

For enzyme immunoassay (EIA), supernatant was collected after 20 h, clarified by centrifugation and concentrated 60-fold using Centripreps (10 000 kDa cut-off; Amicon). The infected cells were pelleted by centrifugation and resuspended in 3 ml 0·15 M NaCl/ 0·5 M Trizma Base (Sigma) and complete proteinase inhibitor (Boehringer Mannheim) at pH 8·8. Cells were disrupted by sonication for 3 min. Cellular debris was removed by centrifugation.

For labelling with [^{35}S]methionine, Vero or HepG2 cells were washed 6 h post-infection (p.i.) with pre-warmed DMEM without methionine (Life Technologies) and then incubated in the same medium for 1 h to induce methionine starvation. For steady-state labelling, infected cells were labelled for the indicated time with 30–50 μCi [^{35}S]methionine (ICN Biochemicals) ml^{-1}, in medium containing 1/40 of the normal concentration of methionine. After labelling, supernatant was collected and infected cells were washed once with ice-cold PBS before lysis (20 min/4 °C) in Tris-buffered saline (TBS) lysis buffer containing 0·5 % Igepal (Sigma). Cell lysates and supernatants were clarified by centrifugation (15 min/4 °C/12 000 ***g***) and then precleared with Protein A–Sepharose after TBS and Igepal concentrations had been adjusted to 1 × and 0·2 % respectively. Immunoprecipitations were carried out as previously described (Dubuisson *et al.*, 1994). The precipitates were solubilized by heating for 3 min at 96 °C in SDS-PAGE sample buffer and run on a 10 or 12·5 % polyacrylamide gel. Separated proteins were transferred onto a nitrocellulose membrane (Protran BA nitrocellulose, Schleicher and Schuell) and exposed to Kodak XAR film.

Endoglycosidase digestion. Immunoprecipitated proteins were eluted from Protein A–Sepharose beads in 0·5 % SDS and 1 % mercaptoethanol by heating at 96 °C for 10 min. The protein eluates were then divided into three equal portions for digestion with peptide *N*-glycosidase (PNGase) F or endo-*β*-*N*-acetylglucosaminidase H (Endo H; both enzymes from New England Biolabs) plus an undigested control. Digestions were carried out for 1 h at 37 °C following the manufacturer's protocol. Digested samples were analysed by SDS-PAGE and autoradiography as described above.

Production of secreted E2-661 by transient transfection of cells with an E2 expression plasmid. Production of secreted recombinant E2-661 by transient transfection of HEK 293 cells was done as described previously (Flint *et al.*, 1999). Briefly, HEK 293 cells were cultured in 145 mm dishes and transfected with a eukaryotic expression vector encoding E2-661 (p14.tE2·661.hiv, HCV genotype 1a, kindly provided by J. McKeating) using Effectene (Qiagen). Supernatant was harvested after 72 h. E2 was enriched from these supernatants using GNA lectin columns (Amersham Pharmacia) or affinity columns, produced by coupling purified anti-E2 antibodies (1/39 or H33) to HiTrap NHS activated columns (Amersham Pharmacia).

Western blot analysis. Samples were separated by SDS-PAGE on 10 % or 12·5 % polyacrylamide gels under reducing or non-reducing conditions. Proteins were then transferred to nitrocellulose membranes (Protran BA Nitrocellulose) and probed with either anti-E2 mAb H47 (a mouse mAb, detected using a peroxidase-conjugated goat anti-mouse IgG; Pierce) or anti-E2 mAb3/11 (a rat mAb, detected using a peroxidase-conjugated anti-rat IgG; Harlan Sera-Lab). Proteins were then visualized using an enhanced chemoluminescence system (Amersham Pharmacia).

The amount of intracellular versus secreted antigen was estimated by EIA, using several dilutions. All samples were analysed on the same plate. Using this analysis, the amount of intracellular antigen corresponded to the amount of secreted antigen of the same preparation when cell pellets were resuspended in an equivalent volume as the concentrated supernatant.

GNA-capture EIA and CD81-capture EIA. EIAs were performed as previously described (Flint *et al.*, 2000). Captured antigen was detected by incubation with the conformation-dependent anti-E2 mAb H53 followed by incubation with an anti-mouse IgG1–HRP antibody (BD/Pharmingen) and a tetramethylbenzidine substrate (TMB; Sigma). As a positive control for CD81 binding to the plates, an anti-CD81 mAb (Autogen-Bioclear) that recognizes both human and mouse CD81 was used in conjunction with an anti-goat IgG–HRP mAb (Santa-Cruz Biotechnology). Absorbance values were determined at 450 nm using an EIA reader (SpectraMax 340, Molecular Devices, UK).

Sera. Coded sera from 55 individuals were tested for reactivity against secreted E2-661. They were obtained from: 38 patients, 19 males and 19 females, 18–70 years old with chronic hepatitis C (CHC); 7 patients with other forms of liver disease, i.e. primary biliary cirrhosis (PBC), chronic hepatitis B (HBV) and chronic hepatitis G (HGV); and 10 healthy individuals negative for anti-HCV antibodies. All sera from patients with CHC were obtained before the beginning of interferon-*α* treatment and were anti-HCV positive by EIA (Ortho) and positive for HCV RNA (Roche, Amplicor). The infected genotype was determined by INNOLiPA (Innogenetics). According to their response to interferon therapy they were divided into three groups: (a) sustained responders (SR) with negative HCV RNA in serum and normal alanine aminotransferase (ALT) levels at the end of the treatment, as well as at the end of a 6 month follow-up after cessation of therapy, (b) relapsed responders (RR) with negative serum HCV RNA and normal ALT levels at the end of therapy, but with virological and biochemical relapse during the 6 months of post-treatment follow-up; and (c) non-responders (NR) with positive serum HCV RNA and increased ALT levels maintained up to the end of interferon-*α* therapy.

Detection of HCV antibodies in serum samples. Immunolon II EIA plates (Dynal) were coated with 50 μl per well of the secreted or intracellular recombinant E2-661 antigen, respectively, in TBS at an estimated final concentration of 0·5 μg per well and incubated for 1 h at 37 °C. Wells were blocked with 100 μl TBS supplemented with 5 % w/v non-fat milk for 1 h at room temperature and subsequently washed once with TBS containing 1 % (w/v) non-fat

milk and 0·1 % (v/v) Tween 20 (TBS-MT). Patient sera diluted 1/100 in TBS-MT were preincubated with HSV glycoproteins (5 μg ml^{-1}) for 1 h at 37 °C in order to remove anti-HSV-specific antibodies from the samples. Pretreated serum samples (100 μl) were added to each well and the plates were incubated for 1 h at 37 °C. After washing, 100 μl rabbit anti-human IgG conjugated to HRP (Dako) at 1/1000 in TBS-MT solution was added to each well and the plates were incubated for 1 h at 37 °C. The final immune complexes were detected by addition of 100 μl TMB substrate solution and colorimetric determination of the absorbance at 450 nm. For each sample, the final absorbance value was calculated by subtracting the A_{450} of the control antigen EIA from the respective A_{450} of the fusion protein EIA. The cut-off point was determined as the mean value of the negative samples plus two standard deviations (SD).

Immunization procedures. The housing, maintenance and care of the animals were in compliance with all relevant guidelines and requirements. Groups of three to five female BALB/c mice (6–8 weeks old) were injected twice at day 0 and 14 intraperitoneally with 5×10^6 p.f.u. of DISC-gEE2^{661} recombinant virus, whereas control animals received 5×10^6 p.f.u. DISC-GFP virus.

Antibody analysis. Sera collected from the immunized animals at the indicated times were analysed for anti-E2 total IgG Ab concentration by standard EIA methodology, using prokaryotic E2 recombinant protein isolated from inclusion bodies. Medium binding EIA plates (Corning Costar) were therefore coated with prokaryotic E2-antigen at a concentration of 0·5 μg per well. Total IgG was detected in mice sera by HRP-conjugated anti-mouse immunoglobulins (Dako) as a secondary antibody and developed with TMB substrate. Analysis of the antibody isotypes was performed according to the manufacturer's instructions using a commercial isotyping kit (BD/Pharmingen).

RESULTS

Construction of recombinant HSV encoding a truncated form of the HCV E2 protein

A recombinant replication-defective HSV-1 recombinant virus lacking the gH-encoding gene (HSV-DISC) and expressing the HCV E2 protein truncated at aa 661 was engineered (as described in Methods) and designated DISC-gEE2^{661} (Fig. 1). To ensure efficient production and secretion of the E2-661 protein in the context of the HSV genome, the virus was designed to contain the signal sequence of the HSV glycoprotein E in place of the native E2 signal sequence (Miriagou *et al.*, 1995; Tsitoura *et al.*, 2002). The transgene was inserted into the gH locus under the control of the CMV promoter and the integrity and proper insertion of the HCV E2 fragments into the HSV-1 genome was checked by PCR and Southern blot analysis (data not shown).

Expression analysis of the HCV E2-661 protein from DISC-gEE2^{661} recombinant virus-infected cells

To investigate the expression of the HCV E2-661 protein, CR1 or Vero cells were infected with DISC-gEE2^{661} at an m.o.i. of 5. Cell lysates and supernatants were harvested periodically over a 24 h period and analysed by Western blot using the conformation-independent E2-specific mAb

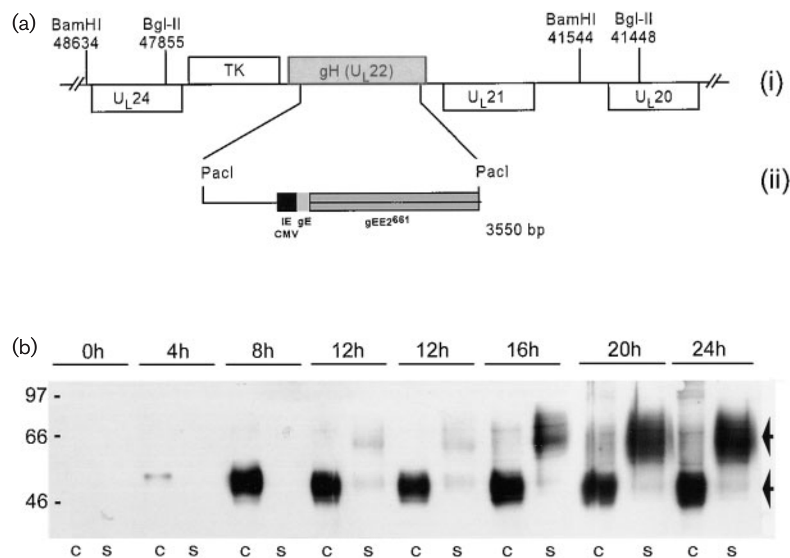

Fig. 1. (a) Construction of DISC-gEE2^{661} recombinant virus. (i) Schematic representation depicting the location of the gH-encoding gene in the HSV-1 viral genome. (ii) The *Pac*I fragment containing the HCV E2-661-encoding sequences (grey box) under the control of the immediately early CMV promoter (black box). gE represents the signal sequences from the gE HSV-1 glycoprotein. (b) Kinetics of E2-661 expression in DISC-gEE2^{661}-infected cells. Western blot analysis of E2-661 expression in CR1 cells infected by the DISC-gEE2^{661} virus. Cell extracts (c) and supernatants (s) were harvested at the indicated times p.i. The blots were probed with anti-E2 mAb H47. Sizes of molecular mass markers are shown on the left. Arrowheads indicate secreted E2 protein (upper band) and the intracellular newly synthesized E2 protein (lower band).

H47. As shown in Fig. 1(b), this antibody detected a protein of 66 kDa in the supernatant (12–24 h p.i.) and 55 kDa in the cell-associated fraction of DISC-gEE2^{661}-infected cells (4–24 h p.i.), consistent with the predicted size of the truncated E2-661 protein. The molecular mass of the secreted E2 glycoprotein was higher than that of the intracellular protein, suggesting additional post-translational modification during secretion. The amount of the E2-661 antigen secreted from cells after 18 h p.i. with DISC-gEE2^{661} ranged between 1–2 mg of protein per 2×10^9 infected cells and a similar amount of antigen could be retrieved from the cell pellets (data not shown).

To analyse the glycosylation status of the HCV E2-661 protein expressed in the context of the HSV-1 viral vector, Vero cells were infected with the DISC-gEE2^{661} virus for 10 h and were subjected to [^{35}S]methionine labelling for the last 4 h of infection. Cell lysates and supernatants were collected and digestions with Endo H and PNGase F on the immunoprecipitates were carried out as described. Endo H removes the chitobiose core of high mannose and some hybrid forms of *N*-linked sugars but not complex forms of *N*-linked sugars, whereas PNGase F removes high-mannose and complex sugars. Therefore, resistance to digestion with Endo H but not with PNGase F indicates that the proteins reached the *trans*-Golgi where complex sugars are added. The analysis of the glycosylation pattern is shown in Fig. 2. Only the secreted E2-661 protein expressed in Vero (Fig. 2b, lanes 4–6) or HepG2 cells (Fig. 2c, lanes 4–6) was resistant to Endo H digestion. In contrast, the intracellular form of E2-661 was Endo H sensitive (Fig. 2b, c, lanes 1–3). These results confirm that the secreted E2-661 protein undergoes Golgi processing prior to secretion when expressed in the context of HSV infected cells.

Finally, we further investigated the folding state of this protein. Normally, a large proportion of the HCV glycoproteins expressed in transiently or stably transfected cell lines form aggregates, stabilized by disulfide bonds. These forms are most likely dead-end products and do not retain functions such as CD81 binding (Choukhi *et al.*, 1999; Deleersnyder *et al.*, 1997; Flint *et al.*, 2000). Therefore, it is important to be able to differentiate between aggregates and correctly folded monomeric proteins. To this end, secreted E2-661 as expressed by DISC-gEE2^{661} was analysed by Western blot analysis under reducing and non-reducing conditions to determine the proportion of the protein present as aggregates. In parallel, E2-661 secreted from HEK 293 cells transiently transfected with an E2-661-expressing plasmid (genotype 1a), which has been previously described to produce a proportion of misfolded aggregates (Flint *et al.*, 2000), was analysed and served as a positive control. As expected, under non-reducing conditions the majority of the transiently expressed E2-661 protein was detected as high molecular mass aggregates (Fig. 3a, lanes 5 and 6), whilst under reducing conditions the higher molecular mass form was markedly reduced and the majority of protein was of a lower molecular mass (Fig. 3a, lanes 2 and 3). In contrast, nearly all the secreted and intracellular forms of E2-661 protein expressed by the recombinant DISC-gEE2^{661} was monomeric under reducing (Fig. 3a, lane 1) and non-reducing conditions (Fig. 3a, lane 4; Fig. 3b,

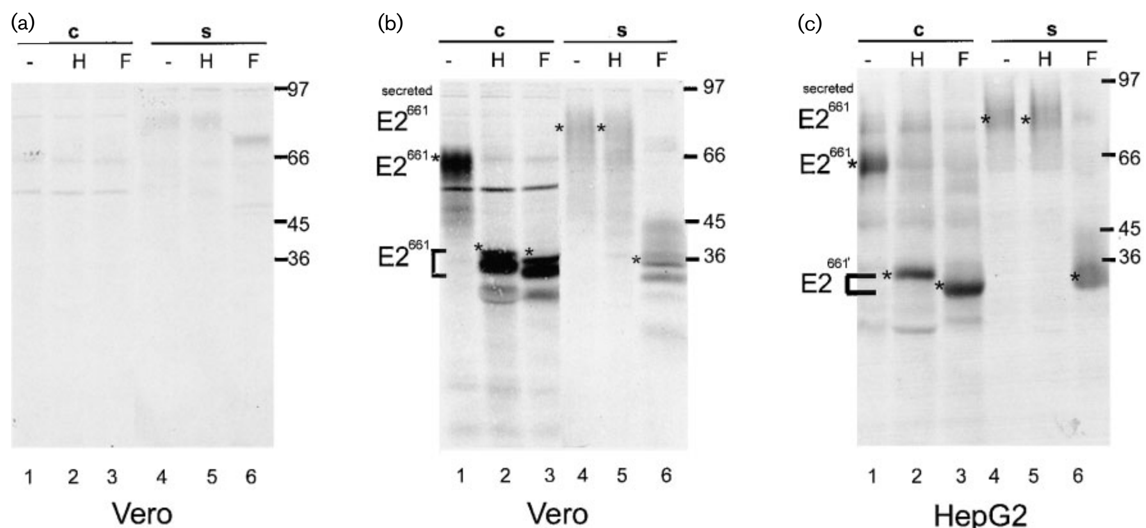

Fig. 2. Glycosylation state of the cellular (c) and secreted (s) truncated HCV E2 glycoproteins. Vero or HepG2 cell monolayers were infected with DISC-GFP (mock control; a), or DISC-gEE2^{661} (b, c) and labelled with [^{35}S]methionine from 10 h p.i. Equivalent portions of the cell lysates (c) or supernatants (s) were immunoprecipitated with mAb H47. Immunoprecipitates were resuspended and treated with Endo H (H), PNGase F (F) or left untreated (-). Samples were separated by 12·5 % SDS-PAGE, transferred to nitrocellulose and visualized by autoradiography. HCV-E2-specific proteins and their deglycosylated forms are indicated by asterisks. Molecular mass markers are shown on the right.

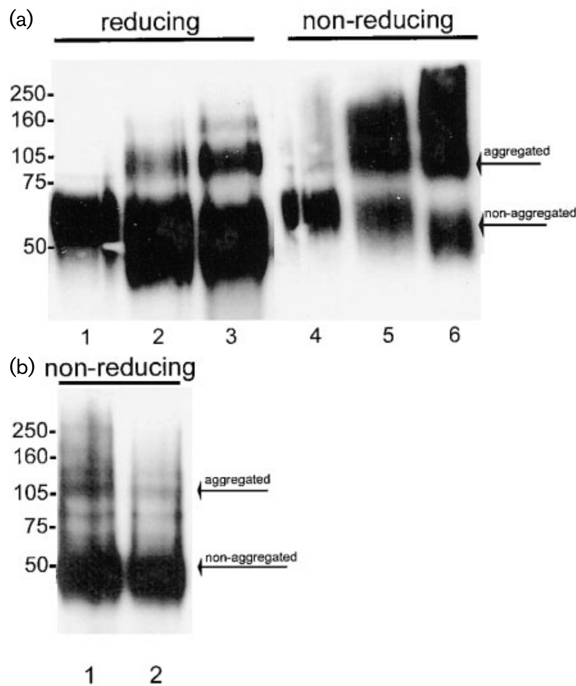

Fig. 3. Analysis of secreted and intracellular forms of the E2-661 protein under reducing and non-reducing conditions. (a) Lanes 1 and 4: supernatant concentrated from Vero cells infected with DISC-gEE2^{661} 18 h p.i. Lanes 2, 3, 5 and 6: supernatants concentrated from 293 cells transfected with a plasmid encoding HCV E2 truncated at residue 661, from which E2 was enriched using either a GNA lectin column (lanes 2 and 5) or an Ab affinity column (lanes 3 and 6). Samples were separated on a 12 % SDS-PAGE gel under either reducing (1–3) or non-reducing (4–6) conditions. E2 protein was identified by Western blotting using anti-E2 mAb 1/39. Molecular mass markers are indicated on the left. Arrows indicate aggregated and non-aggregated E2 protein. (b) Lanes 1 and 2: two different amounts of intracellular E2-661 protein from Vero cells infected 18 h previously with DISC-gEE2^{661} were separated on a 12 % SDS-PAGE gel under non-reducing conditions. E2 protein was identified by Western blotting using anti-E2 mAb 1/39. Molecular mass markers are indicated on the left. Arrows indicate aggregated and non-aggregated E2 protein.

lanes 1 and 2). Size differences observed between E2-661, transiently expressed or expressed by DISC-gEE2^{661}, are most likely due to different processing, e.g. glycosylation of E2-661 in the 2 systems.

Binding of intracellular and secreted E2-661 to CD81 and GNA lectin

Expression of correctly folded E2 glycoproteins by DISC-gEE2^{661} was further assessed by analysis of their ability to bind to CD81. Binding to GNA lectin and to the EC2 extracellular loop of either recombinant mouse or human CD81 was assessed by EIA. Binding of cellular and secreted E2-661 to GNA lectin, as detected using the

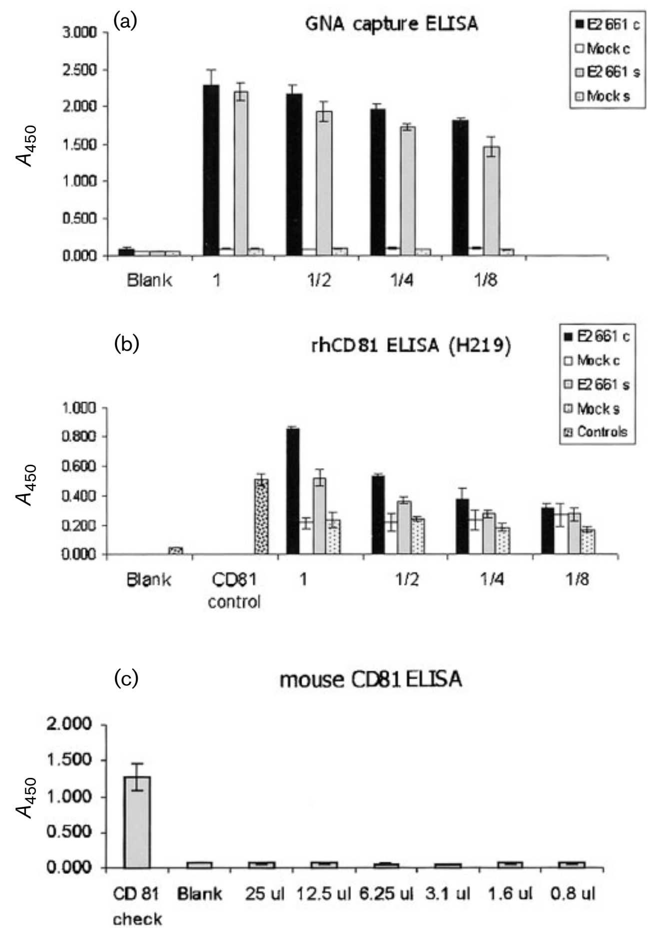

Fig. 4. Comparison of the binding of intracellular and secreted E2-661 to CD81 by EIA. Concentrated supernatants (s; white bars) and cell lysates (c; black bars) from DISC-gEE2^{661}-infected Vero cells were analysed by EIA for their ability to bind to plates coated with (a) GNA lectin, (b) GST-fused human CD81 or (c) GST-fused mouse CD81. Each supernatant was tested at different dilutions: neat (1), 1/2 and 1/4 diluted. Blank represents a medium only control. Binding was detected with anti-E2 conformational dependent mAb H53 followed by a HRP-conjugated anti-mouse IgG1 Ab. The results are shown as the EIA readout values (A_{450}). CD81 control represents the binding of an anti-CD81 antibody which recognizes both human and mouse CD81 to the CD81-coated plates (positive control). Comparable data were obtained in three similar experiments.

conformational mAb H53, was equivalent for both proteins (Fig. 4a). Importantly, both the intracellular and the secreted forms of E2-661 protein were also found to bind to the large extracellular loop of human (Fig. 4b) but not mouse CD81 (Fig. 4c; here shown for secreted E2-661), providing evidence that these proteins have a functionally intact conformation. Notably, intracellular E2-661 bound more efficiently than secreted E2-661 to the EC2 loop of human CD81 (although the two preparations showed equivalent binding to GNA lectin).

Recognition of E2-661 by human sera

A panel of 55 serum specimens including 38 HCV-positive sera from chronic hepatitis patients, 7 sera from patients with non-HCV-associated liver disease and 10 healthy individuals was analysed by EIA for the presence of antibodies able to recognize secreted E2-661 antigen. Thirty (79 %) of the 38 HCV-positive sera showed reactivity against E2-661 (Fig. 5, column 1). None of the 17 negative serum samples showed any reactivity against this antigen (Fig. 5, columns 2–5). 18/24 (75 %) of the sera from patients infected with genotype 1, 9/9 sera from patients infected with genotype 3 and 3/5 sera from patients infected with genotype 2 or 4 viruses were positive in the EIA, indicating considerable subtype cross-reactivity. Thus, the E2 antibodies detected in our assay are not genotype specific and probably recognize conserved epitopes. This has been previously observed, but some genotype-specific regions may exist (e.g. aa 493–576) (Lesniewski *et al.*, 1995; Mink *et al.*, 1994; Psichogiou *et al.*, 1997; Yuki *et al.*, 1996). It should be noted that comparison of the serological profiles of therapy NR (71 %), RR (87 %) and SR (78 %) did not reveal any significant differences in their reactivity to the E2-661 antigen.

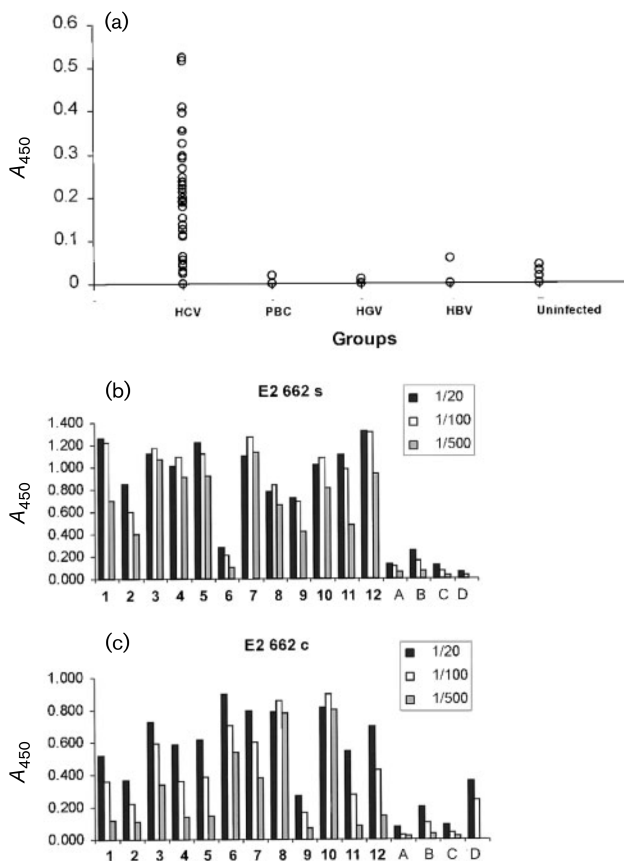

Fig. 5. (a) EIA analysis of the recognition of secreted E2-661 by human sera. EIA binding (A_{450} values) of human serum samples (all tested at 1/100 dilution) to secreted E2-661. Column 1, HCV-positive sera; column 2, sera from patients with PBC; column 3, HGV-positive sera; column 4, HBV-positive sera; column 5, HCV-negative sera. (b, c) EIA analysis comparing the recognition of secreted and intracellular E2-661 by human sera. EIA binding (A_{450} values) of HCV-positive human sera (1–12) or HCV-negative sera (A–D) to secreted (b) and intracellular (c) forms of E2-661. Each sample letter represents a different patient serum. Each serum was tested at three different dilutions (1/20, 1/100 and 1/500 as indicated).

Furthermore, an additional 12 HCV-positive, HSV-negative and 4 HCV-negative serum samples were compared for their reactivity against the intracellular and secreted forms of E2-661 (Fig. 5b, c). 10/12 HCV-positive serum samples showed reactivity against the intracellular E2-661, whilst 11/12 were reactive against the secreted antigen, indicating that both forms of the antigen are recognized by the majority of infected patient sera. Interestingly, the serum samples from some of the patients showed preferential recognition of one, but not both forms of E2-661 (samples 6, 9).

Antibody response in mice vaccinated with the recombinant DISC-gEE2^{661} virus

To analyse the immunogenicity of the DISC-HCV recombinant virus, BALB/c mice were injected intraperitoneally with 5×10^6 p.f.u. of DISC-gEE2^{661} or the parental DISC-GFP virus twice (on days 0 and 14) and the E2-specific IgG responses were measured by reactivity against plate bound recombinant E2 by EIA at days 14 and 28 post-initial (day 0) injection. As shown in Fig. 6(a), all mice immunized with recombinant DISC-gEE2^{661} virus developed high levels of anti-E2 antibodies after the first immunization which increased further following the second inoculation. No antibodies were detected in mice immunized with the control virus. Subsequent subclass analysis of the anti-E2 antibodies showed that the predominant anti-E2 isotype was IgG2a, suggesting a strong Th1-like response. The IgG1 levels were significantly lower (Fig. 6b). Notably, intramuscular immunization with a DNA vaccine 14 days prior to inoculation with the DISC-gEE2^{661} virus improved the levels of the IgG1 isotype (data not shown).

DISCUSSION

We have constructed and characterized a recombinant replication-defective HSV-1 virus that expresses a C-terminal truncated form of the HCV E2 protein (E2-661). We found that E2-661 protein produced using this system was properly glycosylated and was expressed at high levels both within cells and in the cell supernatant. Thus, the efficiency of our system is comparable to that reported for the baculovirus–insect cell system (Lanford *et al.*, 1993), and yet has the advantage that E2 expression from the DISC-HCV recombinant viruses is achieved in mammalian cells. Most importantly, Western blotting under non-reducing conditions showed that most of the intracellular and secreted E2-661 protein was monomeric with only a low

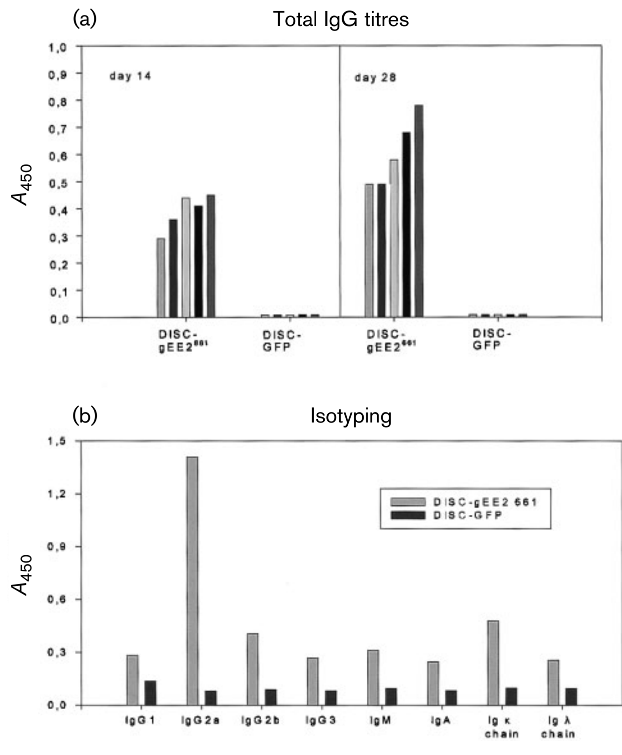

Fig. 6. Anti-E2 antibody responses in DISC-gEE2^{661}-immunized mice. (a) Sera obtained from BALB/c mice immunized with 5×10^6 p.f.u. DISC-gEE2^{661} or parental viruses were evaluated against a recombinant E2 protein by EIA 14 days following the first (day 14) or the second (day 28) inoculation. Anti-E2 absorbance values of serum samples diluted 1 : 100 from each mouse are depicted. (b) IgG isotype distribution in DISC-gEE2^{661}- or DISC-GFP-immunized mice. IgG isotype levels were measured in 1 : 100-diluted sera from the five mice of each group by using the isotype detection kit from BD/Pharmingen.

proportion of disulfide-linked aggregates. This was confirmed by immunoprecipitation experiments with the conformation-sensitive antibody H53, which is able to distinguish between aggregated and non-aggregated E2 protein (data not shown). Recognition of cellular and secreted E2-661 by mAb H53 in EIA assays was as strong as E2 recognition by the other mAbs used in this study (e.g. mAb H47), underlining the low proportion of aggregated protein generated using this expression system. By contrast, when high levels of E2-661 were produced by transient transfection of mammalian cells with a plasmid expressing a truncated version of E2, much of this protein was found to consist of misfolded aggregates (Flint *et al.*, 2000). The reasons for the relative lack of aggregated E2 in our system are unclear and may be due to HSV-induced effects.

As mAb H53 reactivity has been correlated with an E2-conformation capable of binding to CD81 (Flint *et al.*, 2000), we also investigated whether intracellular and secreted E2-661 produced by DISC-HCV recombinants was capable of binding to CD81 by using an EIA-based capture assay. Consistent with results previously obtained using E2-661 produced using a transient transfection system (Flint *et al.*, 2000), we found that both intracellular and secreted forms of E2-661 were able to bind to recombinant CD81 in a concentration-dependent manner, and that intracellular E2 bound nearly twice as efficiently to CD81 as the secreted form. The greater efficiency of binding of the intracellular form of E2-661 to CD81 is thought to be due to the differences in glycosylation between this and the secreted form of E2. Secreted E2-661 passes through the Golgi compartment following the secretory pathway, a process that leads to the acquisition of complex sugars which might then interfere with CD81 binding either directly or by steric hindrance.

Consideration of how the conformation and glycosylation state of recombinant E2 proteins may be related to forms of the protein that are expressed during *in vivo* infection with HCV is of importance with regard to the choice of antigen used both for diagnostic tests and in subunit vaccine design. For diagnostic tests, it is important that the E2 protein used should be recognized by sera from infected individuals. Here, we report that secreted E2-661 antigen was recognized in an EIA assay by anti-HCV antibodies present in the sera of patients persistently infected with HCV (79 %). This is comparable to previous data which have shown that most chronic HCV-sera (80–97 %) contain IgG antibodies reactive with recombinant E2 antigen produced using mammalian expression systems (Chien *et al.*, 1993; Mink *et al.*, 1994; Saracco *et al.*, 1994; Yuki *et al.*, 1996). Anti-E2 specific antibodies are much less efficient at recognition of E2 antigen produced in insect cells (Chien *et al.*, 1992; Hussy *et al.*, 1997; Lanford *et al.*, 1993), yeast (Chien *et al.*, 1993; Lok *et al.*, 1993; Mink *et al.*, 1994) or bacteria (Hussy *et al.*, 1997; Mita *et al.*, 1992; Yokosuka *et al.*, 1993), indicating an important role for protein conformation and glycosylation in antibody recognition. As previously described, an individual's response to interferon therapy was not predictable on the basis of the anti-E2 antibody level measured before treatment (Fournillier-Jacob *et al.*, 1996; Saracco *et al.*, 1994). No false positive results in the controls of uninfected, HBV- or HGV-infected patients, nor in sera of patients with autoimmune cirrhosis (primary biliary cirrhosis), were observed. Furthermore, we compared the ability of human sera from a limited number of infected individuals to recognize the intracellular versus secreted forms of E2-661. Interestingly, some of the sera could discriminate between the two forms of the protein, suggesting that a proportion of infected individuals have an antibody response to epitopes that are differentially expressed in the two different forms of the protein. A previous study also documented preferential reactivity of serum samples from a proportion of infected individuals with the intracellular from of E2 (Flint *et al.*, 2000), and suggests that an EIA assay combining intracellular and secreted forms of E2 may be beneficial for diagnostic purposes.

Although the nature of the protective immune responses against HCV is not well defined, several studies suggest that

HCV E2 protein represents a potential target for vaccine development (Abrignani & Rosa, 1998; Choo *et al.*, 1994; Siler *et al.*, 2002; Zucchelli *et al.*, 2000). To this end, we present preliminary evidence that vaccination of BALB/c mice with a replication-defective HSV recombinant virus expressing a truncated HCV E2 protein (DISC-gEE2^{661}) is capable of eliciting a strong humoral immune response against the E2 protein. It is noteworthy that all animals developed high levels of E2-specific antibodies after the first immunization, which increased further following the second immunization. Isotype analysis of the response against the E2 antigen suggested a strong Th1-type response by the almost exclusive detection of IgG2a. Therefore, the use of attenuated recombinant HSV-based vectors provides an alternative virus-based expression system efficient at producing large quantities of non-aggregated, correctly folded, seroreactive, CD81-binding recombinant protein and may represent a new immunization strategy to induce humoral and cellular immune responses against HCV. Experiments characterizing the cellular immune response are in progress to further evaluate such possibilities.

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