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Abstract

The hepatitis B virus (HBV) envelope protein (S) self-assembles into subviral particles used as commercial vaccines against hepatitis B. These particles are excellent carriers for foreign epitopes, which can be inserted into the external hydrophilic loop or at the N- or C-terminal end of the HBV S protein. We show here that the N-terminal transmembrane domain (TMD) of HBV S can be replaced by the TMDs of the hepatitis C virus (HCV) envelope proteins E1 and E2, to generate fusion proteins containing the entire HCV E1 or E2 sequence that are efficiently coassembled with the HBV S into particles. This demonstrates the remarkable tolerance of the HBV S protein to sequence substitutions conserving its subviral particle assembly properties. These findings may have implications for the design of new vaccine strategies based on the use of HBV subviral particles as carriers for various transmembrane proteins and produced with the same industrial procedures established for the HBV vaccine.

Key words

HBV ; HCV ; viral hepatitis ; envelope protein ; chimeric protein ; fusion protein ; subviral particle ; CHO ; vaccine ;

Introduction

Many viral structural proteins are intrinsically able to assemble into virus-like particles independently of viral nucleic acids. Antigens present as multiple copies on these virus-like particles are effective immunogens, and can elicit potent anti-viral humoral and cellular immune responses against the viruses from which they are derived [1, 2]. In particular, the small (S) envelope protein of the hepatitis B virus (HBV) self-assembles into highly immunogenic, non-infectious subviral particles [3]. The HBV S protein produced in mammalian cells is secreted as a 22-nm subviral particles which has over the last 25 years been used worldwide as a commercial hepatitis B vaccine. As these HBV subviral envelope particles provide a safe antigen delivery system, they have also long been used as a carrier for the presentation of various antigen epitopes. The epitopes presented in this way have included those of the capsid protein of poliovirus [4, 5], the hepatitis C virus (HCV) envelope proteins [6, 7], the human immunodeficiency virus envelope proteins [8-11] and the malaria parasite [12]. According to the current model, the HBV S protein is an intrinsic membrane protein of 226 amino-acids - aa - (*adw* subtype) that spans the endoplasmic reticulum (ER) membrane four times (Fig. 1) [13-15]. This model predicts the exposure of the amino-terminal (N-terminal) and carboxyl-terminal (C-terminal) ends and of a short hydrophilic loop (also known as the antigenic loop) in the middle of the protein to the ER lumen and at the surface of the subviral particle after particle budding. The insertion of small foreign sequences into the luminal loop of the HBV S protein has been shown to be compatible with particle formation [4, 5, 7, 9, 11, 16]. The N-terminal ectodomain [10, 12] and the C-terminal [8] ectodomains of HBV S protein also tolerate the insertion of foreign residues, with no effect on particle formation. A study investigating the incorporation of green fluorescent protein (GFP) into various domains of the HBV S protein demonstrated that the N-terminal ectodomain of HBV S was more permissive for insertions than the C-terminal ectodomain [17].

However, none of these previous studies was designed to incorporate a foreign protein, including its transmembrane domain, into the HBV subviral envelope particle. As the HBV S protein can tolerate major modifications without deleterious effects on self-assembly into subviral particles, we decided to address this question. We chose to use the HCV E1 and E2 envelope proteins as models for this approach for three major reasons:

(i) HCV is a member of the *Flaviviridae* family and it is an important human pathogen. Its envelope proteins E1 and E2 constitute a potential target for the development of a prophylactic HCV vaccine, as they are probably involved in virus-host recognition and antibodies directed against these proteins seem to neutralize HCV [18].

(ii) Both these proteins have a single transmembrane domain resulting in their retention in the ER membrane (Fig. 1), and preventing their purification [19]. Subviral envelope particles lacking a core are observed in other flavivirus infections, but no subviral HCV envelope particles have been described in the serum of infected patients, and it is not possible to generate such particles by producing HCV E1 and E2 proteins in cell lines [20]. The production of HCV envelope proteins as secreted recombinant proteins for vaccine development requires the deletion of the transmembrane domain. However, these truncated forms remain however difficult to purify, requiring the use of processes incompatible with industrial developments [21]. Furthermore, the truncation of these proteins by deletion of their transmembrane domain has been shown to impair their antigenic and functional properties [22].

*(iii)*This strategy could potentially lead to the development of a bivalent HBV-HCV prophylactic vaccine candidate of potential interest as the populations at risk of infection with these two viruses are essentially the same [18].

We thus designed chimeric HBV-HCV envelope proteins in which the HCV E1 or E2 protein was fused to the HBV S protein. The N-terminal transmembrane domain of the HBV S was replaced by the transmembrane domain of the HCV E1 or E2 protein, to preserve the integrity of the HCV E1 or E2 proteins, and to maintain the chimeric proteins in an appropriate membrane topology. We found that the production of these chimeric proteins was compatible with subviral particle assembly and secretion.

Materials and methods

Semliki forest virus (SFV)-derived plasmids construction

The plasmid pSFV-SHBs^{adw} (renamed here pSFV-S) encoding the small (S) envelope protein of a HBV subtype *adw* has been described elsewhere [23]. A second plasmid, pSFV-E1E2, containing the DNA fragment encoding the HCV E1 and E2 envelope proteins was designed from the previously described pSFV-HCV^{dj} plasmid (Genbank AF529293), encoding the core-E1-E2 sequence of a genotype 1a HCV [24]. This amplified HCV^{dj} DNA sequence also contained the upstream signal sequence responsible for translocation of the E1 ectodomain into the ER lumen (aa 166 to 191 from the HCV core protein in the HCV polyprotein) [25]. Briefly, the HCV^{dj} E1-E2 DNA sequence (corresponding to aa 166 to 746 in the HCV polyprotein) was amplified by PCR and inserted into the *Bam*HI restriction site of a pSFV vector (Invitrogen). Using a proofreading *Taq* DNA polymerase (*TaKaRA Ex Taq*, Takara), we constructed by serial PCR amplification the pSFV-E1-S and pSFV-E2-S plasmids, encoding the chimeric HBV-HCV envelope proteins (Fig. 1). For pSFV-E1-S, we first amplified a DNA fragment encoding a truncated form of the HBV S protein (aa 23 to 226) from pSFV-S, using a forward primer (regular typeface) flanked by an HCV "E1" sequence (italic) 5'-*GCTGTTCGCCAGC*ACAAGAATCCTC-3' and a reverse primer flanked by a *Bam*HI restriction site sequence (underlined) 5'-ATAGGATCC**TTA**AATGTATACCCAGAGACAAAGAAAAT-3'. The inserted stop codon is indicated in bold typeface. The same strategy was used to design the pSFV1-E2-S construct, with a forward primer flanked by the HCV "E2" sequence (italics) 5'-*GCTACTCATATCCCAA*ACAAGAATCCTCACAATACC-3'. We then amplified a DNA fragment encoding the HCV E1 protein from pSFV-E1E2, using a forward primer flanked by *Bam*HI restriction site sequence 5'-ATAGGATCC***ATG***ACAGGGAACCTTCCTGGTTGC-3' and a reverse primer flanked by HBV S sequence 5'-TGAGGATTCTTGT*GCTGGCGAACAGCAA*-3'. The inserted start codon is indicated in bold typeface. This DNA fragment encoded aa 166 to 380 of the HCV polyprotein, including aa 166 to 191 from the HCV core protein, serving as an ER translocation signal sequence for E1. The same strategy was used to amplify a DNA fragment encoding the HCV E2 protein, for the construction of pSFV-E2-S, with a forward primer flanked by the *Bam*HI restriction site sequence 5'-ATAGGATCC***ATG***GGGAACTGGGCGAAGGT-3' and a reverse primer flanked by HBV S sequence 5'-GGTATTGTGAGGATTCTTGT*TTGGGATATGAGTAGCA* -3'. This DNA fragment encoded aa 366 to 743 of the HCV polyprotein, including aa 366 to 383 from the HCV E1 protein and serving as an ER translocation signal sequence for E2. The E1 and E2 proteins encoded by these constructs had 3 aa fewer at their C-terminal ends (aa 381 to 383 for E1 and aa 744 to 746 for E2) than the wild-type proteins, to prevent cleavage of the chimeric proteins by the ER signal peptidases. Finally, a third PCR was used to connect the

sequence encoding E1 or E2 to the corresponding S DNA fragment. The PCR product was inserted into pGEM®-T (pGEM®-T Easy Vector System, Promega) and then into the *Bam*HI restriction site of the pSFV vector (Invitrogen). All PCR products were verified by DNA sequencing.

Analysis of BHK-21 cells transiently transfected with pSFV RNAs

Recombinant Semliki RNA synthesis, BHK-21 cells (baby hamster kidney cells) culture and transfection, and western-blot analysis were performed as previously described [23]. Briefly, for recombinant RNA synthesis, pSFV constructs were linearized by digestion at the single *Spe*I site. Linear plasmids were transcribed *in vitro,* using SP6 RNA polymerase, according to the standard protocol provided by the manufacturer (Promega). For the negative control, recombinant RNA encoding β-galactosidase (β-Gal) was synthesized from the pSFV3 expression vector (Invitrogen). For western-blot analysis, the membranes were incubated overnight at 4 °C with a rabbit polyclonal antibody against HBsAg (R247) [26] or a mouse monoclonal antibody against E1 (A4) or against E2 (H52) [27]. For ultrastructural analysis by electron microscopy (EM), cells were fixed directly in the culture dish 16 hours after transfection, by incubation for 48 hours in 4 % paraformaldehyde and 1 % glutaraldehyde in 0.1 M phosphate buffer pH 7.2. Cells were scraped off with a Cell Scraper® (Falcon), washed in PBS, post-fixed by incubation for 1 hour with 1 % osmium tetroxide and dehydrated in a graded series of ethanol solutions. Cell pellets were embedded in Epon resin (Sigma), which was allowed to polymerize for 48 hours at 60 °C. Ultrathin sections were cut, stained with 5 % uranyl acetate and 5 % lead citrate, and deposited on EM grids coated with collodion membrane, for examination under a Jeol 1010 transmission electron microscope (TEM).

For the analysis of intracellular subviral envelope particles, transfected BHK-21 cells were collected 16 h after transfection and treated as previously described [23]. Briefly, cells were lysed by heat shock and homogenized for 10 minutes on ice. The samples were layered onto the top of a discontinuous sucrose gradient (25 to 60 % in 20 mM Tris pH 8), and centrifuged at 4 °C for 16 hours at 28,000 rpm in an SW 41 rotor (Beckman). The collected fractions were analyzed by immunocapture ELISA on plates coated with a mouse monoclonal antibody against S (H25B10, ATCC), and quantified using the biotinylated form of the H25B10 as a detection reagent. Sequential dilutions of a recombinant HBs Ag (HBs Ag *adw* R86872, BioDesign) were used as a standard. Positive fractions were pooled and dialyzed against 20 mM Tris pH 8 at 4 °C, and then concentrated with an Amicon® Ultracell-100k (Millipore) device. These crude preparations were further purified by anti-S affinity chromatography as previously described [23]. The final concentrated preparations were then deposited on carbon-coated EM grids, negatively stained with 1 % uranyl acetate, and analyzed in the TEM. They were also analyzed by western blotting, as for the cell lysates, as described above.

Stable production of the wild-type HBV S and chimeric HBV-HCV envelope proteins in clones of the CHO cell line

Chinese hamster ovary cells (CHO) cells were stably transduced using a strategy based on a lentiviral expression vector, with the pHR' plasmid, described elsewhere [28], and generously provided by Didier Trono (Ecole Polytechnique Fédérale de Lausanne, Switzerland). The DNA sequence encoding the HBV S protein in pSFV-S plasmid was released and inserted into the *Bam*HI restriction site of pHR'^{hph} (constructed from pHR', containing the *hph* gene encoding hygromycin phosphotransferase), to generate pHR'^{hph}-S. Lentiviruses were

produced in HEK-293T cells (human embryonic kidney cells), maintained in Dulbecco's Modified Eagle Medium (DMEM). Twenty-four hours before transfection, 3 x 10^6 cells were used to seed a 75-cm^2 culture dish (Falcon). Cells were transfected with an equimolar mixture (1 pmol of each) of lentiviral pHR'^{hph}-S, pHCMVG plasmid encoding the VSV-G (vesicular stomatitis virus envelope glycoprotein) and the packaging construct p8.74, by the calcium phosphate method. The following day, the transfection solution was removed and replaced with fresh complete medium. After 24 and 48 hours of culture, the supernatant was collected, filtered through a low-protein binding filter with 0.45-µm pores (Sartorius) and concentrated by centrifugation on a 20 % sucrose gradient at 4 °C for 90 minutes at 100,000 X g. The pellet was resuspended in 500 µl of PBS and stored at -80 °C until use. The unit of transduction (UT) titer was determined by quantifying p24 protein (Innotest HIV Antigen mAb Kit, Innogenetics). The DNA sequences of the pSFV-E1-S and pSFV-E2-S plasmids encoding the E1-S and the E2-S proteins, respectively, were transferred into the *Bam*HI restriction site of the pHR'^{gfp} plasmid (constructed from pHR', encoding the GFP as a screening marker) by a similar strategy, to generate the pHR'^{gfp}-E1-S and pHR'^{gfp}-E2-S plasmids. Lentivirus particles were produced as described above.

CHO cells in DMEM-F12 were first transduced with the recombinant HR'^{hph}-S lentivector. One day before transduction, 10^5 cells per well were used to seed in a six-well cell culture plate (Falcon). Cells were incubated with HR'^{hph}-S vectors (multiplicity of infection : 2.5) and 4 µg/ml polybrene (Sigma) in fresh complete medium. The following day, the transduction solution was removed, the cells were rinsed with PBS and then incubated for 48 hours. They were then used to seed a 75-cm^2 culture dish (Falcon) and incubated with 1 mg/ml hygromycin (Euromedex) for 3 weeks. A hygromycin-resistant cell clone named CHO-S was isolated, using a cloning cylinder (Invitrogen), and amplified. This CHO-S clone was then transduced with recombinant HR'^{gfp}-E1-S or HR'^{gfp}-E2-S lentivirus preparations, as described above. Three days after post-transduction, the cells were used to seed a 96-well cell culture plate (Falcon) at a density of 1 cell per well. The plates were incubated for 3 weeks. Two GFP-positive cellular clones - CHO-S+E1-S and CHO-S+E2-S - were amplified. The intracellular production of the HBV S, HBV-HCV chimeric E1-S and E2-S proteins was analyzed by the western blotting of the cell lysates, as described above for BHK-21 cells.

Analysis of the supernatant of CHO cells stably coproducing HBV S and chimeric HBV-HCV envelope proteins

Secreted subviral envelope particles were purified from cell supernatants by centrifugation on a CsCl gradient, as previously described [29]. Briefly, 200 ml of supernatant was clarified and total protein precipitated by adding a 45 % solution of $(NH_4)_2SO_4$ (pH7.5). The precipitate was collected by centrifugation at 4 °C for 15 minutes at 10,000 X g and the pellet was dissolved in a minimal volume of Tris-NaCl-EDTA buffer (TNE) (10 mM Tris/HCl pH 7.5 / 100 mM NaCl / 1 mM EDTA). The solution was dialyzed against TNE buffer and CsCl was added to a density of 1.22 g/cm^3. Two successive runs of isopycnic centrifugation were performed at 15 °C for 24 hours at 40,000 rpm in a 45Ti rotor (Beckman). Fractions were collected from the top and were assayed for HBsAg by ELISA, as described above. Peak fractions were pooled and dialyzed at 4 °C against TNE buffer. The final preparations were analyzed by negative staining and western blotting as described above.

Results

Production of the HBV S, the HBV-HCV chimeric E1-S and E2-S proteins from an SFV vector in BHK-21 cells

Sixteen hours after transfection with the recombinant SFV constructs encoding the S protein, the chimeric E1-S protein or E2-S protein, BHK-21 cells were harvested and lysed for western blot analysis (Fig. 2A). Consistent with our previous findings, the S protein was detected as its unglycosylated (p24) and glycosylated (p27) forms (23) which were produced in similar amounts. The chimeric E1-S protein was detected at 54 kD, by both the anti-HBs rabbit polyclonal antibodies, and the anti-E1 monoclonal antibody (Fig. 2A). As an E1 produced from an SFV construct containing the DNA sequence encoding the E1-E2 proteins migrated at 30 kD on the same blot, this band at 54 kD is consistent with the expected size of the chimeric E1-S protein. Previous studies have shown that the unglycosylated E1 protein migrates at 19 kD [30]. This suggests that our chimeric E1-S protein at 54 kD is glycosylated and therefore has the expected transmembrane topology, with the E1 ectodomain in the ER lumen. The chimeric E2-S protein was detected at around 85 kD, by both the polyclonal anti-HBs antibodies and the monoclonal anti-E2 antibody (Fig. 2A). As E2 produced from an SFV construct containing the DNA sequence encoding the E1-E2 proteins migrated at 65 kD, this band around 85 kD is consistent with the expected size of the chimeric E2-S protein. Previous studies have shown that an unglycosylated form of E2 migrates at 38 kD [31]. As above, this suggests that our chimeric 85 kD E2-S protein is also glycosylated, thus retaining the expected transmembrane topology, with the E2 ectodomain in the ER lumen. Although the anti-HBs and anti-E2 antibodies gave a weak signal for the chimeric proteins as compared to the wild-type proteins, the anti-E1 antibody detected efficiently the E1-S chimeric protein. This suggests that the E1-S chimeric protein was produced at a good level and that its weak detection by the anti-S was more related to a poor recognition by the antibody than its expression level.

Ultrastructural changes induced by the production of HBV S or of the HBV-HCV chimeric E1-S and E2-S proteins from a SFV vector in BHK-21 cells

Sixteen hours after transfection with the recombinant SFV constructs encoding the S protein, the chimeric E1-S protein or the chimeric E2-S protein, BHK-21 cells were investigated by EM. Consistent with our previous findings [23], cells producing the S protein displayed significant ultrastructural modifications on EM, with respect to the cells producing β-Gal (Fig. 2B). Intracellular vesicles packed with HBV subviral filaments, were abundant in the perinuclear area of these cells (Fig. 2B). We have previously demonstrated that these filaments act as the precursor of spherical HBV subviral envelope particles [23]. However, despite intensive observation and repeated experiments, no ultrastructural changes and no filaments were observed in the cells producing the E1-S or E2-S proteins, suggesting that these chimeric proteins were unable to self-assemble into subviral filaments.

Cotransfection of BHK 21 cells with the recombinant SFV constructs containing DNA sequences encoding the S and chimeric E1-S or E2-S proteins, and purification and analysis of the intracellular HBV subviral fiaments

Initial analysis by negative EM staining of the crude BHK-21 cell lysates subjected to centrifugation on a sucrose gradient led to the observation of long subviral filaments 22 nm in diameter and frequently branched, as previously described [23] (data not shown). These

filaments were purified by affinity chromatography with anti-HBs antibody. They were then systematically unbranched, and their ends tended to dissociate into subviral spherical particles (Fig. 3A), as previously described [23]. Western blots of purified subviral filaments showed that the filaments induced by production of the S and E1-S proteins together contained both the wild-type S protein (p24 and p27, Fig. 3B) and the E1-S chimeric protein (p54, Fig. 3B) which was detected by both anti-HBs polyclonal antibodies and the anti-E1 monoclonal antibody. The chimeric E1-S protein was therefore efficiently incorporated into intracellular HBV subviral filaments. However, it was more difficult to observe the incorporation of the E2-S protein into the filaments obtained following the production of the S and E2-S proteins together, as p85 was barely detectable with both polyclonal anti-HBs and monoclonal anti-E2 antibodies (data not shown).

Coproduction of the chimeric E1-S or E2-S proteins with the wild-type HBV S protein in stably transduced CHO cells

Western blot analysis of a lysate of the CHO cells stably producing the HBV S protein (CHO-S) showed a high intracellular concentration of p24 and p27 (Fig. 4). Similar intracellular levels of HBV S protein were observed in the two CHO subclones obtained by transduction of the parental CHO-S cell line with lentiviral constructs encoding E1-S or E2-S (subclones CHO-S+E1-S and CHO-S+E2-S, respectively). In addition, large amounts of intracellular E1-S protein (p54) were detected with the anti-E1 antibody in the CHO-S+E1-S subclone, and a large amounts of intracellular E2-S protein (p85) were detected with the anti-E2 antibody in the CHO-S+E2-S subclone (Fig. 4). Thus, we were able to establish two clones of the CHO cell line that continuously produce the HBV S protein together with either the E1-S or the E2-S chimeric HBV-HCV envelope protein.

Purification and analysis of the HBV subviral particles released into the supernatant of the CHO-S, CHO-S+E1-S and CHO-S+E2-S clones

The supernatants of the three clones were found to contain similar amounts of HBs Ag (around 100 ng/ml, determined by ELISA), and these amounts remained stable over time during passages of the different clones (58^{th} passage at this time). The supernatant (200 ml) of these 3 clones was subjected to protein precipitation and centrifugation on an isopycnic CsCl gradient. We collected 20 fractions from the bottom of the gradient and tested them individually for HBsAg by ELISA (Fig. 5A). For all the three clones, fraction 9 (1.18 g/cm^3 in CsCl) corresponded to the peak fraction for HBsAg levels in the supernatant. Fractions 8, 9 and 10 of each of the 3 clones were pooled and dialyzed, before further analysis by negative EM staining and western blotting. EM analysis demonstrated that these 3 supernatants contained spherical HBV subviral particles. The subviral particles present in the supernatant of the CHO-S+E1-S and CHO-S+E2-S clones were similar in size (about 22 nm in diameter) and shape to the subviral particles present in the supernatant of the CHO-S cell line producing the wild-type HBV S protein (Fig. 5B). A sample corresponding to one microgram of HBs Ag (determined by ELISA) for each of the three preparations was subjected to western blotting with the polyclonal anti-HBS and the monoclonal anti-E1 and anti-E2 antibodies, as described above. The HBV S protein was detected in the form of p24 and p27 in all three preparations (Fig. 5C). The amount of p24 was higher than for p27 in the purified subviral particles, as observed in previous studies (5, 9). Moreover, significant amounts of E1-S protein (p54) and E2-S protein (p85) were detected with anti-E1 and anti-E2 antibodies in the samples corresponding to the CHO-S+E1-S and CHO-S+E2-S clones, respectively (Fig. 5C). Thus, the chimeric E1-S and E2-S proteins were efficiently incorporated into the spherical HBV

subviral particles stably secreted by the CHO-S+E1-S and CHO-S+E2-S subclones. Moreover, these chimeric HBV-HCV envelope subviral particles were similar in appearance to the wild-type HBV envelope subviral particles.

Discussion

For optimal immunogenicity, epitopes should ideally be presented as several copies on a defined particulate structure. HBV subviral envelope particles are used worldwide in the form of commercial vaccines for hepatitis B prevention. Based on their particulate structure and intrinsic immunogenic potential, these particles have also been thoroughly investigated as a possible carrier matrix for foreign epitopes exposition. For such strategies, the external hydrophilic loop of the HBV S protein is the preferred site of insertion [16], but many studies have also reported the insertion of foreign protein sequences at the N-terminus or C-terminus of the HBV S protein, as the ends of the protein are also exposed at the surface of the subviral particle. We report here another strategy leading to the insertion of foreign protein sequences at the HBV S N-terminus and demonstrate, for the first time, that the N-terminal transmembrane domain of the HBV S protein may be replaced by the transmembrane domain of a foreign protein, to generate a chimeric protein suitable for assembly into and secretion as a subviral particle. Indeed, although our chimeric HBV-HCV envelope proteins S-E1 and S-E2 were unable to self-assemble into a subviral particle on their own, these proteins were efficiently assembled into subviral particles when produced together with the wild-type HBV S protein. We made use of our recently established model of HBV S production with an SFV vector [23], which constitutes a powerful tool for studying the assembly or coassembly properties of modified HBV S proteins. Most previous studies focusing on the assembly of HBV S mutants were limited to investigation of the release of these mutants into the cell supernatant [17, 32], without determining whether complete or partial defects were due to the impairment of assembly or secretion.

Our study of HBV-HCV chimeric proteins shows that the HBV S N-terminal hydrophobic region is remarkably tolerant to sequence substitutions preserving its ability to assemble into subviral envelope particles. Previous studies have demonstrated that the N-terminal hydrophobic region of S (aa 8 to 22) is a type I signal sequence not cleaved by signal peptidase. This domain seems to be sensitive to mutations as it is not possible to delete even part of this sequence without deleterious effects on the assembly and secretion of subviral envelope particles [17, 32]. An HBV S mutant lacking amino acids 9 to 22 cannot self-assemble or assemble with the wild-type HBV S [33]. As demonstrated by efficient glycosylation, the second hydrophilic domain of this mutant was translocated across the ER membrane but remained arrested in a membrane-associated configuration. Targeting to and anchoring in the ER membrane were probably mediated by the second hydrophobic transmembrane domain of S (aa 80 to 98), corresponding to a type II signal. In another study, a chimeric protein consisting of the β-lactamase signal sequence, the α-globin and the HBV S protein deleted of its N-terminal transmembrane domain was constructed [32]. This chimeric protein was not secreted on its own, but was secreted into the culture medium in very small amounts if produced together with the wild-type HBV S protein. The authors suggested that the N-terminal transmembrane domain of HBV S might not be absolutely essential for coassembly and cosecretion with the wild-type HBV S protein [32]. Our results are consistent with those of this previous study, although our chimeric HBV-HCV envelope proteins seemed to be efficiently coassembled and secreted with the HBV S protein. It remains unclear whether this efficient coassembly was due the specific contribution of the HCV E1 or E2 transmembrane domains. Lipids account for only 25 % by weight of the HBV subviral

particle [34], and are therefore unlikely to be organized into a conventional membrane bilayer. Instead, lipid reorganization and tight lateral interaction between protein subunits are probably required for subviral particle formation [13]. It would be interesting to determine in the future whether coassembly is equally efficient with other foreign transmembrane proteins. This is of importance, as similar strategies with other foreign proteins could lead to new vaccine strategies for other infectious agents, based on vaccine candidates produced with the same industrial procedures used for the HBV vaccine. Our model of HBV S production with an SFV vector could be very useful for screening various chimeric proteins for their coassembly properties with the wild type HBV S protein.

We were here able to establish clones of the CHO cell line stably producing high levels of the HBV S together with the chimeric HBV-HCV S-E1 or S-E2 proteins. CHO is a relevant cell line as it is widely used for medicinal recombinant protein production. The subviral envelope particles secreted by these two clones were similar to the wild-type HBV S subviral particles and contained large amounts of chimeric HBV-HCV S-E1 or S-E2 proteins. Thus, the entire HCV E1 or E2 envelope protein, including the ectodomain and transmembrane domain can be incorporated in large amount into subviral envelope particles resembling the HBV vaccine. The ectodomains of E1 and E2 are presented at the surface of these subviral particles, as shown by the size of these chimeric proteins in the purified particles, consistent with the expected glycosylation pattern. As previous studies have shown that HCV envelope proteins lacking their transmenbrane domain have impaired antigenic and functional properties [22], our chimeric HBV-HCV subviral envelope particles may open new possibilities for testing the entire HCV E1 and E2 proteins (individually or as a mixture) for original vaccination strategies. Our study may also have implications for the design of new vaccine candidates based on the use of the HBV subviral particle as a carrier for various transmembrane proteins from other infectious agents.

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Figure 1

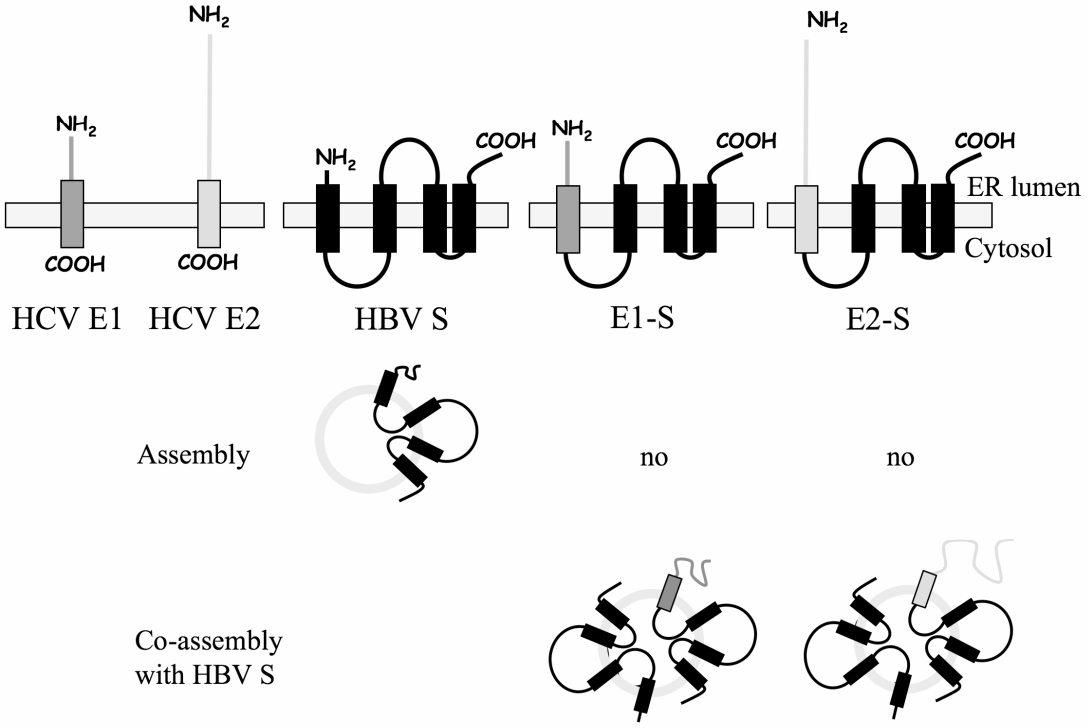

Fig1. Transmembrane topology of the wild-type HBV S and HCV E1 and E2 envelope proteins and of the HBV-HCV E1-S and E2-S chimeric proteins used in this study. The boxes indicate the hydrophobic domains of these proteins, anchored in the ER membrane. Assembly refers to the ability of the HBV S or the HBV-HCV chimeric proteins to self-assemble into a subviral envelope particle. Coassembly with HBV S refers to the ability of the HBV-HCV chimeric proteins to coassemble with the wild-type HBV S into a subviral envelope particle, following the production of the chimeric protein together with the HBV S-protein.

Figure 2

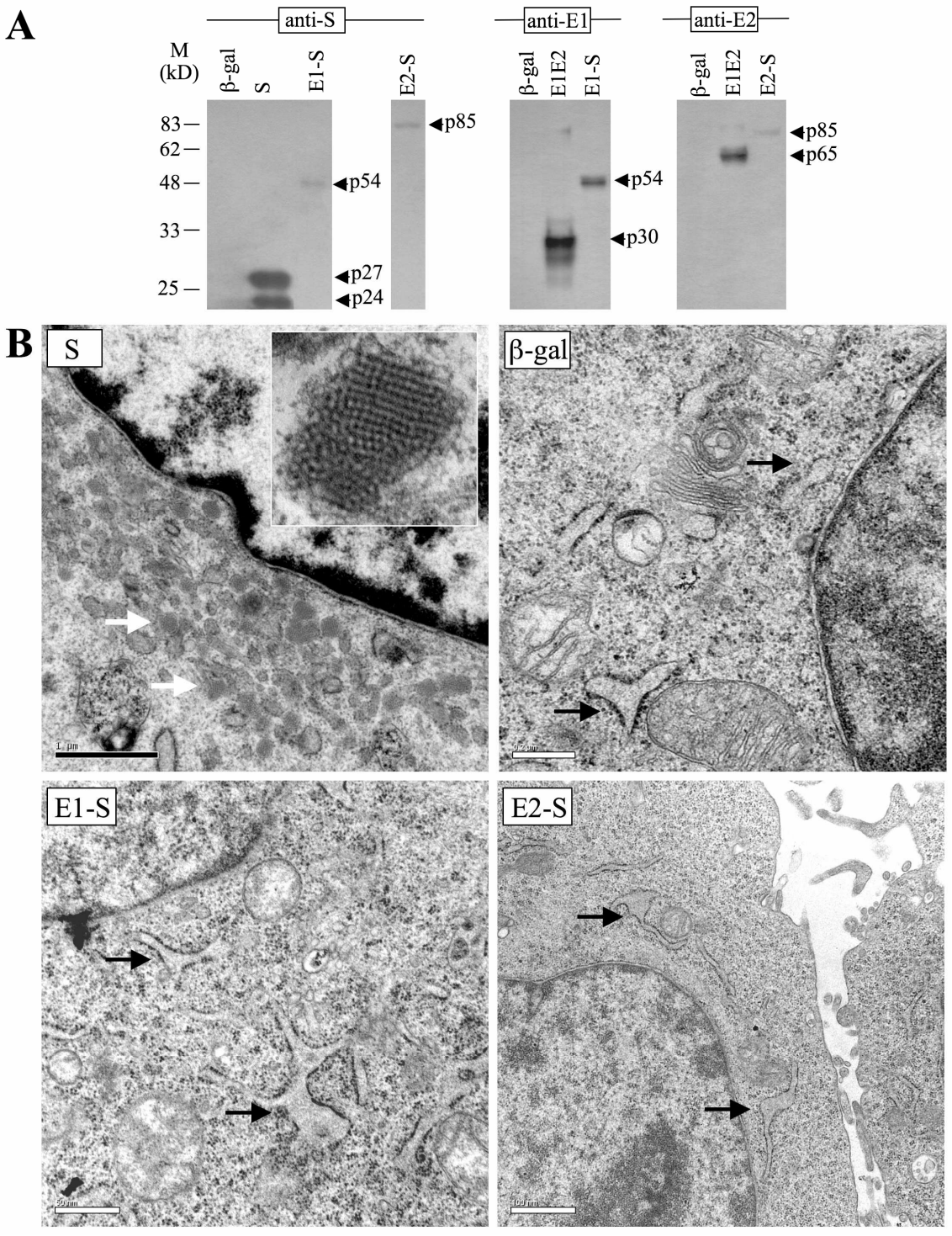

Fig2. Analysis of the HBV and HCV wild-type envelope proteins and HBV-HCV chimeric protein production within BHK-21 cells transfected with the various pSFV constructs, by western blotting (A) and electron microscopy (B). A : Western blots probed with polyclonal anti-HBs antibodies showed that transfection with the pSFV-S construct led to production of the unglycosylated (p24) and glycosylated (p27) forms of the HBV S protein. Western blots probed with anti-E1 and anti-E2 monoclonal antibodies showed that transfection with pSFV-E1E2 led to production of the HCV E1 (p30) and E2 (p65) proteins. Cell transfection with

pSFV-E1-S led to the production of a 54 kD protein, recognized by both anti-HBs and anti-E1 antibodies with the expected size of the chimeric E1-S protein. The transfection of cells with pSFV-E2-S led to the production of a protein at 85 kD that was recognized by both anti-HBs and anti-E2 antibodies and had the expected size of the chimeric E2-S protein. BHK-21 cells transfected with pSFV-β-gal were used as controls. Molecular weight markers (M) are indicated on the left of the blots. B : BHK-21 cells producing the wild-type HBV S protein were characterized by the presence of numerous vesicles 0.2 to 0.3 mm in diameter (white arrows), packed with 22 nm large subviral filaments (enlarged view in the inset) in the perinuclear area. No specific ultrastructural changes were observed in cells producing β-Gal, the chimeric E1-S, the chimeric E2-S, or the E1 and E2 HCV envelope proteins (not shown on the figure). In these cells, no particular change in the ER compartment (black arrows) could be identified. Bars: 1 micrometer (for S), 0.2 micrometers (for β-gal), 50 nanometers (for E1-S), 100 nm (for E2-S).

Figure 3

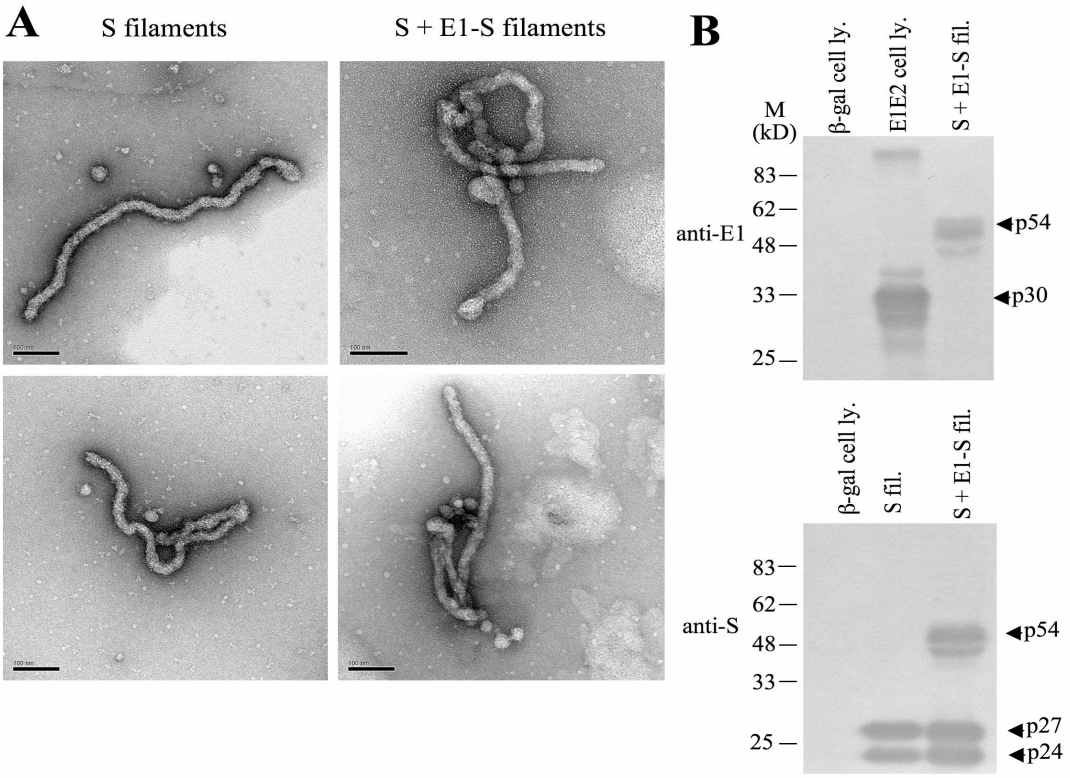

Fig 3. Purification by affinity chromatography and analysis by negative EM staining (A) and western blotting (B) of the intracellular subviral filaments recovered from BHK-21 cells transfected with the pSFV-S construct or cotransfected with the pSFV-S and pSFV-E1-S constructs. A : Long filaments with a diameter of 22 nm dissociating into subviral spherical particles at their ends were observed in both cases (bars : 100 nm). B : Subviral filaments recovered from cells producing the HBV S protein (S fil.) were rich in S protein, as shown by the large amount of p24 and p27 detected in these filaments with anti-HBs antibodies. Subviral filaments recovered from cells producing both HBV S and the chimeric E1-S proteins (S+E1-S fil.) were also rich in S protein, as the same bands were identified with anti-HBs antibodies. These filaments also contained the E1-S (p54) protein, which was recognized by both anti-HBs and anti-E1 antibodies. Lysates (cell ly.) of BHK-21 cells transfected with pSFV-E1E2 or pSFV-β-gal were used as controls

Figure 4

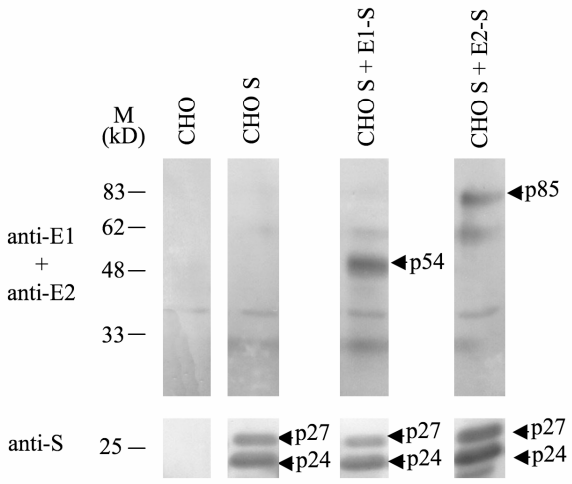

Fig4. Western blot of HBV S production in a CHO clone stably transfected with a lentiviral construct encoding HBV S (CHO-S), and of HBV-HCV chimeric proteins produced in two derived subclones (CHO-S+E1-S and CHO-S+E2-S) stably cotransfected with lentiviral constructs encoding the chimeric E1-S or E2-S proteins. All CHO clones displayed similar levels of HBV S protein production, in the form of p24 and p27, as revealed with polyclonal anti-HBs antibodies. The CHO-S+E1-S subclone produced a p54 kD protein recognized by the anti-E1 monoclonal antibody and corresponding to the E1-S chimeric protein. The CHO-S+E2-S subclone produced a p85 kD protein recognized by the anti-E2 monoclonal antibody, and corresponding to the E2-S chimeric protein. Untransfected CHO cells were used as control.

Figure 5

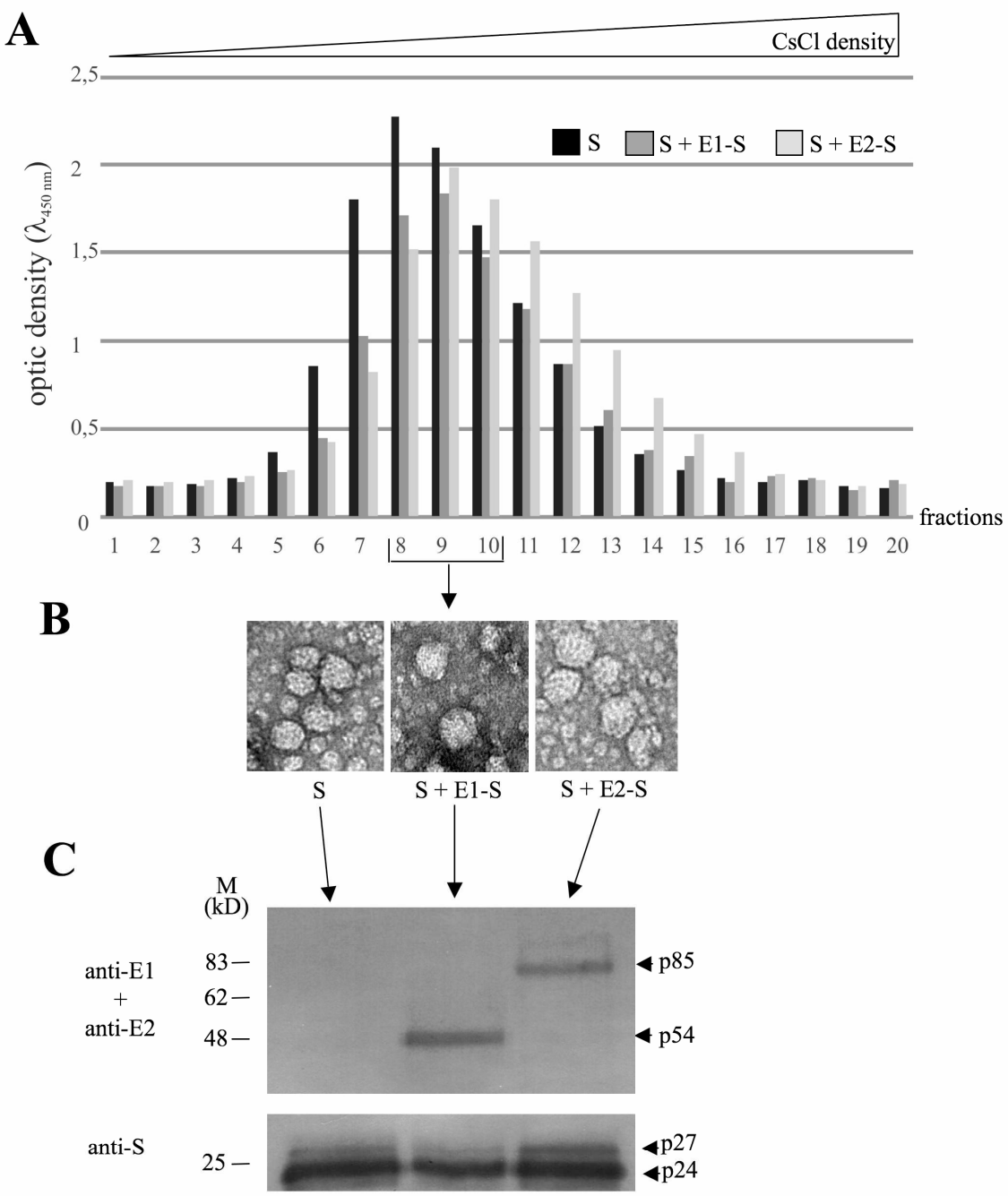

Fig5. Analysis of the subviral envelope particles secreted into the supernatant of the CHO-S, CHO-S+E1-S or CHO-S+E2-S clones, by CsCl gradient centrifugation (A), negative EM staining (B) and western blotting (C). A: For the three supernatants, HBs Ag concentration, as determined by ELISA, peaked in fractions 8, 9 and 10. B: Negative EM staining performed after the pooling and dialysis of fractions 8-9-10 for the three supernatants demonstrated the presence of similar 22 nm-diameter spherical subviral envelope particles. C : All preparations

contained similar amounts of HBV S protein, in the form of p24 and p27, detected with polyclonal anti-HBs antibodies. The S+E1-S particles contained the p54 kD protein, which was recognized by the anti-E1 monoclonal antibody and corresponded to the E1-S chimeric protein. The S+E2-S particles contained the p85 kD protein recognized by the anti-E2 monoclonal antibody, corresponding to the E2-S chimeric protein.