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Original Article

Signal peptide replacements enhance expression and secretion of hepatitis C virus envelope glycoproteins

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A large number of researches focused on glycoproteins E1 and E2 of hepatitis C virus (HCV) aimed at the development of anti-HCV vaccines and inhibitors. Enhancement of E1/E2 expression and secretion is critical for the characterization of these glycoproteins and thus for subunit vaccine development. In this study, we designed and synthesized three signal peptide sequences based on online programs SignalP, TargetP, and PSORT, then removed and replaced the signal peptide preceding E1/E2 by overlapping the polymerase chain reaction method. We assessed the effect of this alteration on E1/E2 expression and secretion in mammalian cells, using western blot analysis, dot blot, and *Galanthus nivalis* agglutinin lectin capture enzyme immunoassay. Replacing the peptides preceding E1 and E2 with the signal peptides of the tissue plasminogen activator and Gaussia luciferase resulted in maximum enhancement of E1/E2 expression and secretion of E1 in mammalian cells, without altering glycosylation. Such an advance would help to facilitate both the research of E1/E2 biology and the development of an effective HCV subunit vaccine. The strategy used in this study could be applied to the expression and production of other glycoproteins in mammalian cell line-based systems.

Keywords hepatitis C virus; signal peptide; envelope glycoprotein; expression; secretion

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Introduction

Hepatitis C virus (HCV) is a small, enveloped, positive-stranded RNA virus that belongs to the *Hepacivirus* genus in the *Flaviviridae* family [1]. HCV infection often leads to chronic hepatitis, liver cirrhosis, and hepatocellular carcinoma. Despite its clinical significance, the functional characterization of HCV glycoproteins, and thus vaccine development, has been hindered by a paucity of knowledge relating to the major structural glycoproteins E1/E2 [1,2].

The HCV genome encodes a single polyprotein that is processed by cellular and viral proteases to generate 10 polypeptides [3]. The HCV virion is thought to have a diameter of ~50 nm [4]. It is composed of a nucleocapsid surrounded by a host cell-derived membrane envelope that contains the viral glycoproteins E1 (polyprotein residues 192–383) and E2 (residues 384–746) [5]. Because they are exposed at the surface of the virion, the envelope proteins are the target of neutralizing antibodies. Therefore, E1 and E2 represent the most immunologically significant HCV antigens. For this reason, E1 and E2 were the main focus of HCV vaccine development [4,6].

HCV glycoproteins are type I membrane proteins with a C-terminal transmembrane domain (TMD) anchored in the virion phospholipid envelope. In their functional form, E1 and E2 are thought to form a non-covalent heterodimer, and their TMDs are essential for heterodimerization [5,7]. The ectodomains of the HCV envelope glycoproteins E1 and E2 are highly modified by N-linked glycans, which have been shown to play a major role in protein folding, virus entry, and protection against neutralizing antibodies [5,7–11].

The enhancement of E1/E2 expression and secretion is vital for the characterization of E1/E2 structure and function and for the development of an effective E1/E2-based subunit vaccine [4–7]. To our knowledge, native E1/E2 expressed in mammalian cells is unsuitable for this purpose. However, some studies have shown that the optimization of signal peptide may enhance the levels of expression and secretion of these glycoproteins [12–14]. Similar strategies also have been described in researches of HIV and some other viruses [15–19].

In this study, we constructed several plasmids encoding E1 and E2 to assess the effect of the replacement of the signal peptide sequences located upstream of the *E1* and *E2* genes. Our results suggested that replacing the signal sequence preceding the *E1* and *E2* genes with three signal peptide sequences significantly enhanced expression and secretion of eE1 (a secreted form of E1) and eE2 (a secreted form of E2) in mammalian cells. Such modifications represent a significant advance in the technique available to investigate the E1 and E2 structure and their function and will facilitate the development of an HCV entry inhibitor and/or effective vaccine.

Materials and Methods

Cell culture and plasmid construction

Human embryo kidney 293T cells were grown in Dulbecco's modified essential medium (Invitrogen, Carlsbad, USA) supplemented with 10% fetal bovine serum (FBS) and 100 U/ml penicillin/streptomycin. Cells were routinely maintained at 37°C with 5% CO_2.

Wild-type HCV 1b *E1* and *E2* genes were amplified from the plasmid as described previously [20]. To replace their signal peptide regions, the *E1* and *E2* genes minus the signal peptide were amplified by polymerase chain reaction (PCR) using long upstream primers. These primers were used to add the signal peptide of self-designed [21,22], tissue plasminogen activator (tPA) [23,24] or Gaussia luciferase (Gluc) [25] (**Table 1**), to the *E1* and *E2* genes. Each of these primers contained 15 nucleotides of the *E1* or *E2* sequence spanning the signal peptidase cleavage site. An *Eco*RV site and a *Bst*EII site were added in those primes for clone construction. To facilitate downstream purification, a *Bam*HI restriction site and a DNA sequence encoding a His_6-tag peptide were synthesized and added to the 3′ end of the *E1* and *E2* sequences by PCR (**Fig. 1**). Standard recombinant DNA techniques were used to generate all constructs. After purification, fragments containing the desired signal sequence were digested with *Eco*RV/*Bam*HI and then cloned into the same sites in the pVRC8301 vector. The general scheme of plasmid construction is shown in **Fig. 1** and **Table 2**.

Cell transfection

Transient transfections were performed by plating 2×10^5 cells/well in a 12-well plate 12 h prior to initiation of the study. When cells were ~80–90% confluent, the experiments were performed. On the day of transfection, the original growth medium was replaced with a serum-free medium and DNA transfection was performed using the FUGENE HD Transfection Reagent (Roche, Basel, Switzerland) according to the standard protocols with slight modifications. Briefly, DNA solution containing

Table 1 Prediction results for different signal peptides

Signal peptide (Sp)	Sequence and cleave site prediction[a]	Extracellular rate in k-NN prediction	
		Sp + E1 (%)	Sp + E2 (%)
E1	M G C S F S I F L L A L L S C L T T P A S A ↓ . . .	44.4	
E2	M V G N W A K V L I V M L L F A G V D G ↓ . . .		55.6
Self-design (sd)	MD A M K V L L L V F V S P S Q V T G↓ . . .	66.7	77.8
Gluc	M G V K V L F A L I C I A V A E V T G ↓ . . .[b]	66.7	66.7
tPA	M D A M K R G L C C V L L L C G A V F V D S V T G↓ . . .	66.7	66.7

[a]Protein cleavage sites are indicated by ↓.

[b]The SignalP-NN result is AVA ↓ EV, but the SignalP-HMM result is VTG ↓, and the SIG-Pred result conforms to HMM.

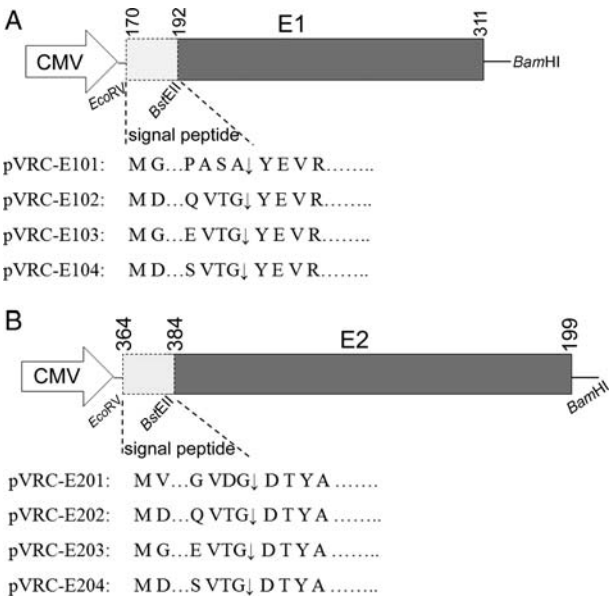

Figure 1 Schematic representation of HCV E1 (A) and E2 (B) expression vectors Arrows indicate signal peptide cleavage sites.

1 μg of pVRC-E1/E2 was diluted in 50 μl of Opti-MEM; and the ratio of transfection reagent:DNA was 6:2. Mixtures were incubated at room temperature for 15 min. Culture medium in 12-well plates was removed and the cells were washed with Opti-MEM. A total of 900 μl of

Table 2 Plasmids used in this study

Plasmid	Relevant characteristic
pVRC8301	Efficient expression vector in eukaryotic cells
E1	
pVRC-E101	pVRC8301 derivative containing the wild-type HCV(1b) E1 (amino acids 170–311)
pVRC-E102	pVRC-E101 derivative with the signal peptide sequence replaced with a self-designed signal sequence
pVRC-E103	pVRC-E101 derivative with the signal peptide sequence replaced by that of tPA
pVRC-E103	pVRC-E101 derivative with the signal peptide sequence replaced by that of Gluc
E2	
pVRC-E201	pVRC8301 derivative containing the wild-type HCV(1b) E2 (amino acids 364–661)
pVRC-E202	pVRC-E201 derivative with the signal peptide sequence replaced with a self-designed signal sequence
pVRC-E203	pVRC-E201 derivative with the signal peptide sequence replaced by that of tPA
pVRC-E203	pVRC-E201 derivative with the signal peptide sequence replaced by that of Gluc

fresh serum-free Opti-MEM and 50 μl of transfection mixture were mixed and added to each well. After 48 h of incubation, the culture medium was collected and cell pellets were separated by centrifugation at 1200 *g* for 5 min. Pellets were washed with PBS and then lysed in 200 μl of lysis buffer for 30 min on ice and centrifuged at 14,500 *g* for 5 min; the clarified cell lysate was collected. Clarified lysate and culture medium were used for expression analysis [20]. Identical cDNA amount of pVRC was transfected as a control.

PNGase F digestion for analysis of E1 and E2 glycosylations

Cell lysates and culture medium were diluted in sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) loading buffer and heated at 100°C for 10 min. PNGase F (NEB) digestion was performed in 1× G7 buffer, with 1% NP-40 at 37°C for 1 h. Enzyme-treated samples were analyzed by resolving on 10–13% SDS–PAGE gels and western blot analysis.

Western blot analysis

After separation by SDS–PAGE (10–13%), proteins were electroblotted onto a nitrocellulose membrane (Whatman, Florham Park, USA). Monoclonal antibodies to E1 (A4) or E2 (AP33) (GeneTech, Redwood, USA) were used as the primary antibody with appropriate dilution (1:1500). The horseradish peroxidase (HRP)-conjugated AffiniPure goat anti-rabbit immunoglobulin G (IgG; 1:5000 dilution; Sigma, St Louis, USA) was used as the secondary antibody. Proteins were revealed on Thermo film in a dark room using the SuperSignal West Pico Chemiluminescent Substrate (Pierce, Rockford, USA) as recommended by the manufacturer.

Dot blot assay

A nitrocellulose membrane was pre-wetted with PBS and the Dot blot apparatus (Bio-Rad, Hercules, USA) was assembled according to the manufacturer's introductions. Culture media and diluted cell lysates were added to wells and vacuum was applied to load products onto the membrane. Wells were washed twice with 200 μl of PBS in a low vacuum. The apparatus was disassembled and the membranes were blocked with blocking buffer (PBS + 3% FBS). After extensive washing, the primary antibody, HCV-infected human serum diluted 1:200 in FBS buffer, was added. Then, the secondary antibody, HRP-conjugated goat anti-mouse IgG (Sigma) diluted 1:5000, was added.

Galanthus nivalis agglutinin lectin capture EIA

Galanthus nivalis agglutinin (GNA) lectin at 1 μg/ml (Sigma) was used to coat enzyme immunoassay/radioimmunoassay (EIA/RIA) plates (Costar Group, Cambridge, USA) overnight at 4°C. After being washed with PBS, plates were blocked with 5% milk powder, and then supernatants or cell lysates were added for binding at room temperature. Plates were washed with a washing buffer (PBS + 0.05% Tween-20) four times, then 100 μl of primary antibody monoclonal antibody (MAbs) A4 or AP33, dilution at 1:1500) was added, followed by incubating for 90 min at 37°C. Unbound antibody was washed off with the washing buffer. HRP-conjugated goat anti-mouse IgG (secondary antibody, 100 μl) was added to each well at 1:5000 dilution in 5% milk powder and incubated for 45 min at 37°C. Finally developed with a tetramethylbenzidine substrate and absorbance values at 450 nm were determined.

Results

Replacement of the sequence encoding the signal peptide of E1 and E2

The online programs SignalP, TargetP, and PSORT were used to determine cleavage sites and the expression localization of various signal peptides [21,22]. Our goal was to select the peptide that could facilitate higher extracellular expression of the E1 and E2 proteins compared with the native signal peptide. *E1* and *E2* genes were modified to enable a series of translational fusions with different signal

peptide-encoding regions. An *Eco*RV site and a *Bst*EII site were inserted at each end of the signal peptide-encoding region (**Fig. 1**). The *Bst*EII insertion created a mutation in the C-terminal of each signal peptide, in which the terminal amino acid was replaced with V-T-G (**Fig. 1**). Software analysis indicated that these replacements did not alter the cleavage site of E1/E2 (**Table 1**). For expression, the fragment obtained by digesting each PCR product with *Eco*RV/*Bam*HI was inserted at the same sites in the pVRC8301 vector. Plasmids containing the wild-type signal peptide sequence were named pVRC-E101 and pVRC-E201; plasmids containing the computer-designed signal peptide sequence were named pVRC-E102 and pVRC-E202; plasmids containing the Gluc signal peptide sequence were named pVRC-E103 and pVRC-E203; and plasmids containing the tPA signal peptide sequence were named pVRC-E104 and pVRC-E204 (**Table 2**). In each case, successful insertion was confirmed by sequencing.

Effects of signal peptide replacement on intracellular expression of E1 and E2

The signal peptide sequence of the HCV E1/E2 glycoproteins was substituted with three heterologous signal peptide sequences. Intracellular and extracellular expression levels of E1 and E2 proteins were assessed using a monoclonal antibody specific for either E1 or E2. Compared with the control (pVRC-E101/pVRC-E201), all of the clones (pVRC-E102/pVRC-E202, pVRC-E103/pVRC-E203, and pVRC-E104/pVRC-E204) successfully expressed the E1 or E2 glycoproteins. HCV E1 expressed in untreated cells had an apparent molecular mass of 33 kDa and the molecular weight (MW) was reduced to 18 kDa after deglycosylation, and the MW of E2 is 60–70 kDa in nature and 31 kDa after deglycosylation [3]. Our results showed that the MW of each modified signal peptide attached to E1 and E2 did not differ significantly from the wild type (**Fig. 2**). Furthermore, after digestion with N-glycosidase F, the MWs of E1 and E2 with the modified signal peptide were not significantly different from that of wild type, suggesting a pattern of identical glycosylation (**Fig. 3**).

Native E1 glycoprotein was detected with the least expression, despite its expression conditions was identical with others. Only by increasing the loading quantity to 30 μg (six times of the loading quantity than that of other samples), the band of native E1 glycoprotein can be detected (**Fig. 2**). However, band intensity remained noticeably lower than any of the modified E1 proteins. Thus, production of E1 with a wild-type signal sequence was particularly low, at least less than one-sixth, that of E1 with either the tPA or Gluc signal peptide.

Effect of signal peptide replacement on secretion of E1/E2

After confirming the intracellular expression of E1/E2 with the replaced signal peptide, we assessed the extracellular concentrations of the E1 and E2 proteins. As predicted by software, the ratios of secretion were different among the various signal peptides. To assess the true expression level of the modified E1 and E2 glycoproteins, we chose methods that could enrich the non-denature productions for more sensitive detection, such as dot blot and GNA capture EIA, both the semi-quantitative methods. They were used to detect glycoproteins E1 and E2 in all cell lysates and culture media in parallel. Results from these two methods showed no obvious disparities (**Fig. 4**). The expression level of the E1 native signal peptide was particularly low, and three of the modified signal peptides, particularly tPA and Gluc, showed significantly increased expression than the native

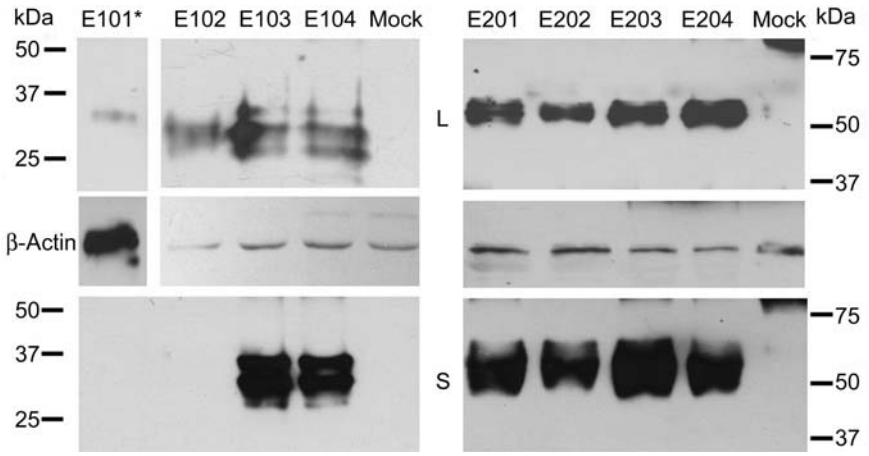

Figure 2 Western blot analysis of E1/E2 produced by 293T transfected with different plasmids Cell lysate samples (L, 5 μg/sample) and supernatants (S, 20 μg/sample) were electrophoresed separately. All samples were collected after 48 h cultivation with non-BSA opti-MEM. The blot was stripped and re-probed with β-actin. Asterisk denotes that the loading volume of this sample was six times greater (30 μg) than that of other lysate samples. 293T cells transfected without a plasmid was used as a control (mock).

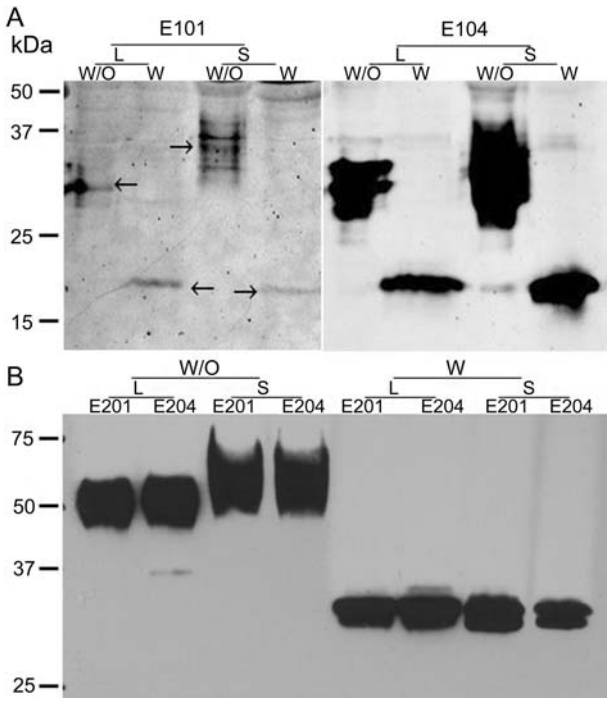

Figure 3 Different signal peptide have no apparently effect on the expression of E1 and E2 in 293T cells 293T cells contain pVRC-E101/104 and pVRC-E201/204 plasmids were collected 48 h after transfection. Cell lysate (L) and supernatant (S) of each samples were treated with PNGase F (W) or left untreated (W/O). After being separated by SDS–PAGE (E1 in 13% and E2 in 10% acrylamide) under reducing conditions, E1 and E2 were immunoprecipitated with monoclonal antibodies A4 and AP33. (A) In the same loading quantity (20 μg), bands detected from E101 (arrow indict) were much fainter than those of E104, but their distributions were the same under the similar treatment. (B) The distinction of band grayscale between E201 and E204 is not obvious, but the MW and distribution of their bands are identical under the same treatment.

signal peptide. Such distinction was not so obvious in E2, but the expression level of HCV E2 glycoprotein with a native signal peptide was still lower than that of E2 modified with any of the exogenous signal peptides. Otherwise, the expression level of pVRC-E102/pVRC-E202, whose signal peptide was predicted to have the greatest secretion ability, was lower than the sample with tPA or Gluc signal peptides. Furthermore, modified with the tPA or Gluc signal peptides, neither the expression nor the secretion levels of glycoproteins showed any marked differences (**Fig. 4**). In our experiment, E2 could be more easily detected than E1. This might be caused by different primary antibody, or because E1/E2 were glycosylated at different levels leading to distinct GNA-binding ability.

Discussion

More than 120 million people worldwide are chronically infected with HCV, making HCV infection to be the leading cause of liver transplantation in developed countries. Treatment options are limited, and the efficacy depends on both the infecting strain and the initial viral load [1]. During the translation of HCV, the nascent E1 and E2 polypeptides are targeted to the host endoplasmic reticulum membrane for modification by N-linked glycosylation [5]. E1 and E2 are released from the polyprotein through cleavage by a host signal peptidase [3] and are anchored in the viral lipid envelope as a heterodimer, which plays a major role in the HCV entry [7,11]. Deletion of the TMDs of E1 and E2 results in the secretion of these truncated forms of HCV glycoproteins into the extracellular medium. E2 antigen in its secreted

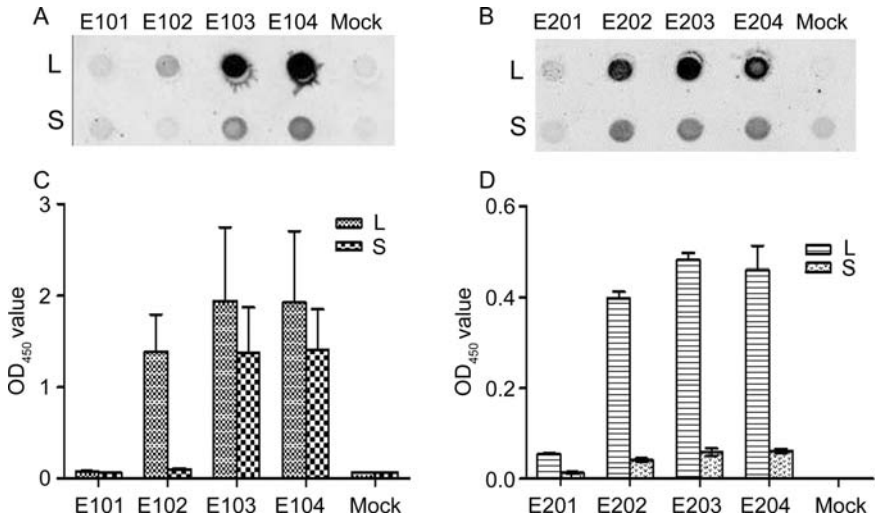

Figure 4 Analysis of the production levels of E1 and E2 glycoproteins containing altered signal peptides Human embryo kidney 293T cells contained the following plasmids were indicated, and 293T cells transfected without a plasmid was used as a control (mock). Cell lysates (L) and supernatants (S) were collected 48 h after transfection. (A) and (B) represent the dot blot results of E1 and E2 glycoproteins detected using HCV-infected human serum as the primary antibody. GNA lectin capture EIA data are shown in (C) and (D). Data obtained from both the dot blot and EIA methods showed no marked discrepancies.

form leads to an increased humoral response in mice [26]. HCV E1 and E2 are primary determinants of entry and pathogenicity. HCV E2 glycoproteins are involved in receptor binding, virus–cell fusion, and entry into host cells [11]. However, its role in membrane fusion and immune evasion remains uncharacterized. The function of E1 is unknown; however, it is a target for neutralizing antibodies and its association with E2 is essential for viral entry [11]. Increasing knowledge of the nature and the function of HCV E1 and E2 glycoproteins help to develop antiviral drugs and vaccine candidates.

The production of large quantities of functional and secreted E1 and E2 proteins will allow us to perform comprehensive biochemical and biophysical analysis, which will lead to the development of a vaccine that is effective against HCV infection. In this study, we developed a novel expression system for producing the secreted form of E1 (eE1) and E2 (eE2) ectodomains from mammalian cells via a signal peptide sequence replacement strategy and performed a comprehensive biochemical characterization. These data enhance our understanding of HCV envelope glycoproteins and may assist in the design of HCV vaccines and entry inhibitors.

Our data showed that the replacement of the signal peptide sequences located at the upstream of *E1* and *E2* genes altered their secretion and expression levels, and most importantly, the replacement did not affect glycosylation. Furthermore, replacing them with tPA and Gluc resulted in the greatest increases at expression and secretion levels of eE1 and eE2 in mammalian cells. We believed that the strategy used in this study could be applied to the expression and production of other glycoproteins in mammalian cell line-based systems.

The next challenge is to use our novel strategy to generate large quantities of HCV E1 and E2 envelope glycoproteins. This study will allow us to elucidate their structural biology and the mechanism(s) involved in the interactions between these glycoproteins and other cellular factors, eventually to facilitate the development of an HCV vaccine and/or entry inhibitor.

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