Purification and application of C-terminally truncated hepatitis C virus E1 proteins expressed in *Escherichia coli*

**Jing Liu, Li-Xin Zhu, Yu-Ying Kong, Guang-Di Li, Yuan Wang**

**INTRODUCTION**

Hepatitis C virus (HCV) is the major etiological agent of both community-acquired and post-transfusion non-A, non-B hepatitis[1,2]. It is estimated that 3% of world population have been infected with HCV[3]. Approximately 85% of patients develop chronic infection, and about 20% of chronic cases will progress into cirrhosis and/or hepatocellular carcinoma[4,5]. Presently, there is no vaccine against HCV[6] and the only available therapy, interferon-alpha on its own or in combination with ribavirin, is effective in only a minority of patients and carries the risk of serious side effects[7,8]. There is a pressing need to develop effective prophylactic and therapeutic measures against HCV in order to combat this global public health threat.

Envelope proteins of HCV (E1 and E2) are predicted to be type I membrane glycoproteins, and generally believed to constitute the protein components of virion membrane[9,10]. Various studies have implicated both E1 and E2 in important steps of HCV entry into target cells, such as receptor binding and membrane fusion[11,12]. Vaccination of chimpanzees with E1 and E2 glycoproteins resulted in limited but measurable protection against homologous virus challenge[13]. Therefore, E1 and E2 have become two major targets in HCV vaccine research.

Evaluation of HCV envelope protein-based vaccines requires an effective method for antigen detection and an inexpensive supply of large quantities of antigens. Although E1 glycoproteins expressed in mammalian cell systems in theory would best reflect properties of E1 proteins present on HCV virion membrane, low yield as well as difficulty in purification and scaling up makes such systems unsuitable for large-scale applications[14]. In order to circumvent this problem, we attempted to express E1 proteins in *E. coli*. Bacterial expression systems, compared to other higher organism expression systems, usually offer higher yield at considerably lower cost. Our previous work has shown that *E. coli*-derived recombinant E2 proteins and rabbit antisera against them are sufficient for these applications, and could partly substitute expensive mammalian system-expressed envelope proteins and infectious HCV patients’ sera[15,16]. In this work, C-terminally truncated E1 was expressed in *E. coli* as hexa-histidine-tagged fusion proteins and purified under denaturing conditions using immobilized-metal affinity chromatography. Rabbit anti-sera raised against purified recombinant E1 specifically reacted with mammalian cell-expressed E1 glycoproteins in Western blot. Furthermore, *E. coli*-derived E1 protein was able to detect animal antibodies elicited by E1-based DNA immunization.

**CONCLUSION**

These results demonstrate that the prokaryotically expressed E1 proteins share identical epitopes with eukaryotically expressed E1 glycoprotein. The *E. coli*-derived E1 proteins and corresponding antisera can become useful tools in anti-HCV vaccine research.

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**Key words:** HCV envelope protein 1; Recombinant Fusion Proteins; *Escherichia coli*

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**MATERIALS AND METHODS**

**Plasmids and bacterial host**

pUC18/CE1E2-W carrying C, E1 and E2 coding sequences of HCV (subtype 1b) was provided by professor Yu Wang of...
were performed as previously described[20]. pUC19/E1E2-Z carrying E1 and E2 coding sequences of a different HCV isolate (also subtype 1b) was provided by Dr. Xin-Xin Zhang of Ruijin Hospital, Shanghai. pQE8 is an N-terminal hexa-histidine fusion expression vector from Qiagen GmbH, Hilden, Germany. E. coli strain TG-1 was used as cloning and expression host.

**Construction of recombinant expression plasmids**
Polymerase chain reactions and recombinant cloning were performed according to standard protocols[21]. DNA sequences coding for E1 amino a-cid (aa) 192-340 and aa 192-383 were amplified from pUC19/E1E2-W and pUC18/CE1E2-Z, and cloned into pQE8 between the BamHI and HindIII sites to create pQE8/E1Z, pQE8/E1Z, pQE8/E1W, and pQE8/E1W respectively. Sequences between BamHI and SalI were removed from pQE8/E1Z, by double digestion and used to replace corresponding sequences in pQE8/E1W. The resultant plasmid carrying chimeric E1 coding sequences was designated as pQE8/E1Z. Deletion of E1 C-terminal hydrophobic region (aa 341-383) in pQE8/E1W or pQE8/E1Z was conducted by double digestion with BsrEII and HindIII, blunting of the resultant ends, removal of unwanted small fragments and self-ligation of large fragments. The obtained subclones were designated as pQE8/E1W and pQE8/E1Z, respectively. Structures of these plasmids are illustrated in Figure 1.

**Expression and purification of recombinant E1 proteins**
Freshly saturated recombinant TG-1 culture was inoculated into fresh LB media at 1:100. Two hours after inoculation, expression was induced by adding isopropyl-β-D-thiogalactopyranoside (IPTG) to a final concentration of 1 mmol/L. Cells were harvested 6 h later by centrifugation and stored at -20 °C.

Solubility analysis and purification of expression products were performed as previously described[15-17]. Briefly, harvested bacteria were resuspended in phosphate-buffered saline (PBS, containing 137 mmol/L NaCl, 2.7 mmol/L KCl, 4.3 mmol/L Na2HPO4, 1.4 mmol/L KH2PO4, pH 7.3), sonicated on ice-bath, and centrifuged at 15 000 r/min at 4 °C. The soluble and insoluble fractions after centrifugation were analyzed for the presence of expression products. Insoluble recombinant E1 proteins were extracted with 6 mol/L GuHCl/20 mmol/L β-NTA/PBS (pH 8.0), centrifuged at 15 000 r/min at 4 °C, and loaded onto pre-activated Ni2+-NTA agarose (Qiagen). The gel matrices were sequentially washed with 6 mol/L GuHCl/20 mmol/L β-ME/PBS (pH 6.3) and 8 mol/L urea, 20 mmol/L β-ME/PBS (pH 6.3), and then eluted with 8 mol/L urea, 20 mmol/L β-ME/PBS (pH 4.3) or boiled in reductive SDS-PAGE sample buffer for elution.

**E1 glycoprotein expression in mammalian cells**
Expression of HCV structural proteins C, E1 and E2 was performed as previously described[22]. Recombinant vaccinia virus vCEH-2 contained coding sequences of HCV polyprotein aa 1-730 under the control of T7 promoter, whereas vTT7 encoded the T7 polymerase required for expression. HeLa cells were co-infected with vTT7 and vCEH-2 at a multiplicity of infection of 4:41 (vTT7:vCEH-2-cell) and cultured for 48 h. Cells were collected by scraping, washed with PBS at 4 °C and stored at -20 °C.

**Protein analysis**
SDS-PAGE under reducing or non-reducing conditions and Western blot were conducted according to standard protocols[23]. In Western blot, first antibody was diluted at 1:500 and second antibody [HRP-labeled protein A (Sigma, St. Louis, MO, USA) or swine anti-rabbit Ig (Dako, Denmark)] was diluted at 1:1000. Blots were developed using the enhanced chemi-luminescent (ECL) method (PerfectBio, Shanghai, China).

For deglycosylation analysis, cell samples were treated with PNGase F (New England Biolabs Inc., Beverly, MA, USA) according to manufacturer’s instructions and then subjected to reductive SDS-PAGE/Western blot analysis.

**Animal immunization, antisera preparation and antibody analysis**
Female rabbits (Shanghai Laboratory Animal Center) were immunized subcutaneously on the back with 300 μg of purified recombinant protein emulsified in complete Freund’s adjuvant (Sigma) and boosted twice at an interval of 4 wk with the same quantity of antigen emulsified in incomplete Freund’s adjuvant (Sigma). One week after final boosting, total blood was collected through the carotid artery and serum was prepared using standard procedure[21].

Anti-E1 antibodies in post-immune animal sera were analyzed in standard ELISA using purified recombinant E1 protein as coating antigen. Microplates were coated with the antigen used for immunization at 0.1 μg/hole. Serially diluted post-immune sera were analyzed as previously described[15-17] with pre-immune sera diluted at 1:100 as negative control. The highest dilution giving a positive reading was taken as the antibody titer of corresponding antisera. All tests were done in duplicate.

**RESULTS**

**Construction of expression plasmids**
For the expression of HCV E1 protein in E. coli, full-length or C-terminally truncated E1 sequences from two subtype 1b isolates were used (Figure 1). Chimeric constructs carrying sequences derived from both isolates were also used (Figure 1). Corresponding coding sequences were cloned into N-terminal hexa-histidine fusion expression vector pQE8 as described in MATERIALS AND METHODS.

**Expression and purification of recombinant E1 proteins**
TG-1 cells transformed with recombinant expression plasmids were induced with IPTG and analyzed on SDS-PAGE (Figure 2). C-terminally truncated pUC19/E1E2-Z-derived pQE8/E1Z, pQE8/E1Z, pQE8/E1Z, and pQE8/E1Z were removed from pQE8/E1Z, pQE8/E1Z, and pQE8/E1Z, respectively. Structures of these plasmids are illustrated in Figure 1.

**Figure 1** Schematic representation of E1 fragments selected for 6×His fusion expression. The original E1 coding sequences from two subtype 1b isolate cDNA plasmids are aligned at the top and bottom, respectively. Numbers indicate positions on the HCV polyprotein. Positions of restriction endonuclease recognition sites used for cloning are also shown. HR: hydrophobic region.

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expressed high levels of recombinant E1 protein of predicted apparent molecular weight (Figure 2A, lane 2), whereas pUC18/CE12-W-derived full-length pQE8/E1W or C-terminally truncated pQE8/E1W326 exceeded no obvious expression of recombinant proteins (Figure 2C). However, when N-terminal sequences (aa 192-262) in pQE8/E1W326 were replaced by corresponding pUC19/E1Z2-derived sequences, the resultant chimeric pQE8/E1Z2-W326 showed prominent expression of recombinant E1 proteins upon induction (Figure 2B, lane 2). Expression products from pQE8/E1Z2 and pQE8/E1Z2-W326 were designated as E1Z340 and E1Z262W326, respectively (Figure 2A, lane 5; and Figure 2B, lane 5). These minor quantities of higher and lower mobility bands, in addition to corresponding pUC19/E1E2-Z-derived sequences, the resultant truncated pQE8/E1W326 displayed no obvious expression of both oxidized and reduced E1 glycoproteins (Figure 4A). Both E1Z262W326N and E1Z262W326N were recognized deglycosylated E1 glycoproteins, as shown representatively in Figure 4B.

Preparation and analysis of rabbit anti-E1 antisera

Purified E1Z340, E1Z262W326N and E1Z262W326R respectively. Their anti-E1 titers were determined by ELISA to be 1: 1×10^8, 1: 8×10^8 and 1: 1.6×10^8 respectively.

Detection of prokaryotic and eukaryotic E1 proteins using rabbit anti-E1 antisera

All three rabbit anti-E1 sera displayed similar recognition of E. coli-expressed E1 proteins with high sensitivity and specificity. Representative data obtained from E1Z340 and E1Z262W326N are shown in Figure 3. For glycosylated E1 proteins expressed using recombinant vaccinia virus system, E1Z262W326N showed no specific recognition (data not shown), whereas E1Z262W326R displayed specific recognition of both oxidized and reduced E1 glycoproteins (Figure 4A). Both E1Z262W326N and E1Z262W326N recognized deglycosylated E1 glycoproteins, as shown representatively in Figure 4B.

Figure 3 Specificity analysis of rabbit antisera against E. coli-derived E1 proteins. Western blot analysis was carried out using (A) E1Z340 and (B) E1Z262W326N as primary antibody against (A) E1Z340 and (B) E1Z262W326N, respectively. Lane 1: induced TG-1(pQE8) as negative control; lane 2: whole-cell sample after induction; lane 3: purified products.

Figure 4 Detection of mammalian E1 glycoproteins using rabbit antisera against E. coli-derived E1 proteins E1 glycoproteins expressed using recombinant vaccinia virus system were analyzed on standard SDS-PAGE followed by Western blot using E1Z340 (A&B) and E1Z262W326N (C) as primary antibody. In (A) and (C), lanes 1 and 3: HeLa cells infected with vvT7 alone; lanes 2 and 4: HeLa cells co-infected with vvT7 and vCEH-2. Positions of recognized E1 bands are indicated. Prefixes ‘g’ or ‘d’ indicates glycosylated or deglycosylated E1, respectively. Subscript ‘ox’ or ‘re’ indicates oxidized or reduced forms of E1, respectively.
DISCUSSION

Various reports have suggested that, in addition to being a structural component of HCV virion membrane, E1 also plays important roles in viral attachment and entry into target cells as well as virus-host immune interactions[11-14]. As a result, both virologists and vaccinologists are paying considerable attention to this protein.

It has been reported that the C-terminal hydrophobic region (aa 341-383) of E1 hinders its expression in E. coli, possibly through interference with normal bacterial membrane functions[20-23]. In our work, we also found that constructs carrying the hydrophobic region were unable to express recombinant proteins to a level detectable by Coomassie brilliant blue staining (Figure 2C). Truncation of the hydrophobic region resulted in expression of high levels of recombinant proteins upon induction, but only for those constructs carrying N-terminal sequences (aa 192-262) derived from pUC19/E1E2-Z, i.e., pQE8/E1Z262 and pQE8/E1Z262W326. The construct carrying pUC19/CE1E2-W-derived N-terminal sequences (pQE8/E1W326) showed no difference in its full-length predecessor pQE8/E1W (Figure 2). Comparison of pUC19/E1E2-Z and pUC19/CE1E2-W sequences in the aa 192-262 region revealed 14 transitions and 4 transversions, resulting in four amino acid residue changes (data not shown), suggesting that nucleotide and/or amino acid sequences play an important role in E1 expression in E. coli. A similar phenomenon was also observed when we attempted to express HCV E2 in E. coli[27]. The exact mechanism of sequence difference causing different expression is still under investigation.

Expression products induced from pQE8/E1Z262 and pQE8/E1Z262W326 are largely insoluble, which is in agreement with reports by other researchers[28,29]. Large-scale expression and purification using denaturing immobilized-metal affinity chromatography could produce large quantities (>1 mg/L) of both E1Z262 and E1Z262W326, with fairly high purity (>85%).

Rabbit sera raised against these proteins not only recognized well as virus-host immune interactions important roles in viral attachment and entry into target cells as structural component of HCV virion membrane, E1 also plays reducing conditions (R
during extraction-purification procedures fold E1 polypeptide to present non-glycosylated forms of E1 glycoproteins (Figure 4C). This result suggests that some disulfide bonds formed in E. coli or during extraction-purification procedures fold E1 polypeptide to present non-linear epitopes identical to those found on mammalian E1 glycoproteins. Such reactivity against non-reduced E1 glycoproteins makes these sera promising candidates for the histological detection of E1 in liver biopsy samples.

Since bacteria-derived E1 proteins share identical epitopes with mammalian E1 glycoproteins, we used E1Z262W326 to detect anti-E1 antibodies in E1-based DNA immunization studies. BALB/c mice immunized with E1-expressing plasmids developed anti-E1 antibodies detectable by E1Z262W326 in ELISA[28]. This result further demonstrates the immunogenic/antigenic similarity between bacterial and mammalian E1 proteins. We also tested purified E1 proteins for their ability to react with two homologous patients’ sera in Western blot or ELISA. With the two sera we used, no specific reaction could be obtained. The failure of E. coli-derived E1 proteins to react with homologous patients’ sera might indicate that these sera do not contain any anti-E1 polypeptide antibodies, or the antibody titer is too low to be detected. In chronic patients, it has been reported that the prevalence of anti-E1 polypeptide antibody is only about 51.5%[26] or even lower[29].

In summary, this work produced large quantities of E. coli-derived C-terminally truncated E1 proteins with high purity, obtained highly specific rabbit antisera against these proteins, and demonstrated the applicability of these sera for the detection of E1 proteins expressed in both prokaryotic and eukaryotic systems. These results indicate that E. coli-derived E1 proteins are immunologically similar to mammalian E1 glycoproteins. E1 antigens and antisera reported here can serve as useful tools in the development of E1-based HCV vaccine as well as other E1-related studies.

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