RAPID COMMUNICATION

Hepatitis C Virus E1 Protein Induces Modification of Membrane Permeability in E. coli Cells

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The E1 gene of hepatitis C virus (HCV) has been cloned and expressed in BL21(DE3)pLys Escherichia coli strain by pET3a vector to analyze changes in membrane permeability produced by this protein. We showed that the expression of E1 (aa 192–383), as well as of two C-terminal fragments (aa 331–383 and aa 341–383) corresponding to the transmembrane (TM) region of this protein, induced a rapid lysis of cells. On the contrary, the expression of a mutant of E1 (aa 192–340), lacking the last 40 amino acids, did not cause cell lysis. The analysis of permeability changes revealed that modification of membrane permeability to several compounds were observed only in clones expressing E1 and C-terminal fragments, while the synthesis of the C-terminal-deleted mutant had little or no effect on permeability. These findings demonstrate that the TM domain of E1 protein has membrane-active properties that may be involved in some aspects of virus-cell interaction.

INTRODUCTION

Hepatitis C virus (HCV) genome encodes one long open reading frame that is translated into a single polyprotein from which all structural and nonstructural proteins of the virus derive. The structural proteins, a capsid protein and two envelope glycoproteins designated E1 and E2, are released from the N terminus of the polyprotein by the action of a host-derived signal peptidase (11).

The E1 gene encodes for a 191 amino-acid-long (aa 192–383) protein (11). The expression in eukaryotic systems indicates that E1 is translocated into the endoplasmic reticulum (ER) where interacts with the other envelope protein E2 to form a membrane-associated complex. The expression of the E1/E2 complex is restricted to the ER of the expressing cells where both molecules become glycosylated (6, 8, 18).

The hydrophobicity plot of E1 shows a C-terminal hydrophobic regions (aa 341–383) that may contain the transmembrane (TM) domain for E1 and the signal sequence for E2. It has been suggested that the E2 signal sequence remains part of the mature E1 protein (9). In this respect, E1 protein would be quite similar to flavivirus prM in which both regions are required for stable membrane anchoring (17).

Recent data indicate that the full-length E1 could not be expressed in E. coli; however, when the C-terminal region was truncated, the protein was synthesized to high level (12, 27, 28). According to the membrane-associating properties of this region, we propose that the insertion of this domain into membranes may produce modifications of permeability that are toxic for bacteria.

Several viral proteins that modify membrane permeability have been identified (1, 3, 21, 24). Irrespective of the specific function that these proteins have during viral replication, it has been suggested that they form hydrophilic pores into the membrane by oligomerization of two or more molecules. Computer analysis predicts that viral proteins with channel-activity usually contain long hydrophobic regions that may form α-helical domains into the membrane. Though the helices are largely hydrophobic, they do contain some hydrophilic residues that presumably face the aqueous lumen of the channel (3).

Among the viral proteins that modify membrane permeability, gp41 of HIV shows some similarities with the E1 protein of HCV. They are both glycoproteins that form oligomeric structures into the viral envelope (8, 22). In addition, the TM region of these proteins is rich in hydrophobic amino acids but also contains conserved basic residues (1, 2), which are very unusual in TM domains of glycoproteins. The analysis of the effect produced by the interaction of gp41 with membrane revealed that the TM region is the fragment that most drastically modified cell permeability, suggesting that this domain is functionally involved in the alteration of membrane integrity and...
perhaps is mainly responsible for cell lysis during HIV infection (1).

To determine whether the E1 protein of HCV induces permeability changes, we expressed this protein in E. coli by an inducible system developed for the synthesis of toxic polypeptides (3, 26).

We report that a low level expression of E1 and of fragments corresponding to the C-terminal region was lytic for E. coli and altered membrane permeability to several compounds. Conversely, a C-terminal truncated mutant of E1, which was expressed to high level, did not cause cell lysis and significant permeability changes.

To evaluate the effect of E1 on membrane permeability, we cloned full-length E1 and its fragments in pET3a vectors and expressed the corresponding proteins in E. coli BL21(DE3)pLysS cells. This is an inducible system in which the expression of foreign gene is achieved by the T7 RNA polymerase. The synthesis of this enzyme requires the addition of IPTG, which derepresses the lacUV5 promoter. In this system, low levels of uninduced T7 polymerase are blocked by a constitutive expression of T7 lysozyme, a natural inhibitor of the T7 polymerase.

In that way, even low-level expression of toxic protein is completely avoided before the induction (26).

The positions of the cloned fragments respect to the hydrophobicity plot (15) of E1 are described in Fig. 1.

Fragment AP (aa 341–383) contains the last 40 amino acids (Fig. 1B), which, according to their hydrophobic properties (Fig. 1A), probably form the TM domain of E1. The AP region is long enough to span the membrane more than one time by α-helical domains. Computer analysis (4) showed that it has the tendency to α-helical formation but also includes a positive charged residue (Lys 370), which may be important for the channel activity. Fragment VP overlaps the AP domain but contains also a short hydrophobic region of 10 amino acids preceding the first residue of AP (Fig. 1B). Because this region shows a predictive α-helical structure (4) and contains one hydrophilic amino acid (Arg 339), it may contribute to alter membrane permeability. Fragment SUB (aa 192–340) contains the E1 gene deleted of the C-terminal region (Fig. 1B).

We analyzed the kinetic of expression of the recombinant clones at different times after the induction with

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**FIG. 1.** Cloning and expression of E1 fragments. (A) Hydrophobicity plot (15) of the E1 protein. (B) The E1 fragments studied are indicated as open boxes. Amino-acid residues are indicated for each fragment. (C) Kinetics of protein expression of the E1 recombinant clones. BL21(DE3)pLysS cells transformed with plasmid pET3a-ENV, pET3a-SUB, pET3a-VP, and pET3a-AP were induced as described in text. At the indicated times (hours) postinduction, the expressed proteins were labeled and analyzed by 15% SDS-PAGE. Markers in kDa are indicated on the left.
IPTG in the presence of rifampicin (Fig. 1C). Rifampicin blocks E. coli transcription, which leads to diminished synthesis of E. coli proteins. This allows an easier detection of the cloned protein. The expression of the C-terminal truncated form of E1 (SUB), which is 149 amino acids long, gave rise to a polypeptide that migrated as a band of the expected molecular size (about 15 kDa). This product was synthesized at high level in the 4 h after induction analyzed (Fig. 1C, SUB). The expression of full-length E1 (ENV), which is 192 amino acids long, gave rise to a polypeptide of the expected size (about 20 kDa) and to a series of higher molecular weight polypeptides that probably were oligomers of E1. The ENV product was synthesized at lower level respect to SUB. Also, the kinetic of expression of this clone clearly showed a gradual decreasing of protein synthesis in the hours after induction (Fig. 1C, ENV). A lower molecular weight polypeptide of identical size (<15.3 kDa), was also produced by the expression of ENV and SUB clones (Fig. 1C, compare ENV and SUB). This molecular form is probably due to cleavage in the E1 region proceeding the amino acid 340. Fragments AP and VP, which contain 43 and 53 amino acids, respectively, migrated as a diffuse band of the expected molecular size (about 8.7 kDa).

These products were expressed to a very low level and only for the first hour upon induction (Fig. 1C, AP and VP). We cannot exclude that the poor expression of AP and VP could be due to the reduced number of methionine residues that they contain (2 and 3 residues) with respect to ENV and SUB (8 and 6 residues). However, the results obtained from growth curves and permeabilization assays (see below) suggest that this faint expression is much likely related to their toxicity in E. coli cells. These results indicate that the E1 protein and fragments containing the C-terminal region are poorly expressed and only for a short period after induction, suggesting that their synthesis is not tolerated by E. coli cells.

Several membrane-active proteins are lytic when cloned in E. coli strains expressing T7 lysozyme (1, 10, 16, 25). In this case the alteration of membrane permeability allows the passage of T7 lysozyme to the periplasmic space where it could exert its lytic activity on bacterial cell wall. To test the effect of E1 expression on BL21(DE3)pLysS cell growth, we measured the optical density at 600 nm (O.D. 600 nm) of recombinant cultures at different times after induction (Fig. 2). As rifampicin blocks E. coli transcription, the addition of IPTG and rifampicin to pET3a clone (control) led to growth arrest. The expression of SUB produced the same effect on cell growth, as indicated by comparison with cells bearing plasmid pET3a (Fig. 2, compare SUB to pET3a). By contrast, the expression of fragments VP and AP was clearly lytic for E. coli. Soon after induction of VP synthesis, cell density began to drastically decrease (Fig. 2, VP). The effect was slightly less rapid upon induction of AP fragment (Fig. 2, AP). Cell lysis was also observed in culture expressing ENV, but in that case the gradual decrease of cell density required longer postinduction times (Fig. 2, ENV).

To explore the possibility that the insertion of E1 into membrane increases membrane permeability, we analyzed membrane selectivity of E1 recombinant cultures by four different assays. We first tested the entry of hygromycin B in cells. Hygromycin B acts by blocking protein synthesis, but the entry of this antibiotic is detected only in cells in which membrane permeability has been modified (10, 16, 25). Figure 3A shows incubation with hygromycin B of recombinant cultures after induction with IPTG plus rifampicin. We observed inhibition of protein synthesis in cells expressing ENV (Fig. 3A, compare lane to + of ENV), VP, and AP (data not shown). Recombinant cultures expressing SUB revealed only a partial inhibition of protein translation after treatment with hygromycin B. We estimated a reduction of about 50% of radioactivity (Fig. 3A, compare lane to + of SUB). This effect was obviously more drastic on lower expressed bands (see the cleavage product below 15.3 kDa).

In a second step we analyzed the entry of ONPG into cells. ONPG is a substrate of the β-galactosidase that is normally excluded by membrane of intact cell (10, 24). We tested β-galactosidase activity of recombinant cultures by measuring the appearance of ONPG cleavage product (O.D. at 420 nm) at different times after induction. After 1 h from the addition of IPTG, synthesis of AP and VP caused an increase of ONPG entry into cells as compared with control (Fig. 3B, left, AP and VP). The same effect was observed upon induction of ENV clone while expression of SUB caused only a moderate increase of ONPG entry respect to the control (Fig. 3B, right, ENV and SUB).

To test the possibility that expression of E1 and its fragments leads to the exit of nucleotides (1, 10, 16), we loaded recombinant clones with labeled uridine and measured the release of radioactivity at different times after induction. As shown in Fig. 3C, the release of [3H]uridine to the medium was first observed after 30 min of induction of ENV synthesis and after 1 h of induction of VP and AP synthesis. Clones expressing SUB, as well as clones bearing the parental plasmid, did not cause a significant exit of uridine even after longer postinduction times.

The effect on membrane permeability produced by E1 expression was further analyzed by testing the entry of propidium iodide (PI) into cells at different times after induction. Binding of PI to double-stranded DNA is followed by a fluorescent emission. Because the entry of propidium iodide (PI) into cells required alteration of membrane selectivity (7, 14), flow cytometer analysis of PI incorporation represents a useful method to study alteration of permeability (1). The uptake of PI by clones expressing AP and VP was detected after 1 h postinduction. We observed a strong increase in PI fluorescence...
as compared to cells before induction (0 h) used as control. The same values of fluorescence were observed at 2 and 3 h postinduction times (Fig. 4, AP and VP). Even cells expressing ENV showed a modification of membrane selectivity to PI but the fluorescence signal was lower with respect to AP and VP and remained the same at different postinduction times (Fig. 4, ENV). Even after 3 h postinduction, incorporation of PI was not detected in cells expressing SUB as well as in cells bearing the parental plasmid (Fig. 4, SUB and control).

In this report we show that the E1 protein of HCV is able to modify membrane permeability and was lytic
when expressed in E. coli cells. The results indicate that the interaction of the C-terminal hydrophobic region with membrane is mainly responsible for the permeability changes and cell lysis induced by E1.

The expression of proteins by pET vectors in BL21(DE3)pLysS cells has been a useful approach for the identification of viral channel proteins (1, 3, 21, 24). At least for the HIV gp41 and influenza virus M2 proteins, the permeabilization activity identified by E. coli system (1, 10) was confirmed by their expression in eukaryotic
cells. It was reported that M2 protein has an ion channel-activity when expressed in *Xenopus laevis* oocytes (23) and that the lentivirus lytic peptide, comprising the last amino acids of gp41, is able to alter membrane permeability in both prokaryotic and eukaryotic cells (1, 20). Expression studies in the bacterial system demonstrated that the efflux of compounds through the membrane was directly due to an intrinsic ability of these proteins to modify membrane permeability. In fact the simple expression of hydrophobic proteins to high level in bacteria does not cause the entry or the exit of molecules which are normally excluded by the membrane (1, 16). On the contrary, even low-level expression of membrane-active proteins produces a drastic effect on membrane selectively (1, 10, 16, 25).

Our present findings indicate that the C-terminal region is an important domain for the interaction of E1 with *E. coli* membrane. Therefore it seems likely that the hydrophobic sequences plus the basic residues associated to this domain (Arg 339 and Lys 370) are responsible for the lytic activity and for the alteration of membrane permeability of the ENV, AP, and VP fragments.

Sequence analysis of different genotypes of HCV shows that the structural organization of E1 C-terminal region is strongly conserved. This protein showed a high variability in the amino-acid sequence (68.7%) but mutations in the C-terminal domain were prevalently oriented to a conservation of hydrophobicity with the 77.3% of the hydrophobic residues invariant or showing conservative variations. Moreover the positive charged amino acids,
Arg 339 and Lys 370, were highly conserved among all HCV isolates (2).

According to our results, previous works showed that secretion of E1 in eukaryotic cells has been observed only after the rimotion of the last 40 amino acids (5, 13). However, other authors indicated that both an internal hydrophobic region (aa 263-289) that the C-terminal domain must be deleted for secretion of E1, suggesting that more than one region may be responsible for membrane association (18). Our present findings indicated that most of the SUB protein, containing the internal region but lacking the last 40 amino acids, was not associated with membrane fraction (data not shown). This result correlates well with data from cell lysis and permeabilization assays. In fact, SUB protein failed to induce lysozyme and uridine exit and did not allow propidium iodide entry. Further clones expressing SUB showed a reduction of the signal, respect to the ENV clones, in the β-galactosidase and hygromycin B assay.

Specific functions have been proposed for membrane-active proteins of other RNA viruses. The ion-channel activity of influenza M2 protein is expected to have a critical role in the uncoating of virus particle (23), while the permeabilization activity of poliovirus 3AB, Semliki Forest virus 6K, and HIV gp41 proteins has been linked to the lytic potential of these viruses (1, 3).

The lack of an efficient cell system for replication of HCV has hampered studies on specific protein functions involved in viral replication and HCV pathogenesis. The identification of the E1 membrane-activity may open new directions for the analysis of the replication cycle and for new antiviral strategies.

By analogy with flavivirus, it is possible that HCV enters cells by endocytosis. As was reported for influenza virus M2 (23), the channel activity of the E1, activated by the low pH of the endosome, could drive the uncoating of virus by allowing the ions to enter the virion particle.

On the other hand, it could be speculate that E1 protein play a role in the mechanisms of virus-cell interaction. In this regard, it could be relevant to note that E1 is localized to the ER membrane, which is a membrane that have the potential to influence metabolic processes by regulation of ion permeability.

The ability of E1 to form channel may be modified by the interaction with proteins that may regulate its membrane insertion and/or oligomerization. Recently, experimental data indicate that the folding of E1 is affected by the expression of E2 (6, 19). This chaperon-like activity of E2 may regulate conformational changes of E1 relevant for its channel activity. In conclusion, this study demonstrates, for the first time, a biochemical function of the E1 protein of HCV, which may play an important role in specific steps of HCV replication and/or pathogenesis.

The recombinant clones encoding E1 and different fragments of this protein were obtained by a RT±PCR method from a HCV-positive human serum. The nucleotide sequence of the clones was determined and classified the isolate as type 1a (2). The oligonucleotide primers used for the PCR amplification of E1 clones were designed to contain start and stop codons and a unique NdeI restriction site. The PCR products were purified, digested, and ligated with dephosphorylated pET3a vector (Stratagene) previously digested with NdeI enzyme. After transformation of E. coli BL21(DE3)pLysS competent cells (Stratagene), the sequence of the recombinant clones were confirmed by the modified dideoxynucleotide method with ABI 373A automatic sequences.

For the induction of protein expression in E. coli cells, overnight cultures of recombinant clones were grown in LB medium with 100 μg of ampicillin and 34 μg of chloramphenicol per milliliter. The cultures were diluted 50-fold in M9 medium supplemented with 0.2% glucose and antibiotics and incubated at 37°C and 250 rpm. The cultures were induced at an A600 of 0.5 with 1 mM isopropylthiogalactopyranoside (IPTG). Thirty minutes after induction, 150 μg of rifampicin (Sigma) per milliliter was added to block transcription by E. coli RNA polymerase.

Analysis of expressed proteins were performed by labeling 4 ml of each recombinant cultures with 1 μCi of [35S]methionine (145 Ci/mmole; Amersham Corp.) per milliliter for 10 min at 37°C. After centrifugation the pellet was resuspended in electrophoresis sample buffer (12.5 mM Tris-HCl, 2% glycerol, 0.4% SDS, 1% B-mercaptoethanol, 0.1% bromphenol blue). Labeled proteins were analyzed by 15% SDS±PAGE and autoradiography.

To assay the entry of hygromycin B into recombinant cultures, 50 min after induction, the cells were incubated with 1 mM hygromycin B and 1 μCi of [35S]methionine per milliliter for 10 min at 37°C. The labeled proteins were analyzed as described.

To test the entry of α-nitrophenyl-β-D-galactopyranoside (ONPG) into cells, at given times after induction, pellet from 1 ml of induced cultures was resuspended in 1 ml of M9 medium containing 50 μg/ml of streptomycin to stop translation and mixed with 0.2 ml of 12 mM ONPG. Reactions were incubated for 10 min at 30°C and stopped by the addition of 0.4 ml of 1 M sodium carbonate. After this incubation the cells were pelleted, and the absorbance at 420 nm of the cleaved product was measured.

For uridine incorporation into E. coli cells, 2 h before induction cultures were loaded with 2 μCi of [3H]uridine (27.3 Ci/mmol; Amersham Corp.) per milliliter. The cells were washed three times with uridine-free growth medium and induced as described. Aliquots of 0.2 ml were pelleted at different times after induction. To quantify the released radioactivity, the supernatants were mixed with L-929 scintillation cocktail (DuPont) and analyzed.

For analysis in the flow cytometer (18), 8 μl of cells were collected at different hours after induction and stained with 0.005% propidium iodide (PI) in 500 μl phosphate-buffered...
saline (PBS) for 10 min at 37°C. Cells were analyzed in a FACScan flow cytometer (Becton Dickinson, San Jose, Calif.) equipped with an argon ion laser tuned to 480 nm and power emission of 25 mW. To detect signals from bacteria, the side scatter and the forward scatter were set in log mode. The emitted PI fluorescence was collected by a BP 630/22 filter and direct to the photomultiplier (PMT, with a voltage of 600 V). PI fluorescence signals were displayed in log scale. All measurements were gated by the forward scatter channel. Signals from 10,000 cells were collected as a sample.

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