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Mutagenesis of hepatitis C virus E1 protein affects its membrane-permeabilizing activity

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The E1 glycoprotein of hepatitis C virus is a transmembrane glycoprotein with a C-terminal anchor domain. When expressed in *Escherichia coli*, E1 induces a change in membrane permeability that is toxic to the bacterial cell. The C-terminal hydrophobic region (aa 331–383) of E1 is mainly responsible for membrane association and for inducing changes in membrane permeability. These observed changes are similar to those produced in *E. coli* by influenza virus M2, human immunodeficiency virus gp41 and poliovirus 3AB proteins, whose hydrophobic domains are thought to cause pore formation in biological membranes. To further characterize the activity of E1 at a molecular level, the membrane-permeabilizing ability of a second internal hydrophobic region (aa 262–291) was examined by expressing different deletion mutants of E1 in an *E. coli* system that is widely used for analysing membrane-active proteins from other animal viruses. Moreover, highly conserved amino acids in the C-terminal hydrophobic region were mutated to identify residues that are critical for inducing changes in membrane permeability. Analysis of cell growth curves of recombinant cultures and membrane-permeability assays revealed that synthesis of this fragment increased the flux of small compounds through the membrane and caused progressive cell lysis, suggesting that this domain has membrane-active properties. Furthermore, analysis of C-terminal mutants indicated that the conserved amino acids Arg^{339}, Trp^{368} and Lys^{370} play a critical role in protein function, as both cell lysis and changes in membrane permeability induced by the wild-type clone could be blocked by substitutions in these positions.

Introduction

Infection with hepatitis C virus (HCV) occurs worldwide and represents a major cause of liver disease (Hoofnagle, 1997).

The structural region of the HCV genome encodes two proteins, E1 and E2, which constitute the virus envelope glycoproteins (Grakoui *et al.*, 1993; Hijikata *et al.*, 1991). Studies using protein expression systems indicate that E1 and E2 interact to form complexes that are probably the functional envelope subunits of HCV (Deleersnyder *et al.*, 1997; Dubuisson *et al.*, 1994; Lanford *et al.*, 1993; Ralston *et al.*, 1993). These complexes are retained in the endoplasmic reticulum (ER), where both proteins are modified by *N*-linked glycosylation (Dubuisson *et al.*, 1994; Duvet *et al.*, 1998). A critical role in HCV envelope biogenesis seems to be played by the hydrophobic regions found at the C-terminal ends of both E1 and E2. Indeed, these domains are involved in multiple functions, such as E1 and E2 retention in the ER, membrane anchoring and complex formation (Ciccaglione *et al.*, 1998*b*; Cocquerel *et al.*, 1998, 1999; Dubuisson, 2000).

Recently, we reported that expression of E1 in *Escherichia coli* modifies membrane permeability (Ciccaglione *et al.*, 1998*a*). This effect is mainly due to the interaction of a C-terminal hydrophobic region (aa 331–383) with the cell membrane (Ciccaglione *et al.*, 2000), suggesting that insertion of this domain into biological membranes may alter their selectivity to ions or small compounds. Several reports indicate that proteins that form pores in the membrane are equally active in both prokaryotic and eukaryotic cells (Carrasco, 1995; Dempsey, 1990). For this reason, the inducible synthesis of proteins in *E. coli* is a useful system for the identification and analysis of several membrane-active proteins from other animal viruses, such as poliovirus 2B and 3AB (Aldabe *et al.*, 1996; Lama & Carrasco, 1995, 1996), influenza virus M2 (Guinea & Carrasco, 1994) and human immunodeficiency virus gp41 and Vpu (Arroyo *et al.*, 1995; Gonzalez & Carrasco, 1998). In several cases, it has been demonstrated that changes in membrane permeability induced by these proteins are directly related to important alterations in the membrane observed

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during specific steps of virus infection, such as cell fusion, virus release and glycoprotein biogenesis (Dubay *et al.*, 1992; Lee *et al.*, 1989; Doedens & Kirkegaard, 1995; Henkel *et al.*, 2000; van Kuppeveld *et al.*, 1997).

Analysis of membrane-active proteins from virus and non-virus origins indicates that a specific structural organization is required for the formation of pores in the membrane. All these proteins show one or more hydrophobic domains that contain internal, charged hydrophilic residues (Carrasco, 1995). Interestingly, we found that a similar structural organization is highly conserved in the internal (aa 262–291) and C-terminal (aa 331–383) hydrophobic regions of E1 (Bukh *et al.*, 1993). As E1 has a highly variable amino acid sequence (68·7%), it is likely that these structurally conserved domains could play a specific function during the virus replication cycle.

Alteration of biological membranes by E1 is a potentially important mechanism that may be relevant to studies on HCV biology and for the design of antiviral compounds. In order to demonstrate that this function may be directly related to the intrinsic characteristics of E1, we evaluated the role of the internal hydrophobic domain in modifying membrane permeability in *E. coli* and analysed the effects that the mutation of conserved amino acids in the C-terminal domain have on membrane-permeabilizing activity.

Methods

■ **Construction of recombinant clones.** Recombinant clones encoding different fragments of E1 (Fig. 1) were obtained by RT–PCR from the serum of an HCV-positive human. The nucleotide sequences of the clones were determined and the isolate was classified as HCV type 1a (Bukh *et al.*, 1993). Site-directed mutagenesis was carried out by PCR. After purification, PCR products were digested and ligated into pET-3a (Stratagene), previously digested with *Nde*I and dephosphorylated. After transformation into *E. coli* BL21(DE3)pLysS competent cells (Stratagene), the sequences of the recombinant clones were confirmed by modified dideoxynucleotide sequencing using an ABI 373A automatic sequencer.

■ **Induction of protein expression in *E. coli*.** Overnight cultures of *E. coli* BL21(DE3)pLysS containing the recombinant clones were grown in LB medium supplemented with 100 μg/ml ampicillin and 34 μg/ml chloramphenicol. Cultures were diluted 50-fold in M9 medium supplemented with 0·2% glucose and antibiotics and incubated at 37 °C, with shaking at 250 r.p.m. When the cultures reached an OD_{600} of 0·5, protein expression was induced with 1 mM IPTG. After inducing protein expression for 30 min, 150 μg/ml rifampicin (Sigma) was added to block transcription by *E. coli* RNA polymerase.

■ **Cell lysis assay.** Cell lysis was analysed by measuring cell growth (OD_{600}) at different times post-induction. All data from cell lysis and membrane-permeability assays are the result of at least three independent experiments.

■ **Uridine incorporation into *E. coli*.** Cells were incubated with 2 μCi/ml [^{3}H]uridine (27·3 Ci/mmol; Amersham) for 2 h before inducing protein expression. Cells were then washed three times with uridine-free growth medium and protein expression was induced as described above. Aliquots of 0·2 ml were pelleted at different times post-induction. To quantify the release of radioactive uridine, supernatants were mixed with L-929 scintillation cocktail (Dupont) and analysed. Radioactivity corresponds to low-molecular-mass compounds that cannot be precipitated by trichloroacetic acid.

■ **Flow cytometry analysis.** Flow cytometry was carried out as described previously (Ciccaglione *et al.*, 1998*a*; Arroyo *et al.*, 1995). Briefly, 8 μl of cells were collected at different times (h) post-induction and stained with 0·005% propidium iodide (PI). Cells were analysed in a FACScan flow cytometer (Becton Dickinson). Each sample for analysis contained from 5000 to 10000 cells.

Results

Cloning of E1 fragments containing the internal hydrophobic region

The hydrophobicity plot (Kyte & Doolittle, 1982) of the E1 protein shows internal (aa 262–291) and C-terminal (aa 331–

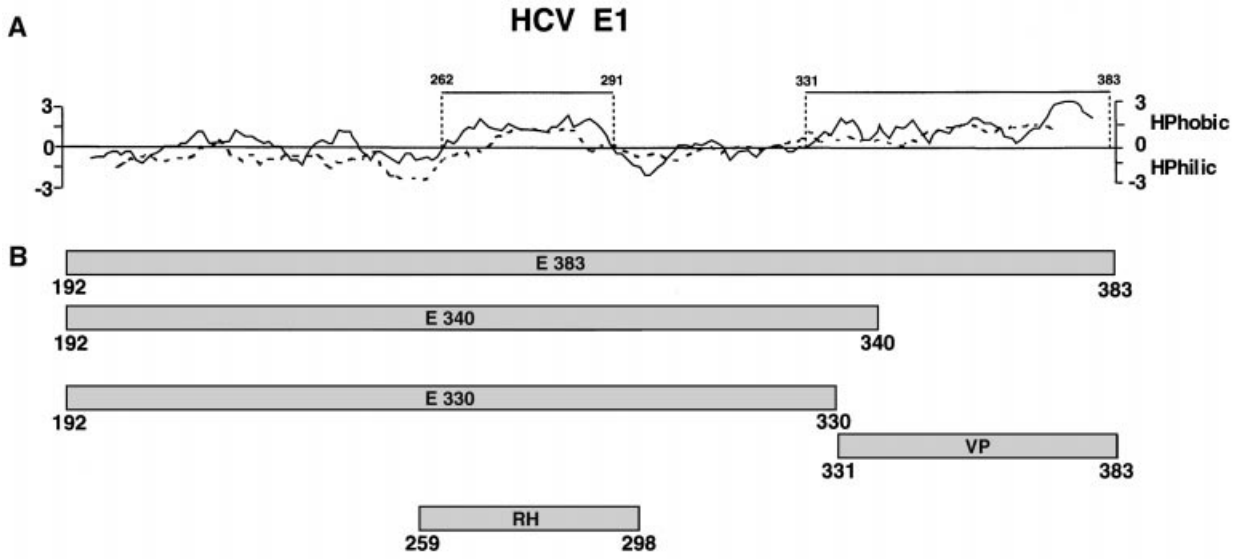

Fig. 1. (A) Hydrophobicity plot (Kyte & Doolittle, 1982) of the E1 protein. (B) E1 clones are indicated as boxes. Amino acid residues are indicated for each clone.

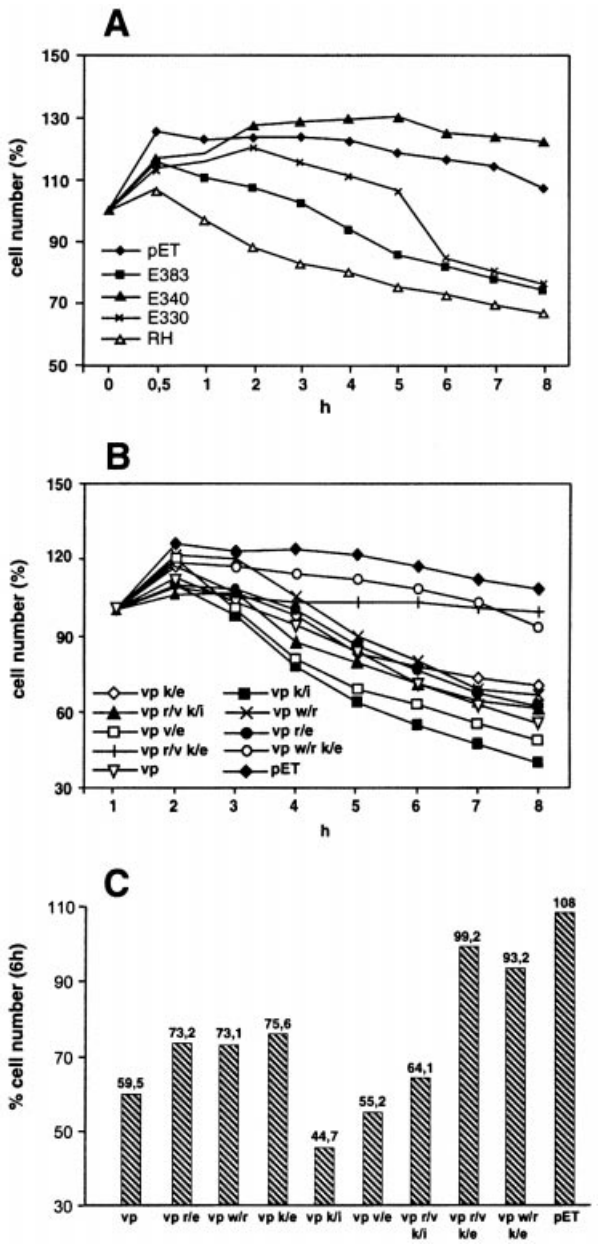

Fig. 2. Lysis of cultures expressing recombinant fragments. Cell number at the indicated times (h) post-induction was calculated by measuring the OD_{600} of cells transformed with (A) pET-3a (control), E383, E340, E330, RH or (B) VP mutants. (C) Cell number at 6 h post-induction was reported for the indicated VP mutants.

383) hydrophobic regions (Fig. 1A). In *E. coli*, the C-terminal domain of E1 seems to be involved in changing membrane permeability. In order to evaluate the ability of the internal hydrophobic region of E1 to modify membrane permeability, we expressed different fragments of E1 containing the internal hydrophobic region using the pET-3a vector and *E. coli* BL21(DE3)pLysS. This system is well-suited for the inducible expression of toxic genes (Studier & Moffatt, 1986; Studier, 1991) and for analysing the function of proteins that modify membrane permeability (Aldabe *et al.*, 1996; Gonzalez & Carrasco, 1998; Guinea & Carrasco, 1994). The positions of the cloned fragments, with respect to the hydrophobicity plot of E1, are shown in Fig. 1(B). Fragment RH (aa 259–298) overlaps the internal hydrophobic region. Fragments E383 (aa 192–383), E340 (aa 192–340) and E330 (aa 192–330) contain the entire E1 gene or an E1 gene with deletions of different fragments of the C-terminal region. The VP fragments (aa 331–383) encompassing the C-terminal region have also been examined.

Lysis of recombinant cultures expressing the internal hydrophobic region

Membrane-active proteins are lytic when expressed in *E. coli*. Cell lysis is more pronounced when *E. coli* strains expressing T7 lysozyme, such as *E. coli* BL21(DE3)pLysS, are used. In this case, the change in membrane permeability caused by the expressed protein may permit the passage of T7 lysozyme into the periplasmic space, where it exerts lytic activity on the bacterial cell wall (Aldabe *et al.*, 1996; Arroyo *et al.*, 1995; Guinea & Carrasco, 1994; Lama & Carrasco, 1996). To test the effect of fragments containing the internal hydrophobic region on membrane permeability to T7 lysozyme, we estimated cell lysis by measuring the OD_{600} of recombinant cultures at different times post-induction (Fig. 2A). Expression of fragment RH was clearly lytic for *E. coli*. Cell lysis was also observed in cultures expressing the entire E1 gene. The expression of mutant proteins lacking different fragments of the C-terminal region of E1 produced contrasting results. While synthesis of E340 did not alter cell growth, even after extended times post-induction, the expression of E330 did not influence cell growth in the first hours after induction, but clearly showed a negative effect on the cells if analysed at later times post-induction (Fig. 2A).

Uridine release in recombinant cultures expressing E1 fragments

The ability of the E1 clones to alter membrane permeability was assessed by testing the release of radioactive nucleotides at different times post-induction from [^{3}H]uridine-loaded cells. As shown in Fig. 3(A), uridine release was observed in cultures expressing RH as well as E383 and E330. In contrast, uridine was not detected in the supernatant of cells expressing E340 (Fig. 3B).

Flow cytometry analysis of recombinant E1 clones

Changes in membrane permeability induced by the expression of fragments containing the internal hydrophobic region were also analysed by testing the entry of PI into cells at different times post-induction. The uptake of PI by clones expressing RH and E383 was detected 1 h post-induction. We observed an increase in PI fluorescence as compared to cells

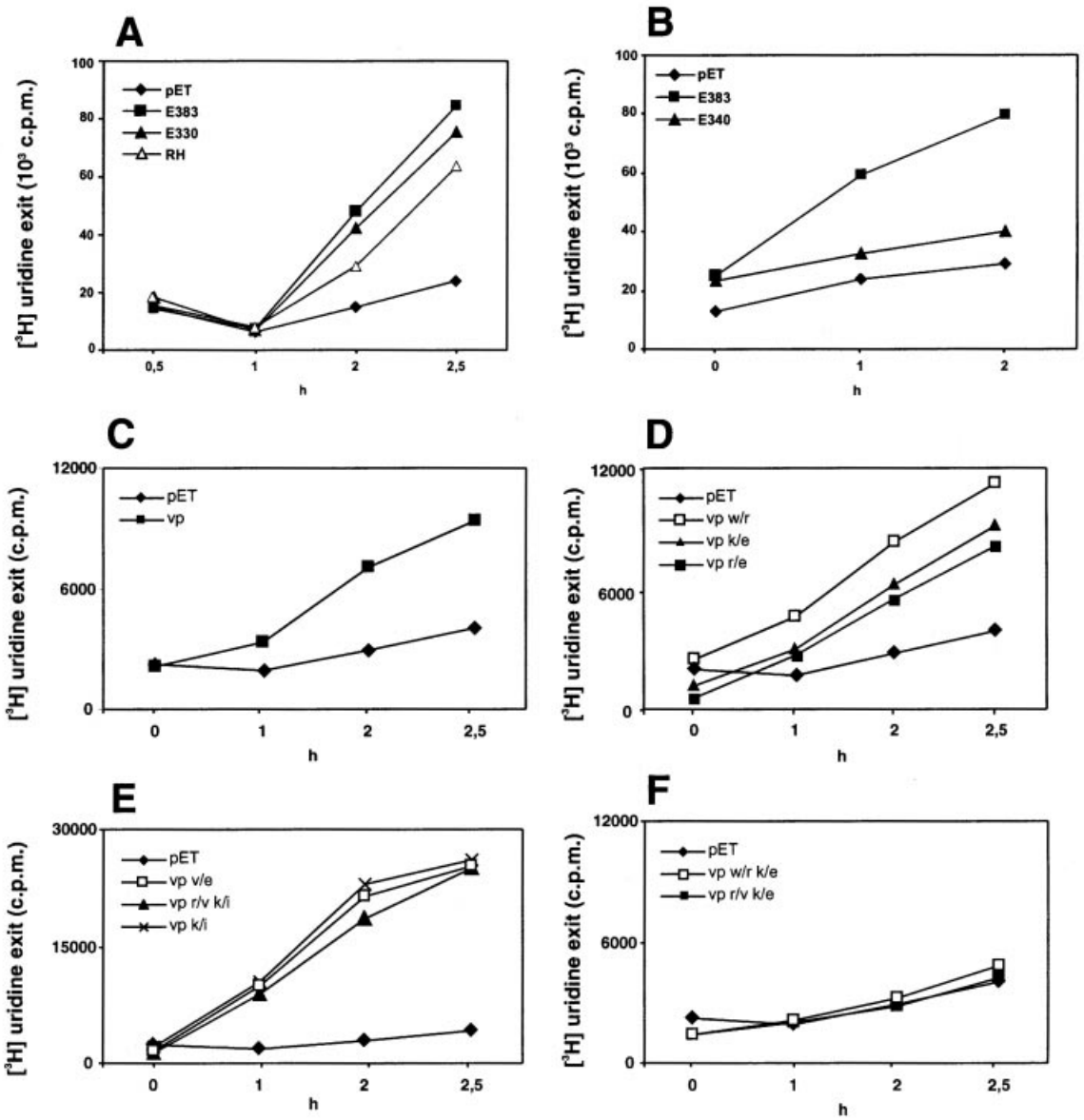

Fig. 3. [^{3}H]Uridine release from cultures expressing mutated E1 fragments. Cells containing (A) pET-3a (control), E383, E330, RH, (B) E340 or (C)–(F) VP mutants were loaded with [^{3}H]uridine as described in Methods. At the indicated times (h) post-induction, the radioactivity of the supernatant was measured.

before induction (0 h). The same fluorescence signal was detected at different post-induction times (Fig. 4, RH and E383). Synthesis of E330 produced the highest signal for PI, which was particularly evident 2 h post-induction. Cellular heterogeneity was observed at this time, with a percentage of cells exhibiting a high level of PI accumulation and a percentage of cells exhibiting a low level of PI accumulation (Fig. 4, E330). In contrast, even at 3 h post-induction, the uptake of PI was not detected in cells expressing E340 as well as in cells expressing the parental plasmid (Fig. 4, E340 and control).

According to cell lysis and permeability assays, E383 and RH were expressed at a low level and only for the first hour after inducing protein expression, suggesting that even low level expression of membrane-active proteins could have a drastic effect on the cell membrane. In contrast, E340 and E330 were synthesized at higher levels. However, the expression of E330 gradually decreased 4 h post-induction (Ciccaglione *et al.*, 1998*a*; data not shown).

All together, our findings indicate that RH, which overlaps the internal hydrophobic region of E1, shows membrane-permeabilizing activity in *E. coli* and could be considered to be an additional membrane-active domain of E1. However, results indicated that not all fragments containing this region are active on membranes. In this regard, analysis of membrane

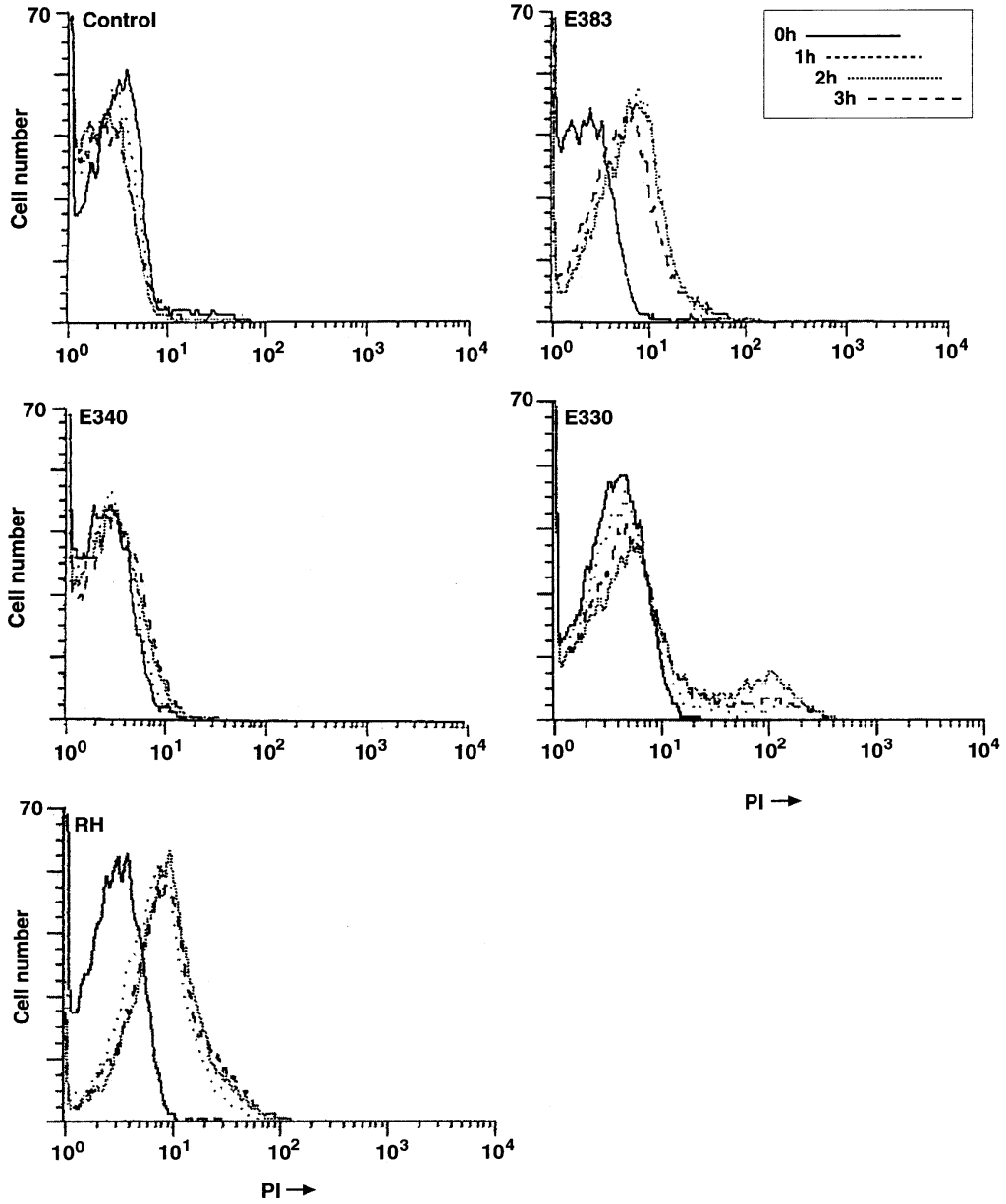

Fig. 4. Flow cytometry analysis of E1 recombinant clones. At different times (h) post-induction, recombinant cultures expressing pET-3a (control), E383, E340, E330 or RH were labelled with 0·005% PI and analysed in a FACScan flow cytometer as described in Methods. Alterations in membrane permeability are indicated by the uptake of PI.

association indicated that E340 was found mainly in the soluble phase of the cell, while E383 and E330 co-purified with the cell membrane (data not shown), suggesting that there may be conformational constraints for the membrane association of E340.

Site-directed mutagenesis of the C-terminal hydrophobic region of E1

The C-terminal hydrophobic region of E1 (aa 331–383), unlike the internal one, contains amino acids that are strictly conserved in all HCV genotypes (Bukh *et al.*, 1993). Four conserved residues, Arg^{339}, Trp^{368}, Lys^{370} and Val^{371}, are located close to or inside hydrophobic stretches that show a high propensity to fold into α-helical configuration (Chou & Fasman, 1978) and have been predicted to be transmembrane (TM) regions (Hofmann & Stoffel, 1993). It may be possible then that these residues are inserted into the membrane and play a role in pore formation and/or correct membrane–protein interaction. To establish the role of single conserved amino acids in the membrane-permeabilizing activity of E1, we mutated the four conserved amino acids in the C-terminal region of wild-type E1 (Fig. 1, VP), which is the most

Table 1. E1 C-terminal region VP mutants

Amino acid substitutions compared to wild-type are indicated for each clone.

Clone	Mutation
VPr/e	$Arg^{339} \rightarrow Glu$
VPr/v+k/e	$Arg^{339} \rightarrow Val + Lys^{370} \rightarrow Glu$
VPr/v+k/i	$Arg^{339} \rightarrow Val + Lys^{370} \rightarrow Ile$
VPw/r	$Trp^{368} \rightarrow Arg$
VPw/r+k/e	$Trp^{368} \rightarrow Arg + Lys^{370} \rightarrow Glu$
VPk/e	$Lys^{370} \rightarrow Glu$
VPk/i	$Lys^{370} \rightarrow Ile$
VPv/e	$Val^{371} \rightarrow Glu$

membrane-active fragment (Ciccaglione *et al.*, 1998*a*). The amino acid substitutions of the VP mutants are indicated in Table 1. Membrane-permeabilizing activity was analysed by cell lysis and uridine-release assays.

Lysis of recombinant cultures expressing VP mutants

Cell number at different times post-induction was calculated by measuring the OD_{600} of cultures transformed with VP and VP mutants. A gradual decrease in cell number was observed for wild-type and VP mutants with single amino acid mutations. In contrast, the expression of fragments containing the double mutations Arg → Val + Lys → Glu and Trp → Arg + Lys → Glu did not influence cell growth, as indicated by comparison with the growth of cells expressing the parental plasmid (Fig. 2B). Analysis of cell number at 6 h post-induction indicated that clones expressing the double mutants Arg → Val + Lys → Glu and Trp → Arg + Lys → Glu showed a significant reduction in cell lysis with respect to the wild-type clone (Fig. 2C). In these cultures, 99·2 and 93·2% of cells, respectively, were still alive 6 h post-induction, a percentage that is close to the value reported for the negative control (Fig. 2C, compare mutants Arg → Val + Lys → Glu and Trp → Arg + Lys → Glu to pET-3a). In contrast, cell lysis was pronounced in clones expressing the wild-type VP and mutants Lys → Ile, Val → Glu and Arg → Val + Lys → Ile. Moreover, the Lys → Ile and Val → Glu mutants showed an increase in the cell lysis phenotype with respect to the VP clone (Fig. 2C). Clones containing the amino acid substitutions Arg → Glu, Trp → Arg and Lys → Glu showed only a moderate attenuation in cell lysis (Fig. 2C).

Uridine release in recombinant cultures expressing VP mutants

The activity of VP mutants on membrane permeability was analysed by measuring the release of [^{3}H]uridine from preloaded cultures after treatment with IPTG. As in the cell lysis assay, clones expressing the Arg → Val + Lys → Glu and Trp → Arg + Lys → Glu mutations, as well as clones expressing the parental plasmid pET-3a, did not cause the release of uridine (Fig. 3F). In contrast, radioactive uridine was detected in the supernatant of cultures expressing wild-type VP (Fig. 3C) and mutants Lys → Ile, Val → Glu, Arg → Val + Lys → Ile (Fig. 3E) and Trp → Arg (Fig. 3D). Even in this assay, mutants Lys → Ile, Val → Glu and Arg → Val + Lys → Ile showed an increase in uridine release with respect to the wild-type clone (Fig. 3C, E). Finally, the Lys → Glu and Arg → Glu mutants induced a slower release of uridine with respect to the wild-type clone (Fig. 3C, D).

According to the results obtained from membrane-permeability assays, analysis of protein expression revealed that VP and all the membrane-active mutants were expressed at a very low level and only for the first hour after induction. This suggests that very low expression of these fragments in *E. coli* is toxic. In contrast, fragments containing the Arg → Val + Lys → Glu and Trp → Arg + Lys → Glu mutations were synthesized at a higher level and their expression had not decreased 4 h after induction (Ciccaglione *et al.*, 1998*a*; data not shown).

In conclusion, our findings indicate that clones with the double mutations Trp → Arg + Lys → Glu and Arg → Val + Lys → Glu lose the ability to make membranes permeable to different compounds and their synthesis is well-tolerated by *E. coli*, suggesting that the mutated residues could play a crucial role in the membrane activity of E1.

Discussion

In this paper, we have identified a new hydrophobic region in the E1 protein which shows membrane-altering properties. In addition, we demonstrated that substitutions of single amino acids in highly conserved positions of the C-terminal hydrophobic domain of E1 may modulate its function.

In the past, inducible protein expression in *E. coli* has been used successfully for analysing the membrane-permeabilizing activity of several proteins from other animal viruses (Aldabe *et al.*, 1996; Arroyo *et al.*, 1995; Guinea & Carrasco, 1994). These studies have demonstrated that the effluence of compounds through the membrane was directly due to an intrinsic ability of these proteins to modify membrane permeability (Lama & Carrasco, 1992*b*, 1995). Consequently, the results obtained by expressing E1 in *E. coli* can be considered to be a significant indication of a new biochemical function.

The E1 protein of HCV is a type 1 TM glycoprotein with a C-terminal anchor domain. However, computer prediction of a second internal TM domain, in addition to the C-terminal one, indicates that E1 may interact with the membrane through a more complex mechanism involving multiple regions. Our findings show that the RH fragment, which overlaps the internal TM region, is able to modify the permeability of the membrane to different compounds. As permeability changes

are detected immediately after inducing RH expression, it is likely that a direct interaction of RH with the membrane may cause an alteration of its integrity.

It has been proposed that the internal hydrophobic region may contain the HCV fusion peptide (Flint *et al*., 1999). Changes in membrane permeability induced by RH indicate that this segment has the ability to disturb the architecture of the lipid bilayer, a property that is intrinsic to viral fusion peptides. Nevertheless, two conserved features of RH, the hydrophobicity and the positively charged ends, as well as the presence of another membrane-active fragment in E1, lead us to favour the hypothesis that considers RH as a domain which co-operates with the C-terminal fragment in modifying membrane permeability. Further studies are, in any case, required to investigate the role of this region in virus fusion.

The hydrophobic region found at the C-terminal end of E1 is 53 amino acids in length (aa 331–383). The structure of such a putative TM region is difficult to predict, but it is long enough to traverse the membrane two or three times. Computer analysis reveals the presence of two putative TM segments in this region, which contains the conserved amino acids Arg^{339}, Trp^{368} and Lys^{370}. Our data demonstrate that these residues are critical for membrane-permeabilizing activity, as mutations affecting these positions abolish the phenotype. It is likely that these amino acids may participate in the formation of the hydrophilic lumen of a membrane pore (Arg and Lys) and/or correctly position the protein into the membrane (Trp), thus allowing permeability.

Recently, it has been reported that Lys^{370} is required for retaining a chimeric CD4–E1 protein in the ER: CD4–E1 contains a C-terminal fragment (aa 353–383) of E1 fused to a CD4 ectodomain (Cocquerel *et al*., 2000). Our results, reporting that mutations of Lys^{370} affect membrane activity, support the concept that the C-terminal TM region of E1, whose N-terminal limit still requires a final definition, is involved in more different activities and that specific amino acids, such as Lys^{370}, could play a key role in more than one function. We retain that the exact position that Lys^{370} assumes, with respect to the membrane environment could be critical for stable association and may influence more E1 activities which are structurally linked to membrane interaction.

The molecular characterization of E1 activity may have important implications for the design of therapeutic agents that specifically block this function. It is interesting to note that amantadine, an antiviral reagent that inhibits influenza virus M2 channel-formation activity, seems to be effective in 18% of patients who previously failed to respond to interferon-α therapy (Smith, 1997). As membrane-permeabilizing activity has been reported only for the E1 protein of HCV, the inhibition of such a function by amantadine is currently under investigation in our laboratory.

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