Viroporin activity of murine hepatitis virus E protein

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Received 1 April 2005; revised 20 May 2005; accepted 27 May 2005

Available online 8 June 2005

Edited by Hans-Dieter Klenk

Abstract The viroporin activity of the E protein from murine hepatitis virus (MHV), a member of the coronaviruses, was analyzed. Viroporins are a growing family of viral proteins able to enhance membrane permeability, promoting virus budding. Initially, the MHV E gene was inducibly expressed in *Escherichia coli* cells, leading to the arrest of bacterial growth, cell lysis and permeabilization to different compounds. Thus, exit of labeled nucleotides from *E. coli* cells to the cytoplasm was apparent upon expression of MHV E. In addition, enhanced entry of the antibiotic hygromycin B occurred at levels comparable to those observed with the viroporin 6K from Sindbis virus. Mammalian cells are also readily permeabilized by the expression of MHV E protein. Finally, brefeldin A powerfully blocks the viroporin activity of the E protein in BHK cells, suggesting that an intact vesicular system is necessary for this coronavirus to permeabilize mammalian cells.

Keywords: Viroporin activity; Coronavirus E protein; Membrane permeability; Brefeldin A; Viral membrane protein

1. Introduction

Injury to the cellular plasma membrane is a common feature observed with the vast majority of animal viruses. Alterations of membrane permeability constitute part of the strategy to facilitate the release of virions from infected cells [1]. A number of viral proteins alter membrane functioning of host cells: (i) viral glycoproteins, some of them endowed with membrane fusion activity [2]; (ii) viral proteases, such as HIV-1 PR [3]; and (iii) a group of proteins known as viroporins. These proteins share a number of features in their structure and function. Viroporins are integral membrane proteins which vary in size from about 50–120 aminoacids, possessing at least one hydrophobic stretch able to form an amphipatic α-helix. There also may be a second hydrophobic domain integrated into membranes. A major activity of viroporins is to act at late stages of the viral cycle promoting the exit of new virus particles from the cell [4]. Recently, another domain in the alphavirus 6K protein consisting of two interfacial motifs at the N-terminal end, rich in aromatic residues, has been described. This motif is important both for virus budding and to enhance membrane permeability [5].

Viroporins interact with membranes to increase permeability to ions and other small molecules [1,4]. After their insertion into membranes, viroporins tend to oligomerize to create a hydrophilic pore [6–9]. Many viroporins are not required for viral replication, although their presence significantly increases the formation of the virus particles, but they are excluded from them [10–15]. In the last few years, several viral proteins have been assigned to the viroporin family [4]. Following this trend, we have now analyzed the viroporin activity of the E protein from murine hepatitis virus (MHV). MHV is an enveloped, positive-strand RNA coronavirus that belongs to the order *Nidovirales* [16,17]. The MHV envelope contains three integral proteins: the spike protein (S), the membrane glycoprotein (M) and the E protein. Virus budding takes place at intracellular membranes [18,19]. The M protein is the most abundant envelope component. It contains three membrane-spanning domains and plays essential roles in virus morphogenesis, while E is the minor protein component of the virion. E is a short and hydrophobic protein of 83 amino acids that localizes at the budding compartment with its C-terminal hydrophilic region extending into the cellular cytoplasm or the virion interior. It contains a large hydrophobic N-terminal region, but its orientation and topology are not yet well defined. The coronavirus E protein may span either side of the membrane, although recent evidence suggests that a tag at the N-terminus is exposed at the cytoplasm [20,21]. The E gene is not essential for viral replication, but is required for efficient virus budding and morphogenesis [22]. The expression of M and E proteins from several coronaviruses induces the production of virus-like particles (VLPs), while the sole expression of the E protein promotes the release of membrane vesicles akin to those observed in MHV-infected cells [23,24]. In addition, the E protein also induces apoptosis [25]. More recently, it was described that severe acute respiratory syndrome (SARS) E protein has the ability to form pores in artificial membranes, as well as to permeabilize bacterial cells after its expression [26,27]. Our present findings agree well with these reports and further reveal the capacity of the MHV E protein to permeabilize *E. coli* and mammalian cells, supporting the idea that the E protein from different coronaviruses is endowed with viroporin activity. This work was initially presented at the 2nd European Congress of Virology Eurovirology 2004 (Madrid, Spain).

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Abbreviations: IPTG, isopropyl-β-D-thio-galactopyranoside; MHV, murine hepatitis virus; SV, sindbis virus

0014-5793/$30.00
doi:10.1016/j.febslet.2005.05.046

2. Materials and methods

2.1. Cells

Baby hamster kidney (BHK-21) cells were grown at 37 °C in Dulbecco's modified Eagle's medium (DMEM) supplemented with 5% fetal calf serum and nonessential amino acids.

2.2. Bacterial expression plasmids

To generate the bacterial expression plasmids pET11 6Kwt [5] and pET11.E, the *Nde*I/*Bam*HI fragment in pET11 plasmid [28] were replaced by PCR-amplified products encoding 6K protein from sindbis virus (SV) and the E protein from murine hepatitis virus (MHV-A59), respectively. E gene was obtained from plasmid pB59 that was kindly provided by Dr. P. Masters (State University of New York at Albany, New York). Plasmids were checked by sequencing the inserts from PCR amplifications by standard sequencing techniques.

2.3. SV replicons

A SV replicon, pT7repC+E, was obtained by cloning the *Nde*I/*Bam*HI-digested PCR fragment encoding the E protein after the sequence of SV capsid protein (C) in the plasmid pH3′ 2J-C [29], using the same restriction sites. In a second step, the fragment digested with *Aat*II and *Xho*I was inserted into an *Aat*II/*Xho*I digested pT7SVwt vector [29]. Replicons repC and repC+6K were previously described [5,29]. As a result of the cloning strategy, three codons (UCCGCA-CAT) encoding aminoacids SAH were introduced between C and E.

2.4. Growth and induction of protein expression in E. coli

Bacterial plasmids were transfected into *E. coli* strain BL21(DE3)-pLys [30]. Individual colonies were grown overnight in LB medium in the presence of 100 μg/ml ampicillin and 34 μg/ml chloramphenicol. Then the cells were diluted 100-fold in M9 medium with antibiotics and grown at 37 °C. Once the cultures reached an absorbance of 0.6 at 660 nm, they were induced with 1 mM isopropyl-1-thio-β-D-thiogalactopyranoside (IPTG). Twenty minutes after induction 150 μg/ml rifampicin (Sigma) was added to inhibit the transcription by *E. coli* RNA polymerase. At the indicated times 1 ml-culture samples were taken to determine the bacterial density by measuring the light scattering at 660 nm.

2.5. Uridine release and hygromycin B assays

Permeability changes in bacteria were tested by measuring the release of [^{3}H] uridine preloaded cells and the entry of the antibiotic hygromycin B (HB). Cells were grown as described before and loaded with 5 μCi of [^{3}H] uridine per ml (28.0 Ci/mmol; Amersham Biosciences) for 30 min. Cells were then washed and resuspended in uridine-free M9 medium and incubated at 37 °C for 15 min. The cells were induced to express the target proteins as described above. Aliquots of 0.2 ml were pelleted at different times post-induction. To quantify the radioactivity released to the medium, the supernatants were mixed with an L-929 scintillation cocktail (Dupont-New England Nuclear) and analyzed in a liquid scintillation counter.

The entry of HB was measured as described previously [5]. Briefly, at 30 and 60 min after induction, aliquots of 1 ml were labeled for 15 min with 4 μCi of [^{35}S] Met/Cys (15 mCi/ml; Amersham Biosciences) in the presence or absence of 1 mM HB (Clontech). Cells were harvested and the radioactively labeled proteins analyzed by SDS–polyacrylamide gel electrophoresis (PAGE), fluorography and autoradiography. Protein synthesis was quantified by densitometric analysis of the bands corresponding to 6K, E or a cellular protein.

2.6. Transfection of BHK cells

BHK cells were electroporated with in vitro synthesized RNAs from the different plasmids as described previously [13].

2.7. Permeabilization assay in BHK cells

10^6 cells were electroporated with 110 μg of RNA synthesized in vitro from the different constructs or with only transcription buffer and further seeded in four wells of an L-24 plate. At 8 and 15 h post-electroporation (h.p.e.), cells were pretreated with 1 mM HB for 20 min at 37 °C, or left untreated. Next, proteins were radiolabeled for 40 min with 10 μCi of [^{35}S] Met/Cys in methionine/cysteine free DMEM in the presence or absence of 1 mM HB. Finally, cells were collected in a buffer sample, boiled for 4 min and analyzed by SDS–PAGE and fluorography. Western blot analysis was carried out using an antibody against the MHV-A59 E protein (a gift from S. Makino, University of Texas Medical Branch at Galveston, TX) at a dilution of 1:1000 and a polyclonal rabbit serum against the SV C protein diluted 1:10.000. Goat anti-rabbit IgG coupled to peroxidase (Pierce) was used at 1:10.000 dilution. Treatment with the antibiotic brefeldin A (BFA) was carried out as follows: Cells were incubated with 5 μg/ml BFA (Sigma) at 4 h.p.e. for 4 h. BFA was also added to the radiolabeling medium with or without HB and the samples were processed as described above. The percentage of protein synthesis was quantified by densitometric scanning, using a GS-710 calibrated Imaging Densitometer (Bio-Rad) and calculated by dividing the densitometered values obtained for HB-treated samples by the corresponding values from untreated cells. Either C protein band or a cellular protein band was densitometered.

3. Results

3.1. Expression of the MHV-A59 E protein in E. coli cells

Initially, we tested whether or not the expression of the MHV E protein affects bacterial cell growth. The well-known viroporin 6K protein from SV was used as a control [5]. For this purpose the corresponding genes were cloned under the control of a lac/T7 hybrid promoter containing a lac operator before the nucleotidic sequence of E or 6K protein, generating the plasmids pET11.E and pET11 6Kwt. BL21 (DE3)pLys *E. coli* cells were transformed with these plasmids. Several membrane-active proteins previously analyzed were lytic for this *E. coli* strain [31–33]. To assess whether or not MHV E protein affects bacterial cell growth, we measured the optical density at different times after induction with IPTG and rifampicin, which inhibits *E. coli* transcription, but not transcription by T7 polymerase (Fig. 1A). Notably, cell density started to fall at 30 min post-induction in *E. coli* expressing the E protein. On the other hand, SV 6K brought about a decrease in cell growth slightly higher than the E protein at the same times post-induction (Fig. 1A). Thus, both proteins exhibit lytic activity on *E. coli* cells, though with different kinetics. As a control, bacterial cultures non-induced with IPTG did grow, illustrating that indeed MHV E and SV 6K proteins caused arrest and lysis of the cells.

3.2. Alteration of E. coli membrane permeability by the expression of E protein

Next, we tested to see if the lysis of bacterial cultures by the E protein was the consequence of modifications on membrane permeability. To address this question, two assays were carried out: (i) the release of radioactivity from [^{3}H] uridine-preloaded cells; (ii) the entry of the non-permeant translation inhibitor hygromycin B (HB) [5,31,32]. The release of [^{3}H] uridine was first observed at 30 min of induction and increased to significant amounts at 90 min after induction of either E or 6K proteins (Fig. 1B). The release of labeled uridine was a bit lower with E protein as compared to 6K. Inhibition of host translation by HB entry is only detected in cells whose membrane permeability has been modified, but not in cells in which the membrane remains intact [34]. The expression of both proteins E and 6K was assayed by bacterial labeling with [^{35}S] Met/Cys. Addition of IPTG and rifampicin induced the expression of a protein migrating with the expected mobility for E protein (9.66 kDa) (Fig. 1C). Synthesis of the E protein takes place

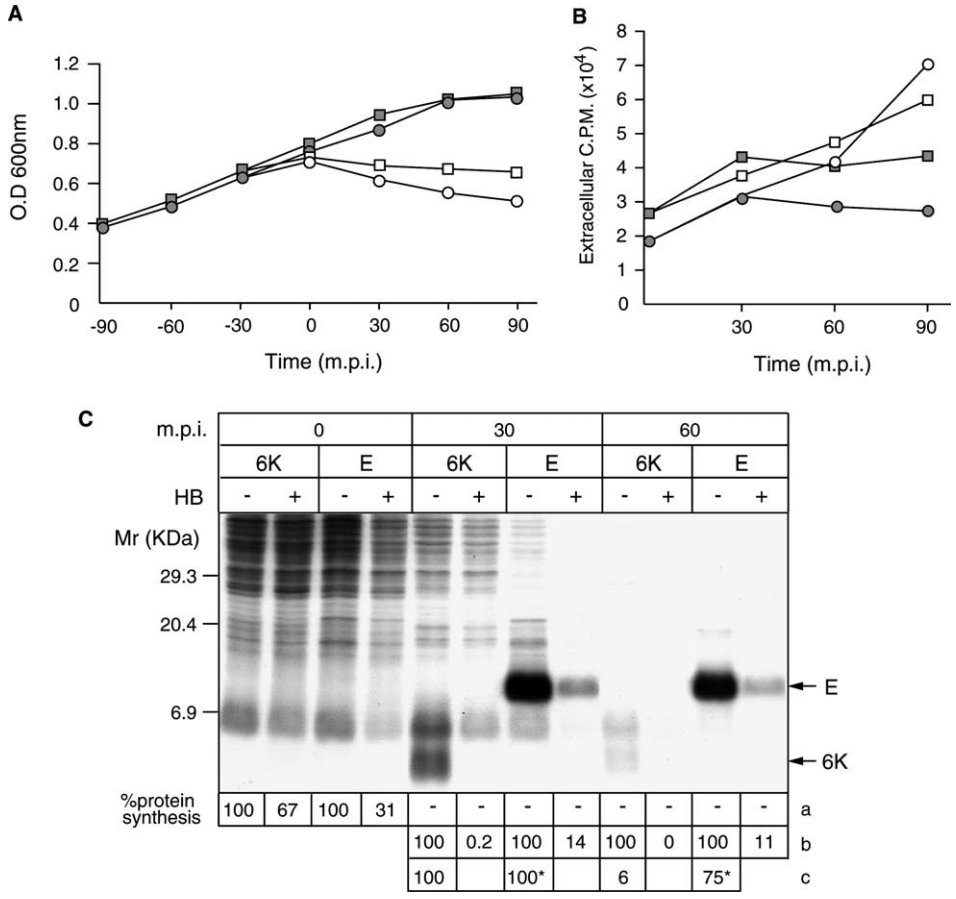

Fig. 1. Effects of the MHV E protein on *E. coli* cells. *E. coli* BL21 (DE3)pLys cells transformed with pET11.E (squares) and pET11 6Kwt (circles) were induced with 1 mM IPTG and 150 μg/ml of rifampicin at 20 min post-induction (m.p.i.). (A) Growth kinetics of BL21 (DE3)pLys cells expressing E and 6K genes. Cells were induced (white symbols) or not (gray symbols) with 1 mM IPTG at zero time. Cell density (O.D. 660 nm) was measured at the indicated times post-induction. (B) Release of [^{3}H] uridine from cells. *E. coli* transformed cells were preloaded for 30 min with [^{3}H] uridine. At different times post-induction the radioactivity in the supernatant was measured. (C) Hygromycin B assay in bacterial cells. Cultures of transformed bacteria were induced to express E and 6K as described above. At 30 or 60 min after induction, cells were labeled for 15 min with [^{35}S] Met/Cys in the presence (+) or absence (−) of 1 mM HB, and then processed by SDS–PAGE, fluorography and autoradiography. The numbers in lines a and b below the gel represent the percentages of protein synthesis calculated by dividing the densitometered values obtained for HB-treated cells by the values from untreated cells. Line c shows the percentages obtained by dividing the values of untreated cells at 60 m.p.i. by the corresponding values at 30 m.p.i. (*, values for E). In line a, a cellular protein band was densitometered. In lines b and c, E protein and 6K protein bands were quantified.

at higher levels than the SV 6K protein, and it was detected after more than 150 min post-induction (m.p.i.) (data not shown). By contrast, synthesis of SV 6K is very toxic in the presence of lysozime in such a way that it was not observed from 60 min post-induction (Fig. 1C, line c). These results indicate that the E protein is less toxic than SV 6K for bacterial cells. Fig. 1C shows that translation in *E. coli* cells that express the E protein was deeply inhibited by HB at 30 min after induction, and only small amounts of E protein synthesis (line b) could be observed at 30 and 60 m.p.i. in the presence of HB (14% and 11% E protein synthesis, respectively). Thus, the permeabilizing ability of E protein is similar to that found with 6K.

3.3. Entry of HB promoted by MHV E protein in BHK cells. Effects of Brefeldin A

To analyze the permeabilization to HB by the E protein, a system has been developed based on a SV replicon. Thus, the replicon repC + E was generated. This SV replicon was obtained by cloning the E sequence after the SV capsid (C) gene. In this manner, upon translation of the subgenomic mRNA, the C protein is detached by its autocatalytic processing and the remaining protein is made at normal levels [13]. This system synthesizes E protein in large amounts in mammalian cells. In vitro transcribed RNAs from the pT7repC + E plasmid were electroporated in BHK cells, and at 9 and 16 h.p.e., protein synthesis was estimated in the absence or presence of HB (Fig. 2A). To detect E protein expression at 9 h.p.e., a Western blot analysis was performed by using an anti-E protein antibody against the C-terminus of the E protein. Fig. 2B shows the presence of protein bands corresponding to the E monomer and the C–E non-proteolyzed product. The identity of the C–E non-proteolyzed product was determined by using a rabbit serum against the C protein. We also detected several specific bands possibly resulting from post-translational modifications or different oligomerization forms of the E protein. Notably, the entry of HB promoted by MHV E powerfully inhibited protein synthesis to an extent comparable to that observed with 6K (Fig. 2A and E). HB did not enter into BHK cells transfected with transcription buffer or with RNA encoding only C protein

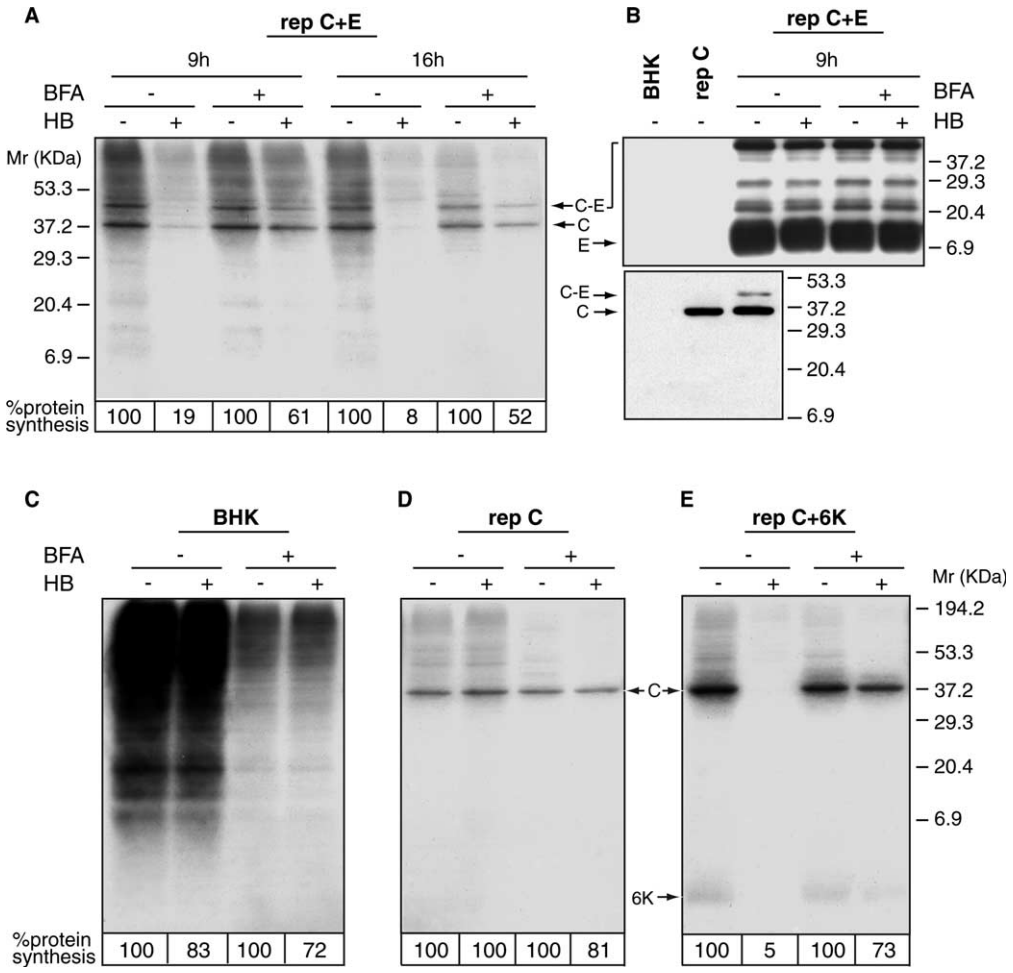

Fig. 2. Membrane permeabilization induced by E protein in BHK cells. Effect of BFA. BHK cells were electroporated with in vitro transcribed RNAs from different constructs. Cells were treated or not with 5 μg/ml BFA. At 8 h.p.e. cells were pretreated with 1 mM HB for 20 min or left untreated. Next, proteins were labeled with [^{35}S] Met/Cys in the absence (−) or presence (+) of 1 mM HB and BFA, as indicated in the figure. Samples were processed by SDS–PAGE, fluorography and autoradiography. (A) BHK cells electroporated with RNA from pT7repC+E plasmid. (B) Analysis of the expression of E protein. The expression of E protein at 9 h.p.e. was analyzed by Western blotting using an anti-E antibody (upper panel). The C–E non-proteolyzed product was also revealed using a polyclonal anti-C serum (lower panel). BHK cells transfected with transcription buffer or with RNA from pT7repC were used as controls. (C) Control BHK cells electroporated only with transcription buffer. (D) BHK cells electroporated with RNA from pT7repC plasmid. (E) BHK cells electroporated with RNA from pT7repC+6K plasmid. Numbers below each lane indicate the percentages of protein synthesis obtained by dividing the densitometered values for HB-treated cells by the values for untreated cells. In (A, D and E) the SV C protein band was quantified by densitometric scanning. In (C), a cellular protein band was quantified.

(Fig. 2C and D). This result indicates that the E protein from MHV possesses the capacity to permeabilize mammalian cells.

The MHV E protein localizes at the ER-Golgi intermediate compartment (ERGIC) and at the plasma membrane [21,35]. Expression of E induced the formation of structures termed tubular bodies [21]. Budding of coronaviruses occurs at ERGIC membranes [18]. Finally, we analyzed the effect of brefeldin A (BFA) on the permeabilization induced by the E protein. BFA is a macrolide fungal antibiotic that disorganizes the Golgi apparatus and redistributes *cis*-, medial and *trans*-Golgi markers into the ER [36,37]. As observed in Fig. 2A, addition of BFA at 4 h after transfection blocked the entry of HB induced by E protein. BFA treatment did not decrease protein synthesis in HB-treated cells as compared to untreated cells. On the other hand, BFA by itself is not able to induce membrane permeabilization to HB in BHK cells (Fig. 2C) [13]. In agreement with Raamsman et al., electron microscopic studies of E-expressing BHK cells revealed that the characteristic curvated membranous structures induced by E were insensitive to BFA treatment (data not shown). These findings indicate that the correct functioning of the intracellular vesicular system is essential for the E protein to enhance membrane permeability, but not to produce membrane curvature.

4. Discussion

The existence of small viral proteins that disturb membrane integrity leading to enhanced permeability has been reported for both enveloped and non-enveloped viruses. These proteins, known as viroporins, are able to form hydrophilic pores in biological membranes and play an essential role in virus budding [1]. The findings reported in this work support the idea that the MHV E protein modifies membrane permeability in both *E. coli* and mammalian cells. These results agree well with

recent reports on the ability of SARS-CoV E protein to open pores in artificial membranes as well as to enhance permeability in bacterial cells [26,27]. Despite the low aminoacid sequence homology between the E proteins from MHV and SARS, their functionality could be similar [38]. Therefore, the coronavirus E protein can now be classified also as a member of the viroporin family. So far the group of viroporins include: poliovirus 2B, 2BC and 3A, coxsackievirus 2B, togavirus 6K protein, HIV-1 Vpu, human respiratory syncitial virus SH, influenza A M2, avian reovirus p10, hepatitis C virus p7, bovine ephemeral fever virus alpha p10, hepatitis A virus 2B and 2BC, infectious bursitis virus VP5, japanese encephalitis virus NS proteins, bluetongue virus NS3 and the E protein from SARS-CoV [4,26,39–43].

The exact mechanism by which viroporins alter the permeability of the plasma membrane is as yet unknown. Two possibilities to account for their action can be put forward: direct permeabilization by the viroporin located at the plasma membrane, or an indirect mechanism that involves the opening of a cellular pore at the plasma membrane triggered by the viroporin placed at the intracellular membrane. In favour of the first possibility is the fact that viroporins open hydrophilic pores in artificial membranes of a size similar to that observed in the infected cells [8,9,27,44–46]. Moreover, BFA interferes with viroporin activity, including MHV E protein as shown in this work [13,43,47–49]. It is possible that BFA blocks the trafficking of E protein from the ER to the plasma membrane. Alternatively, the E protein may remain at an intracellular compartment, provoking a signal that travels through the vesicular system to open a cellular pore. Two additional lines of evidence also support the idea that viroporins themselves can reach the plasma membrane. One of them is that the expression of some viroporins induces the formation of vesicles from the plasma membrane that contain the viroporin [24]. In addition, the budding of some animal viruses from the plasma membrane is greatly enhanced by the expression of the corresponding viroporin. However, it is puzzling that the greater proportion of most viroporins analyzed remains at intracellular membranes, without little or no sign of their presence at the plasma membrane. One reason for this behaviour is that the low proportion of viroporin at the external membrane makes it almost undetectable. Moreover, it may also be that soon after viroporin arrival to the plasma membrane, vesicles are secreted to the culture medium.

It is even possible that viroporins act both at the plasma membrane and on intracellular organelles. Thus, permeation to HB is exerted at the plasma membrane, but additional effects at intracellular membranes cannot be ruled out. Already some viroporins like rotavirus NSP4, poliovirus 2BC and coxsackie virus 2B have been found to be capable of disrupting Ca^{2+} homeostasis from ER [50–52]. The mobilization of calcium by NSP4 involves phospholipase C activation and inositol trisphosphate production [53]. The alteration of subcellular ion homeostasis could trigger an internal signal that would travel through the secretory pathway to finally act on cellular channels or pores as well as on phospholipases to disrupt the plasma membrane integrity. The expression of several small integral membrane proteins of both viral and cellular origin, at least in the oocytes of *Xenopus laevis*, is capable of regulating some channels endogenous to the host cell [54]. So far, the existence of interactions between the E and cellular proteins has not been described. A viroporin, HIV-1 Vpu, was found to interact and alter the normal function of the widely expressed cellular two-pore K^+ channel TASK-1 [55]. The E protein abundantly localizes in membranes of intermediate compartment where it could be modifying the permeability leading to the disruption of ion gradients at intracellular organelles. Further studies would be necessary to gain greater insight into the molecular mode of action of the different viroporins thus far described.

Acknowledgments: The financial support of Grants from DGICYT No. BMC2003-00494, FIPSE (Fundación para la Investigación y la Prevención del SIDA en España) and the Institutional Grant to the Centro de Biología Molecular "Severo Ochoa" from the Fundación Ramón Areces are acknowledged. V.M. is a recipient of an I3P predoctoral fellowship from CSIC and Ministerio de Ciencia y Tecnología, Spain. M.J.G. was the recipient of a CSIC short-term fellowship.

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