Viroporin activity of murine hepatitis virus E protein

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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 higromycin 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.

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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 amino acids, possessing at least one hydrophobic stretch able to form an amphipathic 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 protein 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).

Abstract

Abbreviations: IPTG, isopropyl-β-d-thiogalactopyranoside; MHV, murine hepatitis virus; SV, sindbis virus

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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 pH11 E, the NdeI BamHI 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 NdeI BamHI-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 AarI and XhoI was inserted into an AarI/XhoI digested pT7SVwt vector [29]. Replicons repC and repC+E 6K were previously described [5,29]. As a result of the cloning strategy, three codons (UCCGA-CAT) encoding amino acids 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-galactopyranoside (IPTG) 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.

2.5. Uridine release and hygromycin B assays

Permeability changes in bacteria were tested by measuring the release of [3H] uridine preloaded cells and the entry of the antibiotic hygromycin B (HB). Cells were grown as described before and loaded with 5 µCi of [3H] uridine per ml (28.0 Ci/mmol; Amersham Biosciences) for 30 min. The 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.

2.6. Transection 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

10⁶ 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 treated with 1 mM HB for 20 min on 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 antibody 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 to see 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 [3H] uridine-preloaded cells; (ii) the entry of the non-permeant translation inhibitor hygromycin B (HB) [5,31,32]. The release of [3H] 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 [35S] 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...
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 lysozyme 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.

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.
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 ER-GIC 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 curved 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
from the plasma membrane that contain the viroporin [24]. In evidence also support the idea that viroporins themselves vesicular system to open a cellular pore. Two additional lines of evidence 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 trafficking of E protein from the ER to the plasma membrane. One reason for this behaviour is that 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. Additionally, 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 coxsackievirus 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 triphosphate 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.

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