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Ion channel activity of the CSFV p7 viroporin in surrogates of the ER lipid bilayer

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ABSTRACT

Viroporins comprise a family of non-structural proteins that play significant and diverse roles during the replication cycle of many animal viruses. Consequently, they have become promising targets for inhibitory drug and vaccine development. Structure–function traits common to all members of the family are their small size (ca. 60–120 aa), high hydrophobicity, and the presence of helical domains that transverse the membrane and assemble into oligomeric-permeating structures therein. The possibility that viroporins show in particular conditions any kind of specificity in the transport of ions and small solutes remains a point of contention in the field. Here we have approached this issue using the Classical Swine Fever Virus (CSFV) protein p7 viroporin as a model. We have previously reported that CSFV-p7 induces release of ANTS (MW: 427.33) from lipid vesicles that emulate the Endoplasmic Reticulum (ER) membrane, and that this process is dependent on pH, modulated by the lipid composition, and recreated by a C-terminal transmembrane helix. Here we have assayed CSFV-p7 for its capacity to form ion-conducting channels in ER-like planar lipid membranes, and established whether this activity is subject to regulation by the same factors. The analysis of electrophysiological recordings in ER membrane surrogates suggests that CSFV-p7 forms pores wide enough to allow ANTS release. Moreover, we were able to discriminate between two pore structures with slightly different sizes and opposite ion selectivities. The fact that the relative abundances of each pore type depend crucially on membrane composition strengthens the view that the physicochemical properties of the lipid bilayers present in the cell endomembrane system modulate viroporin activity.

1. Introduction

Modulation of the cellular ion balance in order to take over the cellular machinery seems to be a common feature for viruses. Several viruses encode at least one protein displaying ion channel (IC) activity. These proteins are known as viroporins, the term being first proposed when it was observed that several proteins involved in virus-promoted cell membrane permeabilization shared common characteristics [1]. The list of identified viroporins is constantly growing, especially in RNA viruses [2,3]. Thus, the M2 of influenza virus (IFV) [4–7], the p7 of hepatitis C virus (HCV) [8–12], the vpu of human immunodeficiency virus (HIV) [13–17], and the poliovirus (PV) 2B [18–21] proteins are only some members of this list found in prominent human pathogens. Accordingly, viroporin activity blocking by specific inhibitors, and viroporin-defective live attenuated vaccines offer new therapy approaches to treat and prevent viral infection, which reinforces the importance of investigating these proteins [2].

Viroporins are overall small (they are comprised of some 60–120 amino acids) and highly hydrophobic. They usually contain one (class I) or two (class II) α-helices long enough to transverse the lipid bilayer [2,22]. These features enable viroporin insertion and oligomerization in cell membranes forming ion conductive pores, which alters the cell permeability allowing the transport of ions and other small molecules. The IC activity of these proteins is expected to be involved in virus entry, trafficking, morphogenesis and maturation [23,24]. In fact, research work supports the relevance of the IC activities of IFV M2 [25–28], HCV p7 [29–31], or SARS-CoV E [32] proteins in virulence and pathogenesis.

IC activity of viroporins usually involves weakly selective nanopores that alter unspecifically the membrane permeability, i.e. they allow the transport of ions and small molecules irrespective of their chemical nature [32]. However, in some particular cases like IFV M2 and HCV p7, they can form highly selective channels displaying specificity for physiologically relevant cations [4–7,33,34]. Given the varied functions ascribed to viroporins and the various cell environments sampled by these products, it is not unlikely that different functional structures can emerge at different stages of the viral cycle. One consequence of this functional diversity is the possibility that depending on the conditions

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of the lipid bilayer the same viroporin may have the potential either to establish nanometer-sized ion channels, or alternatively, participate in additional membrane permeabilization mechanisms allowing the transport of larger solutes exceeding the nanometer scale [35,36].

Interestingly, it has been observed that lipid charge influences the IC activity of some viroporins like the E protein of SARS-CoV [32], while vesicle permeabilization assays suggest that PV 2B pore-forming activity can be regulated by the negatively charged phospholipid species existing at the cytofacial monolayers of the target membranes [37]. However, studies addressing in parallel and systematically (i.e., using same lipid compositions and assessing dependence of these activities on the same regulatory factors) planar membrane electrophysiology vs. vesicle permeability assays for the same viroporin are missing. Here, to carry out such a comparative assessment, we have taken the advantage that CSFV-p7-induced release of ANTS from lipid vesicles mimicking the Endoplasmic Reticulum (ER) membrane is subject to pH, lipid composition and amino acid sequence regulation [38]. Thus, we have first established IC activity of CSFV-p7 in ER-like lipid planar bilayers and then analyzed its dependence on the same factors.

2. Materials and methods

2.1. Materials

The CSFV p7 protein and its derived peptides (sequences displayed in Fig. 1) were commercially synthesized (Thermo Scientific). The purified peptides were dissolved in dimethyl sulfoxide (DMSO; spectroscopy grade), and their concentrations were determined by bicinchoninic acid microassay (Pierce, Rockford, IL). Small, diluted aliquots were stored frozen upon use. Phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylinositol (PI), and cholesterol (Chol) were purchased from Avanti-Polar Lipids (Birmingham, AL). The 8-aminonaphthalene-1,3,6-trisulfonic acid sodium salt (ANTS) and p-xylenebis(pyridinium)bromide (DPX) were obtained from Molecular Probes (Junction City, OR, USA).

2.2. Planar lipid membranes formation

Two monolayers were made from 5 mg/ml pentane solutions of lipid mixture buffered with 5 mM HEPES with KCl at both sides of Teflon chambers partitioned by a 15 μm thick Teflon film with 70–100 μm diameter orifices. Planar lipid bilayers were formed by monolayer apposition on the orifices previously treated with a 1% solution of hexadecane in pentane. Protein and peptides dissolved in DMSO were supplemented to the lipid solutions prior to monolayer formation only in one of the chamber sides, the *cis* side. Bilayer formation was directly detected and its thickness can be estimated by capacitance measurements.

2.3. Channel conductance measurements

An electric potential was applied using Ag/AgCl electrodes in 2 M KCl, 1.5% agarose bridges assembled within standard 250 μl pipette tips. Potential is defined as positive when it is higher at the side of the protein addition (the *cis* side), while the *trans* side is set to ground. An Axopatch 200B amplifier (Molecular Devices, Sunnyvale, CA) in the voltage-clamp mode was used for measuring the current and applying potential. The membrane chamber and the head stage were isolated from external noise sources with a double metal screen (Amuneal Manufacturing Corp., Philadelphia, PA).

2.4. Reversal potential measurements

Lipid bilayers were formed with different salt concentrations at each side. Once the protein was inserted, a net ionic current appeared due to the concentration gradient, and it was manually set to zero by adjusting the applied potential. The potential needed to achieve zero current was then corrected by the liquid junction potential calculated from Henderson's equation to obtain the zero current potential or reversal potential (E_{rev}) [39].

2.5. Vesicle permeability assays

Large unilamellar vesicles (LUVs) were prepared according to the extrusion method [40]. Vesicle permeabilization was assayed by monitoring the release to the medium of encapsulated fluorescent ANTS (ANTS–DPX assay) [41]. LUVs containing 12.5 mM ANTS, 45 mM DPX, 20 mM NaCl and 5 mM HEPES were obtained by separating the unencapsulated material by gel-filtration in a Sephadex G-75 column that was eluted with 5 mM HEPES and 100 mM NaCl (pH 7.4). Internal and external osmolarities were measured in a cryoscopic osmometer (Osmomat 030, Gonotec, Berlin, Germany) and adjusted by adding NaCl. Fluorescence measurements were performed in an SLM Aminco 8100 spectrofluorimeter (Spectronic Instruments, Rochester, NY) by setting the ANTS emission at 520 nm and the excitation at 355 nm. A cutoff filter (470 nm) was placed between the sample and the emission monochromator. The baseline leakage (0%) corresponded to the fluorescence of the vesicles at time 0, while 100% leakage was the fluorescence value obtained after the addition of Triton X-100 (0.5% v/v).

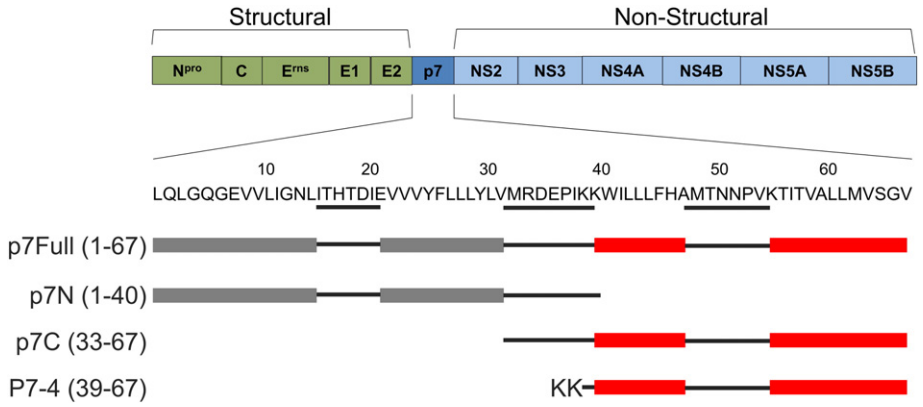

Fig. 1. CSFV p7 sequence and derived peptides. Top: Direct translation of pestivirus ssRNA genome gives rise to a single, large polyprotein, which is processed into individual viral proteins (Top panel). Bottom: CSFV p7 sequence includes two main hydrophobic regions (gray and red blocks, respectively) intervened by sections with propensity for establishing turns in membrane (black, thick lines). The panel displays in schematic representation the sequences covered by the peptides used in this study.