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Pore-forming activity of pestivirus p7 in a minimal model system supports genus-specific viroporin function

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ABSTRACT

Viroporins are small integral membrane proteins functional in viral assembly and egress by promoting permeabilization. Blocking of viroporin function therefore constitutes a target for antiviral development. Classical swine fever virus (CSFV) protein p7 has been recently regarded as a class II viroporin. Here, we sought to establish the determinants of the CSFV p7 permeabilizing activity in a minimal model system. Assessment of an overlapping peptide library mapped the porating domain to the C-terminal hydrophobic stretch (residues 39–67). Pore-opening dependence on pH or sensitivity to channel blockers observed for the full protein required the inclusion of a preceding polar sequence (residues 33–38). Effects of lipid composition and structural data further support that the resulting peptide (residues 33–67), may comprise a bona fide surrogate to assay p7 activity in model membranes. Our observations imply that CSFV p7 relies on genus-specific structures–mechanisms to perform its viroporin function.

1. Introduction

The *Flaviviridae* family comprises of positive-strand RNA viruses, classically classified into three genera: Flavivirus, Pestivirus, and Hepacivirus (Lindenbach et al., 2007). These genera include highly diverse viruses with increasing relevance as both human and animal pathogens. Pestiviruses and hepaciviruses show a high similarity in genome structure and replication mechanisms, and to a lesser extent similarity to flaviviruses (Lindenbach et al., 2007). All viruses belonging to this family translate their RNA into a single polyprotein precursor, which is subsequently cleaved by a combination of host and viral proteases to render structural and non-structural proteins. Specific to the Pestivirus and Hepacivirus genera is the existence of an α-helix-turn-α-helix integral membrane hairpin that connects the structural proteins encoded in the N-terminal portion of the polyprotein with the C-terminal non-structural proteins. After full proteolytic processing of the precursor, the released product, termed p7 (Fig. 1A), remains associated to membranes as an integral membrane protein of approximately 60–70 amino acids (Harada et al., 2000; Lin et al., 1994).

In the last decade an intense research effort has focused on the p7 protein derived from hepatitis C virus (HCV) (reviewed in Steinmann and Pietschmann 2010). This protein is required for production of infectious viruses, but dispensable for viral entry or RNA replication (Steinmann et al., 2007a; Steinmann and Pietschmann, 2010). Moreover, HCV p7 is a viroporin, whose membrane-permeabilizing activity has been proven essential for viral propagation (Jones et al., 2007; Nieva et al., 2012; Wozniak et al., 2010). Consistent with this idea, several compounds have been shown to block HCV p7 pores and inhibit virus production (Foster et al., 2011; Griffin et al., 2003; Steinmann et al., 2007b).

Similarly to hepaciviruses, p7 plays an essential role in the life cycle of pestiviruses and contributes to their pathogenicity (Gladue et al., 2012; Griffin et al., 2004; Luscombe et al., 2010). It has been argued that the pestivirus p7 constitutes a general model for the HCV p7 viroporin, therefore making it useful in the characterization of anti-HCV compounds (Griffin et al., 2004; Luscombe et al., 2010). Here, we have aimed at establishing the determinants of the pestivirus p7 pore-forming activity in a liposome-based model system. Accordingly, we first sought to establish the minimal CSFV p7 sequence required for a pH-dependent pore-forming activity that could be inhibited by channel blockers. Establishment of a shorter active sequence, more amenable for synthesis and purification than the full-length protein, subsequently allowed determining the lipid dependence of the process, and the structural characterization of the pore-forming domain. Together, our data disclose the

Abbreviations: BVDV, bovine viral diarrhea virus; CD, circular dichroism; Chol, cholesterol; CL, cardiolipin; CSFV, classical swine fever virus; HCV, hepatitis C virus; PC, phosphatidylcholine; PE, phospahtidylethanolamine; PS, phosphatidylserine; PI, phosphatidylinositol; SM, sphingomyelin.

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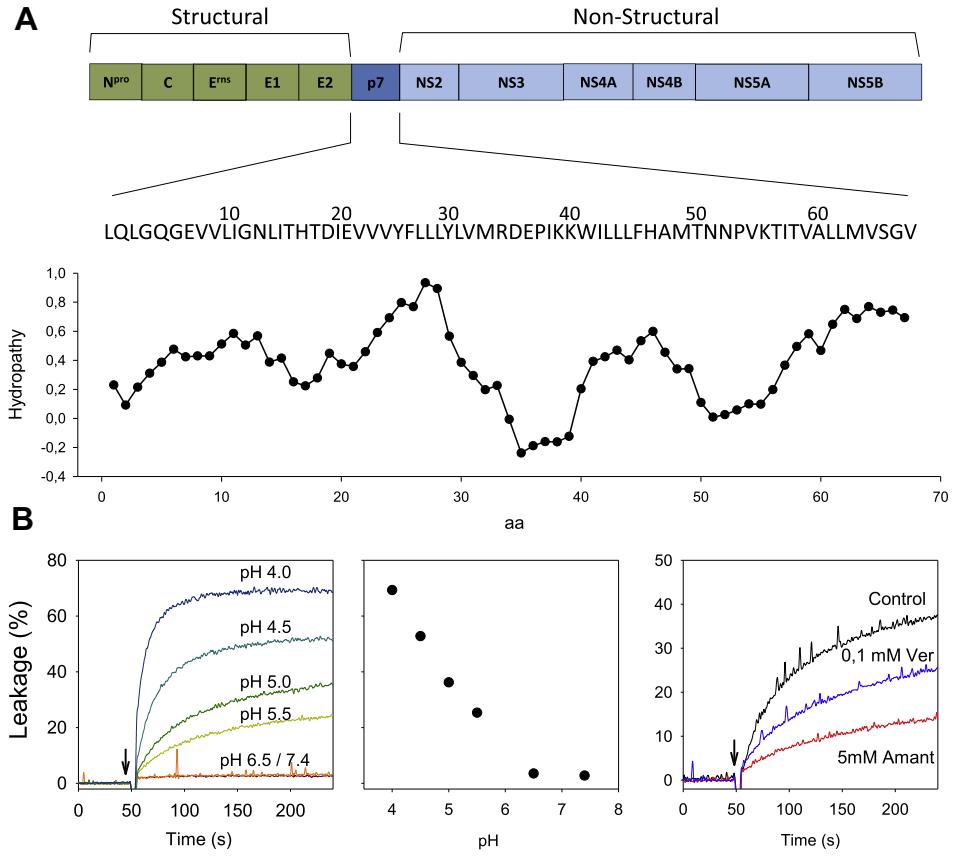

Fig. 1. CSFV p7 sequence and pore-forming activity. (A) Direct translation of pestivirus ssRNA genome gives rise to a single, large polyprotein, which is processed into individual viral proteins. Membrane-integral p7 originates from processing by the host signal peptidase (Top). The hydropathy plot below CSFV p7 sequence is based on the Eisenberg scale. Plotted values represent means for a sliding window of 11 amino acids. (B) Membrane permeabilization induced by p7 (ANTS/DPX assay). The protein dissolved in DMSO was injected (indicated by the arrow) into stirring solutions of PC:PE:PI (5:3:2 mol ratio) liposomes at a protein-to-lipid ratio of 1:100 (mol:mol). Leakage of vesicular internal aqueous contents was monitored as a function of time. Lipid concentration was 100 μM. The effect of lowering the pH from 7.4 to 4.0 is illustrated in the left and central panels. In the latter the levels of permeabilization after 200 s was plotted as a function of the assayed pH values. Addition of peptide solvent alone did not have any effect on vesicle stability (not shown). The right panel shows the effect of adding amantadine (5 mM) or verapamil (100 μM) on pore-forming activity (red and blue curves, respectively) at pH 5.0. The black curve corresponds to the control without inhibitor. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

determinants of the p7 permeabilizing activity, and suggest a new potential means for isolation of antivirals targeting p7.

2. Materials and methods

The p7 protein and derived peptides displayed in Table 1 were produced and assayed as previously described (Gladue et al., 2012). Phosphatidylcholine (PC), phospahtidylethanolamine (PE), phosphatidylinositol (PI), phosphatidylserine (PS), sphingomyelin (SM), cardiolipin (CL) and cholesterol (Chol) were purchased from Avanti Polar Lipids (Birmingham, AL, USA). Dodecylphosphocholine (DPC) was from Anatrace (Maumee, OH, USA). The 8-aminonaphtalene-1,3,6-trisulfonic acid sodium salt (ANTS) and *p*-xylenebis(pyridinium)bromide (DPX) were obtained from Molecular Probes (Junction City, OR, USA).

Large unilamellar vesicle (LUV) production, ANTS/DPX leakage assays, and circular dichroism (CD) measurements, were essentially conducted as previously reported (Sanchez-Martinez et al., 2008). The leakage assay protocol was adapted as described (Gladue et al., 2012).

Penetration into lipid monolayers was measured to estimate the capacity of p7 peptides for inserting into membranes that mimic different target organelles. In brief, changes in surface pressure were monitored as a function of time in a fixed-area circular trough (μTrough S system, Kibron, Helsinki) measuring 2 cm in diameter and with a volume of 1 ml. The aqueous phase consisted of 1 ml of 5 mM Hepes, 100 mM NaCl (pH 7.4). Lipids, dissolved in chloroform, were spread over the surface and the desired initial surface pressure (π_0) was attained by changing the amount of lipid applied to the air–water interface. Peptides were injected into the subphase with a Hamilton microsyringe (Fig. S1).

Table 1
Peptide sequences used in this study.

Name	Sequence[a]	CSFV p7 numbering[a]
p7	LQLGQGEVVLIGNLITHTDIEVVVYFLLLYLVMRDEPIKK WILLLFHAMTNNPVKTITVALLMVSGV	1–67
p7-1	LQLGQGEVVLIGNLITHTDIE	1–21
p7-2	THTDIEVVVYFLLLWLVMRDEPIKK-KK	16–40-KK
p7-3	MRDEPIKKWILLLFHAMTNNPVK	33–55
p7-4	KK-KKWILLLFHAMTNNPVKTITVALLMVSGV	KK-39–67
p7-N	LQLGQGEVVLIGNLITHTDIEVVVYFLLLYLVMRDEPIKK	1–32
p7-C	MRDEPIKKWILLLFHAMTNNPVKTITVALLMVSGV	33–67

[a] Sequences and numbering based on Brescia strain. Underlined residues correspond to the polar turn proposed to connect the two transmembrane domains.