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Review

Viral proteins function as ion channels

Kai Wang [a], Shiqi Xie [a], Bing Sun [a,b,*]

[a] Laboratory of Molecular Virology, Institut Pasteur of Shanghai, Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences, 225 South Chongqing Road, Shanghai 200025, China
[b] Laboratory of Molecular Cell Biology, Institute of Biochemistry and Cell Biology, Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences, 320 Yueyang Road, Shanghai 200031, China

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ABSTRACT

Viral ion channels are short membrane proteins with 50–120 amino acids and play an important role either in regulating virus replication, such as virus entry, assembly and release or modulating the electrochemical balance in the subcellular compartments of host cells. This review summarizes the recent advances in viral encoded ion channel proteins (or viroporins), including PBCV-1 KcV, influenza M2, HIV-1 Vpu, HCV p7, picornavirus 2B, and coronavirus E and 3a. We focus on their function and mechanisms, and also discuss viral ion channel protein serving as a potential drug target.

Contents

1. Introduction

Since M2 protein of influenza virus A was reported to have ion channel activity in 1992 [1], several ion channel proteins encoded by viruses have been discovered. This new family of virus proteins that enhance membrane permeability was named viroporins, and includes small proteins that contain at least one hydrophobic transmembrane (TM) domain [2]. Typically, viroporins are comprised of 50–120 amino acids (Table 1), which tend to form homo-oligomers, most of them frequently forming tetramers. Oligomerization of these subunits produces a hydrophilic pore which facilitates ion transport across host cell membranes. The functions of these viral channel proteins may be involved in many stages of the virus infection cycle.

2. Function of viral ion channels

2.1. PBCV-1 Kcv

Chlorella virus PBCV-1 encodes a 94 aa protein, Kcv, which has two transmembrane domains separated by 44 amino acids that contain

* Corresponding author. Institut Pasteur of Shanghai, Chinese Academy of Sciences, 411 He Fei Road, Shanghai 200025, China. Tel.: +86 21 63851929.
E-mail address: bsun@sibs.ac.cn (B. Sun).

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Table 1
List of several viral encoded ion channel proteins.

Virus family	Viral channel	AA residues	TM	References
Picornaviridae	Poliovirus 3A	87	1	[3–5]
	Poliovirus 2B	97	2	[4–6]
	Coxsackievirus B3 2B	99	2	[7,8]
	EV71 2B	99	2 (predicted)	
Togaviridae	SFV 6K	60	2	[9]
	Sindbis virus 6K	55	1 (predicted)	[10]
	Ross River virus 6K	62	1 (predicted)	[11]
Retoviridae	HIV-1 Vpu	81	1	[12–14]
Paramyxoviridae	HRSV SH	64	1	[15]
Orthomyxoviridae	Influenza A virus M2	97	1	[1,16,17]
	Influenza B virus NB	100	1	[18–20]
	Influenza B virus BM2	115	1	[21,22]
	Influenza C virus CM2	115	1	[23–26]
Reoviridae	ARV p10	98	1 (predicted)	[27]
Flaviviridae	HCV p7	63	2	[28,29]
Phycodnaviridae	PBCV-1 Kcv	94	2	[30–32]
	ATCV-1 Kcv	82	2 (predicted)	[33]
Rhabdoviridae	BEFV alpha-1 protein	88	1 (predicted)	[34]
Coronaviridae	SARS-CoV E	76	1	[35,36]
	MHV E	83	1 (predicted)	[37,38]
	SARS-CoV 3A	274	3	[39,40]

EV71, Enterovirus 71; SFV, Semliki forest virus; HIV-1, human immunodeficiency virus type 1; HRSV, human respiratory syncytial virus; ARV, avian reovirus; HCV, Hepatitis C virus; PBCV-1, Paramecium bursaria chlorella virus; ATCV-1, Acanthocystis turfacea chlorella virus; BEFV, bovine ephemeral fever virus; MHV, murine hepatitis virus; SARS-CoV, Severe acute respiratory syndrome-associated coronavirus.

the K^+ channel signature sequence TXXTXGFG. When expressed in *Xenopus* oocytes [30] or mammalian HEK293 [32] and CHO cells [41], Kcv shows a potassium-selective conductance, which is sensitive to the typical K^+ channel blockers Ba^{2+}. The PBCV-1 Kcv channel has an overall K^+ channel architecture, which is similar to the bacterial channels KcsA and KirBac as well as to eukaryotic Kir channels [42,43]. Kcv alignment with KcsA (a pH-gated bacterial K^+ channel) indicates 42% aa similarity and 19% aa identity [41].

Six Kcv-like proteins forming functional K^+-selective channels were identified from 40 additional chlorella viruses [44]. Recently a K^+ channel protein (82 amino acids, named ATCV-1 Kcv) was found in another chlorella virus, acanthocystis turfacea chlorella virus, which was demonstrated in *Xenopus* oocytes and K^+ uptake deficient *Saccharomyces cerevisiae* [33]. It seems that chlorella viruses are a rich source of ion channel genes [45].

Infection of chlorella NC64A cells by PBCV-1 produces a rapid depolarization of the host cell. Kcv is considered responsible for the depolarization and is related to modulation of the efflux of potassium ions [46]. The host membrane depolarization may have two functions during PBCV-1 infection. Firstly, the virus-triggered K^+ efflux occurs at the same time as the host cell wall is broken and viral DNA is ejected. It was postulated that loss of K^+ is associated with an ejection of DNA from the virus particles into the host. Secondly, when the unicellular green alga is inoculated with two viruses, usually only one virus replicates in a single cell [47]. Timo Greiner et al. demonstrated that virus-induced host membrane depolarization, presumably triggered by a virus-encoded K^+ channel (Kcv) located in the virus internal membrane and by the rapid release of K^+ from the cell, is at least partially responsible for this mutual exclusion phenomenon [48].

2.2. Influenza virus M2

The influenza A virus M2 protein, the most well-studied viral proton channel, is a 97 residue single-pass membrane protein [49] which forms homo-tetramers [50]. Pinto et al. first demonstrated the ion channel activity of M2 in *Xenopus laevis* oocytes [1]. Its channel activity was further studied in mammalian cells [51,52] and lipid bilayers [17]. Schroeder et al. indicated its proton transfer activity by functional reconstitution of M2 in lipid vesicles [53], and its selective proton permeability also detected in mouse erythroleukaemia (MEL) cells expressing M2 protein [52]. The BM2 of influenza B virus also shows proton channel activity [21]. However, the CM2 of influenza C virus forms a voltage-activated ion channel permeable to chloride ion in *Xenopus laevis* oocytes [26].

The transmembrane domain of influenza A virus A/M2 and influenza B virus BM2 both contain the motif HXXXW. The high proton selectivity of A/M2 is conferred by histidine residue 37 [54], and the channel gate is conferred by tryptophan 41 [55] . At low pH, the His37 H^+ selectivity filter becomes protonated and the indole ring of Trp41 rotates to permit protons to pass. The function of M2 channel activity is to promote virus uncoating in endosomes [56] and to affect glycoprotein processing and trafficking [57,58].

The cytoplasmic domain of influenza A virus M2 protein consists of 54 amino acid residues from 44 to 97aa. Distinct domains of this part of M2 mediate its binding to M1 protein and facilitate virus production [59]. The cytoplasmic part of A/M2 also found to interact with caveolin-1 [60], is a host a cholesterol-binding protein enriched in lipid raft [61]. However, the effect of this interaction needs further investigation.

2.3. HIV-1 Vpu

Vpu is a 16 kDa protein expressed by HIV-1 gene *vpu*, which is absent from HIV-2 and other simian immunodeficiency viruses except for the simian immunodeficiency virus of the chimpanzee (SIVcpz) [62,63]. The Vpu was encoded by the Rev-dependent bicistronic mRNA and has three α-helices: one in the transmembrane domain at the N-terminal and the other two in the cytoplasmic domain [64,65]. The two serines at positions 52 and 56 within the cytoplasmic domain were found to be phosphorylated by casein kinase (CK-2), a process important to the CD4 degradation function of Vpu [66].

In some cell lines, Vpu is essential for the release of virus particles [62,67,68]. The absence of Vpu in a viral genome can lead to the retention of virus particles in the cell plasma membrane [68]. In early studies, it was unclear how the deficiency of Vpu might affect virus release. Nevertheless, some functions of Vpu were determined. Firstly, Vpu can lead to the degradation of CD4 [69], which seems to be irrelevant to the activity of Vpu in enhancement of virus release [65,70]. Next, the transmembrane domain at the N-terminal of Vpu was found to have ion channel activity in vitro and to be involved in the regulation of virus release [14]. The mutant Vpu with no CD4 degradation activity can also facilitate virus release. Moreover, additional expression of Vpu protein of HIV-1 enhances the release of several other viruses, including Sindbis virus, murine leukemia virus (MLV), HIV-2 and visna virus [14,71]. However, with these limited data, we cannot understand the detailed mechanism that how Vpu facilitates virus release.

Subsequently, investigators' attention was drawn to the mechanism by which Vpu can induce CD4 degradation. Briefly, h-βTrCP, a human WD protein, can interact with Vpu as it connects to CD4; this βTrCP–Vpu-CD4 complex then interacts with sKp1p, a targeting protein for ubiquitin-mediated proteolysis, and finally leads to the degradation of CD4 by the ER-associated ubiquitin pathway [72,73]. At nearly the same time, Vpu was found to have high homogeneity with the first transmembrane domain TASK-1, a mammalian endogenous potassium channel [74]. It was reported that Vpu could interact with TASK-1 and form dysfunctional hetero-multimers which may reduce the activity of TASK-1 and lead to a degradation of this potassium channel. On the

other hand, the first transmembrane domain of TASK-1 could be regarded as a functional equivalent for Vpu in virus release. However this work has never been confirmed in any later work.

The most recent studies were based on some interesting phenomenon. It was observed that the dependence of Vpu in HIV release is present in some cell lines (HeLa etc.) but absent in others (COS etc.) [75]. In additions, this dependence could be induced by interferon-α in 293T cells or enhanced in Jukat cells [76]. A study on fusion of between HeLa and COS-7 cells suggested the existence of an inhibitor to virus release which could be antagonized by Vpu protein in HeLa cells but not in COS-7 cells [75]. Finally this inhibitor, CD317/BST2, was revealed by using RNA microarray and was named 'tetherin' [77]. Later studies demonstrated that the inhibition by CD317/BST2 of virus release is a common mechanism through which host cells protect themselves. A similar phenomenon could be found in Lassa and Marburg virus [78], SIV [79] and Kaposi's sarcoma-associated herpesvirus (KSHV) [80]. Direct interactions between tetherin and HIV virions have also been noted [81]. However, there is still some controversy concerning the detailed mechanisms by which Vpu can downregulate tetherin [82–84].

Varthakavi et. al. reported another cellular factor, calcium-modulating cyclophilin ligand (CAML) , a second cellular restriction factor that Vpu antagonizes to enhance HIV-1 release [85]. Currently, the manner in which Vpu overcomes CAML restriction effect is not clear. Although it was proved that the Vpu function is associated with virus release, the question of whether the ion channel activity of the transmembrane domain is required for the viral release is still debated. TASK-1 offered a very good opportunity to link them together. However, available evidence remains inadequate to demonstrate whether TASK-1–Vpu interaction is really important for the regulation of HIV release. We trust that future evidence will illuminate the detailed relationship between the functions of Vpu and the HIV life cycle.

2.4. HCV p7

The p7 protein from HCV is the cleavage product from an E2-NS2 intermediate which consists of only 63 amino acids [86]. It has two transmembrane domains and is mainly located on ER, mitochondrial and plasma membranes [87,88]. Its ion channel activity was first reported in 2003 by using lipid bilayer system [28,29]. Little is known about the function of p7 in the virus life cycle. It was found to be essential for HCV infection in an *in vivo* study [89]. After the discovery of the HCV cell culture system, more data suggested that p7 could facilitate virus assembly and release, functioning mainly at the late stage of virus propagation [90]. Recently, the establishment of a trans-complementary system of p7 provided us a powerful tool with which to study the function of p7 in the HCV life cycle. It also led to the hypothesis that p7 could affect virus in two aspects: its own function and the cleavage of an E2- p7-NS2 intermediate [91]. However, the study of p7 is just at its beginning, the links between p7 function and virus propagation remain to be found and their mechanisms remain to be illuminated.

2.5. Picornavirus 2B

Picornaviruses are small positive strand RNA viruses which express their mature proteins from a poly protein that will be cleaved by protease. 2B protein is one of these mature proteins which consists of about 99 amino acids (varies in different viruses). Until now, most studies have been focused on 2B from polovirus and coxsackievirus B3.

2B protein is mainly localized on ER and Golgi [8,92]. It can form dimers and tetramers, as has been proven by yeast two-hybrid [93,94] and fluorescent resonance energy transfer microscopy [95]. At first, it was reported that 2B, along with its precursor 2BC and another matured protein 3A, could modify membrane permeability to hygromycin B [4,5,96]. These data led to studies indicating that 2B can form a viroporin and contribute to the viral life cycle.

2B proteins are involved in several cellular and viral life stages. 2B in poliovirus was found to be associated with cellular protein secretion [5] and the disassembly of the Golgi complex, the same phenomenon that can be induced by poliovirus infection [97]. Moreover, some *in vitro* studies showed that insertion of poliovirus 2B protein into lipid bilayers can modulate the permeability of the membrane, even allowing the passage of small molecules [98] . In coxsackievirus B3, 2B protein was reported to facilitate virus release by inducing a calcium efflux from endoplasmic reticulum into the cell plasma [7]. The hypothesis is that 2B protein can suppress cell apoptosis by manipulating calcium homeostasis and thus can protect virus from some host anti-viral mechanisms [99]. However, it is still unclear whether 2B protein can adjust the calcium concentration by itself or by affecting some other host proteins. Our recent studies on enterovirus 71 2B protein showed that 2B could induce a chloride-selective current in *Xenopus* oocytes and could aid in virus release. Its detailed mechanisms need further investigation [Xie et al. in submission].

2.6. Coronavirus E and 3a

In Coronaviridae, it was demonstrated that the envelope (E) proteins of MHV, SARS-CoV, HCoV-229E and IBV exhibit viroporin activity [35,37,38,100–103]. The E protein of SARS-CoV is 76 amino acids long, contains one predicted TM domain and forms a pentameric ion channel [35,36,103,104]. The SARS-CoV E protein is more selective for Na^+ than for K^+ ions, status similar to that of Alphavirus 6 K proteins [11]. Although E protein is not essential for MHV replication, the E gene deleted MHV recombinant virus showed low growth rate and low titer, and produced small plaques [105]. Previously, E protein was demonstrated to play a role in viral assembly and morphogenesis [106–108], but how its ion channel activity mediates these functions is still unknown.

Our laboratory found that another protein, 3a of SARS-CoV, could form an ion channel and modulate viral release [39]. The ORF3 gene of SARS-CoV encodes a 274aa protein, containing three TM domains [40] and forms homo-tetramers [39]. When 3a is expressed in Xenopus oocytes, the K^+ channel activity was detected and could be blocked by Ba^{2+}. The siRNA knockdown experiment showed that 3a protein plays a role in viral release. We predicted the existence of 3a protein homologue in different human coronaviruses, and its location between coronavirus Spike (S) gene and Envelop (E) gene on the viral genome. For convenience, we called this gene SNE [40]. Our recent work demonstrated that other SNE genes, such as PEDV ORF3, also encode an ion channel protein [Wang et. al. in submission]. Since it contains three TM domains and a relative long cytoplasmic domain, SARS-CoV 3a protein is larger than previously reported viroporins, including coronavirus E protein. It was well documented that the N terminus of HIV-1 Vpu encoding the TM domain forms ion channels and modulates viral release, while its cytoplasmic domain has many other functions, such as CD4 degradation [65],. The cytoplasmic part of 3a may also have many interesting functions, and it has been already reported that 3a protein contains putative binding sites for caveolin-1 [109], and their interaction has been demonstrated by Padhan et al. [110].

3. Viral channel as drug target

One of the main reasons for the focus on virus ion channels was that they may serve as ideal anti-viral drug targets. Amantadine was one of the most studied and famous inhibitors for the influenza A virus M2 proton channel. Although encumbered with severe side effects, amantadine has been used clinically as an anti-influenza drug since 1966 [111]. Then in 1985, a study indicated that several mutations, which could lead to resistance to amantadine, were located in a hydrophobic sequence in M2. This data suggested that M2 might be the target for amantadine [112]. Then, in 1992 direct evidence was offered indicating that amantadine could inhibit an early M2-dependent stage

in influenza virus replication [113]. In the following decade, M2 and amantadine were regarded as a very good model in which to study the structure of M2 and mutations induced by drug-related selection pressure. There were two papers explaining the structure-based mechanism of the manner in which mutations in M2 can lead to drug resistance to amantadine [114,115].

Amiloride derivatives were reported to block HIV-1 Vpu and coronavirus E protein ion channel activity [38,116]. The newly discovered HCV ion channel protein p7 was likely to become the first anti-viral drug screen target used in practice. After the identification of p7 ion channel activity, several studies revealed that this activity can be inhibited by amantadine [28], hexamethylene amiloride (HMA) [117] and long-alkyl-chain iminosugar derivatives [29]. Interestingly, although the amino acid sequences of p7 vary in different genotypes of HCV, all of them have a specific reaction in response to these drug treatments [118], indicating that p7 might be a good target for anti-HCV drug screening. Furthermore, an in vitro liposome-based drug screen system has been established [119,120], appears very helpful in investigation of viral ion channel activity and may be developed into a suitable method for anti-viral drug screening.

4. Summary

The ion channel proteins were found in many different virus families, play important roles in the life cycle of viruses, and serve as promising anti-viral drug targets. PBCV-1 Kcv and influenza A virus M2 are highly selective channel components, which form potassium channel and proton channel, respectively. In contrast, other viral ion channel proteins, such as HIV-1 Vpu and HCV p7 are permeable to both Na^+ and K^+ ions, and display relatively low ion selectivity. As the smallest K+ channel, Kcv contains a motif of eight amino acids, TXXTXGFG, which is common to all the potassium channels. The channel structure and function studied in influenza A virus M2 are well defined. However, these elements remain unclear with respect to other viral ion channel proteins.

Although great effort has been spent in this field, there are still many questions remaining to be answered. Firstly, as the ion channel activity of most viroporins was identified by using an *in vitro* system such as lipid bilayer or Xenopus oocytes, their real biological function in virus-infected cells or animals remains to be investigated. Only the ion channel activity of Kcv and A/M2 was directly associated with a viral life cycle stage [46,112,113]. The function of the ion channel activity for different viroporins (such as HIV-1 Vpu, HCV p7, coronavirus E and 3a protein) is related to promotion of virus release, but the detailed mechanism remains to be investigated. However, Vpu was reported to act against host factor; and 2B and 3A from picornaviruses were also found to be inhibitory with regard to cell trafficking. None of these functions was proved to be directly related to its ion channel activity.

Secondly, the manner in which viral ion channel proteins interact with other host factors is also an important issue. As most of the ion channel proteins have both a transmembrane domain and cytoplasmic domain, it is quite likely that the cytoplasmic domain may interact with some host molecules and have some interesting functions. The study in Vpu has suggested that its function is associated with βTrCP and the ER-associated protein degradation pathway, a notion that can be used to explain many important functions of Vpu, such as CD4 degradation and Tetherin antagonism. Both SARS-Cov 3a protein and influenza A virus M2 have been reported to interact with caveolin-1. It is generally believed that such interaction with host factors could reflect a newly discovered function of viral ion channel proteins.

Finally, drug screening through the use of viral ion channel proteins has drawn more and more attention. In the past most inhibitors were first checked on virus and then on ion channel proteins. Now high throughput methods have been developed to achieve the same goal by directly testing compounds on viral channels. Given that ion channels have very important functions in the cells and that inhibitors blocking viral ion channels might therefore be harmful to cells, it might be better to use these inhibitors over a short period to transiently reduce virus titer in vivo but not to regard them as long-term treatment. On the other hand, some chemical modifications may also reduce the toxicity of these potential drugs. Nevertheless, viral ion channel proteins have provided us an alternative new target with which to discover new drugs and fight viral infection.

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