2015

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Virology 479-480 (2015) 38–45

Contents lists available at ScienceDirect

Virology

journal homepage: www.elsevier.com/locate/yviro

Review

Large dsDNA chloroviruses encode diverse membrane transport proteins

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ARTICLE INFO

Article history:
Received 25 November 2014
Returned to author for revisions
20 January 2015
Accepted 7 February 2015
Available online 9 March 2015

Keywords:
Chloroviruses
Ion channels
Ion transporters
Giant viruses
Algal viruses

ABSTRACT

Many large DNA viruses that infect certain isolates of chlorella-like green algae (chloroviruses) are unusual because they often encode a diverse set of membrane transport proteins, including functional K^+ channels and aquaglyceroporins as well as K^+ transporters and calcium transporting ATPases. Some chloroviruses also encode putative ligand-gated-like channel proteins. No one protein is present in all of the chloroviruses that have been sequenced, but the K^+ channel is the most common as only two chloroviruses have been isolated that lack this complete protein. This review describes the properties of these membrane-transporting proteins and suggests possible physiological functions and evolutionary histories for some of them.

Contents

Introduction

Viruses infecting algae included in the family *Phycodnaviridae*, together with viruses in the *Poxviridae*, *Iridoviridae*, *Ascoviridae*, *Asfarviridae*, *Mimiviridae* and *Marseilleviridae* families are believed to have a common evolutionary ancestor and are often referred to as nucleocytoplasmic large DNA viruses (NCLDV) (Iyer et al., 2001, 2006; Yutin et al., 2009; Yutin and Koonin, 2012). It has been proposed that these giant viruses be assigned to a new order called Megavirales (Colson et al., 2012, 2013). NCLDV members infect animals, including humans, and diverse unicellular and multicellular eukaryotic microorganisms and either replicate exclusively in the cytoplasm of their host cells or encompass both nuclear and cytoplasmic stages in their life cyles.

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http://dx.doi.org/10.1016/j.virol.2015.02.025
0042-6822/

PMID: 25766639
https://www.ncbi.nlm.nih.gov/pubmed/25766639

The phycodnaviruses represent a diverse group of dsDNA viruses that have genomes ranging from 160 kb to 560 kb and an outer capsid of icosahedral morphology. As the name suggests phycodnaviruses infect eukaryotic algae. Their host range includes both unicellular and multicellular algae that grow in either fresh water or marine environments. It is important to note that some of their algal hosts are only distantly related, e.g., *Chlorophyceae* (green algae), *Phaeophyceae* (brown algae) and coccolithiphores have different evolutionary histories. Both lytic and lysogenic life styles occur in the phycodnaviruses (Van Etten et al., 2002).

The chloroviruses are probably the most intensively studied group among the *Phycodnaviridae*. These viruses infect certain unicellular, eukaryotic, exsymbiotic chlorella-like green algae, which are often called zoochlorellae. They are associated with either the protozoan *Paramecium bursaria*, the coelenterate *Hydra viridis* or the helizoon *Acanthocystis turfacea* (Van Etten and Dunigan, 2012). Three such zoochlorellae are *Chlorella* NC64A, recently named *Chlorella variabilis* (Pröschold et al., 2011), *Chlorella* SAG 3.83 (renamed *Chlorella heliozoae*) and *Chlorella* Pbi (renamed *Micractinium conductrix*). Viruses infecting these three zoochlorellae are referred to as NC64A-, SAG-, and Pbi-viruses, respectively.

The prototype chlorovirus *P. bursaria* chlorella virus (PBCV-1) is an iscosahedron (190 nm in diameter) that contains a 34 nm spike-like structure at one vertex (Cherrier et al., 2009; Zhang et al., 2011). Chloroviruses have a phage-like lytic replication cycle that typically requires 6 to 20 h to complete depending on the virus. Genomes from 41 chloroviruses have been sequenced and annotated (Jeanniard et al., 2013). Collectively, the 41 viruses encode members in 632 protein families. Since any one virus encodes at most about 410 proteins, the chloroviruses exhibit large genetic diversity, including a variety of membrane transport proteins homologous to cellular organisms. In this review we summarize the present state of knowledge on these viral encoded membrane transporters in the virus family *Phycodnaviridae* with respect to their activity, structural aspects and possible evolution.

Classification of membrane transporters

Transport proteins are divided into two main categories, namely proteins for active transport and those for passive transport (Fig. 1). Active transport requires ATPases, which hydrolyze ATP and use the resulting energy to transport ions against their electrical gradient across membranes. The primary ATPases, which are active in cells, are the Na^+/K^+-ATPases in animal cells and the H^+-ATPases in plants and fungi (Morth et al., 2011). Both ATPases, which are classified according to their molecular architecture as P-type ATPases, are essential for the electrical charging of the plasma membrane of cells. This energy is then used by passive transporters for translocating other molecules across membranes. In addition to the aforementioned active transporters there are several other P-type ATPases, which pump a variety of ions against their electrochemical gradient. One type is Ca^{2+}-transporting ATPases (Carafoli and Brini, 2000),

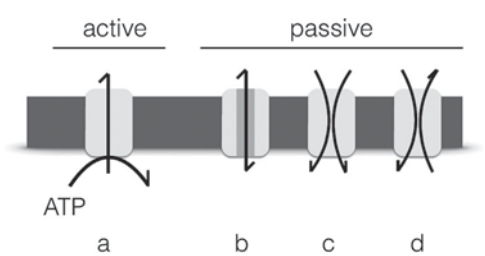

Fig. 1. Overview of the two primary classes of transport proteins in eukaryotes. They require ATPases: (a) for active transport as well as uniporters (b), symporters (c) and antiporters (d) for passive transport.

which are responsible for maintaining a low concentration of free Ca^{2+} in the cytosol by pumping the divalent cation out of the cell or into intracellular organelles. In addition to P-type ATPases, cells also use so-called V-type ATPases for ion transport. These multi-subunit proteins have the same architecture as the ATP-synthases in mitochondria and chloroplasts, which convert the protomotive force across a membrane to synthesize ATP. The best-characterized ATPase of this type is the V-type ATPase, which transports H^+ across the tonoplast membrane in plants (Seidel et al., 2013).

The proteins, which mediate passive transport in eukaryotes, are structurally and functionally diverse. Uniporters are proteins, which facilitate the diffusion of molecules across membranes down a thermodynamic gradient. A specific class of uniporters is ion channels. They allow very fast and highly selective diffusion of ions across membranes (Hille, 2001). In comparison to all other transporters, ion channels have the highest transport capacity; they have a turn over of up to $10^8\ s^{-1}$ ions passing through a channel at a velocity that is close to diffusion in water. Ion channels are classified according to the best-transported ion. Channels discriminate between anions and cations (Hille, 2001). Within the family of cation channels there are proteins that are highly selective for K^+, Na^+ and Ca^{2+}. Anion channels contain proteins that select between Cl^- and NO_3^-. In addition to ion conducting channels there are also channels that conduct water and/or other small molecules such as glycerol, urea or H_2O_2 (Hachez and Chaumont, 2010). Like ion channels, these proteins allow very fast passive movement of molecules across membranes.

In contrast, there is a large and diverse family of proteins that are classified as transporters, which catalyze the slow movement of molecules across membranes. These proteins generally function by transporting one ion down its thermodynamic gradient. The energy available from this downhill transport is then used to transport a second molecule across the membrane against its thermodynamic gradient. The high affinity of these proteins for the transported molecules results in a selective and substantial accumulation of molecules in cells. When both molecules pass the membrane in the same direction the proteins are classified as symporters; when the transport is in opposite directions they are termed antiporters (Hediger et al., 2013).

The plasma membrane of eukaryotes and the membranes of their intracellular organelles simultaneously contain many types of transport proteins for active and passive transport. Their concerted activities are often coupled in a network like fashion by the membrane potential and they are involved in many basic processes of life. This includes the transport of ions, nutrients, water and toxic products in and out of cells. All of these activities are important for basic cellular processes such as growth, osmo-regulation and cell movement. In many cases, the transduction and processing of environmental signals to the interior of cells are also mediated by membrane proteins. This need for selective communication across membranes made it imperative that transport proteins emerged early in evolution together with membranes (Franciolini and Petis, 1989; Pohorille et al., 2005).

Comparative analyses of membrane transport proteins in eukaryotes indicate that the basic structures are present in prokaryotes. The primary P-type ATPases, ion channels and transporters from eukaryotes all have homologs in bacteria and/or archaea (Saier, 2000; Anderson and Greenberg, 2001; Thever and Saier, 2009). Collectively, these similarities suggest a long evolutionary history for transport proteins to a time before the separation of the major domains of life.

Virus encoded ion channels

The discovery of small hydrophobic proteins in viruses, which function as ion channels, dates back to the early 1990s when the M2 protein from influenza virus A was shown to be a H^+ selective ion channel (Pinto et al., 1992). After this seminal work several

proteins with channel function and significance in viral infection and/or replication were reported from a variety of viruses (e.g., Fischer and Sansom, 2002; Wang et al., 2011; Nieva et al., 2012; DiMaio, 2014). However, most of these reported proteins, often referred to as viroporins, have no obvious sequence similarity to either prokaryotic or eukaryotic ion channels.

The one exception is channel proteins coded by the phycodnaviruses that have the structural and functional hallmarks of canonical K^+ channels. Extensive research on the structure/function relationships of these channels, which were named Kcv (K^+ channel of chlorella viruses) and on their relevance in early steps of virus infection, have been reviewed elsewhere (Thiel et al., 2010, 2011). Here we only summarize some of the key features of these interesting viral K^+ channels. Genes that encode K^+ channel-like proteins have been detected in nearly 100 phycodnaviruses from different genera (Fig. 2). The presence of K^+ channel sequences in viruses is independent of the host and genes for K^+ channels can be found in viruses that infect both fresh water and marine algae (Siotto et al., 2014). The hosts for the viruses with K^+ channel genes are as different as green algae and brown algae. A detailed phylogenetic analysis of the viral channels and a comparison with the K^+ channels from some of their hosts indicates that the genes encoding the viral K^+ channels are not acquired from their current hosts (Hamacher et al., 2012; Thiel et al., 2013) and that they must have originated from another source.

In fact in a recent study we suggested that the viral-encoded K^+ channel protein might be the ancestor of all K^+ channels (Thiel et al., 2013). Evidence to support this statement included: the small size of the viral channels, which is basically the pore-forming segment, their functional simplicity, and their self-assembly into tetramers. Also two examples of how complex K^+ channels could have formed from a single genetic event involving the viral encoded Kcv channel were described. Current phylogenetic algorithms were unable to support or disprove this hypothesis.

The common structural feature of all viral K^+ channels is that they are essentially a substructure of more complex prokaryotic- and eukaryotic K^+ channels; i.e., they resemble the pore module of all known K^+ channels (Thiel et al., 2011). Like prokaryotic- and eukaryotic K^+ channels, the monomers of the viral K^+ channels have two transmembrane domains, which are linked by a small pore helix and a selectivity filter. The filter contains the canonical signature sequence of eight amino acids [TXXTXG(Y/F)G], which with a few modifications, is present in all K^+ channel proteins from prokaryotes and eukaryotes.

The most interesting feature of the Kcv type channels is their small size. While K^+ channels from prokaryotes and eukaryotes are often several hundred amino acids long, the viral proteins are less than 100 amino acids long. The smallest functional viral K^+ channel protein discovered to date is coded by *Micromonas pusilla virus 12T*, it has a monomer size of only 78 amino acids (Siotto et al., 2014). From a structural standpoint this is about the size limit for a functional K^+ channel protein. Molecular dynamic simulations of a slightly larger chlorovirus K^+ channel with 82 amino acids indicates that this protein is quasi fully embedded in the lipid bilayer (Braun et al., 2013). Therefore, it is unlikely that further truncation of the protein will be possible without losing its transmembrane orientation.

From an experimental perspective, ion channels have the advantage of high unitary conductance. This allows detailed characterization of their function using electrophysiological methods. To date all of the predicted phycodnavirus K^+ channels tested are functional (e.g., Plugge et al., 2000; Kang et al., 2004; Gazzarrini et al., 2006, 2009; Balss et al., 2008; Braun et al., 2013; Siotto et al., 2014). In spite of their small size the viral proteins exhibit the typical behavior of complex K^+ channels; they are selective for K^+ over Na^+ and they are inhibited by some of the typical blockers of more complex K^+ channels.

The sensitivity of the viral K^+ channels to blockers also contributes to understanding the functional role of the Kcv type proteins in chlorovirus infection. The fact that both channel activity and virus infection are inhibited by a membrane impermeable channel blocker like Ba^{2+} implies that Kcv function is required outside of the cells, i.e., before viral DNA enters the host cell (Mehmel et al., 2003). Experimental results with chlorovirus PBCV-1 support the following scenario: the Kcv gene is transcribed in the host as both an early and a late gene (Fig. 3) and the protein is packaged in the inner membrane of the virus particle (Romani et al., 2013). To initiate infection the virus attaches specifically to the external surface of its host cell wall, presumably the initial contact is made by the spike-like structure (Zhang et al., 2011). Attachment results in digestion of the cell wall at the point of attachment followed by fusion of the virus membrane with the host membrane, which activates Kcv. The large conductance of Kcv then contributes to the overall conductance of the host cell, which results in rapid membrane depolarization (Frohns et al., 2006) and a loss of K^+-salts and water from the host (Neupärtl et al., 2008). Together, these events decrease the internal turgor pressure of the host cell and make it easier for the virus to eject its DNA genome into the host (Thiel et al., 2010). The importance of the K^+ channel in this scenario is supported by experiments, which show that inhibitors of the channel inhibit the transfer of DNA from the virus particle into the host. Depolarization of the host membrane also prevents infection by additional chloroviruses (Greiner et al., 2009).

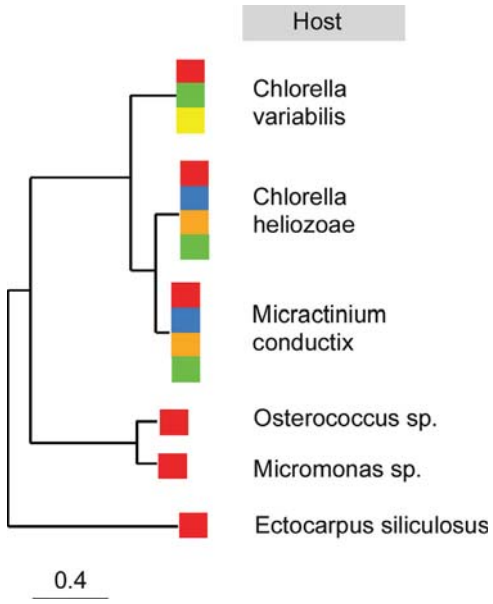

Fig. 2. Occurrence of transport proteins in different phycodnaviruses. The phylogenetic tree is constructed from the viral encoded DNA polymerases, which infect the host algae indicated on the right. The color-coding indicates the presence of genes, which code for K^+ channels (red), K^+ transporters (orange), aquaglycerporins (blue), Ca^{2+}-ATPases (green) and glutamate receptor type proteins (yellow) in the genomes of fully sequenced viruses. Sequence alignments were performed with T-Coffee algorithm at http://www.phylogeny.fr. The phylogenetic tree was calculated with the maximal likelihood algorithm implemented on the same platform.

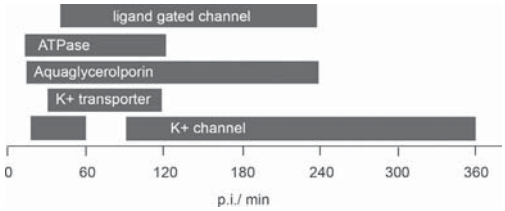

Fig. 3. Time course of transcription of chlorovirus transport proteins after host infection (p.i.).

Virus encoded aquaglyceroporins

Chlorovirus MT325 encodes a 270 amino acid protein (M030R) with the structural features of a Major Intrinsic Protein (MIP) (Fitzgerald et al., 2007a). MIPs are present in all cellular organisms and serve to transport water (aquaporins) and/or glycerol (aquaglyceroporins) across membranes (Abascal et al., 2014). Structural predictions of the viral protein, named AQPV1 (aquaglyceroporin virus 1), detected six transmembrane domains and the signature sequence Asn-Pro-Ala, located in both the amino and carboxyl-terminal portions of the protein (Gazzarrini et al., 2006). These features exist in the channel pores of both aquaporins and aquaglyceroporins. This amino acid motif is essential for the orientation of water and glycerol molecules in the permeation pathway. The selectivity filter of MIPs is formed by four conserved amino acids, which determine the selectivity of the protein i.e., whether they have a preference for water (aquaporins) or glycerol (aquaglyceroporins). Sequence comparisons between AQPV1 and well-studied representatives of either aquaporins or aquaglyceroporins indicate that the viral proteins resemble the selectivity filter of aquaglyceroporins better than aquaporins (Gazzarrini et al., 2006). In addition to a better match between the selectivity filters, AQPV1 also contains five amino acid residues, which are conserved among aquaglyceroporins, but are variable in aquaporins. Finally, a phylogenetic analysis also indicates that AQPV1 fits into the cluster of aquaglyceroporins (Gazzarrini et al., 2006).

AQPV1 not only looks like an aquaglyceroporin it also functions like one. In swelling assays with *Xenopus* oocytes, AQPV1 expression resulted in five times higher water conductance than in control oocytes (Gazzarrini et al., 2006). This water permeability is low compared to canonical aquaporins like AQP1 but still high when compared to aquaglyceroporins. The latter exhibit, with few exceptions, low water permeability. The function of AQPV1 was confirmed by radiotracer experiments, which showed that expression of the viral protein in *Xenopus* oocytes increased glycerol accumulation by a factor of 5. These results are qualitatively and quantitatively similar to those reported for other aquaglyceroporins and hence confirm the viral protein as a functional aquaglyceroporin (Gazzarrini et al., 2006).

Orthologs of AQPV1 exist in 24 chloroviruses representing two chlorovirus types (Fig. 2); they are present in viruses that infect *C. heliozoae* (SAG viruses) and *M. conductrix* (Pbi viruses) (Jeanniard et al., 2013). All 14 sequenced chloroviruses that infect *C. variabilis* (NC64A viruses) lack AQPV1-like sequences. The 24 viral aquaglyceroporins exhibit some diversity with 54% amino acid identity and 80% amino acid similarity among the AQPVs. This diversity is also reflected in protein size, which varies between 269 and 344 amino acids. However, the larger viral AQPVs have an internal Met in about the same position as the shorter AQPVs and so this site may be the actual translational start site of the protein.

A BLAST search against the entire data bank shows that the most closely related proteins to the viral aquaglyceroporins are from bacteria, primarily from *Clostridium* species (Table 1). The viral proteins have about 40% amino acid identity with their bacterial orthologs. A BLAST search against green algae, i.e., the group of algae that are the viral hosts, revealed that the viral aquaglyceroporins also have about 40% amino acid identity with orthologs from *C. variabilis* and *Coccomyxa subellipsoidea*. This similarity between the viral and green algae proteins would seem to have no immediate implications on the question of the origin of the viral proteins because NC64A viruses, which infect *C. variabilis*, do not encode aquaglyceroporins.

Chlorovirus MT325 not only encodes an aquaglyceroporin but it also encodes a functional Kcv type K^+ channel (Fitzgerald et al., 2007a). Since K^+ channels and aquaporins often operate in a concerted manner (Maurel, 1997; Nagelhus et al., 2004), this possibility was investigated with the viral proteins (Gazzarrini et al., 2006). Expression of the Kcv channel and AQPV1 together in oocytes increased the water permeability of the oocytes by a factor of 1.5. The presence of the Kcv channel presumably generates a greater osmotic driving force in oocytes than those that only express AQPV1.

Thus the viral aquaglyceroporin is a functional protein and the question is: does it have a function, either alone or in cooperation with the K^+ channel, during virus replication? Both genes are expressed in infected cells and their expression overlaps. *Aqpv1* is expressed as an early/late gene followed by an even later expression of *kcv* (Fig. 3). The timing of their expression suggests that both proteins might be packaged in the virion. This was experimentally confirmed for a related Kcv protein in virus PBCV-1 (Romani et al., 2013). However, M030R was not detected in a proteome analysis of virus MT325 (Dunigan et al., unpublished results). Currently the role of AQPV1 during the MT325 replication cycle is unknown.

Virus encoded K^+ transporters

A previous section described the importance of the viral encoded K^+ channel Kcv during chlorovirus infection. The key role of the channel in virus infection infers that Kcv channels should be present in all chloroviruses. However, Pbi chlorovirus FR483 lacks a *kcv* gene (Fitzgerald et al., 2007a). Still infection by FR483 resembles infection by chloroviruses that encode Kcvs. This similarity includes inhibition of FR483 induced membrane depolarization by Ba^{2+} (Greiner, unpublished results); Ba^{2+} inhibition is interpreted as being related to blockage of the Kcv channel. One explanation for this conundrum is that FR483 uses another protein to substitute for Kcv. In this context FR483 encodes a 660 amino acid protein (N110R) that resembles a potassium transporter of the HAK/KUP/KT-type with respect to size and topology (Grabov, 2007; Gierth and Mäser, 2007). It was speculated that this protein might substitute for the missing Kcv channel (Fitzgerald et al., 2007a).

Hydropathy analysis predicted that N110R had 12 transmembrane domains with a large gap between transmembranes 2 and 3 and a long C-terminal intracellular domain of ~160 amino acids. The amino acid sequence identity with its closest relative from a plant, HAK5 from *Arabidopsis thaliana*, is 36% (Table 1).

Like the viral encoded K^+ channels, the FR483 K^+ transporter not only resembles HAK/KUP/KT-type transporters, it functions in yeast complementation assays (Greiner et al., 2011). A mutant of *Saccharomyces cerevisiae*, which lacks its endogenous potassium uptake system, does not grow on a medium with low K^+ concentrations. This defect can be overcome by expressing the viral protein in the yeast mutant (Greiner et al., 2011). This positive complementation of the yeast mutant, along with an increase in Rb^+ import into the mutant, provides strong evidence for K^+ conductance by the viral protein. However, expression of the protein in *Xenopus* oocytes failed to generate a measurable current. This result suggests that the protein is not a channel but functions like other HAK/KUP/KT proteins as an electro-neutral transporter. The protein was named HAKCV for High affinity K^+ transporter from chlorella virus (Greiner et al., 2011).

One common feature of all HAK/KUP/KT potassium transporters, including the viral protein, is a sequence motif V(F/Y)GD. This motif is similar to the G(F/Y)GD sequence in the selectivity filter of K^+ channels and this motif may also determine the selectivity of potassium transporters (Rigas et al., 2001). An Ala scanning experiment in which each of these four key amino acids in the viral protein (V39 – D42) was exchanged with Ala indicates that none of the mutants compensate for the defect in the K^+ yeast mutants (Fig. 4). The results of these experiments support the view that the viral protein HAKCV is indeed a canonical HAK/KUP/KT-like transporter. This hypothesis is further supported by recent data, which identified essential anionic amino acids in the transmembrane domains of the HAK/KUP/KT transporter from *Escherichia coli* (Sato et al., 2014). An alignment of the viral and bacterial transporter indicates that most of

Table 1
Results from BLAST searches with different viral transport proteins against the entire NCBI databank and against green algae. The table shows the four bests hits for each search with an e-value threshold at < 0.8.

Viral transporter	Hit	Max score	Total score	Query cover (%)	*E*-value	Identity (%)	Acession number
K^+ channel Kcv_{PBCV1}	K^+ channel *Ectocarpus siliculosus*[a]	57.7	54.7	56	9e−8	56	CBN80308.1
	NAD-binding protein of Kef-type K^+ transporter *Mycobacterium sp 141*	53.5	53.5	73	2e−6	40	WP019971286.1
	Hypothetical protein Marine GroupII euryachaeote SCGC AB-629-J06	48.5	48.5	85	1e−5	34	Wp018035727.1
	K+ channel *Bathycoccus prasinos*	42	72.4	60	4e−5	49	XP007513359.1
	Voltage gated ion chanel superfamily *Micromonas pusilla CCMP1545*	41.6	41.6	62	7e-5	44	Xp003061621.1
	Predicted protein C. reinhardtii	41.2	41.2	85	1e−4	31	XP01690634.1
	Hypotehtical protein *Volvox cateri*	40.8	40.8	65	2e−4	40	XP002955535.1
Aquaglyceroporin AQPV1	Glycerol uptake facilitator *Clostridium sacharobutylicum*	171	171	94	3e−48	41	YP_008674590.1
	Glycerol uptake facilitator *C. butyricum*	159	159	94	6e−44	40	WP003368938.1
	Hypotehtical protein *C. variabilis*	174	174	93	6e−52	39	Xp005844923.1
	Aquaporin like protein *C. subelipsoidea*	107	179	91	6e−26	41	Xp005644521.1
	Hypothetical protein *C. variabilis*	58.9	58.9	16	9e−11	55	Xo005851961.1
	Aquaporin-like protein *C. subelipsoidea*	41.6	41.6	77	0.001	26	Xp0056432225.1
Ca^{2+}-ATPase M535L &C785L[b]	plasma membrane Ca^{2+} ATPase *C. reinhardtii*	681	681	96	0.0	43	XP001690816.1
	Hypothetical protein *C. variabilis*	658	658	88	0.0	43	XP005849646.1
	Ca^{2+} translocating p-type ATPase *C. subellipsoidea*	620	620	96	0.0	41	XP005644036.1
	Hypothetical protein *Volvox carteri*	618	677	96	0.0	42	XP002948264.1
K+ transporter HAKCV	Hypothetical protein *Rhizophagus irregularis*	407	407	96	4e−128	35	ERZ98732.1
	Predicted protein *Arabidopsis lyrata*	372	372	88	4e−114	36	XP002863130.1
	K^+ transporter *A. thaliana*	369	369	88	4e−113	36	NP567404.1
	K^+ transporter *C. subellipsoidea*	345	345	88	2e−105	35	Xp005649047.1
	K^+ transporter *C. reinhadtii*	335	335	85	2e−103	35	Xp001700451.1
	Hypothetical protein *C. variabilis*	33.9	33.9	24	0.66	22	Xp005848144.1
Ligand gated like channel A162L	Glutamate receptor subunit epsilon-2 *Cricetulus griseus*	49.7	49.7	59	0.003	22	ERE66598.1
	Glutamate receptor ionotropic NMDA 2A-like *Lepisosteus oculatus*	48.5	48.5	59	0.006	22	XP006637219.1
	Glutamate receptor subunit epsilon Camelus ferus	46.6	46.6	59	0.026	21	EQB78971.1
	Hypothetical protein C. variabilis	41.6	41.6	37	0.003	24	XP005851272.1
	Gluatmate-gated ion chnanel neurotransmitter receptor family *Micromonas sp.*	35.8	35.8	22	0.58	21	XP002507960.1
Ligand gated like channel A163R	Hypothetical protein *Branchiostoma floridae*	43.5	43.5	30	0.23	26	XP002606449.1
	Hypothetical protein *Daphnia pulex*	43.1	43.1	35	0.26	26	EFX79746.1
	Hypothetical protein *Capitella teleta*	43.5	43.5	68	0.27	20	ELU11964.1
	Gluatamate receptor *Crassostrea gigas*	43.1	43.1	25	0.30	26	EKC18142.1
	Predicted protein *Micromonas sp*	33.5	33.5	21	0.67	24	Xp002508478.1

[a] The K^+ channel detected in *Ectocarpus siliculosus* originates from the K^+ channel sequence of the virus Esv1: Esv1 integrates its genes as an lysogenic virus into the genome of its host.
[b] Data for Ca^{2+}-ATPase M535L and C785L were pooled because both generated the same search results.

the crucial amino acids in the bacterial transporter are well conserved in the viral protein. Alignment of the first transmembrane domain, which contains the V(F/Y)GD motif, is shown in Fig. 4A. Collectively, all the experiments indicate that the viral protein is a functional HAK/KUP/KT-like K^+ transporter and most likely functions in the same manner as its homologs in prokaryotes and eukaryotes.

As noted above, HAKCV is coded by a chlorovirus, which lacks a K^+ channel gene. This situation led to the hypothesis that HAKCV might replace the essential function(s) of the K^+ channel. However, this hypothesis was rejected for several reasons. First, one indirect argument against the hypothesis is that seven other chloroviruses, two members in the Pbi group and 5 in the SAG group, have a gene encoding a HAKCV-like protein (Fig. 2). However, all of these viruses also encode Kcv channels suggesting that the transporter is not a substitute for the channel. Second, northern blots indicate that the *hakcv* gene is expressed early during infection, with a peak at about 30–45 min p.i. (Fig. 3) (Greiner et al., 2011); typically early gene products are not packaged in virions and indeed a proteomics analysis of FR483 failed to detect the HAKCV protein in the FR483 virion (Dunigan et al., unpublished results). In contrast, the PBCV-1 *kcv* gene is expressed as an early/late gene and the Kcv protein is packaged in the virion (Romani et al., 2013), a prerequisite for their function in early infection. Third, the amount of K^+ transported by HAKCV is very low compared to a channel like Kcv and it is unlikely that it is involved in the rapid depolarization of the host plasma membrane response observed during PBCV-1 infection.

Thus the function of the HAKCV protein is unknown. The fact that the transporter is not present in all viruses suggests that it only has an auxiliary function and is not essential for virus replication. Another important question that remains unanswered is: what protein (s) substitutes for the missing Kcv channel in virus FR483? If the potassium efflux is as important for virus infection as the data suggest, Kcv could be replaced in virus FR483 by a host K^+ channel protein, which the virus acquires during its formation. An alternative scenario

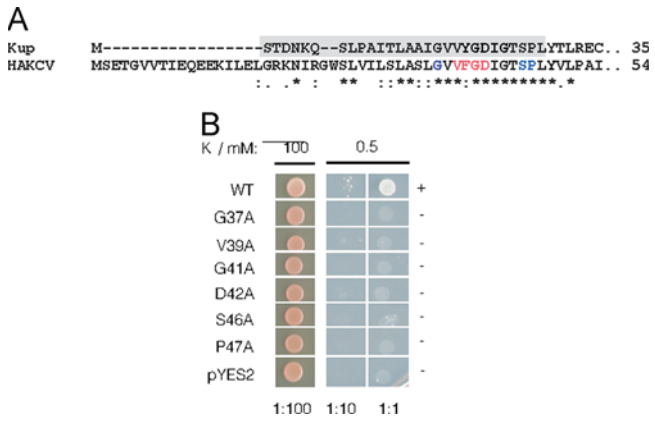

Fig. 4. Similarity between Kup/HAK/KT-type transporter from *Escherichia coli* (Kup) and viral K^+ transporter HAKCV. (A) Alignment of N-terminus of both transporters. The position of the first transmembrane domain in Kup (Sato et al., 2014) is marked in grey. Amino acids, which form the putative selectivity filter V(F/Y)GD in these proteins, are marked in red in HAKCV. The viral K^+ transporter becomes inactive when these critical amino acids or other conserved amino acids in this domain (blue) are mutated to Ala (B). The activity of the viral transporter and the aforementioned mutants in (B) is assayed by a yeast complementation assay (Greiner et al., 2011). While the wild type protein is able to complement yeast growth on selective medium the mutants are not.

is that the virions contain another protein(s) with channel function. Several small putative membrane proteins exist in the FR483 virion that might serve this function (Dunigan et al., unpublished results).

Ca^{2+} transporting-ATPases

Sequencing the chlorovirus MT325 and AR158 genomes (Fitzgerald et al., 2007a, 2007b) revealed two proteins (M535L, 872 amino acids and C785L, 871 amino acids) with sequence similarities to prokaryotic and eukaryotic Ca^{2+}- transporting ATPases (Ca^{2+}-ATPase) (Bonza et al., 2010). M535L and C785L have 37% amino acid identity and 56% similarity to the well-studied Ca^{2+}- transporting ATPase ACA8 from *A. thaliana* (Bonza et al., 2010). The latter protein belongs to the auto-inhibited, calmodulin binding type-IIB Ca^{2+}-ATPase, which complements the *S. cerevisiae* deficient mutant K616.

The two putative viral Ca^{2+}-ATPases contain all the characteristic motifs of type-II P-type ATPases (Møller et al., 1996; Axelsen and Palmgren, 1998), except that they lack the typical calmodulin regulated auto-inhibitory domain. Structural predictions indicate the protein has ten transmembrane domains and an estimated molecular mass of 95 kDa. Because of its short N-terminus and the missing calmodulin binding site the predicted molecular weight of the chlorovirus proteins are about 21 kDa less than *At*-ACA8 (Bonza et al., 2010).

Experiments to determine if the two viral proteins are functional Ca^{2+}- transporting ATPases were more difficult than the other viral transport proteins. Initial experiments established that M535L complemented growth of mutant yeast K616 on Ca^{2+}-depleted medium (Bonza et al., 2010). This assay is routinely used to test for the function of eukaryotic Ca^{2+}-transporting ATPases and the positive complementation by the viral M535L protein suggests that it is a fully active Ca^{2+} pump. This conclusion was supported by the finding that M535L also complemented growth of the K616 mutant on toxic concentrations of Mn^{2+}. Transport of Mn^{2+} and Ca^{2+} is a property of many Ca^{2+}-transporting ATPases and this may also be true for M535L even though the amino acid sequence of the viral protein differs from other Mn^{2+}/Ca^{2+}-transporting ATPases (Bonza et al., 2010).

While the complementation studies suggest an ATPase function for the viral proteins, neither Ca^{2+} nor Mn^{2+} dependent ATPase activity was detected with the purified protein (Bonza et al., 2010). For other ATPases the latter assay is considered the gold standard for proof of activity. One plausible explanation for this negative result is that the viral protein is rather non-selective and may also transport Mg^{2+}. Since Mg^{2+} is an unavoidable ingredient in ATPase assays the pump may already be active in the absence of Ca^{2+} or Mn^{2+} so that no additional activity is detected after adding the two divalent cations. Another possibility is that the kinetic cycle of the ATPase includes some uncoupling; thus ATP may be cleaved without ion translocation. This process, referred to as slippage, occasionally occurs in ATP-driven ion pumps (Dalton et al., 1999) and slippage might also occur in the viral pump when tested in vitro. The ability of *m325l* to complement the yeast K616 phenotype in Ca^{2+}-depleted media, however, indicates that the protein is at least working as an active ion pump in vivo, which is not completely uncoupled.

The interpretation of the functional assays that M535L forms an active pump agrees with other indirect evidence, which also support a function for the viral ATPases. One argument in favor of a function is that the *m535l* gene is expressed during viral replication. Northern blot analysis indicated strong transcription of the *m535l* gene by 15 min p.i., followed by a decrease with time (Bonza et al., 2010) (Fig. 3). Further evidence for a functional role is that the gene is present in 45 of 47 Pbi chloroviruses examined. The gene is also present in some members of all three chlorovirus types. A common, but not universal, presence of the gene in the chloroviruses suggests that the function of the protein is supportive but not mandatory for virus infection/replication. The suggestion of an auxiliary function is consistent with the fact that the gene is not present in all chloroviruses that infect different hosts.

A BLAST analysis shows that the proteins most similar to the viral Ca^{2+}-transporting ATPases are from green algae *Chlamydomonas reinhardtii* and *C. variabilis*; the algal proteins have 43% amino acid identity with the viral proteins (Table 1).

Ligand gated channel like proteins

Ligand gated ion channels open and conduct currents in response to binding specific chemical messengers like neurotransmitters (Hille, 2001). Surprisingly, chlorovirus PBCV-1 encodes two proteins (A162L and A163R), which resemble typical ligand-gated ion channels (Kang et al., 2003). Structural predictions indicate that A162L and A163R have three transmembrane domains with spacing that resembles glutamate receptors. In neurons glutamate receptors mediate an influx of cations including K^+, Na^+ and Ca^{2+} after binding glutamate to a specific, extracellular attachment site (Traynelis et al., 2010). Comparative analysis of the viral proteins with eukaryotic glutamate receptors indicates that this binding domain is missing in the two viral proteins. Hence the two virus PBCV-1 genes encode part of the channel pore of their eukaryotic homologs but lack the glutamate-binding domain. They may bind another unknown ligand.

Currently no experimental evidence exists that indicates the two viral proteins function as ion channels. No currents were detected that correlated with the presence of either of the two proteins (Kang et al., 2003) when the two mRNAs were injected into *Xenopus* oocytes. These results do not necessarily mean that the viral proteins do not function as channels per se because the experimental conditions might not have been suitable for stimulating channel function. For example, a putative plant glutamate receptor is activated by methionine and other bulky amino acids, but not by glutamate (Tapken et al., 2013). Also it is possible that the channels only function as heteromers.

The two PBCV-1 genes were transcribed, beginning at 45 min p.i., and reached a peak at about 90 min p.i., before their mRNAs disappeared between 180 and 240 min p.i. (Fig. 3) (Kang et al.,

2003). This expression pattern classifies the genes as early/late genes and sometimes early/late gene products are incorporated into newly synthesized virions. However, a proteomic analysis did not detect either protein in the PBCV-1 virion (Dunigan et al., 2012).

The putative ligand gated channels are restricted to the NC64A viruses, which infect *C. variabilis* (Fig. 2). Gene *a162l*, is only present in virus PBCV-1, whereas gene *a163r* homologs are present in nine NC64A viruses. A BLAST search of Genbank did not identify any proteins with strong similarity to A162L and A163R. The closest relatives to the two proteins are not from algae or from higher plants but from animals (Table 1). Hence there is no evidence of a relationship between the two viral proteins and those of the current algal host *C. variabilis*.

Chlorovirus transport proteins and the evolution of giant viruses

As noted in the introduction the chloroviruses are believed to have a common evolutionary origin with several other large DNA virus families and collectively these viruses are referred to as NCLDVs (Iyer et al., 2001, 2006; Yutin et al., 2009; Yutin and Koonin, 2012). Although it is reasonably well accepted among virologists that NCLDVs have a common evolutionary origin, the origin of these giant viruses has been the subject of a heated debate between proponents suggesting that giant viruses evolved from smaller viruses by adding genes (e.g., Filee et al., 2008, Filee, 2013; Moreira and Brochier-Armanet, 2008; Yutin et al., 2014) versus scientists proposing that they evolved from larger entities by loosing genes (e.g., Raoult et al., 2004; Boyer et al., 2010; Claverie and Abergel, 2009, 2010, 2013; Legendre et al., 2012). Proponents of the latter hypothesis have suggested that they might represent a fourth domain of life. An extended version of this latter viewpoint is that their ancestor was originally a "cell-like" organism (e.g., Claverie and Abergel, 2013). A discussion of the pros and cons of these various hypotheses is beyond the purview of this paper and will not be discussed here. However, we do want to point out that if the NCLDV ancestor was a "cell-like" organism, the ancestor(s) was presumably enclosed in a membrane and it would have been necessary to transport ions and other molecules across this membrane. In fact, the appearance of proteins involved in the transport of ions and molecules across membranes is considered to have been an early essential step in the evolution of the most primitive forms of life (e.g., Franciolini and Petis, 1989; Pohorille et al., 2005).

If the NCLDVs did evolve from some ancient "cell-like" organism, one might predict that the organism was autonomous, not only with respect to its genetic machinery but also with respect to energy metabolism, membranes and transport of ions. The presence of a variety of chlorovirus-encoded transport proteins, e.g., an ATPase for active transport and different proteins for selective passive transport, is consistent with the view that the NCLDV ancestor was surrounded by an external membrane. The viral ATPase would be able to generate a membrane voltage and this voltage could have been used for selective ion fluxes via K^+ channels and transporters. Aquaglyceroporins could have been employed to catalyze fluxes of small solutes. Hence the set of membrane transport proteins, which are encoded by chloroviruses, would have been sufficient to provide the essential transport properties essential for a membrane-enclosed simple form of cellular life.

However, there are problems with this scenario. If this scenario is correct then all of the chlorovirus transport proteins would be expected to be near the base of their phylogenetic trees. Support for this hypothesis is provided by the virus-encoded K^+ channel Kcv because we have suggested that Kcv or something similar to Kcv was the ancestor of all K^+ channels (Thiel et al., 2013). Currently the two putative ligand-like gated channels do not have any close relatives in Genbank (Table 1) and they could possibly be near the base of a phylogenetic tree. However, phylogenetic analyses of the three other chlorovirus encoded transport proteins do not support this hypothesis. The chlorovirus Ca^{2+}-transporting ATPase is clearly related to algal Ca^{2+}-transporting ATPases (Table 1, Bonza et al., 2010), the K^+ transporter protein is similar to proteins from plants, algae and fungi (Table 1, Greiner et al., 2011), and the aquaglyceroporin is similar to proteins from bacteria and algae (Table 1, Gazzarrini et al., 2006). Therefore, we conclude that the chlorovirus encoded transport proteins are interesting and presumably serve an important role during virus infection. However, their existence cannot be used as evidence in support of the concept that these viruses evolved from a "cell-like" organism.

Conclusions

The chloroviruses are unusual in that collectively they encode four, and possibly six, proteins involved in moving "materials" across membranes. These include a K^+ channel protein, a K^+-transporting protein, an aquaglyceroporin channel protein, a Ca^{2+}-transporting ATPase protein, and two potential ligand channel proteins. With the exception of the two putative ligand channel proteins, the other four proteins are functional because they form active transporters in heterologous systems. None of the six transporters are encoded by all 41 chloroviruses that have been completely sequenced and annotated (Jeanniard et al., 2013); therefore one can question how essential their roles are in the viral life cycles. The one possible exception is the K^+ channel protein because an entire gene is present in 39 of the 41 viruses and the Kcv channel appears to serve an important function early in viral infection (Thiel et al., 2010; Romani et al., 2013).

Acknowledgments

This work was supported by the Deutsche Forschungsgemeinschaft Grant TH558127-1 (GT), the Stanley Medical Research Insitute Grant 11R-0001 (JVE, DDD), National Science Foundation-EPSCoR Grant EPS-1004094 (JVE), the COBRE program of the National Center for Research Resources Grant P20-RR15635 (JVE) and by PRIN (Programmi di Ricerca di Rilevante Interesse Nazionale) 2010CSJX4F and MAE (Ministero Affari Esteri) 01467532013-06-27 (AM).

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