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Fast and selective transport of water through cell membranes is facilitated by water channels. Water channels belonging to the major intrinsic proteins (MIPs) family have been found in all three domains of life, Archaea, Bacteria, and Eukarya. Here we show that Chlorella virus MT325 has a water channel gene, apg1, that forms a functional aquaglyceroporin in oocytes. apg1 is transcribed during infection together with MT325 kcv, a gene encoding a previously undescribed type of viral potassium channel. Coexpression of AQPV1 and MT325-Kcv in Xenopus oocytes synergistically increases water transport, suggesting a possible concerted action of the two channels in the infection cycle. The two channels operate by a thermodynamically coupled mechanism that simultaneously alters water conductance and driving force for water movement. Considering the universal role of osmosis, this mechanism is relevant to any cell coexpressing water and potassium channels and could have pathological as well as basic physiological relevance.

Transport proteins facilitate fast passage of water, ions, and nutrients through membranes and are found in all living cells. Transport proteins are also encoded by some viruses, which are noncellular biological entities. Because viruses take over host cell functions to reproduce, transport proteins are probably used to modify the host cell environment to benefit virus replication. Viral transport proteins, known as viroporins, also exist in human pathogens such as HIV and influenza viruses (1). Viroporins are usually short peptides (<100 aa) with no structural analogies to prokaryotic and eukaryotic transport proteins. However, viroporins encoded by the Phycodnaviridae family (2) of nucleocytoplasmic large dsDNA viruses that infect algae are an exception. Genes encoding prokaryotic and eukaryotic-like membrane channels are present in some of the phycodnaviruses. We have previously shown that Paramecium bursaria Chlorella virus (PBCV-1), the prototype Chlorella virus (genus Chlorovirus), encodes a gene for a functional potassium ion (K⁺) channel, Kcv (3, 4). Functional Kcv-like channels exist in 40 other Chlorella viruses infecting the same host (Chlorella NC64A) (5), as well as in another phycodnavirus, Ectocarpus siliculosus virus (6), which infects a marine filamentous brown alga. Here we report that phycodnavirus MT325, which infects Chlorella Pbi, has a functional water channel (ORF M30R) and a previously undescribed type of Kcv-like functional K⁺ channel (ORF M183R), and that together these two channels improve water transport in Xenopus oocytes.

Results and Discussion

Virus MT325 ORF M30R encodes a 270-aa protein with homology to the major intrinsic proteins (MIPs) family. MIPs are present in all living organisms (7, 8), where they form water channels either selective for water (aquaporins) or glycerol-conducting channels, which also transport water, although to a lesser extent (aquaglyceroporins) (9). The viral MIP-like protein, named AQPV1 (aquaglyceroporin virus 1), was aligned and compared with the best-studied member of each group, aquaporin AQP1 (10, 11) from human and aquaglyceroporin GlpF (12) from Escherichia coli (Fig. 1a). AQPV1 has the main features of MIPs: six transmembrane domains (Fig. 1b) and the signature sequence Asn-Pro-Ala (NPA, highlighted in Fig. 1a) in both the amino- and carboxyl-terminal portions of the protein. These sequences are located in the channel pore of AQP1 and GlpF and play a crucial role in the orientation of water and glycerol molecules (13, 14). The narrowest part of the pore (the selectivity filter) is formed by four conserved amino acids with different physicochemical properties in aquaporins and aquaglyceroporins (12–15). AQPV1 has one polar (R217) and three nonpolar residues (F49, V202, and I211) (arrowheads in Fig. 1a) resembling the selectivity filter of aquaglyceroporins in these positions (15). Furthermore, AQPV1 contains five residues (in gray in Fig. 1a) that are conserved in aquaglyceroporins, but not in aquaporins (16, 17). Accordingly, a phylogenetic tree of aquaporins and aquaglyceroporins, places AQPV1 in the aquaglyceroporin cluster (Fig. 1c).

We performed hypotonic swelling assays in Xenopus oocytes injected with apg1 cRNA to determine whether AQPV1 forms a functional water-conducting channel. The volume changes in oocytes were monitored in an osmotic gradient (∆Osm = 105 milliosmolar) to quantify water permeability (Pᵢ). Fig. 2a shows that the Pᵢ value (in 10⁻⁶ cm/s) of AQPV1-injected oocytes was 5 times higher than control oocytes (AQPV1 58 ± 3.5; control 11.2). However, it was only ~40% as high as AQP1-injected oocytes (151 ± 5), which were used as a reference for aquaporin-mediated water permeability (18). The water permeability of AQPV1 is high for aquaglyceroporins, which, with few exceptions (19), generally have ~1/5 the water permeability of aquaporins (14, 20).

A point mutation N214A in the second AQPV1 NPA motif abolished the increase in Pᵢ (6 ± 0.6). This result confirms the conclusion that the enhanced water permeability in AQPV1-injected oocytes is specifically induced by expression of this protein. Mercury, a classic inhibitor of water channels, reduced the Pᵢ value of AQPV1-injected oocytes about 50% (Inset in Fig. 2a). This inhibition was partly reversed by the reducing com-

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Abbreviations: AQPV1, aquaglyceroporin virus 1; Osm, osmolar; PBCV-1, Paramecium bursaria Chlorella virus.

Data deposition: The sequences reported in this paper have been deposited in the GenBank database (accession nos. DQ195162 for AQPV1 and DQ195163 for MT325-Kcv).

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and AqpZ, E. coli Arabidopsis thaliana TIP1.1, glycerol uptake rate (V of AQPV1 abolishes both water and glycerol transport. The 15 min). Hence the same mutation in the conserved pore domain were obtained for oocytes expressing AQP1 (66 oocyte per 15 min), a water channel not permeable to glycerol consistent with simple membrane diffusion. Similar low values in control oocytes (53 oocyte per 15 min) (Fig. 2). A comparative alignment, predicted membrane topology, and phylogeny of AQPV1. (a) Multiple sequence alignment performed with CLUSTALW (1.82) of the deduced amino acid sequences of AQPV1 (GenBank accession no. DQ195162), the human aquaporin AQP1 (NP.932766) and the aquaglyceroporin GlpF from E. coli (NP.418362). Bold lines indicate the six transmembrane domains, TM1–TM6. The two NPA sequences, conserved in all water-channel proteins, are highlighted in black. Arrowheads identify four residues, F49, V202, I211, and R217, that are predicted to be part of the AQPV1 selectivity filter. Highlighted in gray are five amino acids highly conserved among aquaglyceroporins (16, 17). The asterisk indicates the cysteine residue responsible for AQP1 sensitivity to mercury (21). (b) Hydropathy profile of the deduced AQPV1 amino acid sequence predicted by the TMHMM program (www.cbs.dtu.dk/services/TMHMM/). The mean hydrophobicity index was computed according to the algorithm of Kyte and Doolittle (29) with a window of 17 residues. Putative membrane-spanning domains are numbered from I to VI and correspond to TM1–TM6 shown in a. (c) Neighbor-joining unrooted phylogenetic tree of 15 water channel protein sequences, including AQPV1. Multiple alignment was performed by using CLUSTAL with default settings. Phylogenetic analysis was performed by using PAUP 4.0b10. Numbers above the nodes are bootstrap values (1,000 replicates). Sequences are as follows: GlpF, E. coli (NP.418362); AQP3, Homo sapiens (CAI13311); TbAQP1, Trypanosoma brucei (AA697889); GLA, Lactococcus lactis (P22094); cGlpF, Clostridium acetobutylicum (AA79288); AQPV1, MT325 Phycodnaviridae (DQ195162); MbAqp, Methanosarcina Barkeri (ZP.00077803); AqpM, Methanobacterium thermautotrophicus (AB055880); THaAqp, Archaeoglobus fulgidus (NP.070255); TIP1.1, Arabidopsis thaliana (P25818); AQP8, H. sapiens (NP.001160); AQP1, H. sapiens (NP.932766); PIP1.1, A. thaliana (P61837); AqpX, Brucella abortus (Q9LA79); and AqpZ, E. coli (AAC34318). All proteins (except cGlpF) have been determined experimentally to be aquaporins or aquaglyceroporins.

Fig. 1. Comparative alignment, predicted membrane topology, and phylogeny of AQPV1. (a) Multiple sequence alignment performed with CLUSTALW (1.82) of the deduced amino acid sequences of AQPV1 (GenBank accession no. DQ195162), the human aquaporin AQP1 (NP.932766) and the aquaglyceroporin GlpF from E. coli (NP.418362). Bold lines indicate the six transmembrane domains, TM1–TM6. The two NPA sequences, conserved in all water-channel proteins, are highlighted in black. Arrowheads identify four residues, F49, V202, I211, and R217, that are predicted to be part of the AQPV1 selectivity filter. Highlighted in gray are five amino acids highly conserved among aquaglyceroporins (16, 17). The asterisk indicates the cysteine residue responsible for AQP1 sensitivity to mercury (21). (b) Hydropathy profile of the deduced AQPV1 amino acid sequence predicted by the TMHMM program (www.cbs.dtu.dk/services/TMHMM/). The mean hydrophobicity index was computed according to the algorithm of Kyte and Doolittle (29) with a window of 17 residues. Putative membrane-spanning domains are numbered from I to VI and correspond to TM1–TM6 shown in a. (c) Neighbor-joining unrooted phylogenetic tree of 15 water channel protein sequences, including AQPV1. Multiple alignment was performed by using CLUSTAL with default settings. Phylogenetic analysis was performed by using PAUP 4.0b10. Numbers above the nodes are bootstrap values (1,000 replicates). Sequences are as follows: GlpF, E. coli (NP.418362); AQP3, Homo sapiens (CAI13311); TbAQP1, Trypanosoma brucei (AA697889); GLA, Lactococcus lactis (P22094); cGlpF, Clostridium acetobutylicum (AA79288); AQPV1, MT325 Phycodnaviridae (DQ195162); MbAqp, Methanosarcina Barkeri (ZP.00077803); AqpM, Methanobacterium thermautotrophicus (AB055880); THaAqp, Archaeoglobus fulgidus (NP.070255); TIP1.1, Arabidopsis thaliana (P25818); AQP8, H. sapiens (NP.001160); AQP1, H. sapiens (NP.932766); PIP1.1, A. thaliana (P61837); AqpX, Brucella abortus (Q9LA79); and AqpZ, E. coli (AAC34318). All proteins (except cGlpF) have been determined experimentally to be aquaporins or aquaglyceroporins.

pound 2-mercaptoethanol. The moderate inhibition of AQPV1 by this high (1 mM) concentration of mercury can be partly explained by the absence of a key cysteine residue (21) located near the carboxyl NPA motif of AQP1 (asterisk in Fig. 1a).

Glycerol uptake in AQPV1-injected oocytes was measured by incubating the cells in a solution containing 1 mM radiolabeled glycerol (1 μCi/ml [U-14C]glycerol; 1 μCi = 37 kBq). AQPV1-injected oocytes accumulated glycerol rapidly (271 ± 8 pmol per oocyte per 15 min) (Fig. 2b). In contrast, glycerol uptake was low in control oocytes (53 ± 5 pmol per oocyte per 15 min), consistent with simple membrane diffusion. Similar low values were obtained for oocytes expressing AQP1 (66 ± 6 pmol per oocyte per 15 min), a water channel not permeable to glycerol and for the AQPV1 N214A mutant (23 ± 3 pmol per oocyte per 15 min). Hence the same mutation in the conserved pore domain of AQPV1 abolishes both water and glycerol transport. The glycerol uptake rate (V, pmol per oocyte per min) was linearly related to glycerol concentration in the solution (Inset in Fig. 2b).

These results are qualitatively and quantitatively similar to those reported for other aquaglyceroporins (22).

 Often water and K+ channels are physically and functionally related to serve specific physiological functions in animal and plant cells (23, 24). The best-studied example is glial cells (23), where disturbances in the coupling between K+ and water fluxes results in pathological formation of edema in retina and brain (25). Virus MT325 ORF M183R encodes a 95-aa protein, named MT325-Kcv, with ≈50% amino acid identity to the Kcv family of viral K+ channels (3). Therefore, we determined if AQPV1 and MT325-Kcv might exhibit a functional relationship. However, it was first necessary to determine whether MT325-Kcv produced a functional K+ channel in oocytes. The predicted structure of the 95-aa MT325-Kcv differs significantly from the 94-aa prototype viral K+ channel PBCV-1-Kcv (Fig. 3a). MT325-Kcv lacks a functional K+ channel in oocytes. The predicted structure of the 95-aa MT325-Kcv differs significantly from the 94-aa prototype viral K+ channel PBCV-1-Kcv (Fig. 3a). MT325-Kcv lacks a functional K+ channel in oocytes. The predicted structure of the 95-aa MT325-Kcv differs significantly from the 94-aa prototype viral K+ channel PBCV-1-Kcv (Fig. 3a). MT325-Kcv lacks
Despite these apparent structural differences, MT325-Kcv expression in oocytes produces a functional outwardly rectifying ion channel (Fig. 3b and c). It is inhibited in a voltage-dependent manner by the classical K+ channel blocker Ba2+ (n = 10). (b) Glycerol uptake kinetics in oocytes expressing AQPV1 (●), AQPV1 (▲), N214A AQPV1 (○), and control (water-injected) (□) oocytes. External glycerol concentration was 1 mM (1 μCi/ml [14C]glycerol). Data are means (n = 10) ± SE of three independent experiments. When absent, SEs are within the symbols. (Inset) Effect of substrate concentration on glycerol uptake in AQPV1-injected oocytes. Initial velocity (V_i, pmol per oocyte per min) was determined from the initial slope of the time course of glycerol uptake experiments performed with 1, 3, and 5 mM glycerol. Values are means of five oocytes; SEs are within the symbols.

Fig. 2. Osmotic water permeability and glycerol uptake in AQPV1-injected oocytes. (a) Osmotic water permeability (P_f) of AQPV1-injected oocytes and comparison with human AQP1-injected oocytes. The mutation N214A in the highly conserved NPA motif reduces the P_f values of AQPV1-injected oocytes below basal level (control: water-injected oocytes). Data are means (n = 16) for AQP1, AQPV1, N214A AQPV1, and water, respectively ± SE of seven different experiments. (Inset) Pretreating AQPV1-injected oocytes with 1 mM HgCl2 for 10 min reduced the P_f value by 50%. Partial recovery was observed after exposing the oocytes to 5 mM 2-mercaptoethanol (dMe) (n = 10). (b) Glycerol uptake kinetics in oocytes expressing AQPV1 (●), AQPV1 (▲), N214A AQPV1 (○), and control (water-injected) (□) oocytes. External glycerol concentration was 1 mM (1 μCi/ml [14C]glycerol). Data are means (n = 10) ± SE of three independent experiments. When absent, SEs are within the symbols. (Inset) Effect of substrate concentration on glycerol uptake in AQPV1-injected oocytes. Initial velocity (V_i, pmol per oocyte per min) was determined from the initial slope of the time course of glycerol uptake experiments performed with 1, 3, and 5 mM glycerol. Values are means of five oocytes; SEs are within the symbols.
higher than the sum of the \( P_i \) values from individual MT325-Kcv- and AQPV1-injected oocytes. Barium inhibited the synergistic effect produced by the two channels, indicating that MT325-Kcv stimulated water uptake (Fig. 3d).

How does an open \( K^+ \) channel increase water flow? In principle water flow can be elevated either by an increase in water conductance or by an increase in the osmotic driving force. MT325-Kcv does not transport water directly because \( K^+ \) channel conductance in the virus infection and hybridized to AQPV1. To study this possibility, RNA was extracted from cells at various times after MT325 infection and hybridized to AQPV1 and MT325-Kcv. The 0.35-kb band, which is the size expected for a 95-aa gene, also has a \( K^+ \) channel clamps the \( E_{m} \) value at \( 120 \) mV, assuming that both channels are required for virus infection, AQPV1 and MT325-Kcv genes should be present in other viruses that infect \( Chlorella \) Pbi (Pbi viruses). To address this question we hybridized RNA from all of the Pbi viruses, but not the host (Fig. 5, which is \( K^+ \) channel, which in our conditions is close to zero. Net efflux of \( K^+ \) and \( Cl^- \) is allowed and endogenous osmoregulation can occur. In coinfected oocytes the presence of an open \( K^+ \) channel clamps the \( E_{m} \) value at \( 15 \) min postinfection (p.i.), after which it decreased. Assuming the virus MT325 replication cycle is similar to that of virus PBCV-1, MT325 DNA synthesis begins 60–90 min p.i. Thus AQPV1 is expressed as both an early and late gene. The \( 1.2 \)-kb transcript is an appropriate size for a 270-aa protein. In comparison with transcription of \( aqpv1 \), that of the MT325 kcv gene is more complex. A kcv gene probe hybridized with four RNA bands, 1.8, 1.5, 0.65, and 0.35 kb, beginning about 45–60 min p.i. The three larger bands increased in intensity to 120 min p.i., after which they decreased. The 0.35-kb band, which is the size expected for a 95-aa protein, peaks in intensity at 480 min p.i. (Note: The PBCV-1 kcv gene also has a complex transcription pattern; however, a 0.35-kb RNA is expressed late and is the only one that is capped, indicative of a functional mRNA [32].)

Fig. 5. Transcription pattern of \( aqpv1 \) and MT325 kcv in the host \( Chlorella \) Pbi infected with virus MT325. (a) \( Chlorella \) Pbi (host) and Pbi virus MT325 RNAs hybridized with an \( aqpv1 \) dsDNA probe. (b) \( Chlorella \) Pbi (host) and Pbi virus MT325 RNAs hybridized with MT325 kcv dsDNA probe. The \( aqpv1 \) probe hybridized to a 1.2-kb band beginning at 15 min postinfection (p.i.) and increased in intensity to 120 min p.i., after which it decreased. The 0.35-kb band, which is the size expected for a 95-aa protein, peaks in intensity at 480 min p.i. (Note: The PBCV-1 kcv gene also has a complex transcription pattern; however, a 0.35-kb RNA is expressed late and is the only one that is capped, indicative of a functional mRNA [32].)
Conclusions

Recent research has revealed the presence and functional importance of ion-conducting channels in several viruses, including a few that are medically important (1). The present work reveals that a virus encodes a functional water channel with a possible role in virus infection/replication. Our experiments also reveal a synergistic relationship between water and ion channels that provides the virus with a mechanism that controls transmembrane water fluxes by affecting water conductance and driving force. This mechanism could be relevant in any step in the life cycle of this and any other virus that involves osmotically driven processes. Furthermore, the coupling of K+ conductance and water fluxes exemplifies a basic biological principle relevant to any physiological and pathophysiological situation involving cell volume changes and regulation.

Materials and Methods

Oocyte Expression. Capped cRNAs of AQP1, AQPV1, and MT325-Kcv were prepared as described in ref. 3. The point mutation in AQPV1 was created with the QuickChange site-directed mutagenesis kit (Stratagene). Oocytes were prepared from female Xenopus laevis as described (3) and injected with 50 nl of water or 10 ng of cRNAs (0.2 μg/μl). In co-injected oocytes, 10 ng of each cRNA was injected. Oocytes were incubated at 19°C in ND96 solution (96 mM NaCl/2 mM KCl/1.8 mM CaCl2/1 mM MgCl2/5 mM Heps, adjusted to pH 7.5 with NaOH). The experiments were performed 3–4 days after injection.

Standard Oocyte Swelling Assays and Pf Calculations. The oocytes were placed in a chamber with 400 μl of ND96 solution (210 mOsm). At time 0, 200 μl of the solution was replaced by 200 μl of water to give a final osmolarity of 105 mOsm. The increase in oocyte volume was monitored at room temperature under an inverted microscope (Zeiss IM) with a ×2.5 magnifying objective lens and recorded by a charge-coupled device camera (Sony) connected to a computer through a WinTV-USB video-digitizer (Hauppauge, Hauppauge, NY). Two-minute movies of oocyte swelling were made with a sampling rate of one image per second. From sequential images, the surface areas of equatorial sections were estimated using software designed for Research Resources Grant P20-RR15635 (to J.L.V.E.), and by Minority Supplement to the Center for Biomedical Research Excellence program of the National Center for Research Resources Grant P20-RR15635 (to J.L.V.E.), and by Minis-
Figure 6- Effect of hypoosmotic treatment on current/voltage relation of an oocyte co-injected with MT325-Kcv and AQPV1. To determine if MT325-Kcv is activated by cell swelling, we monitored MT325-Kcv currents in oocytes co-expressing MT325-Kcv and AQPV1. Oocytes were exposed to the same osmotic conditions used to estimate P₀. Control solution (○) contained 20 mM KCl, 1.8 CaCl₂, 1 MgCl₂, 5 HEPES, pH 7.4 with KOH; mannitol was used to adjust the osmolarity to 215 mOsm. Hypoosmotic solution (●) same as control but with osmolarity adjusted to 105 mOsm. Measurements were taken 10 min after exposing the oocyte to the indicated solutions. The slight changes in current with the hypoosmotic step are due to a positive shift (mean 8 ± 2 mV, n=5) of the reversal potential; this shift is probably due to the dilution of internal [K⁺] by the incoming water. This shift was reversed completely by restoring control conditions (not shown). Collectively the data show that MT325-Kcv is not stretch-activated.
Figure 7- Presence of *aqpv1* and MT325 *kcv* genes in other viruses that infect *Chlorella* Pbi (Pbi viruses). The genomic DNA from 47 Pbi viruses was hybridized with *aqpv1*- and