A single aquaporin gene encodes a water/glycerol/urea facilitator in *Toxoplasma gondii* with similarity to plant tonoplast intrinsic proteins[☆,1]

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Abstract **We describe a single aquaporin gene in *Toxoplasma gondii* which, surprisingly, has only 28% sequence similarity to the aquaglyceroporin of another apicomplexan parasite, *Plasmodium falciparum*. Sequence comparisons showed 47% similarity to water-specific plant aquaporins and the conservation of typical pore-forming residues. We established that the *Toxoplasma* aquaporin protein is a bifunctional membrane pore with intermediate water and high glycerol permeability. Furthermore, we identified hydroxyurea, an antineoplastic agent with inhibitory effects on parasite proliferation, as a permeant of this channel.**

Key words: Aquaglyceroporin; Water; Glycerol; Hydroxyurea; *Toxoplasma*; *Plasmodium*

1. Introduction

Intracellular parasites have minimized their interface with the host cell to achieve nutrient uptake and waste exit with the least possible number of transporters. For example, the genome of *Plasmodium falciparum* contains only six proteins of the major facilitator superfamily compared to 60–80 members in similarly complex yeast genomes [1]. Few transmembrane proteins favorably lower the antigenicity of the parasite surface but restrict its choice of potential host cells.

Recently, we have biochemically characterized the single aquaglyceroporin from *P. falciparum* (PfAQP) [2]. It is an excellent water channel probably protecting the integrity of the parasite membrane by balancing osmotic stress as it occurs during kidney passage. In addition, it is also a highly permeable glycerol facilitator and thus provides access to the serum glycerol pool.

Toxoplasma and *Plasmodium* parasites are unicells of the phylum Apicomplexa [3]. *Plasmodium* multiplies exclusively in erythrocytes, whereas *Toxoplasma gondii* invades various cells where it replicates rapidly (tachyzoite stage). Later on, brain and muscle cells harbor dormant bradyzoites which can persist life-long in the host [4]. Although *Toxoplasma* has to adapt to conditions of diverse host cells, osmotic stress is rarely encountered. Here, we report the cloning and an initial functional characterization of the single aquaporin encoded in the genome of *T. gondii* and its comparison to the malaria aquaglyceroporin.

2. Materials and methods

2.1. Cloning and expression of TgAQP M_1 and TgAQP M_{39}

The coding sequences of *T. gondii* aquaporin (TgAQP) M_1 and TgAQP M_{39} were amplified by polymerase chain reaction from *T. gondii* genomic DNA and subcloned for cRNA synthesis into pOG2, a pBSTA-derived vector containing parts of the 5′ and 3′ untranslated regions of the *Xenopus* β-globin gene. Further, to the N-terminus of both TgAQP constructs a c-myc epitope tag (MEQKLISEEDL) plus a seven or eight amino acid linker, respectively, for TgAQP M_1 (NSSETAS) and TgAQP M_{39} (NSEHLIFN) was added by insertion of an annealed primer pair. For cRNA synthesis T7 RNA polymerase was used (mMESSAGE mMACHINE transcription kit, Ambion). Defolliculated oocytes of stages V and VI were injected with 5 ng of cRNA (TgAQP M_1 and TgAQP M_{39}; for water permeability) or a combination of 2.5 ng TgAQP with 2.5 ng rat AQP1 cRNA (for solute permeability). Oocytes were incubated in ND96 buffer (96 mM NaCl, 2 mM KCl, 1.8 mM $CaCl_2$, 1 mM $MgCl_2$, 5 mM HEPES, pH 7.4) at 15°C for 3 days before testing.

2.2. Western blot analysis

Oocytes were lysed and homogenized in hypotonic Na_2HPO_4 buffer (7.5 mM, pH 7.5) with protease inhibitors (Complete, Roche Molecular Biochemicals). Oocyte membranes were collected by fractionated centrifugation ($500\times g$, 5 min, and $27\,000\times g$, 30 min, 4°C). Per lane, protein equivalent to a single oocyte was electrophoresed into 15% sodium dodecyl sulfate–polyacrylamide gels. Proteins were transferred to polyvinylidene difluoride membranes (Macherey and Nagel), incubated with anti-c-myc antiserum (1:500, rabbit polyclonal, Santa Cruz Biotechnologies) and detected with horseradish peroxidase-conjugated goat anti-rabbit antiserum (Dianova) using the ECL Plus system (Amersham Bioscience).

2.3. Standard oocyte swelling assays

Oocytes were transferred into 1:3 diluted ND96 for measuring water permeability or into isosmotic ND96 where 65 mM NaCl was replaced by 130 mM of a non-ionic test solute for assaying solute permeability. For ionic solutes 96 mM NaCl was replaced by the salt of the solute. Swelling assays were carried out at room temperature and were video monitored using a 2.5× objective. Osmotic water permeability (P_f, in μm/s) was calculated from the oocyte surface area ($S = 0.045$ cm^2), the initial oocyte volume ($V_0 = 9\times10^{-4}$ cm^{-2}), the initial slope of the relative volume increase ($d(V/V_0)/dt$ in s^{-1}), the molecular water volume (18 cm^3/mol) and the osmotic gradient ($osm_{in}-osm_{out}$) by the following equation: $P_f = V_0\times d(V/V_0)/dt/[S\times V_w\times(osm_{in}-osm_{out})]$. For comparison of solute permeability the initial swelling rates ($d(V/V_0)/dt$) were plotted.

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[1] Nucleotide sequence data are available in the DDBJ/EMBL/GenBank databases under accession number AJ581909.

Abbreviations: AQP, aquaporin; GlpF, *Escherichia coli* glycerol facilitator; TIP, tonoplast intrinsic protein

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3. Results

3.1. Sequence analysis of TgAQP

Using BLAST searches we identified a single putative aquaporin gene in the assembled *T. gondii* genome which we refer to as TgAQP. Two potential ATG start codons were present in the same reading frame at nucleotide positions 1 and 115 which translated into proteins TgAQP M_1 (29.9 kDa) and TgAQP M_{39} (26.3 kDa; Fig. 1A), respectively. The boundaries of the coding region were characterized by an in-frame TAA stop codon 54 bp upstream of the first ATG and a 318 bp purine-rich stretch containing 250 adenine or guanine residues (79%) in the immediate 3′ untranslated region. TgAQP is most similar to water-specific tonoplast intrinsic proteins (TIP) from plants (24% identity, 47% similarity) [5], mammalian orthodox aquaporins, e.g. AQP1 (21% identity, 39% similarity) [6], and to glycerol facilitators, such as *Escherichia coli* GlpF (19% identity, 34% similarity) [7]. The level of similarity is reflected by the 'Expect' (*E*) values from the BLAST analysis [8] which vary by four orders of magnitude (Fig. 1B). The pore-forming residues around the canonical NPA motifs are highly conserved between TgAQP and TIP channels including a characteristic valine at the pore mouth (asterisk in Fig. 1B) where other aquaporins almost exclusively have a positively charged arginine [9].

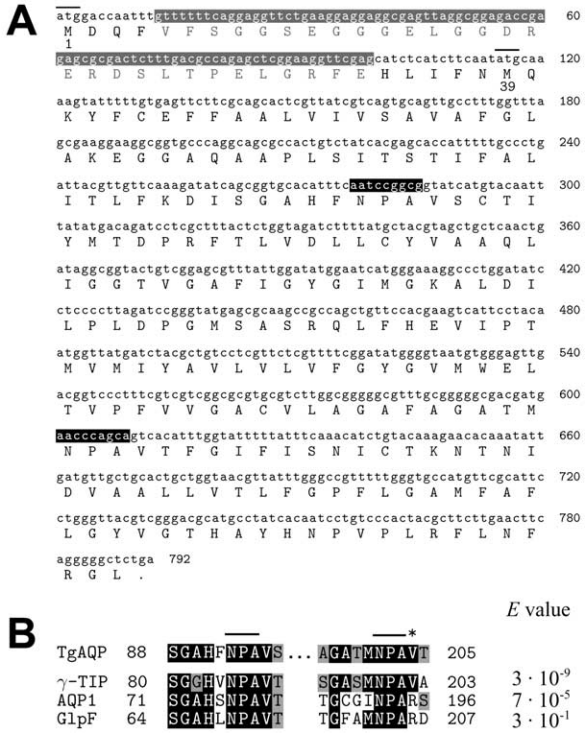

Fig. 1. Sequence analysis of the *Toxoplasma* aquaporin gene. A: The open reading frame is shown with two potential ATG start codons in the same reading frame (overlined). The canonical NPA motifs are shaded black. An in-frame intron is possibly located in the 5′ region (gray shading). B: Sections of a sequence alignment of prototypical aquaporins. NPA motifs are overlined, the asterisk denotes a residue which is usually a highly conserved arginine at the pore mouth but is changed to valine in TgAQP and TIPs. The Expect (*E*) values from a BLAST search with TgAQP as a query are indicated on the right.

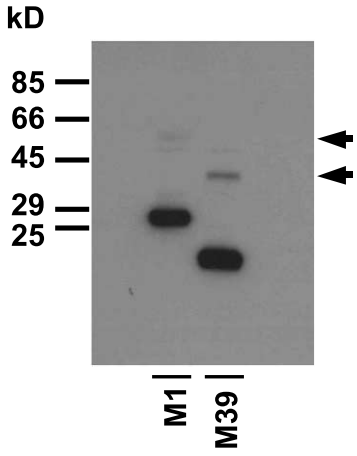

Fig. 2. Immunoblot of c-myc-tagged TgAQP constructs M_1 and M_{39} expressed in *Xenopus laevis* oocytes. Protein dimers are labeled by arrows.

3.2. TgAQP is a water and glycerol facilitator

Constructs of TgAQP M_1 and TgAQP M_{39} with an N-terminal c-myc epitope tag were expressed equally well in *Xenopus* oocytes (Fig. 2). The proteins migrated somewhat below the calculated molecular weight. Protein dimers were slightly visible (arrows in Fig. 2). For the functional characterization untagged proteins were used.

In oocyte swelling assays using 1:3 diluted ND96 medium (140 mOsm/kg gradient) both constructs induced swelling, however, to markedly different extents (Fig. 3A). TgAQP M_{39} water permeability (P_f) was three times higher than that of control oocytes (40 vs. 14 μm/s) whereas expression of TgAQP M_1 led to an eight-fold increase (108 μm/s, Fig. 3A). For comparison, the P_f value of the single aquaporin (PfAQP) from the apicomplexan *P. falciparum* was 323 μm/s classifying TgAQP as a water channel with intermediate permeability.

Having earlier identified PfAQP as a bifunctional channel with high permeability for both water and glycerol [2], we tested glycerol passage through TgAQP M_1 and TgAQP M_{39}. Using 130 mM glycerol we found that TgAQP M_1 was permeated by this solute at rates statistically identical to PfAQP (Fig. 3B). Again, the TgAQP M_{39} construct was functional at about one third of the TgAQP M_1 permeability. In summary, TgAQP constitutes an aquaglyceroporin with intermediate water and high glycerol permeability.

3.3. Permeability of further solutes

The permeation sequence of polyols is a pore characteristic of aquaglyceroporins. Therefore, we measured the permeability of TgAQP M_1 for several solutes (Fig. 4A). Besides urea, which passed the pore as well as glycerol, only erythritol and D-arabitol led to reasonable swelling rates of about one quarter that of glycerol. Larger compounds with a linear backbone hardly passed whereas *myo*-inositol and charged solutes did not pass at all (Fig. 4A).

It is reported that the proliferation of *T. gondii* can be repressed in vitro by the antineoplastic drug hydroxyurea which inhibits ribonucleotide reductase and thus arrests DNA synthesis [10]. The growth of *P. falciparum* parasites is also impaired albeit to a lesser extent [11]. Therefore, we analyzed the hydroxyurea permeability of TgAQP M_1 and PfAQP (Fig.

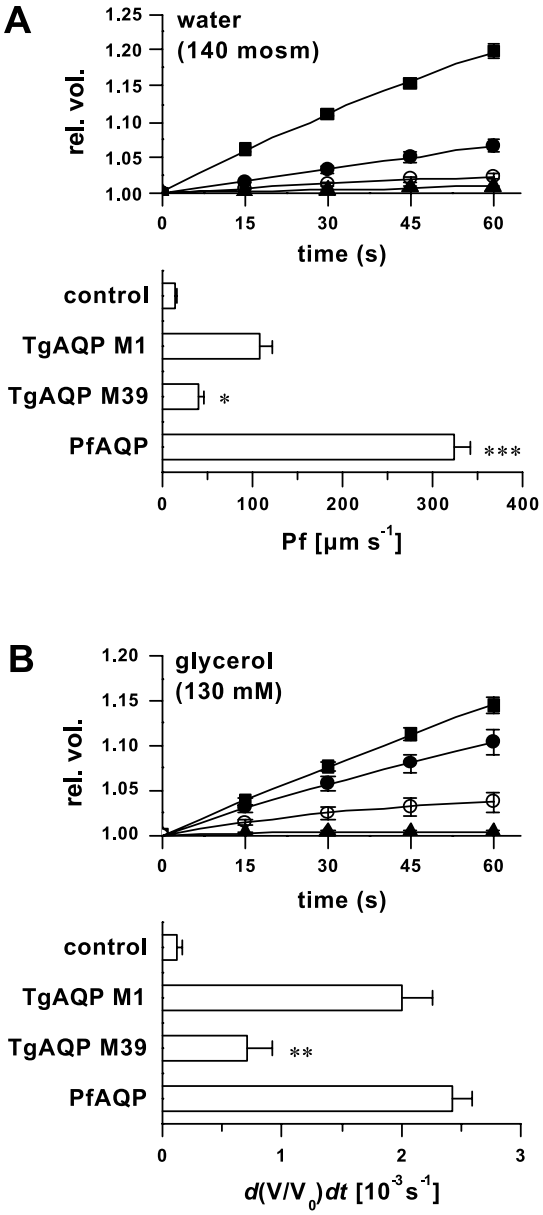

Fig. 3. A: Water permeability of TgAQP M_1, TgAQP M_{39}, and PfAQP compared to control oocytes in 1:3 diluted ND96 medium. The relative oocyte volume increase for the first minute of swelling is shown in the upper panel. Deduced P_f values are plotted below. B: Glycerol permeability of TgAQP M_1, TgAQP M_{39}, and PfAQP. Oocyte swelling curves in a 130 mM glycerol gradient are shown in the upper panel. Below, bars represent the initial swelling rates. Here, control denotes oocytes with AQP1 cRNA injection. All values are mean ± S.E.M. ($n = 5$–8). Statistically significant differences with TgAQP M_1 permeabilities are indicated by asterisks (*$P < 0.05$; **$P < 0.02$; ***$P < 0.01$).

4B). Surprisingly, the drug passed through both aquaglyceroporins with rates 75% of the glycerol permeability. The non-facilitated diffusion of hydroxyurea over the oocyte membrane was negligible.

4. Discussion

The BLAST analysis suggests a connection of the *Toxoplasma* aquaporin with plant TIPs. The presence of a valine at the pore mouth instead of the highly conserved arginine is a strong indicator of a relevant phylogenetic relation. Similar examples of plant-derived genes in *Toxoplasma* have been noted [12] supporting the hypothesis that the phylum Apicomplexa originated from a eukaryotic ancestor that acquired a plastid by secondary endosymbiosis, probably from an alga [13]. Plastid-encoded genes are thought to have entered the parasite's nucleus thereafter. The aquaglyceroporin from *P. falciparum*, in contrast, is similar to the glycerol facilitator of *E. coli* (50%) suggesting that PfAQP is not of plant origin but was derived from bacteria. Apparently, aquaporin channels were adopted from different sources.

For further comparisons, we used aquaporin sequences from recent genome projects of other apicomplexan parasites, i.e. *Eimeria tenella*, *Babesia bovis* (both from www.sanger.ac.uk) and the *Plasmodium* species *berghei*, *chabaudi*, *knowlesi*, and *yoelii* (www.tigr.org). So far, each of these genomes contains only a single aquaporin gene. The structural alignment is

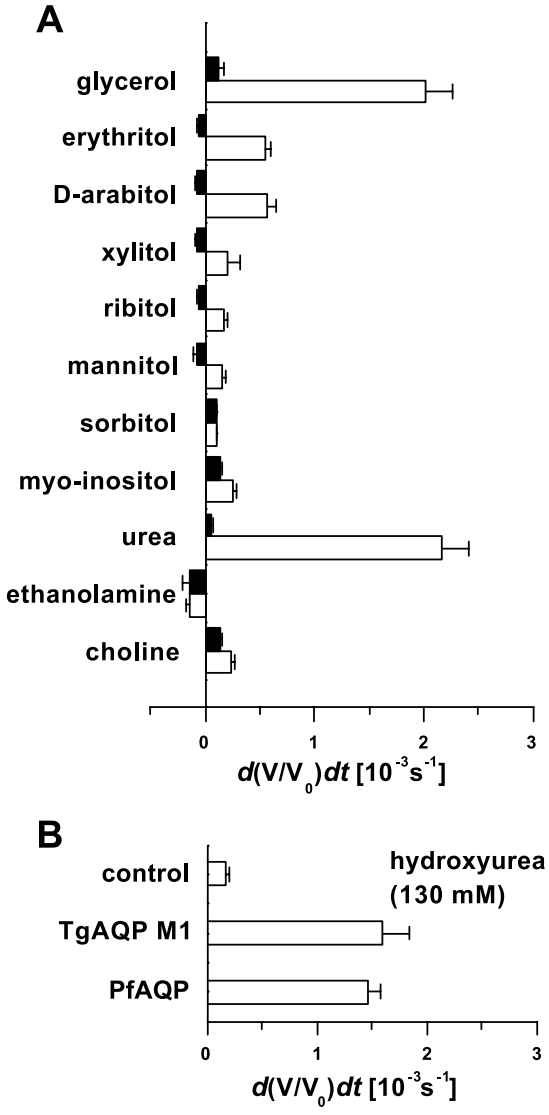

Fig. 4. Pore selectivity of TgAQP M_1. A: The initial swelling rate in isosmotic medium containing diverse solutes is depicted for TgAQP M_1-expressing oocytes (open bars) and AQP1 control oocytes (filled bars). B: Hydroxyurea permeability of TgAQP M_1 and PfAQP. Shown are mean values ± S.E.M. ($n = 5$).

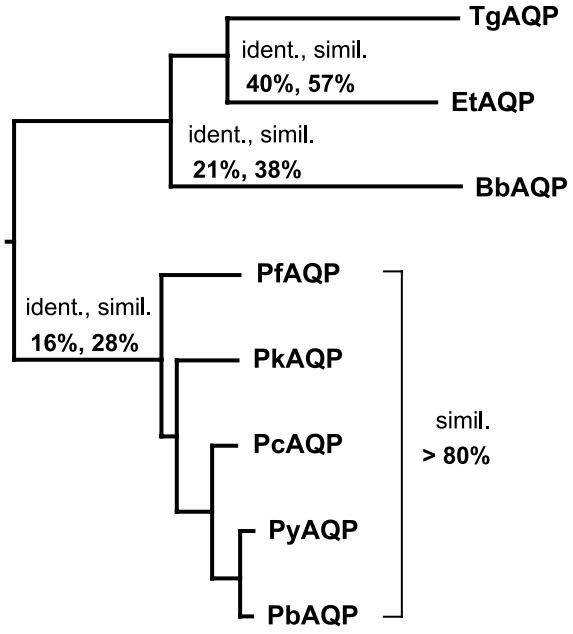

Fig. 5. Phylogenetic representation of sequence similarities among apicomplexan aquaporin proteins (EtAQP, *E. tenella* aquaporin; Bb, *B. bovis*; Pf, *P. falciparum*; Pk, *P. knowlesi*; Pc, *P. chabaudi*; Py, *P. yoelii*; Pb, *P. berghei*). The tree is based on sequence alignments using the Clustal algorithm with the PAM250 residue weight matrix. The level of identity and similarity compared to TgAQP is shown at the branches.

available as supplementary material. A phylogenetic representation based on sequence similarity mirrors the close taxonomic relation between *T. gondii* and *E. tenella*, both from the class Coccidia, and the distance to *B. bovis* (Piroplasmida) and particularly to *Plasmodium* sp. (Haemosporida, Fig. 5). Thus, aquaporin sequences may serve to corroborate the sometimes ambiguous taxonomic classifications within the Apicomplexa.

A length variation of 31 amino acids is apparent in the N-termini of TgAQP and EtAQP. It seems possible that an in-frame intron with classical GT/AG boundaries is present in the TgAQP gene (positions 13–99, gray shading in Fig. 1A). Functionally, modifications of the N-terminus should not affect channel selectivity considering that the ratio of water and glycerol permeability of the TgAQP M_{39} construct was equal to that of TgAQP M_1. Which variant is actually expressed and where it is localized in the pathogen can only be answered when a specific antiserum becomes available. The PfAQP of *P. falciparum* is constitutively expressed in all blood stages suggesting a basic role in osmotic protection (water permeation) and energy metabolism (glycerol uptake) [2]. Similar functions may be attributed to the *T. gondii* aquaglyceroporin.

The high glycerol permeability of TgAQP is particularly intriguing because plant TIPs are water-specific channels. This implies that solute permeability was gained after gene transfer to an ancestor of *Toxoplasma*. The reverse event, i.e. gain of high water permeability of a glycerol facilitator, probably occurred in the *Plasmodium* aquaglyceroporin. In both instances, two physiological functions were combined in a single channel molecule probably to reduce antigenic surface structures. Such adaptations are driven by the enormous selective pressure of an intimate parasite–host relation. Plants, on the other hand, obviously did not realize this solution to the problem of glycerol facilitation. Instead, a horizontal gene transfer from bacteria is proposed to have brought glycerol channels to plants in the form of the nodulin26-like intrinsic protein family [14].

Structural requirements for glycerol passage through TgAQP might be elucidated by identification of differences in the pore-forming amino acids compared to TIPs. Considering the common layout of water and glycerol channels [15], relatively small changes which enhance the pore diameter of a water pore by less than 1 Å should suffice to induce solute permeability. Finding such positions, however, is hampered by the lack of structural data on TIPs. So far, protein structures are available only for mammalian and bacterial aquaporins, i.e. AQP0 [16], AQP1 [17] and GlpF [18].

Hydroxyurea is the first pharmacologically active compound found to pass aquaporins. Due to the miniature size of the pore we expect that the number of aquaporin-permeating drugs will hardly increase. It will be interesting to test whether mammalian aquaglyceroporins, i.e. AQP3, 7 and 9 [19–21], are also permeated by hydroxyurea.

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