

Functional Characterization of Three Aquaglyceroporins from *Trypanosoma brucei* in Osmoregulation and Glycerol Transport

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Key Words

Trypanosoma brucei • Aquaglyceroporins • RNA interference • Osmoregulation • Glycerol transport

Abstract

Previous studies using bloodstream form *Trypanosoma brucei* have shown that glycerol transport in this parasite occurs via specific membrane proteins, namely a glycerol transporter and glycerol channels [1]. Later, we cloned, expressed and characterized the transport properties of all three aquaglyceroporins (AQP1-3) [2], which were found permeable for water, glycerol and other small uncharged solutes like dihydroxyacetone [3]. Here, we report on the cellular localization of TbAQP1 and TbAQP3 in bloodstream form trypanosomes. Indirect immunofluorescence analysis showed that TbAQP1 is exclusively localized in the flagellar membrane, whereas TbAQP3 was found in the plasma membrane. In addition, we analyzed the functions of all 3 AQPs, using an inducible inheritable double-stranded RNA interference methodology. All AQP knockdown cell lines were still able to survive hypo-osmotic stress conditions, except AQP2 knockdown parasites. Depleted TbAQP2 negatively impacted cell growth

and the regulatory volume recovery, whereas AQP1 und 3 knockdown trypanosomes displayed phenotypes consistent with their localization in external membranes. A simultaneous knockdown of all 3 AQPs showed that the cells were able to substitute the missing glycerol uptake capability through a putative glycerol transporter.

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Introduction

Trypanosoma brucei is a protozoan parasite that causes Human African Trypanosomiasis (HAT), also known as sleeping sickness and the animal disease Nagana in domestic livestock. It depends on the tsetse fly for dissemination and possesses a complex life cycle with different developmental stages in both, the mammalian host and the insect vector.

For energy production, the bloodstream form parasite relies exclusively on glycolysis, metabolizing glucose or, alternatively, glycerol [4, 5]. Glycolysis in trypanosomes differs markedly from other cells, because the first seven enzymes of the pathway are localized in a specific

organelle, called glycosome [6]. To maintain a balanced redox potential within the glycosome, the NADH formed by glyceraldehyde-3-phosphate dehydrogenase is re-oxidized by glycerol-3-phosphate dehydrogenase. The resulting glycerol-3-phosphate leaves the glycosome, is oxidized by molecular oxygen to dihydroxyacetone phosphate via a glycerol-3-phosphate oxidase complex within the inter-membrane space of the mitochondrion, and dihydroxyacetone phosphate is re-imported into the glycosome [6]. Under anaerobic conditions, a glycosomal glycerokinase converts glycerol-3-phosphate to glycerol and ATP. Thus, 1 mol glucose is metabolized to 2 mol pyruvate under aerobic conditions and to 1 mol pyruvate and 1 mol glycerol in the absence of oxygen. In the latter case, glycerol has to be secreted out of the glycosome and eventually out of the cell as the glycerokinase has a preference for catalysis of glycerol to glycerol-6-phosphate.

One way trypanosomes may have solved the transport of glycerol across the glycosome and the plasma membrane is by utilizing their aquaglyceroporins. These water and glycerol channels work by simple diffusion. They could also help the trypanosomes to cope with the considerable variations of osmolarity during its life cycle, e.g. when passing through the kidney in the mammalian host.

TbAQP1-3 are very similar possessing sequence identity scores between 66.4% and 68.3%. TbAQP1 and 3 contain the canonical NPA/NPA motive, while TbAQP2 possesses an unusual NSA/NPS motive at the pore forming region. All 3 channels are expressed in both bloodstream and procyclic form trypanosomes [7, 8], but AQP1 and AQP3 show different mRNA levels: compared with the transcript levels in procyclic parasites, AQP1 is twofold down-regulated, while AQP3 is more than fourfold up-regulated [7, 8].

Trypanosomes have the machinery for RNA interference (RNAi), a post transcriptional gene silencing mechanism, providing a fast and robust method to produce conditional mutants [9]. We used an inducible inheritable RNAi system, which allows generation of potentially lethal transfectants to study the function of all 3 different AQPs in bloodstream forms. In addition, their sub-cellular localization was investigated.

Materials and Methods

Culture methods for Trypanosoma brucei

Bloodstream form trypanosomes MITat 1.2 (VSG variant 221) of the monomorphic strain Lister 427 (named 221) were

grown at 37°C in a 5% CO₂ atmosphere using modified minimum essential medium as described previously [10, 11]. ‘Single marker bloodstream form’ trypanosomes [12], called BF SMB, were also cultured at 37°C in 5% CO₂ atmosphere using modified minimum essential medium, but with 2 µg/ml of the gentamicin analog G418 (Roth, Germany) added to preserve the genomically integrated constitutively expressed genes for T7 RNA polymerase and tetracycline repressor. Cell densities were calculated using a Neubauer chamber and kept between 10⁵ and 10⁶ cells/ml. For medium exchange, trypanosomes were pelleted in a centrifuge at 4°C and 1,300 g for 5 min, unless otherwise stated.

Antibodies for AQP1, 2 & 3

In order to obtain primary antibodies for each of the 3 different AQPs, short and as unique as possible amino acid sequences were used, this was for AQP1: KIN VHQ YPS EAD VRG LKA RC; for AQP2: CDV QKH EVA EAQ EKP V; and for AQP3: CEK GTA GVF STY PRD SN. These peptides, all located outside transmembrane domains and without post-translational modification sites, were applied to immunize chickens (BioGenes, Germany) to obtain polyclonal antibodies. Each peptide was then bound to CNBr-Sepharose 4B to isolate every antibody by affinity chromatography.

Immunofluorescence microscopy

5 x 10⁶ trypanosomes were fixed for 2 h at 4°C in 500 µl PBS (pH 7.4) containing paraformaldehyde (2.4%, w/v) and glutaraldehyde (0.06%, w/v). Cells were washed twice with ice cold PBS and incubated thereafter for 15 min in 100 mM phosphate buffer (pH 7.4) containing 100 mM glycine. Cells were permeabilized by adding one volume of 0.2% Triton X-100 in PBS for an extra five minutes, washed in 0.5 ml PBS containing 1% BSA and incubated in PBS dilutions (1:200 each) of respective chicken anti-AQP antibody supplemented with 1% BSA for one hour at 4°C. Cells were washed twice in ice cold PBS before the same incubation regiment was repeated for the FITC-labeled mouse anti-chicken IgY secondary antibody (Sigma-Aldrich, Germany) diluted 1:400. For counterstaining DNA of nucleus and kinetoplast, 100 µg/ml bisbenzimidazole was added to the solution, which was then incubated for another five minutes. Finally, cells were washed twice with ice cold PBS and deionized water, resuspended in 50 µl deionized water and placed onto poly-L-lysine-coated slides to dry at room temperature. A BH 2 fluorescence microscope (Olympus, Germany) and CellF 3.1 imaging software (Olympus, Germany) were used to view and analyze the parasites.

Preparation of aquaglyceroporin RNAi constructs

To knockdown individual aquaglyceroporins, DNA stretches corresponding to the open reading frame of *TbAQP1* and to the 3' untranslated regions of *TbAQP2* and *TbAQP3* were amplified using Omniscript Reverse Transkriptase Kit (Qiagen, Germany) and HotStarTaq Master Mix Kit (Qiagen, Germany) for reverse transcription PCRs. The selection of knockdown fragments and the subsequent primer design was assisted by RNAit [13]. The chosen 567 bp long DNA fragment of *TbAQP1* stretched from nucleotide 130 to nucleotide 696 of

the respective gene, whereas the 360 and 337 bp long DNA fragments used for *TbAQP2* and *TbAQP3* encompassed the nucleotides 175 to 534 and the nucleotides 175 to 511 of the 3' untranslated regions, respectively. The following primers were used (TaaI restriction sites are underlined): AQP1_RNAi_1x_se (5'-GCC AAT CCA CAG GAA AAG AA-3') and AQP1_RNAi_1x_as (5'-ACC GAC GCA AAA GAG AAG AA-3'), AQP2_RNAi_1x_TaaI_se (5'-ACATACTGTGCG TTGAAG TTT TTG CCT TC-3') and AQP2_RNAi_1x_TaaI_as (5'-TAT AAC TGT TCG TTG CGT GGT AAC GTA AA-3'), AQP3_RNAi_1x_TaaI_se (5'-AGG TAC TGT TTT TTG AGT GCA TGA GCT GC-3') and AQP3_RNAi_1x_TaaI_as (5'-ACG CAC TGT TGG TTC AGC GCT ATT CAG TG-3'). The knockdown fragments for AQP2 and 3 were subcloned into plasmid pCR2.1-TOPO (Invitrogen, Germany) and re-liberated via the TaaI restriction sites. TaaI, using these primers, generated 3' adenosine overhangs. All 3 AQP single knockdown fragments were then used for TA-cloning into the Eam1105I sites of the RNAi vector p2T7^{Ti}:Tablue [14]. To knockdown expression of TbAQP1, TbAQP2 and TbAQP3 simultaneously, the three DNA stretches used for the AQP single knockdown fragments were amplified and ligated. The following primers were used (TaaI, NdeI and SalI restriction sites are underlined and the linker sequence is written in small letters): AQP1_3x_link_TaaI_se (5'-GCG TAC TGT GCCAAT CCACAG GAA AAG AA-3') and AQP1_3x_link_NdeI_as (5'-cgt tta aac tta cgg acc gtC ATA TGA CCG ACG CAA AAG AGA AGA A-3'), AQP2_3x_link_NdeI_se (5'-ACG TCA TAT GGC GTT GAA GTT TTT GCC TTC-3') and AQP2_3x_link_SalI_as (5'-TAT GGT CGA CTC GTT GCG TGG TAA CGT AAA-3'), AQP3_3x_link_SalI_se (5'-tga cgg tcc gta agt tta aac gTC GAC TTT TTG AGT GCA TGA GCT GC-3') and AQP3_3x_link_TaaI_as (5'-ACG CAC TGT TGG TTC AGC GCT ATT CAG TG-3'). The PCR fragments for AQP1 and AQP3 were combined (via the complimentary primer sequence) in a linking PCR reaction, amplified by PCR and the resulting construct was cloned into pCR2.1-TOPO. The AQP2 fragment was then ligated into the NdeI and SalI restriction sites of the AQP1-AQP3 fragment. Finally the 1281 bp AQP triple knockdown fragment was released via the TaaI restriction sites and used for TA-cloning into the Eam1105I sites of the RNAi vector p2T7^{Ti}:Tablue. All p2T7^{Ti}:Tablue constructs were validated by sequencing (GATC Biotech, Germany).

Transfection of trypanosomes

The NotI linearised p2T7^{Ti}:Tablue constructs integrated into one of several rDNA spacer regions in the genome via homologous recombination. To generate a stable transfection of cell line BF SMB, the bloodstream form cells were grown in HMI-9 medium [15], which included 10% (v/v) SERUM PLUS (SAFC Biosciences, England). 2.5×10^7 cells were washed in 25 ml ice cold modified cytomix (120 mM KCl, 86.6 mM K_2HPO_4 , 13.4 mM KH_2PO_4 , 25 mM HEPES, 5 mM $MgCl_2$, 0.15 mM $CaCl_2$, 2 mM EGTA, pH 7.6) before they were re-suspended in 440 μ l of the same buffer. 10 μ g NotI linearised plasmid were added to the cell suspension, before electroporation was carried out in ice cold 2 mm cuvettes using a GenePulser XCell (Bio-Rad, Germany) by setting a single pulse (square wave pulse, 1.4 kV,

25 μ F, ∞ Ω). Immediately afterwards, the trypanosomes were transferred into 36 ml HMI-9 medium and grown for six hours, before 2 μ g/ml G418 was added and selection pressure was applied by supplementing with 2.5 μ g/ml hygromycin. 1 ml aliquots were placed into 24-well plates and after five to six days stable clones were observed and used to make stabiliates (2×10^7 cells per ml) in liquid N_2 .

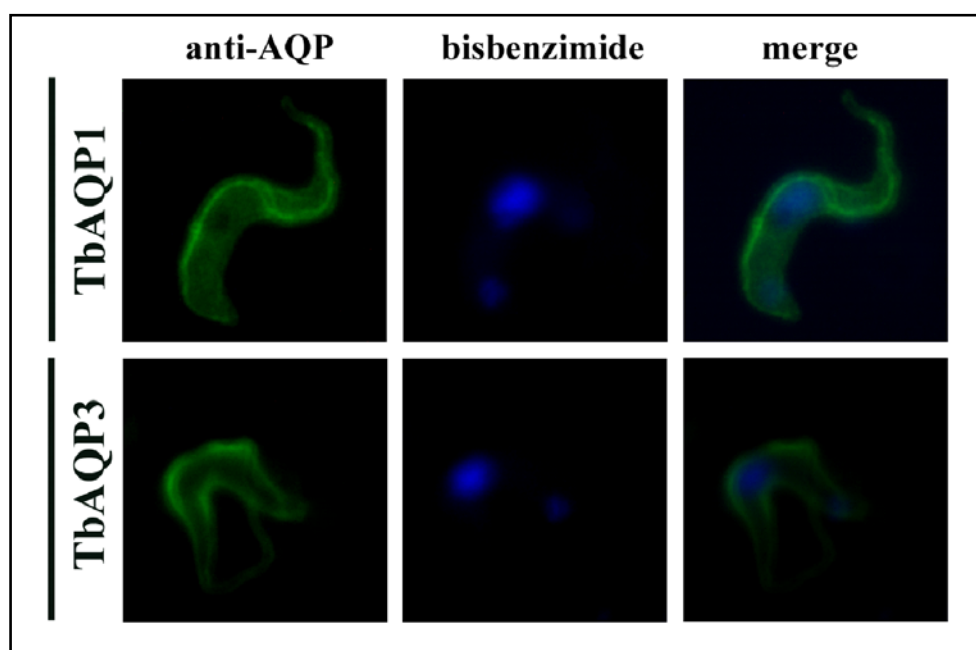
Northern blot analysis

Total RNA from bloodstream form trypanosomes was isolated using the RNeasy Mini Kit (Qiagen, Germany). RNA samples (10–20 μ g per lane) were separated on 1.2% agarose gels (pH 7.0) containing formaldehyde (2.2 M), 3-[N-Morpholino]-propanesulfonic acid (20 mM), sodium acetate (5 mM) and EDTA (1 mM). Ribosomal RNA was used to monitor RNA loading. Gels were blotted onto a Hybond-N+ nylon membrane (Amersham Biosciences, Germany) and cross-linked with a UV Crosslinker CL-1000 (UVP, USA). The next steps were executed in a water bath at 68°C. The blots were blocked with 100 μ g/ml denatured salmon sperm in hybridization buffer (500 mM sodium phosphate buffer containing 7% SDS, 2 mM EDTA and 1% BSA) and hybridized over night by adding α - 32 P labeled DNA probe. DNA probe fragments were labeled with [α - 32 P]dATP (3000 Ci/mmol) utilizing the NEBlot DNA labeling Kit (New England BioLabs, Germany). The DNA probe fragment for *TbAQP1* stretched over the 129 nucleotides of the 5' untranslated region up to ATG and included the first 124 nucleotides of the coding region. For *TbAQP2* the probe stretched from nucleotide 883 of the open reading frame up to the 194th nucleotide in the 3' untranslated region. The *TbAQP3* probe extended from nucleotide 544 to nucleotide 794 within the gene. The β -tubulin (BTUB) probe, used for normalization, extended from nucleotide 1 to nucleotide 1320 of the β -tubulin gene. The following PCR primers were used to amplify the DNA probes: AQP1_probe_se (5'-GGG TCC ACC ACA ATC AAA AA-3') and AQP1_probe_as (5'-GGT TGG GAA TGG GTT CAT TA-3'), AQP2_probe_se (5'-CCA TTC TTT GGA GCT ATC CTT G-3') and AQP2_probe_as (5'-GAA GGC AAA AAC TTC AAC GC-3'), AQP3_probe_se (5'-ACC GCA GGT GTG TTC AGC-3') and AQP3_probe_as (5'-GAA GAG AAG ACC CTG GGA CC-3'), BTUB_probe_se (5'-ATG CGC GAA ATC GTC TGC GTT CAG-3') and BTUB_probe_as (5'-CTC CTC CTC GTC GAA CTC GCC CT-3'). Finally, after the blots were repeatedly treated with washing buffer (40 mM Na_2HPO_4 , 1% SDS, pH 7.2), autoradiographs were obtained using BioMax MR Film (Kodak, Germany) and a SRX-101A tabletop processor (Konica, Germany). If necessary blots were stripped using stripping buffer (1% SDS, pH 7.0) and re-hybridized with additional probes. Film scans were analyzed with Quantity One 4.6.3 Basic (Bio-Rad, Germany).

Hypo-osmotic shock

Relative changes in cell volume after inducing a hypo-osmotic shock were measured using a previously described light-scattering method [16]. Briefly, 5×10^7 trypanosomes were washed with ice cold iso-osmotic Earle's salt buffer (116 mM NaCl, 1.8 mM $CaCl_2$, 5 mM KCl, 0.8 mM $MgSO_4$, 1 mM NaH_2PO_4 , 30 mM HEPES, 30 mM glucose). The buffer was adjusted to pH

Fig. 1. Immunofluorescence micrographs showing the localization of TbAQPs. BF 221 trypanosomes were incubated with chicken anti-AQP1 and anti-AQP3 antibody, respectively. FITC-labeled mouse anti-chicken antibody was used for visualization.



7.4 and its osmolarity was 300 ± 5 mOsm as detected by using a vapor pressure VAPRO 5520 osmometer (Wescor, USA). The cells were resuspended in iso-osmotic Earle's salt buffer at a final density of 10^8 cells/ml. Hypo-osmotic stress was induced by diluting the isotonic cell suspension with one volume of deionized water to a final osmolarity of 150 mOsm. For iso-osmotic control experiments cell suspensions were diluted with one volume of iso-osmotic Earle's salt buffer. Absorbance of the diluted cultures was measured at 550 nm every two seconds for 15 min in a GeneQuant 1300 spectrophotometer (GE Healthcare, Germany). A decrease in absorbance corresponded to an increase in cell volume.

Survival on glycerol

2×10^7 trypanosomes were pelleted (4 min, 1,300 g, 4°C) and washed twice with an ice cold incubation medium (18 mM Na_2HPO_4 , 2 mM NaH_2PO_4 , 5 mM KCl, 80 mM NaCl and 1 mM MgSO_4 , pH 7.4). Afterwards the cells were resuspended in 2 ml incubation medium containing either 10 mM glucose, 10 mM glycerol or 5 mM NaCl and were cultivated in a shaking (150 rpm) incubator at 37°C . For a time course of 120 min, an aliquot was removed every 30 min for counting and sample preparation. In the latter case, 10 μl 70% perchloric acid was added to a 100 μl aliquot for protein precipitation. Samples were stored on ice before pyruvate concentration of the supernatant was determined enzymatically.

Pyruvate measurement

7.5 μl of sample were added to 340 μl potassium phosphate buffer (100 mM, pH 7.0) containing 0.2 mM NADH and 0.5 $\mu\text{g/ml}$ lactate dehydrogenase (Roche, Germany). Each sample was placed in 96-well plates. Following incubation for 20 min at room temperature, the absorption decline at 340 nm was measured using an MRX II ELISA reader (Dynex Technologies, England). All samples were measured in quadruplicate.

Results

Localization of AQPs

We used synthetic peptides, each covering sequence stretches specific for either one of the three AQPs to raise antibodies. The three different antibodies showed no cross reactivity with either one of the two other peptides (results not shown). These peptide-derived anti-AQP antibodies from chicken (BioGenes, Germany) together with FITC-labeled mouse anti-chicken IgY secondary antibody (Sigma-Aldrich, Germany) were applied using immuno-fluorescence staining of BF 221 bloodstream forms (Fig. 1). In case of TbAQP1, a strong fluorescence signal was observed in the flagellar membrane, indicating a preferential localization within this membrane area. For TbAQP3 a clear localization in the plasma membrane of the cell was visible. For TbAQP2 no conclusive immunofluorescence micrographs could be established with our primary antibody. No fluorescence was observed when BF 221 control cells were incubated only with each of the pre-immune sera (data not shown).

Knockdown of AQP genes in Trypanosoma brucei

In order to investigate the importance of aquaglyceroporins for the transport of water and glycerol, we used RNA interference as the method of choice. Each aquaglyceroporin was knocked down individually and, in addition, all three AQPs were targeted simultaneously.

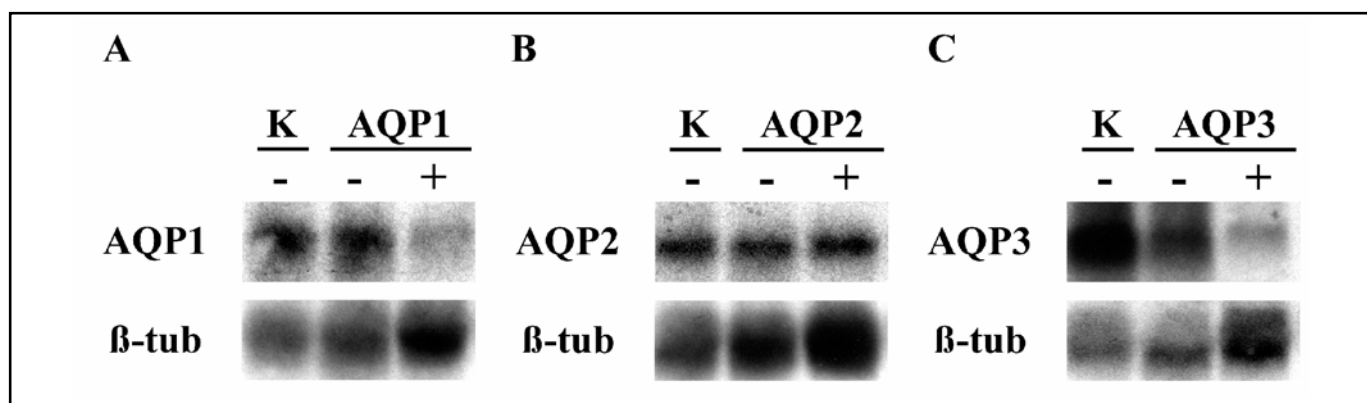


Fig. 2. Northern blot analyses of different BF SMB AQP single knockdown cell lines. Total RNA were isolated from BF SMB control cells (K) and uninduced (-) as well as induced (+) BF SMB AQP1 (A), BF SMB AQP2 (B), and BF SMB AQP3 (C) cells. 20 µg/lane total RNA were separated, blotted and hybridized with radiolabeled DNA probes for *TbAQP1*, *TbAQP2*, *TbAQP3* and *TbBTUB*.

Preparation of aquaglyceroporin RNAi constructs

Because of relatively high sequence similarities between the three AQP genes, it was difficult to find sufficiently long unique RNAi knockdown sequences. We therefore chose stretches from the open reading frame of *TbAQP1* and from the 3' untranslated region of *TbAQP2* and *TbAQP3*. cDNA fragments for the individual knockdown of AQP1, AQP2 and AQP3 and a combination thereof for the simultaneous knockdown of all three AQPs were cloned into the RNAi vector p2T7^{tr}:TAbblue.

Transfection of trypanosomes

Trypanosoma brucei BF SMB cells, expressing T7 RNA polymerase and tetracycline repressor, were transfected with the RNAi constructs directed against AQP1, AQP2, AQP3 or AQP1-3. Hygromycin selection was successfully used to generate the tetracycline inducible stable RNAi cell lines BF SMB AQP1, BF SMB AQP2, BF SMB AQP3 and BF SMB AQP1-3, which were assumed to be clonal, since less than 25% of wells yielded transfectants. Within plasmid p2T7^{tr}:TAbblue, the cloned product was arranged between two opposing T7 promoters with two tetracycline operators each and a T7 terminator on every side. Addition of tetracycline to the medium induced the production of double-stranded RNA triggering RNA interference. AQP knockdown cell lines were grown in the presence of 1 µg/ml tetracycline in the medium for three days, after which no further messenger RNA reduction was observed by Northern blot analysis (data not shown). The bloodstream form trypanosomes had a generation doubling time of ~8 hours, so that after

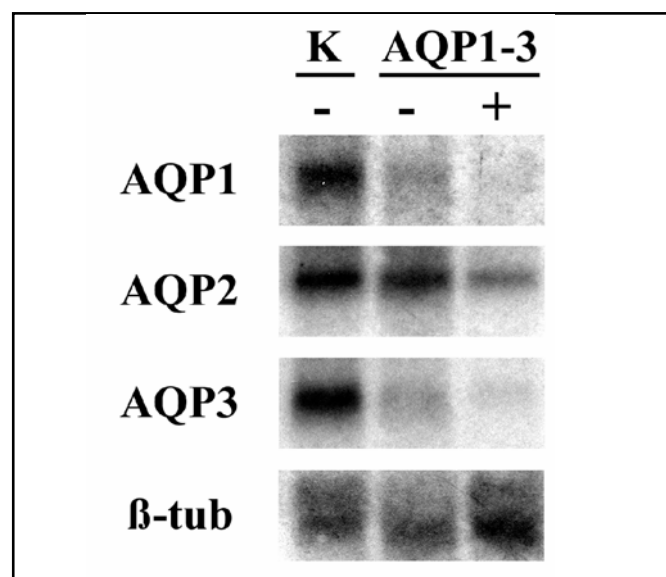


Fig. 3. Northern blot analyses of BF SMB AQP triple knockdown cell lines. Total RNA were isolated from BF SMB control cells (K) as well as from uninduced (-) and induced (+) BF SMB AQP1-3 cells. 20 µg/lane total RNA were separated, blotted and hybridized with radiolabeled DNA probes for *TbAQP1*, *TbAQP2*, *TbAQP3* and *TbBTUB*.

72 h the AQP proteins per cell should diminish to 1/500 of the original amount, disregarding protein degradation and possible new protein synthesis.

Northern blot analysis

To verify the successful mRNA depletion for all AQP knockdown clones, Northern blot analyses for uninduced (-) and induced (+) BF SMB AQP RNAi cell lines as well as for BF SMB control cells (K) were conducted. The intensities of the hybridized bands were determined.

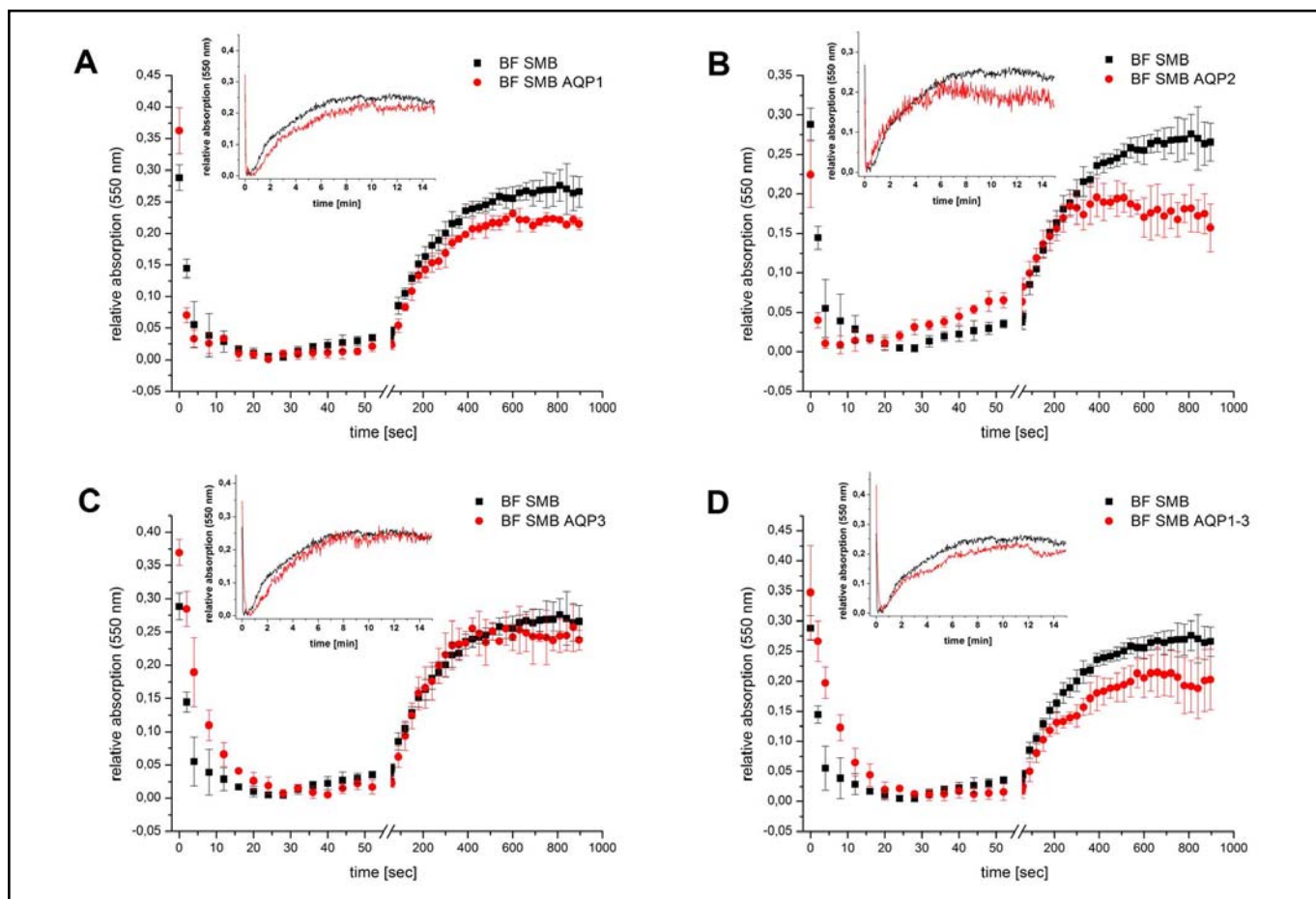


Fig. 4. Volume regulation in different BF SMB AQP knockdown cell lines. Cells were subjected to a hypo-osmotic shock, and the relative changes in cell volume were followed by monitoring the absorbance at 550 nm, as described in Materials and Methods. Results for BF SMB control cells (squares) and tetracycline induced BF AQP1 (A), AQP2 (B), AQP3 (C) and AQP1-3 (D) RNAi knockdown cells (circles) are shown and are expressed in arbitrary absorbance units. A representative trace is shown as an inset.

The values for AQP mRNA were normalized against the loading control mRNA bands of the housekeeping gene β -tubulin and the AQP mRNA value for the BF SMB control cells (K) was set as 100%. The probes used were directed against AQP mRNAs, but not dsRNA knockdown fragments. Radiographs in Fig. 2 revealed that for the three non-induced (-) individual AQP knockdowns leakiness occurred with an mRNA reduction of 26%, 26% and 32% for BF SMB AQP1, BF SMB AQP2 and BF SMB AQP3, respectively. In contrast, the matching induced trypanosomes (+) showed reductions of 83%, 36% and 84% for AQP1, AQP2 and AQP3 transcript levels, respectively. Results for the AQP triple knockdowns are documented in Fig. 3. The non-induced (-) BF SMB AQP1-3 cells showed decreases of 65%, 17% and 63% for AQP1, AQP2 and AQP3 transcripts, respectively. The three day tetracycline induced (+) cells showed reductions of 99% for AQP1 mRNA, 73% for AQP2 mRNA and 94% for AQP3 mRNA.

Growth phenotype of AQP knockdowns

The knockdown cell lines were checked for changes in cell growth. The following results are representative of at least three individual experiments. The measured generation times of BF SMB AQP1 (8.6 ± 1.7 h and 8.0 ± 1.0 h induced), BF SMB AQP3 (8.4 ± 1.7 h and 7.9 ± 1.5 h induced) and BF SMB AQP1-3 (7.7 ± 1.8 h and 7.9 ± 1.3 h induced) were not significantly different from that of the BF SMB control cells (7.6 ± 1.7 h and 7.8 ± 1.3 h induced). Only the doubling time for BF SMB AQP2 (10.3 ± 1.8 h and 13.2 ± 1.7 h induced) showed a significant growth inhibition.

Hypo-osmotic shock phenotype of AQP RNAi knockdowns

The AQP deficient cell lines were tested using hypo-osmotic stress conditions. When BF SMB control cells were subjected to a reduction in osmolarity from 300 to 150 mOsm, a dramatic initial swelling was observed under

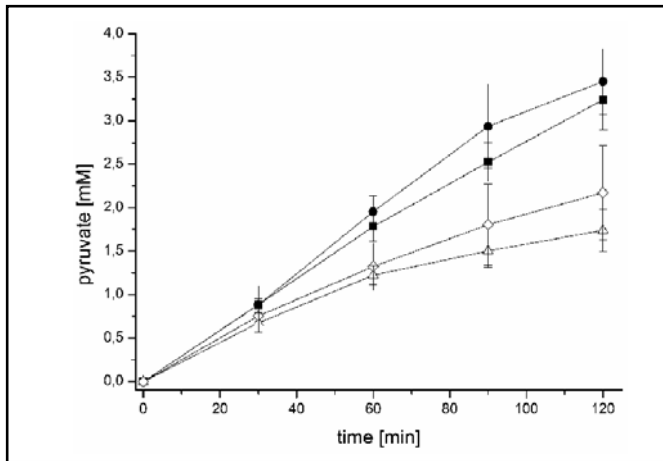


Fig. 5. Survival of BF SMB AQP1-3 trypanosomes on glycerol. Amount of pyruvate produced by BF SMB control cells feeding solely on 10 mM glucose (squares) or 10 mM glycerol (triangles) and tetracycline induced BF SMB AQP1-3 cells nourishing only on 10 mM glucose (circles) or glycerol (diamonds).

the microscope. Within the first three minutes *Trypanosoma brucei* actively ejected amino acids, mainly alanine, glycine and serine, but not sodium or potassium (to be published elsewhere). The regulatory volume decrease led to cell shrinkage within a minute thereafter and rendered the parasites indistinguishable in terms of motility and morphology from control cells maintained under iso-osmotic conditions within ten minutes.

Changes in cell volume after a hypo-osmotic shock were monitored using a spectrophotometer by following the changes in absorbance of the cell suspensions at 550 nm [17]. Fig. 4A-D displays results of at least three independent experiments in each case. A decrease in absorbance corresponded to an increase in cell volume as judged from visual observations using light microscopy. The depleted numbers of AQP proteins in the different AQP knockdown cell lines led to differences in both swelling time and rate of volume recovery. The swelling time was defined as the time passed till the cells were maximally swollen and was determined from fitted curves for each run. The untreated BF SMB control cells, i.e. containing all three aquaglyceroporins, had a swelling time of 18.4 ± 5.2 seconds. Induced BF SMB AQP2 cells showed a similar swelling time of 16.2 ± 2.2 sec. The prolonged swelling times of BF SMB AQP1 (26.0 ± 2.0 sec) and BF SMB AQP3 (34.0 ± 2.0 sec) are consistent with a massively reduced expression of AQP1 and AQP3 in the flagellar membrane and the plasma membrane, respectively. The AQP triple knockdown cells BF SMB AQP1-3 exhibited the longest swelling time with $36.7 \pm$

2.3 sec, consistent with a significantly reduced water exchange rate, but not consistent with a complete stop of water transport across the membranes.

All cell lines, except BF SMB AQP2 knockdown cells, showed a full regulatory volume recovery phase comparable to the control cells. In BF SMB AQP2 cultures about 50% of the cells remained visibly in an enlarged state even after 10 min, while the other half of the population displayed no flagella movement anymore and was obviously dead.

BF SMB AQP1-3 cells, in which AQP2 is also knocked down, showed a very slightly lagging, but full volume recovery. We assume that the simultaneous knockdown of AQP1 and AQP3 might be responsible for the difference in phenotype.

Survival on glycerol

Since AQPs transport small solutes in addition to water molecules, we considered the importance of AQPs for glycerol transport, especially the uptake and intracellular transport of glycerol. For energy production, bloodstream form parasites rely exclusively on glycolysis for ATP generation. As proven earlier [4, 5], glucose can be replaced by glycerol. The metabolic end product in both cases is pyruvate that is released from the cell by a specific pyruvate transporter [18]. Tetracycline ($1 \mu\text{g}/\text{ml}$) induced BF SMB AQP1-3 knockdown cells and BF SMB control cells were grown in the presence of either glycerol, glucose (positive control) or sodium chloride (negative control) as sole energy source. This experiment was designed to demonstrate whether aquaglyceroporin expression in bloodstream form trypanosomes is necessary to survive in the absence of glucose, i.e. for the uptake of glycerol from the medium. Trypanosomes incubated in the absence of glucose or glycerol (negative control) died within a few minutes. In contrast, microscopical observation revealed that trypanosomes metabolizing glucose, showed normal motility over the incubation period of 120 min, whereas parasites relying on glycerol as sole carbon source, started to reduce flagella movements within the first half hour and stayed at that pace for the rest of the trial. BF SMB AQP1-3 knockdown cells and BF SMB control cells showed a comparable constant metabolization of glucose to pyruvate during the 2 h time course, leading to pyruvate concentrations within the medium of 3.24 ± 0.35 mM or 3.45 ± 0.38 mM, respectively (Fig. 5). By changing to glycerol, we saw again no significant difference in pyruvate production between BF SMB AQP1-3 knockdown cells (1.74 ± 0.25 mM) and BF SMB control cells (2.17 ± 0.54 mM), but in

both cases significantly less pyruvate was produced. As inferred from their slower motility, cells of the BF SMB variety relying only on glycerol, showed a reduced metabolism to pyruvate, as compared with glucose. The fact that AQP triple knockdown cells were able to survive solely relying on glycerol implies that these cells have at least one additional transport pathway, that fully substitutes the knocked down glycerol transport capacities.

Discussion

As we reported previously, *Trypanosoma brucei* bloodstream forms express three different aquaglyceroporins. Their capacities for water and glycerol transport are very similar to each other [2]. Next to glycerol transport by simple diffusion through AQPs, they obviously possess a facilitated-diffusion carrier for glycerol, predominating at low concentrations [1]. We were interested in the role AQPs play in osmoregulation and in transporting glycerol across membranes, for example in case of anaerobic growing conditions as simulated by salicylhydroxamic acid treatment [19].

From two studies, one in *Trypanosoma brucei* bloodstream form utilizing microarray analysis [7] and one in *Trypanosoma brucei gambiense* using digital gene expression profiling [8], we know absolute and relative transcript levels for AQP1, 2 and 3. AQP1 mRNA is the most abundant aquaglyceroporin transcript. In bloodstream form trypanosomes, compared with the mRNA level of AQP1, the transcription levels for AQP3 and AQP2 is only 1.3 % and 0.3 %, respectively. This information has to some extent be mitigated by the factor, that trypanosomes rely on gene regulation only in exceptional cases and instead carry out heavy post transcriptional regulation [20].

We investigated the localization of all TbAQPs in bloodstream form trypanosomes using peptide-derived antibodies. These antibodies detected their respective antigen (peptide) specifically and showed no cross reactivity to either one of the other two peptide by dot blot analysis. Unfortunately, they showed no specific band detection in Western blot analysis, but they were successfully employed for TbAQP1 and TbAQP3 using immunofluorescence microscopy. Furthermore we analyzed the functions of all three aquaglyceroporins utilizing an inducible inheritable double-stranded RNA interference methodology in BF SMB cells. The success of the RNAi clones was verified via Northern blot analysis

and the cell lines were tested for phenotype changes like growth inhibition and their response to hypo-osmotic shock conditions.

The micrographs showed TbAQP1 only localized within the flagellar membrane. This corresponds to *Leishmania major*, where LmAQP1 is exclusively found in the flagellar membrane of promastigote forms [21]. Until now no signal peptide for localization to the flagellar membrane is known, but in *Leishmania mexicana* and *Leishmania enriettii*, both encoding three and two glucose transporters respectively, one of the transporters is exclusively located in the flagellar membrane [22, 23]. A possible function of the aquaglyceroporin in the flagellar membrane might be osmotaxis. In *Leishmania major* it was demonstrated that LmAQP1, exclusively situated in the flagellar membrane, plays a role in migrating faster towards an osmotic gradient [21]. The AQP1 RNAi knockdown cells, although AQP1 transcripts were 83% knocked down, exhibited no growth inhibition. When exposed to hypo-osmotic shock conditions, a 41% prolonged swelling time and a full volume recovery could be observed.

TbAQP3 was localized in the plasma membrane, consistent with an analysis of the plasma membrane subproteome of bloodstream form *Trypanosoma brucei* [24]. Although *TbAQP3* is roughly 75 times less transcribed than *TbAQP1*, due to its localization in the plasma membrane, the additional swelling time of AQP3 knockdown trypanosomes is ~100% longer than for AQP1 knockdown cells compared with control cells. In trypanosomes with an 84 % AQP3 knockdown normal growth was observed. The resulting lack of TbAQP3 in the plasma membrane led to an 85% prolonged swelling time under hypo-osmotic stress conditions and the cells presented the usual volume recovery.

For TbAQP2 no conclusive immunofluorescence micrographs could be established with our primary antibody, most likely as a consequence of the very low expression rate (0.3 % of AQP1 level). To gain more insight the AQP2 RNAi knockdown trypanosomes were examined. The cells showed a reduction of AQP2 transcripts to 64 % (i.e. 36 % knockdown) of the control cell level. A complete AQP2 knockdown could not be archived. This is consistent with the result in AQP triple knockdown cells, where mRNA knockdown rates of 99% and 94% for AQP1 and AQP3, but only 73% for AQP2 were found. This partial TbAQP2 knockdown nonetheless caused a significant growth defect and the cells had severe problems recovering in the hypo-osmotic stress test. The maximum swelling time was not significantly different

from the control cells, which let us to the conclusion that AQP2 is only localized to intracellular membranes. Consequently, during the volume recovery phase, one half of the cell population died and the remaining cells needed more time to recover. The reduction of TbAQP2 causes growth impairment and negatively impacted the cells ability to quickly execute the regulatory volume reduction. In *Trypanosoma cruzi* an orthodox aquaporin localizes to acidocalcisomes and the contractile vacuole, which are both involved in osmoregulation in this parasite [25]. *Trypanosoma brucei* do not possess a contractile vacuole complex and the mechanism of the regulatory volume recovery is still unknown. The two genes coding for TbAQP2 and TbAQP3 are arranged directly adjacent to each other on chromosome 10 and probably belong to the same polycistronic transcription unit. Starting from the same number of pre-mRNAs, the amount of AQP2 messenger RNA is down regulated more than fourfold, but only in bloodstream forms not in procyclic form trypanosomes [7, 8].

Similar to the AQP1-3 knockdown trypanosomes, where all aquaglyceroporins are knocked down simultaneously, a *Plasmodium berghei* AQP knockout clone was recently reported. *Plasmodium berghei* expresses one aquaglyceroporin that is located in its plasma membrane. The PbAQP-null cell line was viable although the cells were highly deficient in glycerol transport [26]. The AQP1-3 knockdown trypanosomes showed mRNA reductions for AQP1, AQP2 and AQP3 of 99%, 73% and 94%, respectively. When exposed to hypo-osmotic conditions, these cells displayed a swelling time very similar to the additive single knockdown maximum swelling times. In contrast to the AQP2 single knockdown, a regular volume recovery and no growth inhibition was observed. Although we have no conclusive proof for it, we assume that the simultaneous knockdown of AQP1 and 3 in the triple knockdown cells attenuated the initial cell swelling. The initial swelling is believed to be the initial stimulus for the regulatory volume recovery in *Trypanosoma cruzi* [25].

The AQP1-3 knockdown trypanosomes were subjected to a glycerol survival test. Despite the fact that aquaglyceroporins 1 to 3 were successfully knocked down, the cells were able to live on a minimal medium, containing glycerol as their sole energy source. Obviously, glycerol was able to cross the plasma and glycosomal membranes. Glycerol with its three hydroxyl groups is a polar molecule and cannot pass through a phospholipid bilayer fast enough to remedy this situation. Although it cannot be excluded that AQP1-3 knockdown

trypanosomes, as they are no knockouts, had minimal, but sufficient amounts of TbAQPs left to prevent giving a clear phenotype. Most likely, this experiment showed that other glycerol transporters are present in both membranes. A putative glycerol transporter in the plasma membrane of *Trypanosoma brucei* bloodstream form was reported previously [1]. This carrier, in contrast to simple diffusion transport by aquaglyceroporins, was saturable and predominated at low glycerol concentrations. It could be inhibited by the substrate analogue glyceraldehyde, was sensitive to inhibitors phloretin and cytochalasin B and at 4°C the uptake of glycerol was completely blocked. To date, only a few specific glycerol carriers have been found [27-30]. Although the genome of *Trypanosoma brucei* is fully sequenced, no candidate gene has yet been discovered and the protein responsible for glycerol exchange across the glycosomal membrane is still unknown.

In conclusion, we report on the localization of aquaglyceroporins in *T. brucei* bloodstream forms and show that the knockdown AQP1 and AQP3 cell lines display phenotypes consistent with their localization in external membranes, flagellar and plasma membrane respectively, while that for AQP2 may rather suggest the presence in intracellular membranes. Reduced levels of TbAQP2 stunted cell growth and had a detrimental role on volume reduction under hypo-osmotic conditions. This study also shows that TbAQPs are not essential for glycerol transport via the glycosomal and plasma membrane, implicating the probable presence of other glycerol transporters.

Abbreviations

AQP (aquaglyceroporin); BF (bloodstream form); BTUB (β -tubulin); HAT (Human African Trypanosomiasis); RNAi (RNA interference); SMB (single marker bloodstream form); *T. brucei* (*Trypanosoma brucei*).

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