Molecular & Biochemical Parasitology 175 (2011) 144–153

Contents lists available at ScienceDirect

Molecular & Biochemical Parasitology

Functional analysis of novel aquaporins from *Fasciola gigantica*☆

Amornrat Geadkaew[a], Julia von Bülow[b], Eric Beitz[b], Suksiri Vichasri Grams[c], Vithoon Viyanant[a], Rudi Grams[a,*]

[a] *Graduate Program in Biomedical Sciences, Faculty of Allied Health Sciences, Thammasat University, Phaholyothin Road, Klong Luang, Pathumthani 12121, Thailand*
[b] *Pharmazeutisches Institut, Christian-Albrechts-Universität, Kiel 24118, Germany*
[c] *Department of Biology, Faculty of Science, Mahidol University, Bangkok 10400, Thailand*

ARTICLE INFO

Article history:
Received 14 July 2010
Received in revised form 15 October 2010
Accepted 29 October 2010
Available online 10 November 2010

Keywords:
Platyhelminthes
Fasciola gigantica
Aquaporin
Tegument
Testes
Ovary

ABSTRACT

Fascioliasis, caused by liver flukes of the genus *Fasciola*, is an important disease of ruminants. In order to identify a potential new drug target we have studied aquaporin (AQP) in *Fasciola gigantica*. AQPs facilitate the transport of water, glycerol and other small solutes across biological membranes. The structure, function, and pathology of AQPs have been extensively studied in mammals but data for AQPs from trematodes is still limited. In the present study, we have functionally characterized two closely related AQP isoforms, FgAQP-1 and FgAQP-2, from the trematode *F. gigantica*. Immunohistochemical analysis located the FgAQPs in the tegumental cells, their processes and the tegument itself. In addition, they were present in the epithelial linings of testes and ovary. Expression in *Xenopus* oocytes of these FgAQPs increased osmotic water permeability 3–4-fold but failed to increase glycerol and urea permeability. AQPs have two highly conserved NPA motifs that are important for the function of the channel pore. In FgAQP-1 and FgAQP-2 the first NPA motif is changed to TAA. Substitution of Thr with Asn in the TAA motif of FgAQP-1 increased its water permeability twofold but did not affect urea and glycerol impermeability while the substitution at the pore mouth of Cys204 by Tyr caused loss of water permeability. In addition, the FgAQPs did not increase methylamine and ammonia permeability after expression in yeast. In comparison to rat AQP-1 the described FgAQPs showed low water permeability and further *in vivo* analyses are necessary to determine their contribution to osmoregulation in *Fasciola*.

1. Introduction

Liver flukes of the genus *Fasciola* are important tissue parasites of cattle and sheep worldwide causing severe pathology in these animals and financial losses in agribusiness. Similar to other trematodes they have a complex life-cycle involving an intermediate host, a freshwater snail, in which asexual reproduction of the infectious miracidium takes place leading to the final release of hundreds to possibly thousands of cercariae into the snail's water habitat. Grazing animals take up the infectious metacercariae attached to water plants or floating on the water surface and thereby allow the parasite to continue its development. The encysted parasite becomes activated while in contact with stomach acids and excysts in the small intestine, penetrates the gut wall, migrates through the body cavity towards the liver and enters the liver capsule. Before migrating into the bile ducts as an adult, the juvenile burrows through the liver parenchyma and destroys a substantial amount of tissue. From the life-cycle it should be obvious that the parasite is capable of living in very different environments and must be able to sustain water homeostasis under all conditions. It has been observed that cultured worms rapidly swell or shrink in hypotonic and hypertonic culture medium respectively, but revert to their normal size over time and act as osmotic conformers [1,2]. Interestingly, these studies also showed that *F. gigantica* could withstand a wider range of osmotic pressures compared with *F. hepatica*. Infolds of the basal plasma membrane of the syncytial tegument were the parasite structures primarily affected, and indicated water flux by their swelling/shrinking [3]. At the molecular level this suggests the presence of water channels (aquaporins) which facilitate water transport in these structures. Aquaporins are abundant transmembrane proteins that aggregate into tetramers with each monomer forming an independent channel (for a review see [4]). They are present in bacteria, protozoa, plants and animals, even a virus and have been classified into orthodox aquaporins and aquaglyceroporins, the former conduct only water while the latter additionally conduct other small solutes, such as glycerol and urea. A signature of aquaporins is the presence of two NPA motifs in the primary sequence which are located at the core of the channel in the folded protein. The side chains of the asparagine

☆ *Note:* Nucleotide sequence data reported in this paper is available in the EMBL, GenBank and DDJB databases under the accession numbers HM748644 and HM748645.

* Corresponding author. Tel.: +662 986 9213x7241; fax: +662 516 5379.
E-mail address: rgrams@alpha.tu.ac.th (R. Grams).

0166-6851/$ – see front matter © 2010 Elsevier B.V. All rights reserved.
doi:10.1016/j.molbiopara.2010.10.010

residues are in direct contact with the passing water molecule and are, therefore, especially important for the function of the channel. Higher organisms contain larger sets of distinct isoforms, e.g., 13 aquaporins have been identified in humans. Some human isoforms have been found co-expressed in the same cell type and distinct isoforms have been associated with specific pathologies (reviewed in [5]). In trematodes only a single aquaporin from *Schistosoma mansoni* has been analyzed for its function [6]. It is located in the tegumental layer and blocking the protein's expression with siRNA in cultured schistosomula interrupted the transport of water in hypotonic medium and caused a significantly decreased viability. The authors also demonstrated that this treatment blocked the transport of an antimonial drug into the parasite and suggested that the investigated aquaporin was closer related to aquaglyceroporins by its sequence. In a recent follow up study SmAQP has been shown to also conduct mannitol, fructose and alanine across the tegument and to be responsible for lactate secretion [7].

The present study was carried out to better understand how the liver fluke *Fasciola* maintains water homeostasis as previous investigations of osmoregulation in this parasite date back more than 30 years and lack data to support suggested models at the molecular level. The recent investigation of a schistosome aquaporin and the publicly available sequence data for this parasite class allows us to present a more general conclusion on aquaporins in trematodes. Furthermore, as has been shown for schistosomes, AQPs could be new drug targets in trematodiasis.

2. Materials and methods

2.1. Parasites

Adult *F. gigantica* were collected from the livers and bile ducts of naturally infected cattle sacrificed in a slaughterhouse in Sing Buri Province, Thailand. The parasites were washed several times with 0.85% sodium chloride solution and were either stored frozen or immediately processed for further experiments.

2.2. Molecular cloning and sequence analysis

PCR primers based on a partial cDNA encoding a putative AQP from *F. hepatica* present in a small EST database available at ftp://ftp.sanger.ac.uk/pub/databases/Trematode/Fhep/ were used to amplify a homologous 615 bp cDNA fragment from an adult stage *F. gigantica* cDNA library by PCR (forward primer 5′-TGT GAT TGG TAC CAC ATC AAG-3′, reverse primer 5′-CCA TAC TTT ATT ATA ATC GTT GC-3′). This cDNA was used as a DIG-labeled probe (PCR DIG Labeling Mix, Roche) to isolate full-length AQP cDNAs from *F. gigantica* cDNA libraries (adult and metacercarial stages) by standard plaque lift screening procedures. Incomplete FgAQP-2 was isolated by RT-PCR (forward primer 5′-ATG GGT GAA TAC TAC GAC GAC G-3′, reverse primer 5′-AAG TGA GGA TCA GTC CAC CA-3′) from total RNA of adult *F. gigantica*. A 3′ RACE system (Invitrogen) was used with 2 μg adult stage total RNA to complete the FgAQP-2 cDNA sequence (outer forward primer 5′-TGC TGC TAT CCT GGC GAA AG-3′, inner forward primer 5′-GCG AAA GTA ACC GGC TGT GG-3′) following the supplied instructions. Both strands of the isolated cDNAs were sequenced and primary sequence analyses were performed using EMBOSS version 6 [8]. The prediction of transmembrane regions was done using the TMHMM Server v 2.0 at http://www.cbs.dtu.dk/services/TMHMM/. The NCBI-BLAST server at http://www.ncbi.nlm.nih.gov/BLAST/ was used to search for and to retrieve homologous sequences. Clustal X [9] and TEXshade [10] were used to calculate and format a multiple sequence alignment of mammalian and trematode aquaporins. Clustal X was also used to calculate a multiple sequence alignment and a cladogram of helminth aquaporins using neighbor-joining [11] with bootstrap resampling (1000) for branch-support estimation [12]. Putative trematode aquaporin sequences from *F. hepatica*, *Clonorchis sinensis*, and *Opisthorchis viverrini* were kindly provided by Dr. Neil Young, University of Melbourne. The NCBI accession numbers and contig identification codes of the used AQP sequences are shown in Fig. 2 and the multiple sequence alignment is available as Supplementary data (S1).

2.3. Preparation of nucleic acids

Genomic DNA was extracted from adult parasites and total RNA was extracted from newly excysted, 4-week-old and adult *F. gigantica* as previously described [13]. The concentration of nucleic acids was measured by spectrophotometry at 260 nm. Nucleic acids were stored at −20 °C until use.

2.4. Nucleic acid analyses

For Southern analysis, 20 μg each of genomic DNA from adult *F. gigantica* was digested with restriction endonucleases (Fermentas Life Sciences) *Eco*R I, *Vsp* I, and *Eco*R I/*Vsp* I and size-separated in a 0.7% agarose gel in TBE buffer. The 1 kb DNA ladder (Fermentas Life Sciences) was used as size standard. For Northern analysis, 30 μg total RNA was size-separated in a 1.2% agarose gel containing 2.2 M formaldehyde in 1× MOPS buffer. A high range RNA molecular weight marker (Fermentas Life Sciences) was used to determine sizes of hybridizing RNAs. DNA and RNA were capillary-transferred to nylon membranes (Hybond-N Plus, Amersham Biosciences) and fixed by baking at 80 °C for 1 h. The immobilized nucleic acids were hybridized at 50 °C (DNA) or 56 °C (RNA) in 50% formamide, 5× SSC, 5× Denhardt's solution, 100 μg heat-denatured Herring sperm DNA/ml and 0.5% SDS for 15 h with a DIG-labeled FgAQP-1 DNA probe generated by PCR (PCR DIG Labeling Mix, Roche). Chromogenic detection of the hybridized nucleic acids was performed with NBT/BCIP substrates. Reverse transcriptase PCR with total RNA from newly excysted juveniles, 4-week-old juveniles and adults was performed as previously described [14] at 65 °C annealing temperature (FgAQP-1: forward primer 5′-TCG CAG TCG CTG TGT GGA CTG-3′, reverse primer 5′-AGC GAT TAT CGA GCC AAC CAC-3′; FgAQP-2: forward primer 5′-CAC CCG GAG AGA ATG TAA CG-3′, reverse primer 5′-AGC TAT TAG CGA ACC GAC CA-3′).

2.5. Expression of a recombinant C-terminal FgAQP-1 peptide in E. coli and production of polyclonal anti-C-terminal FgAQP-1 peptide antisera

A FgAQP-1 cDNA fragment encoding the 55 C-terminal amino acid residues (M245–R299, FgAQP-1ct) of the protein was generated by PCR using forward primer 5′-ATT AAT GGC ATG CTC AGT GAC GGT GCA TC-3′ and reverse primer 5′-GGA TCC CAA GCT GAC ATC CCT CAT CG-3′. The PCR product was subcloned into the pPEPTIDE2 vector (MoBiTec GmbH) using the introduced recognition sites for restriction endonucleases *Ase* I and *Bam*H I. *E. coli* BL21pLysS was transformed with pPEPTIDE2-FgAQP-1ct and expression of recombinant protein was induced at an OD_{600} 0.6 by adding isopropyl-β-D-thiogalactopyranoside (IPTG) to a final concentration of 1 mM into the medium. The recombinant product of FgAQP-1 peptide and vector encoded fusion protein (163 amino acid residues) was purified using metal chelating affinity chromatography with Zn^{2+} under denaturing conditions in 3 M guanidinium hydrochloride following the protocol provided in the pPEPTIDE Cloning Vector Kit (MoBiTec GmbH). The rFgAQP-1ct peptide and fusion protein were separated by chemical cleavage with hydroxylamine and the released rFgAQP-1ct peptide was collected in the flow through fraction of a repeated metal chelating affinity chromatography step. Quantity and integrity of rFgAQP-