International Journal for Parasitology 40 (2010) 15–23

Contents lists available at ScienceDirect

International Journal for Parasitology

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

Role of an aquaporin in the sheep tick *Ixodes ricinus:* Assessment as a potential control target ☆

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ARTICLE INFO

Article history:
Received 18 May 2009
Received in revised form 12 June 2009
Accepted 15 June 2009

Keywords:
Tick
Aquaporin
Osmoregulation
Salivary gland
RNA-interference
Knockdown
Ixodes ricinus
In situ hybridisation

ABSTRACT

Ticks undergo tremendous osmoregulatory stress as they take on up to 100 times their body weight in blood, returning about 75% of the ingested water and ions via their saliva into the host. We postulated that water channels, or aquaporins, involved in this mass water transport might be good targets for acaricide development. An aquaporin (*Ir*AQP1) identified in the sheep tick, *Ixodes ricinus*, was present only in tissues involved in mass water flux, namely the gut, rectal sac and especially abundant in the salivary glands. *Ir*AQP1 was localised by in situ hybridisation in specific cell and acini types, possibly Type III acini, but absent from the type I acini that are responsible for rehydration of ticks in the non-feeding phase. Gene knockdown of *Ir*AQP1 in isolated salivary glands completely inhibited dopamine-stimulated secretion. Further, *Ir*AQP1 knockdown adult females had 50% reduced body weight gains over the first 5 days feeding on an artificial feeding apparatus and 21% at the point of engorgement on hosts. Haemolymph osmolarity was increased in the *Ir*AQP1-knockdown ticks. Importantly, the blood volume ingested per body weight was reduced by 30%. Overall, it would appear that water passage from the gut to the saliva was disrupted and tick guts were simply too "full" to ingest more blood. However, double-stranded RNA interference of *Ir*AQP1 did not affect mortality of the ticks which successfully fed to detachment at day 9. Overall, our data indicate that *Ir*AQP1 plays a pivotal role in blood meal water handling through the gut and salivary gland, and although its disruption by double-stranded RNA interference dramatically affects feeding performance, ticks remained feeding on the host with subsequent potential pathogen transmission and, therefore, *Ir*AQP1 is not a suitable candidate target for tick control.

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1. Introduction

Ticks remain major parasites affecting humans, livestock and companion animals, principally through the numerous pathogens they transmit to the host via the saliva produced during feeding. Successful control of ticks is largely via chemical means (acaricides), but there are alarming degrees of resistance to all current acaricides in several important tick species worldwide (Taylor, 2001; Graf et al., 2004; George et al., 2004, 2008). Although most acaricides target components of the central or peripheral nervous system (Lees and Bowman, 2007), an appreciation of critical aspects of tick physiology that differ from that of the vertebrate host may point to non-neuronal targets for acaricide development (Wolstenholme et al., 2007).

Ticks face two phases of water stress leading to osmoregulation being a potential critical acaricide target. Most of the tick life cycle is the off-host phase during which ticks cannot imbibe from free-standing water. Water balance is maintained by absorption of water vapour from the air into hygroscopic droplets secreted by the salivary glands onto the mouthparts (Needham and Teel, 1991; Yoder et al., 2006). Nevertheless ticks are very susceptible to desiccation during the off-host phase. Conversely, during the on-host phase ticks take on an enormous water load along with the blood meal such that adult females increase in body weight by up to 100-fold from their unfed body weight. Dietary water is absorbed across the gut wall and expelled via the paired salivary glands such that about 75% of the volume of the blood meal is excreted back into the host (Sauer and Hair, 1972; Kaufman and Phillips, 1973). Interfering with the tick's osmoregulatory system is predicted to have lethal consequences both during the off-host and on-host phases of the tick life cycle and, thus, components of the osmoregulatory system are potential acaricide targets.

Recently, a water channel or aquaporin (AQP), named *Rs*AQP1, was identified in the brown dog tick (*Rhipicephalus sanguineus*) and shown to be expressed exclusively in tissues associated with high water flux, namely the gut and especially abundant in the salivary gland (Ball et al., 2009). Solute transport studies of *Rs*AQP1 in

☆ *Note:* Nucleotide sequence data reported in this paper is available in EMBL database under the Accession No. FN178519.

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doi:10.1016/j.ijpara.2009.06.010

Xenopus oocytes demonstrated that *Rs*AQP1 was permeable only to water and was inhibited by mercury (Hg^{2+}). Previously, it had been shown that secretion by isolated salivary glands from the sheep tick, *Ixodes ricinus*, was completely abolished by low concentrations of Hg^{2+} (Bowman and Sauer, 2004). We postulated that the tick AQP-type1 is critical to salivation and general osmoregulation and, hence, had potential as a target for acaricide development. Importantly, the AQP field has matured at a fast pace and the development of AQP-blockers for human medicine and as anti-malarial treatment is gaining momentum (Haddoub et al., 2009).

In this paper, we examine the temporal and tissue expression of the *I. ricinus* homologue (*Ir*AQP1) of *Rs*AQP1 and the localisation of *Ir*AQP1 within the salivary gland by in situ hybridisation. Employing a gene knockdown approach, we assessed the contribution of *Ir*AQP1 to water transport in dopamine-stimulated isolated salivary glands. Further, we assessed the consequences of *Ir*AQP1 knockdown on saliva and haemolymph osmolarity and tick feeding parameters both using an artificial membrane feeding apparatus and standard host feeding.

2. Materials and methods

2.1. Ticks and library

Adult *I. ricinus* ticks collected by blanket dragging in June 2008, from a wooded site near Drumnadrochit, UK (latitude, 57.3404; longitude, −4.4961), were maintained in a temperature controlled incubator at 16 °C at 100% relative humidity (RH) on a 16:8 h light:dark cycle.

2.2. Ixodes ricinus library screening for an AQP homologue

A salivary gland cDNA library constructed from partially-fed female *I. ricinus* (Lees and Bowman, unpublished data) using a PCR-amplification approach with a SMART cDNA library construction kit (Clontech, St-Germain-en-Laye, France), according to the protocol provided by the manufacturer was the original template used to obtain the *I. ricinus* AQP. Anchored PCR was carried out on an aliquot of the library with primers designed around conserved regions of an AQP found in the genome of a closely related tick species, *Ixodes scapularis* and homologues to the *Rs*AQP1 (Ball et al., 2009). PCR reactions consisted of 3 µl library plasmid template, 5 µl 10× reaction buffer (160 mM $(NH_4)_2SO_4$, 670 mM Tris–HCl, pH 8.8, 0.1% stabiliser), 2 µl 50 mM $MgCl_2$, 1 µl dNTPs (25 mM each), 1 µl of each primer (10 mM each), 0.5 µl (1.25 U) Taq (Bioline, London, UK) and diethylpyrocarbonate (DEPC)-treated water to give a 50 µl total volume. Primer pairs used were *Ir*AQP1-R2 (TCAACCACGTCTGTTGA) and PT2F1 (AAGTACTCTAGCA ATTGTGAGC), for the 5′ end, and *Ir*AQP1-F1 (ATGCAGCTTTTCGG CAACAC) and PT2R1 (CTCTTCGCTATTACGCCAGCTG) for the 3′ end. PCR cycling conditions were as follows: 1 cycle of 5 min at 94 °C, followed by 36 cycles of 30 s at 94 °C, 30 s at 55 °C and 40 s at 72 °C followed by a final extension time of 15 min at 72 °C. Products were visualised on an agarose gel and specific bands excised and cloned into a pCR4-TOPO TA vector (Invitrogen, Paisley, UK). Purified plasmids were sequenced by Eurofins MWG (Ebersberg, Germany) from the flanking T7 and T3 promoter regions.

2.3. Bioinformatics and phylogeny

Protein sequences of AQPs found by a tBLASTx search in the *I. scapularis* genome database and *Ripicephalus appendiculatus* and *Amblyomma variegatum* expressed sequence tag (EST) databases were aligned in MEGA version 4.1 (Tamura et al., 2007) together with an AQP from *Dermacentor variabilis* (Holmes et al., 2008) and *R. sanguineus* (Ball et al., 2009). Alignments were adjusted manually, where necessary, and amino acid sequences used to estimate phylogeny with the neighbour-joining, minimum evolution and maximum parsimony methods. Phylogenetic trees were constructed with 10,000 bootstrap replicates. All methods gave trees with similar topology and approximate bootstrap values; therefore only the neighbour-joining tree is presented. Percentage homology amongst tick AQPs was calculated using ClustalW (version 1.83).

2.4. Tissue and developmental stage expression of IrAQP1

Female *I. ricinus*, partially fed on guinea pigs for 6 days, were dissected under ice-cold buffer (20 mM Tris (hydroxymethyl) aminomethane (Tris), 5 mM EDTA, 0.9% NaCl, pH 7.4). Salivary glands, Malpighian tubules, synganglia, oviduct, rectal sac and gut were dissected out and stored at −80 °C. Total RNA was prepared from tissue samples using a Mini RNA Isolation I Kit (Zymo Research, Orange, California, USA). Total RNA was extracted from whole larvae (20), nymphs (10) and individual unfed adult male and unfed female ticks using Trizol, according to the manufacturer's instructions (Invitrogen). After isolation, 1 µg of total RNA from stages and tissues was treated with 1 µl RQ1-DNase and 1 µl RQ1 buffer and incubated at 37 °C for 30 min.

One microgram of DNase-treated total RNA was incubated at 70 °C with 0.5 µg of oligo d(T)15 (Promega, Southampton, UK) in a total volume of 10 µl for 5 min. Material was snap-chilled on ice for 5 min prior to the addition of 5 µl 5× reverse-transcription (RT) buffer, 1 µl dNTPs (25 mM each), 0.5 µl Bioscript-reverse transcriptase and 3.5 µl DEPC water. The reaction was incubated at 42 °C for 75 min prior to arrest by heating to 70 °C for 5 min. The presence of the *Ir*AQP1 in tissue and developmental stage cDNA was detected by PCR. The protocol and cycling conditions were carried out as described above using specific primers to give a product of 798 bp (*Ir*AQP1-F1, ATGCAGCTTTTCGGCAACAC and *Ir*AQP1-R2, TCAACCACGTCTGTTGA). Materials and the RT-PCR procedure were validated using *I. ricinus* actin primers (*Ir*ActinF, AGAG CAAGCGTGGTATCCTC and *Ir*ActinR AGCTCGTTGTAGAAGGTGTGG) generating a fragment of approximately 110 bp. Pilot studies determined that the log-phase of the PCR reaction for *Ir*AQP1 and actin were 36 and 34 cycles, respectively, and suitable for semi-quantitative estimation of transcript abundance.

2.5. Preparation of in situ hybridisation (ISH) riboprobes

PCR, as described above, was carried out using a plasmid containing *Ir*AQP1 and specific primers (*Ir*AQP1-ISHF, CTTCCGTCGT CCTCTTCATC and *Ir*AQP1-ISHR, GGGCTACAACTGGTTCTGGGT) to give a product of 394 bp. Product was run on an agarose gel and specific bands excised and gel purified. A TOPO-T7 linker was ligated to the PCR product as above, and secondary PCR reactions were carried out with T7 and either *Ir*AQP1-ISHF or *Ir*AQP1-ISHR primers to generate sense and antisense templates, respectively. Specific products were gel purified and T7 in vitro transcription reactions were performed using a T7-MEGAscript Kit (Ambion, Huntingdon, UK) with UTP mixed with digoxigenin (DIG)-UTP (75 mM stocks, Roche Applied Sciences, Switzerland) at a ratio of 2:1 and incubated at 37 °C for 3 h. *Ir*AQP1 riboprobes were purified using Probequant G-50 micro spin columns (GE Healthcare, Little Chalfont, UK) and quantified with an ND-1000 microspectrophotometer (Nanodrop Technologies Ltd, Wilmington, Denver, USA).

2.6. ISH in I. ricinus salivary glands

Salivary glands were dissected out of partially fed *I. ricinus* ticks under ice-cold buffer and fixed in 4% paraformaldehyde at 4 °C for

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