International Journal for Parasitology 41 (2011) 1053–1061

Contents lists available at ScienceDirect

International Journal for Parasitology

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

Interaction of a *Wolbachia* WSP-like protein with a nuclear-encoded protein of *Brugia malayi*

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ARTICLE INFO

Article history:
Received 14 April 2011
Received in revised form 21 May 2011
Accepted 23 May 2011
Available online 6 July 2011

Keywords:
Filariasis
Brugia malayi
Wolbachia
Symbiotic relationship
Wolbachia surface protein
Phage display panning

ABSTRACT

The *Brugia malayi* endosymbiont *Wolbachia* has recently been shown to be essential for its host's survival and development. However, relatively little is known about *Wolbachia* proteins that interact with the filarial host and which might be important in maintaining the obligate symbiotic relationship. The *Wolbachia* surface proteins (WSPs) are members of the outer membrane protein family and we hypothesise that they might be involved in the *Wolbachia–Brugia* symbiotic relationship. Notably, immunolocalisation studies of two WSP members, WSP-0432 and WSP-0284 in *B. malayi* female adult worms showed that the corresponding proteins are not only present on the surface of *Wolbachia* but also in the host tissues, with WSP-0284 more abundant in the cuticle, hypodermis and the nuclei within the embryos. These results confirmed that WSPs might be secreted by *Wolbachia* into the worm's tissue. Our present studies focus on the potential involvement of WSP-0284 in the symbiotic relationship of *Wolbachia* with its filarial host. We show that WSP-0284 binds specifically to *B. malayi* crude protein extracts. Furthermore, a fragment of the hypothetical *B. malayi* protein (Bm1_46455) was found to bind WSP-0284 by panning of a *B. malayi* cDNA library. The interaction of WSP-0284 and this protein was further confirmed by ELISA and pull-down assays. Localisation by immunoelectron microscopy within *Wolbachia* cells as well as in the worm's tissues, cuticle and nuclei within embryos established that both proteins are present in similar locations within the parasite and the bacteria. Identifying such specific interactions between *B. malayi* and *Wolbachia* proteins should lead to a better understanding of the molecular basis of the filarial nematode and *Wolbachia* symbiosis.

1. Introduction

Filariasis is a serious public health problem worldwide. It is endemic in 83 countries and territories and results in significant economic loss by causing considerable morbidity (Molyneux, 2003). Some 120 million people are infected with the lymphatic filarial nematodes *Wuchereria bancrofti*, *Brugia malayi* and *Brugia timori* (WHO, 2004) with 1.3 billion people at risk. Lymphatic filariasis (LF) provokes acute dermatolymphagioadenitis and lymphedema, often leading to elephantiasis as a result of damage and dysfunction of the lymphatics (Taylor et al., 2010). The related filarial nematode *Onchocerca volvulus* affects 37 million people (Molyneux, 2003) and causes onchocerciasis (river blindness) as well as skin lesions due to inflammation induced by dead microfilaria. The ocular lesions (keratitis, retinal lesions) are caused by microfilaria that migrate to the cornea and penetrate the ciliary body. This may lead to atrophy of the optic nerve and consequent blindness (Taylor et al., 2010).

Currently, the most widely used strategy for the control of filariasis as a public health problem employs mass drug administration of albendazole in combination with ivermectin (IVM) or diethylcarbamazine (DEC) for LF, and IVM alone for onchocerciasis. One of the limitations of this approach is that the drugs are not macrofilaricidal (i.e. they do not kill the adult worms), and thus are not efficient tools for complete elimination of the worms (Nandha et al., 2007; Ottesen et al., 2008).

Most of the human filarial nematode species, including *Brugia* spp., *W. bancrofti* and *O. volvulus*, harbour the obligate intracellular endosymbiont *Wolbachia* (Taylor et al., 2005a). *Wolbachia* bacteria were first identified in insects where they are parasitic and associated with reproductive manipulation (Werren et al., 2008). In filaria, *Wolbachia* is an obligate mutualistic symbiont that plays an essential role in oogenesis and embryogenesis in adult worms

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0020-7519/$36.00
doi:10.1016/j.ijpara.2011.05.008

and during larval development (Smith and Rajan, 2000; Pfarr and Hoerauf, 2007; Ghedin et al., 2008, 2009). Elimination of *Wolbachia* by antibiotic treatment leads to infertility of the female worms, inhibition of larval moulting, and atrophy and death of adult worms (macrofilaricidal effect) (Hoerauf et al., 2000; Casiraghi et al., 2002; Taylor et al., 2005b). This evidence prompted the study of *Wolbachia* as a target for anti-filarial nematode chemotherapy. Multiple in vitro and in vivo studies, including several clinical trials in humans using *Wolbachia* targeting antibiotics, reported anti-filarial effects confirming the essential role *Wolbachia* plays in worm survival and thus vulnerability for elimination (Bazzocchi et al., 2008; Hoerauf et al., 2008; Specht et al., 2008; Specht and Wanji, 2009; Supali et al., 2008; Mand et al., 2009). The endosymbiont was also found to contribute to LF and onchocerciasis pathogenesis and thus morbidity (Taylor et al., 2000; Taylor, 2003). In particular, a member of the *Wolbachia* surface protein (WSP) family was shown to induce an inflammatory response associated with the pathogenesis of onchocerciasis through the activation of an innate immune response (Brattig et al., 2004) and to have anti-apoptotic activity by delaying the apoptosis in human polymorphonuclear cells that are essential for the initiation and execution of the innate immune response against bacterial pathogens (Bazzocchi et al., 2007).

The molecular mechanisms involved in the interaction of the filarial endosymbionts and their hosts remain largely unexplored. With the availability of genomic data of *B. malayi* and its *Wolbachia* endosymbiont (*w*Bm) (Foster et al., 2005; Ghedin et al., 2007), it has become possible to study the underlying mechanisms of this symbiotic system, and it has pointed to some specific pathways and filarial and *Wolbachia* proteins that might be essential in maintaining the *Wolbachia–Brugia* symbiotic relationship. In particular, it was postulated that surface proteins such as the WSP family proteins of the endosymbiont are the most likely to be involved in the *Wolbachia–Brugia* symbiotic interaction (Ghedin et al., 2008). Three WSP-like outer surface proteins (wBm0100, wBm0284 and wBm0432) have been identified in the *Wolbachia* genome (Foster et al., 2005), and these are similar to the outer membrane protein (OMP) family of bacteria known to be involved in bacteria–host interactions (Baldo et al., 2010). In arthropods, a WSP-like protein is thought to be a key player for establishment and persistence of the symbiosis but little is known about the role of this protein and its possible interaction partners in the arthropods and/or nematodes (Baldo et al., 2010).

The WSP-like proteins are highly conserved in *Wolbachia* from filarial nematodes and are also reported to have a heterogeneous pattern of amino acid diversity characteristic of other OMPs (Braig et al., 1998; Baldo et al., 2005, 2010; Serbus et al., 2008). Moreover, through analysis of the *B. malayi* secretome, a number of *Wolbachia* OMPs were found to be secreted or released by the worm (Bennuru et al., 2009). In the work described here, we have examined the role that members of the WSP family might play in the host–endosymbiont relationship, focusing in particular on one member of the family, WSP-0284 (GI: 3266802, locus tag: *w*Bm0284).

2. Materials and methods

2.1. Cloning, expression and purification of the Wolbachia surface protein WSP-0284

The cDNA corresponding to the WSP gene *w*Bm0284 was amplified from positions 73 to 846 of the predicted open reading frame (ORF) by PCR from female *B. malayi* random-primed cDNA using gene-specific primer set: 5′ CACCGAAACAGAAGGATTCTACTT 3′ and 5′ ACAACATGTTTAAACCTTGC 3′. This region consisted of the entire predicted ORF minus the predicted signal sequence. The 787 bp PCR product was first cloned into pENTR™/D-TOPO®, entry cloning for the Gateway® System (Invitrogen, Carlsbad, CA, USA). The first four nucleotides in the forward primers, CACC, are needed for subsequent recombination into the destination vectors. The fidelity of the cloned PCR amplicon and its orientation were confirmed by DNA sequencing. The insert present in a confirmed *w*Bm0284 clone was transferred between specific attachment sites (attL and attR) on the entry clone and the pDEST™15 destination vector mediated by Gateway® LR Clonase™ Plus enzyme mix (LR recombination) following the manufacturer's instructions (Invitrogen). This LR recombination provided a GST Tag at the N-terminal end of the expressed protein. The WSP-0284-pDEST™15 plasmid was transformed into BL21star (DE3) *Escherichia coli*. The ~56 kDa GST-WSP-0284 fusion protein was only expressed in the inclusion bodies even when grown at 16 °C. Therefore, inclusion bodies containing insoluble GST-WSP-0284 were treated with 6 M urea at 4 °C overnight and the urea-soluble GST-WSP-0284 was further purified by a preparative SDS–PAGE on a PrepCell (Bio-Rad, Hercules, CA, USA). The protein-containing fractions eluted in Laemmli buffer (25 mM Tris, 192 mM glycine, 0.1% SDS, pH 8.3) were analysed by SDS–PAGE. Fractions containing the purified GST-WSP-0284 were identified by western blot using mouse-anti-GST antibodies (Thermo Scientific, Rockford, IL, USA) and horseradish peroxidase (HRP) conjugated anti-mouse IgG (KPL, Gaithersburg, MA, USA). The protein concentration of the pooled fractions was determined using the detergent-compatible (DC) protein assay reagents (Bio-Rad).

2.2. Production of antibodies against WSP-0284 protein

A group of five female BALB/c mice were immunised s.c. with 30 μg of recombinant GST-WSP-0284 protein formulated in Sigma Adjuvant System® as recommended by the manufacturer (Sigma–Aldrich, St. Louis, MO, USA) and under an approved protocol (IACUC#224). Boost immunizations were given on days 14 and 28 post-immunization. Blood was collected pre-immunization and on day 14 after the second boost. Pooled serum was analysed by western blot. The corresponding bands of the recombinant GST-WSP-0284 protein as well as the native protein in the *B. malayi* crude extract were detected, whereas there was no recognition of the recombinant proteins when pre-immunization serum was used (data not shown). Anti-GST-WSP-0284 antibodies were also raised in rabbits by Strategic Biosolutions Inc. (USA) following a 70-day standard protocol. The specificity of this serum was identical to mouse serum (data not shown).

2.3. Localisation of WSP-0284 in B. malayi worms by immunoelectron microscopy

Brugia malayi female worms were fixed in 0.25% glutaraldehyde in 0.1 M phosphate buffer, pH 7.4 containing 1% sucrose for 60 min at room temperature and processed for immunoelectron microscopy as described previously (Lustigman et al., 1991). Thin sections of embedded worms were blocked and probed with rabbit antibodies raised against recombinant GST-WSP-0284 (1:50 dilution) followed by 15 nm gold labelled goat anti-rabbit IgG (H+L) (Amersham Biosciences, UK). Pre-immunization serum was used as the control.

2.4. Testing the binding of WSP-0284 to B. malayi crude protein extracts using a modified ELISA-type protein binding assay

Brugia malayi adult female worms (from 120 days p.i. of Mongolian jirds) were obtained from the National Institute of Allergy and Infectious Diseases (NIAID, USA)/National Institutes of Health (NIH, USA) Filariasis Research Reagent Repository Center (FR3; Athens,