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

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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 hypothesize that they might be involved in the *Wolbachia-Brugia* symbiotic relationship. Notably, immunolocalization 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. Localization 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.

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Keywords

Filariasis; *Brugia malayi*; *Wolbachia*; Symbiotic relationship; *Wolbachia* surface protein; Phage display panning

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 dermatolymphagoadenitis 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*, harbor 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 and during larval development (Smith et al., 2000; Pfarr et al., 2007; Ghedin et al., 2008, 2009). Elimination of *Wolbachia* by antibiotic treatment leads to infertility of the female worms, inhibition of larval molting, 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, 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 (wBm) (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: wBm0284).

2. Materials and methods

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

The cDNA corresponding to the WSP gene wBm0284 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 wBm0284 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 analyzed 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 immunized 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 analyzed 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. Localization 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 labeled 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, GA, USA; www.filariasiscenter.org). Soluble PBS crude extracts of *B. malayi* female worms were prepared as described previously (Turner et al., 2006) using Protease Inhibitor Cocktail (Roche, Mannheim, Germany). Soluble crude extract from adult *Acanthocheilonema viteae* female worms was prepared in PBS (pH 7.4) containing N-alpha-p-tosyl-L-lysine chloromethyl ketone (50 µg/ml), N-tosyl-L-phenylalanine chloromethyl ketone (50 µg/ml), and phenylmethylsulfonyl fluoride (1 mM) using a glass hand held homogenizer. The *A. viteae* extract was a gift from Drs. William Harnett and Katrina Houston from the University of Strathclyde, Glasgow, Scotland.

A 96-well polystyrene plate (Corning Inc., Corning, NY, USA) was coated with parasite crude extract (10 µg/ml) in 0.1 ml of PBS (pH 7.2) overnight at 4°C. The wells were then washed five times with PBS-T (PBS plus 0.05% Tween 20) and blocked with 3% BSA in PBS-T for 1 h at room temperature to prevent non-specific binding. After an additional washing step (five times with PBS-T), GST-WSP-0284 recombinant fusion protein was added to duplicate wells at different concentrations (1–10 µg/ml), and was left to react for 2 h at room temperature. BSA was used as a control for non-specific binding of the detecting antibodies to the parasite extracts. Wells were washed three times with PBS-T and bound protein detected by probing with HRP conjugated THE™ Anti-GST Monoclonal Antibody (Mouse) (Genscript, Piscataway, NJ, USA) followed by development with a tetramethyl benzidine substrate (Thermo Scientific), and the absorbance was read at 450 nm using SpectraMAX190 (Molecular Devices, USA). The ELISA was repeated three times using crude extracts prepared from different batches of *B. malayi* worms and one batch of *A. viteae* extract. The BSA background values were subtracted from the GST-WSP-0284 test wells absorbance. The absorbance in the control wells was consistently below 0.07.

2.5. Construction of a T7 select *B. malayi* adult female cDNA phage expression library and panning for putative WSP-0284 interacting proteins

RNA was extracted from 100 *B. malayi* adult female parasites and subjected to two rounds of selection on oligo-dT coated magnetic beads using the Straight A's mRNA isolation system (Novagen, Gibbstown, NJ, USA). A total of 4 g of this purified poly A⁺ mRNA was then used as a template to prepare a random primed cDNA library in the T7 bacteriophage display vector T7select 10-3B, following the manufacturer's protocol (Novagen). T7Select 10-3B allows proteins up to 1,200 amino acids in size to be expressed as fusion proteins with the T7 coat protein, with each recombinant phage expressing five to 15 copies of recombinant proteins on its surface. This library was found to contain 2×10^6 primary recombinants. The library was amplified following the manufacturer's protocol, resulting in an amplified stock containing 4×10^{10} plaque forming units (PFU)/ml. PCR and DNA sequence analysis of 100 randomly selected plaques from the amplified library revealed that 95% of the clones contained an insert, and all of the inserts were derived from *B. malayi*. None of the clones examined were derived from rRNA templates.

For panning, aliquots of the phage display library were first pre-absorbed to remove non-specifically interacting phage. To accomplish this, four wells of an Immulon 2 HB flat-bottomed 96-well ELISA plate (Fisher Scientific, Waltham, MA, USA) were filled with 35 μ l of a solution of 30 μ g/ml of streptavidin in 50 mM Tris-HCl (pH 7.5) and 150 mM NaCl (TBS) and incubated at 4°C overnight. The following morning, the streptavidin solution was removed from one of the plates and replaced with 200 μ l of a solution of 2% (w/v) BSA in TBS (TBS/BSA). The plate was incubated for 30 min at 37 °C. The BSA solution was removed and the wells were washed three times with TBS. A solution containing 2×10^9 PFU of the phage from the above library in TBS/BSA, 0.5% Tween 20 was added to each well, and the plates were incubated by rocking at room temperature for 3 h. The pre-absorbed phage was then removed and stored overnight at 4°C.

For panning, 400 μ g of GST-WSP-0284 fusion protein (prepared and purified as described in Section 2.5) was labeled with biotin using the FluoroReporter Mini-Biotin-XX protein labeling kit (Invitrogen), according to the manufacturer's instructions. A second ELISA plate was coated with streptavidin as described above, and the streptavidin solution replaced with 35 μ l of a solution of 100 nM biotinylated GST-WSP-0284 in TBS. The plates were incubated for 1 h at 37 °C. The plate was then washed three times with TBS plus 0.5% Tween 20 (TBST), and the wash replaced with 35 μ l of a 1 mM biotin solution, to block any unoccupied streptavidin remaining in the wells. The plate was incubated for 5 min at room temperature and the wells washed three times with TBST. The wash was replaced with the pre-absorbed phage solution (see Section 2.5), and incubated for 2 h on a rocking platform at room temperature. The plate was washed six times with TBST and 35 μ l of PBS containing 0.1% Tween 20 and 0.1% SDS was added to each well to elute the bound phage. The plate was incubated for 10 min at room temperature on a rocking platform. The aliquots of the eluted phage were combined and 50 μ l of the combined solution added to 1 ml of a mid-log culture of BLT5403 host cells. The culture was placed on a roller drum at 37 °C and incubated for approximately 2 h or until cell lysis occurred. The lysed cultures were subjected to centrifugation at 4,000 g for 10 min to remove the cell debris and the titer of the phages in the lysate was determined. Subsequent rounds of panning utilized the eluted amplified phage stocks derived from the previous round. The protocol followed in the subsequent panning rounds was identical to that presented here, with the exception that the number of phages added to the plate was reduced to 2×10^7 PFU. Sixteen unamplified plaques were isolated from the last panning round; each of the *B. malayi* inserted regions was PCR amplified using T7SelectUP and T7SelectDOWN primers (Novagen) and sequenced by Sanger DNA Sequencing (Genewiz, South Plainfield, NJ, USA).

The enriched phage contained a fragment of 582 bp corresponding to positions 1,681 to bp 2,574 of the *B. malayi* gene Bm1_46455 (GenBank accession XM_001900718.1, Gene ID: 6104173). This cloned fragment was named Bm-G2. It encoded in frame a polypeptide of 72 amino acids corresponding partially to predicted exon 7 in frame with 14 amino acids encoded by predicted intron 7. Due to incomplete predicted splicing, the 3' end after the stop codon corresponds to the intronic region of positions 1,855 to bp 1,946 of intron 7 and the coding regions of exons 8, 9 and partial exon 10 (2,546–2,575 bp).

2.6. Cloning, expression and purification of the *B. malayi* Bm-G2 protein fragment

A cDNA corresponding to 66 amino acids of the Bm-G2 polypeptide was amplified by PCR from cDNA of female *B. malayi* using the following primer set: 5' CATACCATGTCCATCAGAGC 3'; and 5' CAGAAGCGACACAACACGAC 3' (corresponding to positions 1,700 to bp 2,030 of genomic DNA sequence of *B. malayi* gene Bm1_46455). The 331 bp amplified Bm-G2 fragment was cloned directly into the pCR®4-TOPO® plasmid vector and sequenced after transformation in TOP10 cells according to the manufacturer's instructions (Invitrogen, 25-0276). The Bm-G2 cDNA fragment was then cloned into pTrcHisA vector (Invitrogen) digested with *EcoRI* to generate the pTrcHisA-Bm-G2 expression vector. This cloning strategy added the sequence GAATTCGCCCTT to the 5' end of the Bm-G2 cDNA to keep it in frame with the His tag and for the expression of the amino acids 7–72 with predicted molecular weight of approximately 14 kDa, including the vector's His tag of 50 amino acids. The expression was done using BL21star (DE3) plysS cells (Invitrogen).

The His-Bm-G2 recombinant fusion protein was purified under denaturing conditions in 6 M urea using His•Bind Columns (Novagen), according to the manufacturer's instructions. The purified recombinant His-Bm-G2 protein was dialyzed (50 mM Tris-HCl, 18 mM NaCl, 1 mM EDTA, pH 7.6) and analyzed by SDS-PAGE. The protein concentration was determined using NanoDrop 2000 (Thermo Scientific).

2.7. Localization of Bm-G2 in *B. malayi* worms by immunoelectron microscopy

A group of five female BALB/c mice was immunized as described above using 30 µg of the His-Bm-G2 protein. Anti-His-Bm-G2 monospecific antibodies were affinity-purified from 40 µl mouse anti-His-Bm-G2 serum pre-adsorbed with 160 µl of *E. coli* extract (Promega, Madison, WI, USA) using recombinant His-Bm-G2 transferred to nitrocellulose membrane, following the protocol described previously (Lyon et al., 1988). The eluted monospecific antibodies were concentrated using a 10 kDa cutoff centrifugal filter (Millipore, Billerica, MA, USA) and then examined by western blot analysis for their recognition of the target recombinant protein and native proteins in the *B. malayi* crude extract.

The thin sections of embedded *B. malayi* worms (prepared as described above) were probed with the purified anti-His-Bm-G2 monospecific mouse antibodies (equivalent to 1:2 dilution of serum) followed by 15 nm gold-labeled goat anti-mouse IgG (Fc) (Amersham Biosciences, UK). The pre-immunization mouse serum was used as the control.

2.8. ELISA-type protein binding assay of recombinant WSP-0284 and His-Bm-G2 proteins

For the initial determination of the putative protein-protein interaction between the recombinant GST-WSP-0284 and His-Bm-G2 proteins, 96-well polystyrene plates (Corning Inc.) were coated with the purified recombinant His-Bm-G2 protein at concentrations of 0.2 µg/ml or 1 µg/ml as described above (see Section 2.4). The reactant recombinant GST-WSP-0284 protein or GST, as a control, were then added in duplicates at 1 µg/ml, 5 µg/ml or 20 µg/ml. The bound proteins were detected using the rabbit Anti-GST-tag polyclonal

antibody (Genscript) followed by HRP conjugated goat anti-rabbit IgG (KPL). HRP was detected as described above (see Section 2.4).

2.9. Pull-Down assay to study the interaction between WSP-0284 and Bm-G2

For the pull-down assay, 4 µg of GST-WSP-0284 were incubated with 16 µg of His-Bm-G2 in 1 x TNET buffer (10 x TNET: 0.1 M Tris-HCl, pH 7.5, 0.05 M EDTA, 1.5 M NaCl, 1% Triton) in a final volume of 200 µl for 1 h at room temperature. To immunoprecipitate the putative protein complex, 10 µl of mouse anti-His-Bm-G2 serum or 10 µl of mouse anti-GST-WSP-0284 serum were added to the protein mix for overnight incubation at 4°C. To control for the specific binding of mouse serum to each of the corresponding recombinant proteins, the same amount of mouse anti-His-Bm-G2 or mouse anti-GST-WSP-0284 serum was added to 200 µl TNET buffer containing 16 µg His-Bm-G2 or 4 µg GST-WSP-0284 alone. The next day, 100 µl of prewashed 50% protein G beads (GE Healthcare, Waukesha, WI, USA) in 1x TNET buffer were added to each tube for 1 h at room temperature and the beads were harvested by centrifugation at 40 g for 5 min at room temperature and washed four times with 1 x TNET buffer. The bound proteins were eluted in 2x SDS sample buffer and analyzed by SDS-PAGE, followed by transfer to a nitrocellulose membrane and subsequent western blotting. Detection of the individual proteins was done by probing duplicate western blots with HRP conjugated THE™ Anti-GST or THE™ Anti-His Monoclonal Antibody (Mouse) (Genscript).

2.10. Gene expression analyses using RNA isolated from *B. malayi* worms

The expression of Bm-G2 was analyzed using quantitative Real-Time PCR (qRT-PCR). The primers of Bm-G2 were designed using Primer Express® software (Applied Biosystems, Carlsbad, CA, USA): RT forward primer: 5' ACAGCGGTCATTCATGCTAGAA 3' and RT reverse primer: 5' TCAGCATCAACCACAAGTAATTGA 3'. The qRT-PCR was performed in duplicates using the first-strand random primed complementary DNA samples synthesized from RNA purified from adult *B. malayi* subjected to in vitro antibiotic treatment carried out in triplicate to eliminate their endosymbionts and following the qRT-PCR protocol published by Ghedin et al. (2009). Threshold cycle (C_t) values were then calibrated against *B. malayi* NADH dehydrogenase subunit 1 (NADH), as previously described (Ghedin et al., 2009). Briefly, to characterize the specificity of the amplification, a melting curve analysis was performed at the end of the 40th cycle. Threshold cycle values were then calibrated against *B. malayi* NADH and the relative expression of each transcript in the treated adult female worms versus adult female control worms was calculated using the C_t method. The method involves comparing the C_t values of the samples of interest with a control or calibrator such as a non-treated sample, and calculating $C_t = C_{t\text{-treated}} - C_{t\text{-control}}$. C_t is the C_t value for any sample normalized to the endogenous NADH housekeeping gene. Then C_t values were calculated by subtracting the C_t value of the control group from that of tetracycline-treated samples. Finally, fold changes of tetracycline treated samples over the controls were expressed using the formula; 2^{-C_t} .

3. Results and discussion

3.1. The Wolbachia surface proteins are located on the bacterial cell surface and are also found within filarial tissues

To analyze and compare the ultrastructural localization of WSPs in adult *B. malayi* female worms, we used antibodies raised against two WSP members: WSP-0432 and WSP-0284. WSPs, which contain both trans-membrane domains and a standard signal peptide for secretion, were expected to be found on the surface of the *Wolbachia* of the filarial worms examined, as previously observed for the WSP corresponding to wBm0432 (Kramer et al., 2003; McGarry et al., 2003, 2004; Brattig et al., 2004). However, in our analyses, the WSPs

were not only seen on the surface of *Wolbachia* but also in the *B. malayi* female adult worm tissues (Fig. 1). Immunolocalization using rabbit anti-His-WSP-0432 serum (a gift from Dr. Mark Taylor and Dr. Louise Ford, Liverpool School of Tropical Medicine, Liverpool, UK) established that the WSP-0432 protein was present on the surface of *Wolbachia* (Fig. 1E and G, arrows) as previously shown by McGarry et al. (2004) in the mammalian-stage larvae of *B. malayi*. However, the antibodies also reacted with the protein in the cuticle and the hypodermal region below the cuticle (Fig. 1E) as well as the eggshells surrounding the developing microfilaria (Fig. 1G), and more distinctively to the pseudocoelomic fluid surrounding the gonads (Fig. 1F). In comparison, the WSP-0284 was more abundantly present in the cuticle and the hypodermal region under the cuticle and areas surrounding *Wolbachia* (Fig. 1A) as well as in the nuclei within the embryos (Fig. 1C). There was a similar staining of the eggshells surrounding the developing microfilaria (Fig. 1C). No signal was obtained when pre-immune sera were employed, confirming the specificity of the assay (Figs. 1B and 1D). These results confirmed that these two WSP family proteins are present in the worm's tissues and might potentially have an effect on the filarial host.

3.2. Identification of the WSP-0284 interacting *B. malayi* protein

The broader pattern of the spatial localization of WSP-0284 compared with WSP-0432 suggested that it might be more likely to be involved in the interaction with *B. malayi*. We therefore chose to focus on wBm0284. To determine whether WSP-0284 interacted with *B. malayi* proteins, we used an ELISA-based assay in which we tested whether WSP-0284 binds to immobilized host worm components contained in *B. malayi* soluble crude protein extracts. Crude extracts of *A. viteae*, a filarial nematode that is free of *Wolbachia*, were used as a control for possible non-specific binding (McLaren et al., 1975; Bandi et al., 1998; Hoerauf et al., 1999). As presented in Fig. 2, the GST-tagged WSP-0284 recombinant protein exhibited significant binding to *B. malayi* crude extracts compared with *A. viteae*. The specific binding ($P < 0.005$) was concentration-dependent even though there was some increase in the non-specific binding of GST-WSP-0284 to *A. viteae* crude extracts. These results indirectly confirmed the presence of *Wolbachia* WSP-0284 binding partners within the *B. malayi* crude extract.

To identify *B. malayi* proteins interacting with the *Wolbachia* WSP-0284 protein, a phage display library prepared from poly A+ RNA isolated from adult female parasites was employed. Four rounds of panning were performed using pre-absorbed phage and plates coated with GST-WSP-0284, as described in Section 2.5). Sixteen clones from the last round of panning were eluted, sequenced and searched by BLASTn against the *B. malayi* database in GenBank. Four of the clones were identical and contained a 582 bp cDNA in frame with the phage vector and corresponded to a fragment of *B. malayi* gene Bm1_46455 (accession XM_001900718), which encodes a hypothetical protein. All other clones were empty phages or singletons. The cDNA fragment was named Bm-G2. This result suggested that the Bm-G2 phage clone was enriched during the panning process and thus enabled identification of a portion of a *B. malayi* putative WSP-0284 interacting protein.

The alignment of Bm-G2 cDNA with the Bm1_46455 predicted coding region (Fig. 3A) indicated an ORF corresponding to a portion of the predicted exon 7 (58 amino acids) and the predicted intron located downstream of this predicted exon. Analysis of the ORF located in frame with the phage capsid protein indicated that it encoded a peptide of 72 amino acids followed by a stop codon (Fig. 3B) and out of frame sequences corresponding to predicted intron 7 and exons 8–10. It is thus possible that the Bm-G2 cDNA represents an incompletely spliced mRNA. However, we have found many mRNA clones with a similar structure in various stages of the parasite suggesting that this may represent a splice variant of the predicted Bm1_46455 gene. Work is currently underway to differentiate these possibilities.

In order to confirm the binding of the WSP-0284 and the hypothetical protein coded by *B. malayi* Bm1_46455 gene we focused on the region of Bm-G2 that corresponded to the ORF of 72 amino acids. A recombinant protein encoding 66 amino acids was purified as a His fusion protein, His-Bm-G2, with an expected size of ~14 kDa. This recombinant peptide was employed in the experiments described as follows.

3.3. Validation of the *Wolbachia* WSP-0284 interaction with *B. malayi* Bm-G2 protein fragment

To confirm the interaction between WSP-0284 and Bm-G2, the recombinant GST-WSP-0284 was first tested against His-Bm-G2 using a quantitative ELISA assay. The specific and concentration-dependent binding of GST-WSP-0284 to His-Bm-G2 was verified using rabbit anti-GST (Fig. 4; $P < 0.006$). There was no binding to the control GST protein (negative control) (Fig. 4). These results corroborated the ELISA data using *B. malayi* crude extract and the phage display panning outcome, further signifying the interaction between *Wolbachia* WSP-0284 and *B. malayi* Bm-G2 proteins.

To further confirm the specific interaction between WSP-0284 and Bm-G2, an in vitro pull-down assay was performed using a 1:4 mass ratio of the GST-WSP-0284 (4 µg) and His-Bm-G2 (16 µg) recombinant protein mix. Immunoprecipitation of the mixture with mouse anti-His-Bm-G2 serum or mouse anti-GST-WSP-0284 serum pulled down both target proteins (Fig. 5, Lanes 1 and 4) indicating the functional interaction between both proteins. Notably, Bm-G2 appeared to be polymerized in this reaction, as evident by the presence of multiple reactive bands in the control western blot of His-Bm-G2 used for the immunoprecipitation (Fig. 5, Lane 5). It is possible that this polymerization had some effect on the interaction. Optimally, it would have been preferable to use a monomeric His-Bm-G2 recombinant protein for the assays. Despite this, it appeared that the polymerization did not have a strong influence on the outcomes of pull-down assay; the dimeric His-Bm-G2 protein bound to GST-WSP-0284 to the same extent when it was immunoprecipitated using two distinct antibodies (anti-His-Bm-G2 and anti-GST-WSP-0284; Fig. 5, Lanes 1, 2 and 4, respectively) when compared with His-Bm-G2 without the presence of immunoprecipitating antibodies (Fig. 5, Lane 5). There was some reduction in the binding of the monomeric (~14 kDa) and the trimeric (~42 kDa) molecules, and almost no binding with the tetramer (~56kDa), which might indicate that the Bm-G2 dimer was the most effective in binding the GST-WSP-0284 peptide. This confirmed that WSP-0284 and Bm-G2 peptides were capable of interacting in vitro.

3.4. *Brugia malayi* Bm-G2 protein is expressed in parasite tissues and also found within *Wolbachia*

To further understand the basis of this protein-protein interaction, within *B. malayi* worms we localized the native protein corresponding to Bm-G2 using the anti-His-Bm-G2 antibodies (Fig. 6A-C). As expected, the antibodies reacted strongly with the protein present in worm tissues (cuticle, hypodermis, muscle and nuclei within the embryos) (Fig. 6A and C), but interestingly they also reacted with protein found within the *Wolbachia* present in these tissues (Fig. 6B and C). These signals were not detected in control experiments utilizing pre-immunization sera (Fig. 6D, E and F). Importantly, similar regions within the worms were also recognized by the rabbit antibodies raised against recombinant GST-WSP-0284 (Fig. 1A and C). This indicates that these two proteins are present in similar locations within the parasite and the bacteria, where their potential interaction may have a function in the symbiotic relationship between the parasite host and its endosymbiont. The localization of the *B. malayi* protein corresponding to Bm-G2 within the bacteria is a novel observation and could be a result of its internalization through the surface of the bacterium by mechanisms that are still unknown. Interestingly, pathogenic bacteria in humans were

shown to be able to aggregate and internalize host proteins as part of their invasion process (Garcia-del Portillo et al., 1994). Furthermore, the localization of bacterial WSP and filarial Bm-G2 proteins in the nuclei of the host cell might suggest that these cytoplasmic proteins may be transported to the nucleus of the host cell.

In previous studies we showed that treatment of *B. malayi* with tetracycline regulates a number of *B. malayi* gene transcripts primarily encoding proteins involved in amino acid synthesis and protein translation as well as signaling genes (Ghedin et al., 2009). We therefore tested whether the treatment of adult female worms in vitro over 6 days with 40 µg/ml of tetracycline will have any effect on the regulation of Bm1_46455 gene expression, from which Bm-G2 is derived. Using qRT-PCR, we found that the depletion of *Wolbachia* by tetracycline treatment had a time-dependent effect on Bm1_46455 gene expression (Fig. 7). The transcript levels in the treated parasites were five-fold higher than those in the similarly cultured untreated parasites but this effect only occurred at day four in culture. In our previous studies we showed that by day three after tetracycline treatment in vitro the bacteria within oocytes, embryos and microfilaria were completely degenerated (Ghedin et al., 2009). It is possible that the distinct up-regulation of Bm1_46455 we observed corresponds to these ultrastructural changes of the bacteria within the worm and might represent some kind of feedback loop in this process. Moreover, the combination of the up-regulation of transcript corresponding to Bm1_46455 (Bm-G2) and the localization of this protein in nuclei might highlight a possible regulatory function for Bm-G2 as part of its potential involvement in the relationship between the endosymbiont and its *B. malayi* host.

In summary, we believe this is the first report showing that the symbiotic relationship between *Wolbachia* and its filarial host might be dependent on some interaction between the proteins of both organisms, such as WSP-0284 and the filarial Bm-G2 fragment. The experiments described provide direct evidence that WSP-0284 and Bm-G2 may interact in vitro, and the co-localization of these proteins provides indirect evidence that they may interact in vivo as well. Preliminary western blot studies using *B. malayi* crude extracts and mouse anti-GST-WSP-0284 antibodies have identified two native proteins of ~46 kDa and ~90 kDa, while a ~53 kDa protein was identified using mouse anti-His-Bm-G2 antibodies (Fig. 8). The molecular weight of these native proteins is bigger than their predicted size; 34 kDa for WSP-0284 and ~36 kDa for the full-length protein corresponding to Bm-G2 (encoded by exon 1–7 with the premature stop codon). Unfortunately, we have not yet been successful in immunoprecipitating the WSP-0284 - Bm-G2 complex from crude extracts of *B. malayi* parasites for technical reasons, an experiment that would provide direct evidence that this interaction is also present in vivo. Work to establish this interaction in vivo is ongoing.

The function of each of these proteins in development and survival is not yet known. WSP-0284 belongs to the outer membrane proteins that are known to play important roles in bacterial pathogenesis, including a function in enhancing the adaptability of bacterial pathogens to various environments (Lin et al., 2002). WSP-like proteins have also been shown to correlate with human host immune responses in filarial infection and pathogenesis (Brattig et al., 2004; Ghedin et al., 2008). Further studies incorporating RNA interference approaches will help to define the role of the filarial protein corresponding to Bm-G2, by characterizing the interaction network of this protein within known functional pathways.

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Highlights

- *Brugia malayi* harbors the obligate endosymbiont *Wolbachia*.
- *Wolbachia* surface protein, WSP-0284, is not only located on the surface of bacteria.
- WSP-0284 is released within the worm tissue and binds to the *B. malayi* Bm-G2 protein.
- Bm-G2 partially corresponding to the predicted Bm1-46455 protein is present in similar locations to WSP-0284 within parasite and bacteria.
- Bm-G2/WSP-0284 protein complex is potentially involved in this symbiotic relationship.

Fig. 1.

Localization of *Wolbachia* surface proteins (WSP) wBm0284 and wBm0432 in cross sections of *Brugia malayi* adult female worms. Rabbit antibodies raised against recombinant wBm-GST-WSP-0284 recognize the WSP-0284 protein on the surface of the *B. malayi* and the *Wolbachia* endosymbiont (wBm) (arrows, A and C) in the cuticle (cu; A) and in the nuclei (n) within embryos and eggshells (es) surrounding the microfilaria (C). Rabbit antibodies raised against recombinant His-WSP-0432 recognize the WSP-0432 protein on the surface of the wBm (arrows, E and G), in the hypodermal region (hy, E-F), the cuticle (cu; E) and pseudocoelomic fluid (pf, F) as well as in eggshells (es) surrounding the microfilaria (mf; G). B and D represent Immunogold Electron Microscopy (IEM) using pre-immunization control rabbit serum. The bars in A and C are 1 μ m, the others are 500 nm.

Fig. 2.

ELISA assay to test possible interaction of *Wolbachia* surface protein, wBm-WSP-0284 with crude extracts of parasitic filarial nematodes. Plates coated with 10 µg/ml of crude extracts prepared from *Brugia malayi* (Bm - which contains a *Wolbachia* endosymbiont) or *Acanthocheilonema viteae* (Av - which lacks an endosymbiont) were exposed to varying concentrations of purified recombinant GST-WSP-0284 and bound WSP-0284 was detected as described in Section 2.4. Data represent the mean absorbance at 450 nm ± the S.D. of three independent experiments. The binding of recombinant protein to Bm crude extracts was compared with its binding to Av crude extract by a Student's two-tailed *t* test (** *P* = 0.007).

Fig. 3.

Alignment of *Brugia malayi* gene, Bm1_46455, and *B. malayi* cloned fragment, Bm-G2. A) Representation of the exons that comprise the predicted coding region of the Bm1_46455 gene. The numbers indicate the splice sites in the coding region of the predicted last four exons of the gene and provide a context as to where the Bm-G2 transcript originated. B) Alignment of the partial amino acid sequence of the Bm1_46455 protein and the translated Bm-G2. The box represents the 66 amino acids that were expressed as recombinant fusion protein.

Fig. 4.

Interaction of recombinant Wolbachia surface protein, WSP-0284, with recombinant *Brugia malayi* cloned fragment, Bm-G2, using an ELISA assay. Plates were coated with 0.2 µg/ml or 1.0 µg/ml of His-Bm-G2 and then exposed to varying concentrations (1, 5 or 20 µg/ml) of purified recombinant GST-WSP-0284 or control GST protein. The bound GST-WSP-0284 or GST was detected using anti-GST monoclonal antibodies as described in Section 2.8. –

◆– binding of GST-WSP-0284 to His-Bm-G2 (1.0 µg/ml); –●– binding of GST-WSP-0284 to His-Bm-G2 (0.2 µg/ml); –◇– binding of GST to His-Bm-G2 (1.0 µg/ml); –○– binding of GST to His-Bm-G2 (0.2 µg/ml). The binding of GST-WSP-0284 to His-Bm-G2 at each concentration was compared with the binding of GST to His-Bm-G2 by a Student's two-tailed *t* test (** $P < 0.02$).

Fig. 5. Pull-down assay to study the putative interaction between recombinant *Wolbachia* GST-WSP-0284 and recombinant *Brugia malayi* His-Bm-G2 proteins. Western blot developed using (A) mouse anti-GST antibody to detect GST-WSP-0284 fusion protein (~56 kDa) in the eluates, and (B) mouse anti-His antibody to detect His-Bm-G2 protein (~14 kDa) in the eluates. Lane 1: GST-WSP-0284 and His-Bm-G2 immunoprecipitated using mouse anti-His-Bm-G2; lane 2: His-Bm-G2 immunoprecipitated using mouse anti-His-Bm-G2; lane 3: GST-WSP-0284 immunoprecipitated using mouse anti-His-Bm-G2; lane 4: GST-WSP-0284 and His-Bm-G2 immunoprecipitated using mouse anti-GST-WSP-0284; lane 5: His-Bm-G2; lane 6: GST-WSP-0284. The molecular weights of the polymerized His-Bm-G2 proteins are marked on the right.

Fig. 6.

Localization of *Brugia malayi* Bm-G2 protein in cross sections of *B. malayi* adult female worms. The localization of the native protein corresponding to Bm-G2 in cross sections of *B. malayi* adult female worms using mouse anti-His-Bm-G2 indicates protein localization in worm tissues; hypodermis (hy), cuticle (cu), muscles (mu) (A) and nuclei (n) within embryos (C), as well as within *Wolbachia* (B and C (arrows)). D, E and F represent Immunogold Electron Microscopy (IEM) using control pre-immunization mouse serum. Bars are 500 nm.

Fig. 7.

Changes in mRNA levels corresponding to the Bm1_46455 gene in *Brugia malayi* worms over 6 days of tetracycline treatment. Female *B. malayi* worms were treated with 40 µg/ml of tetracycline for up to 6 days. For each day, the Bm1_46455 specific mRNA levels in treated worms were compared with the untreated control group cultured for the same period of time. Threshold cycle values were calibrated against *B. malayi* NADH and the fold changes in expression levels were determined using Ct method as described in Section 2.10. The means $\pm$ S.D. are represented and statistical analysis utilized the unpaired *t* test (* *P* = 0.02).

Fig. 8.

Western blot showing the specificity of mouse-derived antibodies against *Wolbachia* WSP-0284 (anti-GST-WSP-0284) and against *Brugia malayi* Bm-G2 (anti-His-Bm-G2). The mouse anti-GST-WSP-0284 serum (dilution 1:500) detected the purified recombinant ~56 kDa GST-WSP-0284 (* Lane 1) and two native proteins in the *B. malayi* crude extract (Lane 2; ~46 kDa and ~90 kDa). The monospecific mouse anti-His-Bm-G2 antibodies (dilution 1:1,000) detected the purified monomeric recombinant ~14 kDa His-Bm-G2 as well as its polymers (** Lane 3) and the ~53 kDa native protein in the *B. malayi* crude extract (Lane 4). Lanes 1 and 3 contained 4 µg of recombinant proteins. Lanes 2 and 4 contained 85 µg of crude extract of *B. malayi*.