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Major sperm protein as a diagnostic antigen for onchocerciasis

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ABSTRACT

Onchocerciasis, also known as river blindness, is the second leading infectious cause of blindness worldwide. In order to successfully control this disease, the development of efficient diagnostic tools as well as effective treatments is imperative. A number of proteins have been proposed as vaccine and diagnostic candidates, yet none have been successfully advanced to the point of general clinical use. We have prepared major sperm protein 2 (MSP2) from *Onchocerca volvulus* as a possible diagnostic antigen for onchocerciasis. Importantly, recombinant MSP2 is dimeric in solution, identical to α-MSP from the roundworm, *Ascaris suum*. A panel of sera obtained from Cameroonian individuals afflicted with onchocerciasis positively responded to the recombinant MSP2. Our data suggest that MSP2, like the previously described antigen Ov16, can be utilized as a diagnostic onchocerciasis antigen for monitoring the interruption of transmission.

1. Introduction

An estimated 37 million individuals in Africa, South America, and Yemen currently suffer from onchocerciasis, a debilitating disease caused by the filarial parasite, *Onchocerca volvulus*.[1] Infection with this parasite leads to a range of symptoms from skin lesions and intense pruritus, ultimately resulting in irreversible sclerosing keratitis as a consequence of immune system attack of microfilaria within the eye; indeed, onchocerciasis has led to greater than 700,000 blind individuals, making this disease the second leading infectious cause of blindness in the world. It is commonly known as 'river blindness', as transmission is accomplished by blood feeding of small black flies of the genus *Simulium* which are abundant in riverside areas where they breed. Consequently, fertile riverside areas are occasionally abandoned for fear of onchocerciasis, causing serious socioeconomic problems for endemic regions.

Widespread efforts to control the spread of onchocerciasis in Africa were launched in 1974 with the founding of the Onchocerciasis Control Programme (OCP). Through this and other programs established worldwide, mass distribution of ivermectin (Mectizan™, Merck), the only drug currently available to treat onchocerciasis, has been accomplished. The Special Programme for Research and Training in Tropical Diseases (TDR) now categorizes onchocerciasis as a level III disease, indicating that while the disease burden is falling, several challenges still remain for more effective control and eradication of the disease. Indeed, TDR has been firm on the point that new chemotherapeutic strategies should be developed not only because ivermectin has no macrofilaricidal activity, but also because there is the potential for ivermectin resistance to emerge.[2,3] Additionally, a conduit therapy, treatment with antibiotics aimed at killing the endosymbiotic *Wolbachia* bacteria, is currently undergoing extensive clinical trials.[4]

Arguably, a greater challenge facing onchocerciasis control is the absence of a field-accessible diagnostic tool for detecting current infection.[5,6] Although a previous described antibody test using a recombinant *O. volvulus* protein termed Ov16 is effective, it fundamentally cannot make a distinction between current and historical infections.[7] To resolve this problem, an antigen test using an anti-Ov16 antibody has been proposed; however, the low sensitivity and laborious nature of this and other antigen tests are problematic in practice.[8–10] In sum, since the initial report describing the characterization of Ov16, few nematode-specific proteins have been proposed for the development of an onchocerciasis diagnostic.

Major sperm protein (MSP) makes up ~15% of the total nematode sperm protein.[11,12] It polymerizes into filaments and plays an important role in sperm motility as does the actin cytoskeleton in other metazoan cells, while MSP and actin share little similarity in biochemical properties.[12] Because the concentration of MSP in a given sperm cell is very high (~4 mM), we hypothesized that this protein may serve as a potential diagnostic for *O. volvulus* infection. Additionally, given that MSP only is found in adult male worms, this protein could be considered as an indicator of the presence of live adult worms, a criterion for the diagnosis of active infection. Recent studies have shown that MSP also functions as an extracellular signaling molecule for oocyte maturation and fertilization,[13]

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suggesting not only that there might be higher probability to find MSP outside of nematode sperm, but also that MSP may be intimately involved in nematode reproduction, and thus a chemotherapeutic target to control infection. Indeed, immunization of cattle with recombinant MSP from the worm *Dictyocaulus viviparus* resulted in strong IgG1 responses and decreased the numbers of larvae eleven days after immunization.[14] Here, we report the cloning and characterization of *O. volvulus* MSP2 and the detection of specific antibodies to this protein in the serum of individuals afflicted with onchocerciasis.

2. Results and discussion

Despite the success of ivermectin distribution and treatment programs in controlling the spread of onchocerciasis, the discovery of new therapeutic targets as well as efficient, cost-effective, and field-applicable diagnostics remains a formidable challenge. *O. volvulus* MSP provides an intriguing starting point for the development of diagnostics as well as novel therapeutics, based upon the role of this protein in nematode reproduction.

Recently, α-MSP from *Ascaris suum* was structurally resolved by X-ray crystallography,[15] and it was shown that there are no posttranslational modifications (e.g., glycosylation or phosphorylation) found in this protein. Since α-MSP from *A. suum* and MSP2 from *O. volvulus* share greater than 97% homology at the amino acid level, it is feasible to expect that no posttranslational modifications occur in *O. volvulus* MSP2 as well. Therefore, we chose to use an *Escherichia coli* expression vector system to overexpress recombinant MSP2; for ease of purification, we also constructed a His_6-tagged variant as well as the native MSP2. As shown in Figure 1A, large quantities (>20 mg from a 3 L culture) of relatively pure MSP2-His_6 could be obtained after single metal (Co^{2+}) affinity column chromatography. For purification of MSP2, SP cation exchange chromatography using ionic gradient elution provided a high yield and pure protein (>30 mg from 3 L culture).

Previous biochemical and structural studies showed that MSPs from *A. suum* and *Caenorhabditis elegans* form symmetric homodimers in solution over a wide range of concentrations and conditions.[16,17] In order to confirm that recombinant *O. volvulus* MSP2 was properly folded and dimeric, analytical gel filtration analysis was performed. As shown in Figure 1, MSP2 eluted at an apparent molecular mass of ~28 kDa (K_{av} = 0.203), as previously reported in other proteins from this class. However, we also discovered that MSP2-His_6 eluted at almost the same volume (K_{av} = 0.356) as ribonuclease A (13.7 kDa), indicating that MSP2-His_6 is monomeric and does not dimerize. While curious at first glance, this finding can be explained based upon previous structural data of *A. suum* α-MSP in which it was strongly suggested that the dimer interface of α-MSP includes the C-terminal region of the protein.[15] Thus, the addition of the His_6 affinity tag must interfere with the establishment of native protein–protein interactions in the properly folded dimer. However, the presence of a functional dimer is not indicative of a good immunogen as all antigens are processed prior to MHC display, thus we felt MSP2-His_6 could be a potential diagnostic candidate.

If MSP2 is secreted or released from *O. volvulus* so that it circulates in the bloodstream, it would be reasonable to expect that the human immune system could mount a response against MSP2 and produce the corresponding anti-MSP2 antibodies. To investigate the presence of human anti-MSP2 antibodies in the sera of onchocerciasis-positive individuals, a dot-blot assay using the purified MSP2 was performed. We investigated the sera from a set of nodule-positive Cameroonian individuals (n = 15), using bovine serum albumin (BSA) as a control protein. As shown in Figure 2, all sera from infected patients positively responded to MSP2 with detection as low as 10 μg; however, no signal could be detected against BSA in amounts as high as 120 μg, strongly suggesting that MSP2 is released from *O. volvulus* sperm and is exposed to the human immune system, resulting in the production of specific human anti-MSP2 IgG. Further investigation of sera from skin snip negative individuals (n = 18) showed that 15 of them (83%) had no immune response to either MSP2 or BSA. Individuals who showed positive immunoreactivity to recombinant MSP are likely a result of ongoing transmission in the region where samples were collected. However, it is important to note that the presence of anti-filarial protein antibodies does not indicate current infection, but instead, it is a marker of past exposure to the parasite. Indeed, the presence of anti-MSP2 antibodies indicates not only exposure to the worm, but more correctly, expo-

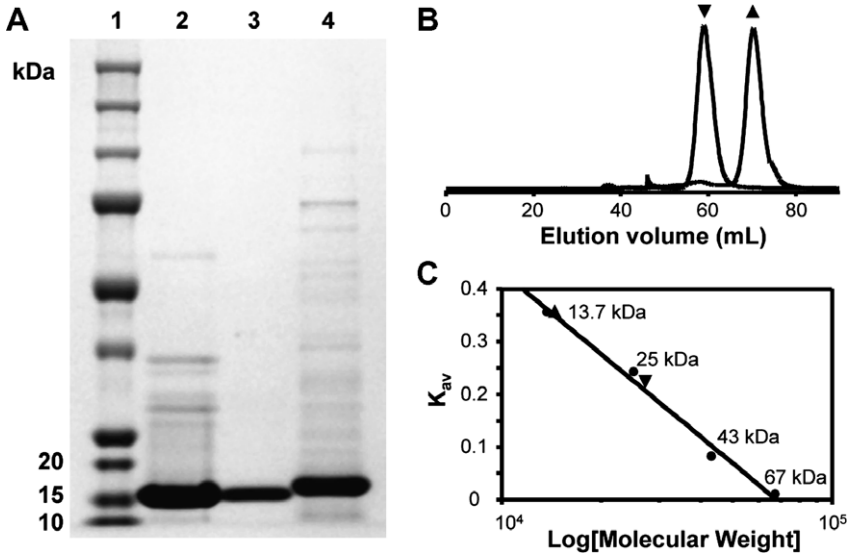
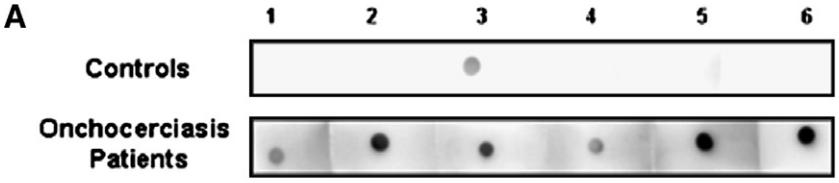


Figure 1. (A) SDS–polyacrylamide gel electrophoresis of the purified MSP2s. 1, marker (Bio-Rad); 2, MSP2-His_6; 3, MSP2 after gel filtration chromatography; 4, MSP2 after SP cation exchange chromatography. (B) Analytical gel filtration analysis. ▼ MSP2, V_e = 59.0 mL; ▲ MSP2-His_6, V_e = 70.4 mL. (C) Molecular weight calibration curve. Protein standards, albumin (67 kDa, V_e = 44.25 mL, K_{av} = 0.01), ovalbumin (43 kDa, V_e = 49.75 mL, K_{av} = 0.083), chymotrypsinogen A (25 kDa, V_e = 62.5 mL, K_{av} = 0.249), and ribonuclease A (13.7 kDa, V_e = 70.7 mL, K_{av} = 0.356) were used to generate calibration curve. Apparent molecular mass (K_{av}) was calculated by the equation, $K_{av} = (V_e - V_o)/(V_t - V_o)$. See Section 3 for full details.

A



B

	Onchocerciasis patients	Controls	LF Patients
# Tested	15	18	3
# Positive with MSP2	15	3	0
# Positive with BSA	0	0	0

Figure 2. Dot-blot assay to detect MSP2-specific antibodies in human serum. (A) Twelve samples, six controls (upper panel) and six patients (lower panel), were tested at the same time using a 12-well tissue culture plate. Control 3 indicates a false-positive signal. See Section 3 for full details. (B) No signal was detected with up to 120 μg BSA. All onchocerciasis patient sera showed positive signals, and ~14% false-positive signal was detected from skin snip negative controls and LF patients.

sure to the sperm produced by active and sexually mature male worms.

Given that parasite MSP proteins share high similarities among species, we also investigated whether *O. volvulus* MSP2 was cross-reactive and could be recognized by the sera from other parasite-infected patients. Gratifyingly, no immune response to *O. volvulus* MSP2 was detectable in the serum from lymphatic filariasis (LF) patient samples acquired from the WHO Filarisis Serum Bank.

In conclusion, here we have reported the expression and characterization of the nematode-specific protein, MSP2 from *O. volvulus*, the causative agent of onchocerciasis. Like the previously reported MSPs from *A. suum* and *C. elegans*, our biophysical analysis using gel filtration chromatography showed that MSP2 also forms a dimer in solution. Interestingly, C-terminal His_6-tagged MSP2 exists as a monomer, suggesting that dimeric interface of *O. volvulus* MSP2 includes the C-terminal region of the monomeric protein, analogous to *A. suum* α-MSP. In an effort to develop MSP2 as an onchocerciasis diagnostic, we performed a dot-blot assay using the serum from onchocerciasis patients. Our data indicated that MSP2 is secreted or released from *O. volvulus* sperm and is exposed to the immune system resulting in the production of human anti-MSP2 IgGs. As demonstrated in our assay, MSP2 has good sensitivity with moderate selectivity (85% total) toward onchocerciasis patient serum. In fact, while the seemingly high false-positive rate might be discouraging in the development of a MSP2-based diagnostic tool, it could be employed with other recombinant nematode-specific proteins as a cocktail antigen, thereby increasing specificity of the analytical result. While antibody detection diagnostics can provide useful information as to the status of ongoing transmission in a given geographic area, these assays only provide a glimpse into the immunological history of an individual and do not directly detect current infection. We are currently examining the potential of MSP2 as an onchocerciasis diagnostic and a new therapeutic target, and these results will be reported in due course.

3. Experimental

3.1. Cloning and expression of *O. volvulus* MSP2

O. volvulus msp2 (381 bp) was amplified by PCR using the clone OvAm Q5 E1 as a template. For MSP2-His_6, primers (Operon) MSP25 (5′-TCGGGCATATGGCGCAATCGGTTCACCCA-3′) and MSP23-His (5′-CCCAAGCTTAAGATTGTATTCGATCGGAAGATTT TTGCGG-3′) were used. The amplified product was digested by the endonucleases, NdeI and HindIII (New England Biolab), and inserted into an *E. coli* expression vector, pET-22b(+) (Novagen). For the native form of MSP2, primers MSP25 and MSP23-STOP (5′-CCCAAGCTTTTAAAGATTGTATTCGATCGGAAGATTTTTGCG-3′) were used and also ligated into pET-22b(+). The integrity of the PCR products was confirmed by DNA sequencing of the recombinant plasmids. To obtain MSP2-His_6 and the native form of MSP2, a single colony of *E. coli* BL21(DE3) transformed with pET-22b(+)-MSP2-His_6 or pET-22b(+)-MSP2 was picked from an overnight-incubated Luria–Bertani (LB) agar plate and grown overnight in LB medium (25 mL) with vigorous shaking. Fresh LB medium (3 L) was prepared and inoculated with the overnight-cultured cells (20 mL). After cells were grown at 37 °C with vigorous shaking for ~3 h ($OD_{600} \approx 0.6–0.8$), IPTG (0.1 mM) was added and protein expression was induced for 4 h at 30 °C. Cells were harvested by centrifugation at 5000 rpm for 15 min, and immediately used for protein purification.

3.2. Purification of MSP2-His_6 and native MSP2 proteins

All protein purifications were performed at 4 °C except analytical gel filtration. MSP2-His_6 was purified by TALON® metal affinity resins following the manufacturer's instructions. The native form of MSP2 was purified by SP cation exchange column chromatography (Pharmacia) followed by gel filtration column chromatography using an ÄKTA FPLC equipped with a HiPrep 16/60 Sephacryl S-100 high resolution column (Amersham Biosciences). Briefly, harvested cells were resuspended in 200 mL of lysis buffer (10 mM sodium phosphate, pH 6.0, 20 mM NaCl, 0.2 mM TCEP, 2 tablets of complete protease inhibitor cocktail (Roche), and 600 mg of prostamin sulfate), and lysed by pulsed-sonication. The lysate was then centrifuged at 14,000 rpm for 20 min and the supernatant loaded onto the SP cation exchange column which was pre-equilibrated with low salt buffer (10 mM sodium phosphate, pH 6.0, 20 mM NaCl). The column was washed with 250 mL of low salt buffer and the protein was eluted by gradient elution generated with low and high salt buffers (10 mM sodium phosphate, pH 6.0, 250 mM NaCl). The fractions containing the

desired protein were confirmed using SDS–polyacrylamide (4–12%) gel electrophoresis.

3.3. Analytical gel filtration

Gel filtration was performed using an ÄKTA FPLC equipped with a HiPrep 16/60 Sephacryl S-100 high resolution column. Partially purified MSP2-His_6 or MSP2 (~5 mg) was eluted with PBS (pH 7.4) at a flow rate 0.5 mL/min and 5 mL fractions were collected. The molecular weights of the proteins were determined by plotting the partition coefficient (K_{av}) against the logarithm of the molecular weight of the protein. The partition coefficient, K_{av}, was obtained by

$$K_{av} = (V_e - V_o)/(V_t - V_o)$$

where V_e is the elution volume for the protein, V_o is the column void volume (43.42 mL), and V_t is the total bed volume (120 mL). A calibration curve was generated using albumin (67 kDa), ovalbumin (43 kDa), chymotrypsinogen A (25 kDa), and ribonuclease A (13.7 kDa).

3.4. Dot-blot assay

Dot-blot assays were performed using the purified native form of MSP2 and onchocerciasis-positive Cameroonian patient sera obtained in July 2005 after patient consent. Control experiments were performed simultaneously using bovine serum albumin, sera from eighteen skin snip negative Cameroonian individuals who have no history of onchocerciasis infection, and three lymphatic filariasis-positive patients. Purified MSP2 (2 μL of 5 μg/mL protein) was spotted onto a nitrocellulose membrane (1.5 cm × 1.5 cm, Invitrogen) and dried at 37 °C for 0.5 h, and then the membranes were blocked by incubating in 2 mL blocking buffer (2% skim milk in TBST, 0.1% Tween 20) for 1 h at room temperature. Membranes were washed once with 2 mL TBST after blocking and incubated with serum (2 μL in 1 mL blocking buffer) for 2 h at room temperature. After this time, the membranes were washed three times for 5 min each with 2 mL TBST. Horseradish peroxidase (HRP) conjugated goat anti-human IgG (H + L) antibody (Pierce) was used as a secondary antibody (0.1% in the blocking buffer) and incubated with the membranes for 1 h at room temperature. After incubation, the membranes were washed five times for 10 min each with 2 mL TBST, and SuperSignal West Femto Maximum Sensitivity Chemiluminescent Substrate (Pierce) was used for detection by chemiluminescence using a FluorChem 8900 imager (Alpha Innotech).

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