

Expression, purification, and human antibody response to a 67kDa vaccine candidate for schistosomiasis japonica

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Abstract

Schistosomiasis remains a leading cause of morbidity and mortality in the developing tropical world, and vaccines to prevent these infections remain a scientific and public health priority. Sj67 is a 67 kDa *Schistosoma japonicum* surface membrane protein homologous to a family of actin-binding proteins. Sj67 is recognized by a mouse monoclonal antibody (mAb 6) that confers resistance to challenge infection in passive transfer experiments. These data support Sj67 as a potential vaccine candidate for schistosomiasis japonica. In the present study, we report the ligation-independent cloning of a cDNA encoding thioredoxin/elastic-like polypeptide (ELP)/rSj67 into a pET-32 Xa/LIC vector. Soluble recombinant fusion protein (Thio-ELP-rSj67) was expressed and purified using anion-exchange and size exclusion chromatography. rSj67 was cleaved from the Thio-ELP fusion partner by digestion with Factor Xa protease and purified using hydroxyapatite column chromatography. Endotoxin was reduced by absorption to a polymyxin support. Purified rSj67 had a molecular weight of 67 kDa and N-terminal sequencing confirmed that the first five amino acids of the recombinant protein matched the predicted sequence for the Sj67 gene. In Western blot analysis, rSj67 was recognized by the Sj67 specific mAb 6 antibody. IgG antibodies in sera from schistosomiasis infected volunteers living in an endemic area of the Philippines ($n = 13$) recognized rSj67 with 4.7-fold greater median fluorescence compared to uninfected North American controls ($n = 5$) ($p < 0.009$). Together, these data confirm the expression and purification of recombinant Sj67 and its immuno-reactivity with sera from *S. japonicum* infected humans.

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Human schistosomiasis, caused by three species of parasitic trematode blood fluke, infects approximately 200 million individuals in 74 countries and remains among the world's most debilitating parasitic diseases [1]. Of the 200 million individuals who are currently infected, 120 million develop symptoms and 20 million suffer severe morbidity due to their infections. Praziquantel, a highly effective and well-tolerated oral drug, has been the cornerstone of control programs in endemic areas for two decades. Unfortunately, rapid reinfection

following drug cure has attenuated the effectiveness of control programs based on Praziquantel treatment. This rapid reinfection, in conjunction with residual high prevalence of infection and morbidity, has compelled research in vaccine development for schistosomiasis [1].

Sj67 is a 67 kDa *Schistosoma japonicum* actin-binding protein which is located on the surface of juvenile parasites [2]. Sj67 is recognized by a mouse monoclonal antibody which adoptively transfers up to 47% reduction in worm burdens following challenge infection in mice [2]. These data support Sj67 as a vaccine candidate for human schistosomiasis japonica. In the present study, we report the cloning, expression, and purification of recombinant Sj67 (rSj67). We specifically report on

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expression of rSj67 as a fusion with an elastin-like polypeptide (ELP) which significantly enhanced the expression level and solubility of rSj67. We also report the recognition of rSj67 by antibodies in sera from infected individuals living in an endemic region of the Philippines.

Materials and methods

Subcloning of Sj67

The cDNA encoding the ORF for Sj67 was cloned using ligation-independent cloning into the pET 32 Xa/LIC expression vector (Novagen, Madison, WI). PCR amplification with Platinum *Taq* DNA polymerase (Invitrogen, Carlsbad, CA) was performed using 10 ng of template plasmid (pJF-10), forward primer 5' GGT ATT GAG GGT CGC ATG CGG AAA ACT GTG TC 3' and reverse primer 5' AGA GGA GAG TTA GAG CCT ACA TAG ATT CGA ATT GA 3'. Reaction conditions were: initial denaturation of 1.5 min at 94°C, 35 cycles of 30 s at 94°C, 30 s at 45°C, 60 s at 72°C, and a 5 min final extension at 72°C. The 1680 bp PCR product was purified by agarose gel electrophoresis and recovered from the gel with Promega Wizard PCR Preps System (Promega, Madison, WI). The PCR fragment was digested with T4 DNA polymerase in the presence of dGTP to produce the LIC overhangs according to manufacturer's instructions (Novagen). This reaction capitalizes on the 3'–5' exo-nuclease activity of T4 DNA polymerase in the presence of limiting nucleotide triphosphates. This digested product was cloned into the LIC overhangs on the commercially available pET32 Xa/LIC vector according to manufacturer's instructions (Novagen). The resulting plasmid, designated pJF-11, was transformed into Novablue (Novagen), the Sj67 cDNA insert was confirmed by colony PCR, and the plasmid was recovered using Quiaprep Spin Mini Prep (Qiagen, Valencia, CA).

Addition of ELP DNA to increase solubility of rSj67

Initial expression attempts with pJF-11 resulted in truncated, insoluble protein. To increase the solubility of rSj67 and provide a rapid method of purification, we used a linker-based strategy to insert a synthetic coding region encoding 90 repeats of a five amino acid elastin-like polypeptide sequence (ELP) [3]. Incorporation of ELP as a fusion partner has been reported to increase solubility and allow rapid purification of fusion proteins via inverse transition cycling [3]. To insert the linker, pJF-11 was cut with *Pin*AI. Two oligonucleotides, 5' CCG GAT GGC CTG GCT GGC CAT 3' and 5' CCG GAT GGC CAG CCA GGC CAT 3', were annealed to form a linker with *Pin*AI overhangs and an internal *Sfi*I site. The linker and digested plasmid were ligated to produce pJF-12. pJF-12 plasmid was prepared from methylation deficient

INV-110 (Invitrogen) cells prior to restriction with *Sfi*I. A plasmid containing the coding sequence for ELP-90 (kind gift of D. Meyer) was cut with *Bgl*II and *Pfl*MI, releasing the ELP-90 sequence. ELP-90 was gel purified and ligated into *Sfi*I restricted, gel purified, pJF-12 to produce the expression plasmid, pJF-13. The resulting construct encoded rSj67 with an N-terminal thioredoxin–ELP fusion partner (see Fig. 1). This construct was designed to produce rSj67 with a native primary amino acid sequence at both the N and C termini following cleavage with Factor Xa protease. Each construct was confirmed by automated sequencing using the BigDye Terminator Cycle Sequencing ready reaction kit v1.0 on an ABI 377 DNA sequencer (Applied Biosystems, Foster City, CA).

Expression of soluble rSj67 in *Escherichia coli*

pJF-13 was transformed into *Escherichia coli* BLR (DE3) (Novagen) and plated on LB agar plates containing 0.5% glucose and 50 µg/ml carbenicillin. Single colonies were inoculated into 150 ml LB medium supplemented with 0.5% glucose and 100 µg/ml carbenicillin. Cultures were grown overnight at 37°C with shaking at 400 rpm. The overnight culture was pelleted and the cells were used to inoculate 3000 ml media divided among six 2 L baffled flasks. These flasks were incubated at 37°C, with shaking at 400 rpm until an OD₆₀₀ nm of 0.5 was reached. The temperature was reduced to 20°C, IPTG was added to a final concentration of 1 mM, and the culture was grown for 18 h. Cultures were centrifuged at 1664g for 1 h and the pellet was resuspended in 1/10 volume of 10 mM Tris and 1 mM EDTA, pH 8.0 (TE). Resuspended material was centrifuged at 10,397g for 10 min and the resultant pellets were stored at –20°C. Cell pellets derived from 9 L of induced culture were thawed, resuspended in 200 ml TE, and bead milled in a dry ice/ethanol jacketed BEAD Beater with 0.1 mm glass beads (BioSpec, Bartlesville, OK) for 8 cycles of 30 s on, 30 s off. The resulting supernatant was cleared by centrifugation at 10,397g for 10 min followed by an additional centrifugation step at 27,476g for 20 min. All centrifugation steps were performed at 4°C.

Purification of rThio-ELP-Sj67 and Factor Xa digestion

The supernatant derived from bead milled lysate was filtered through 0.45 µm cellulose acetate bottle filters and applied to a 215 ml anion-exchange column (XK 26/40, Pharmacia, Uppsala, Sweden) packed with Macro-Prep High Q resin (Bio-Rad, Richmond, CA) as the capture step. The column was equilibrated in 4 column volumes of TE, pH 8.0, and developed in a linear gradient of 0–500 mM NaCl over 10 column volumes. The fusion protein Thio-ELP-Sj67 was contained in the first elution peak, between 135 and 165 mM NaCl. This elution peak was concentrated to a volume of 5 ml using

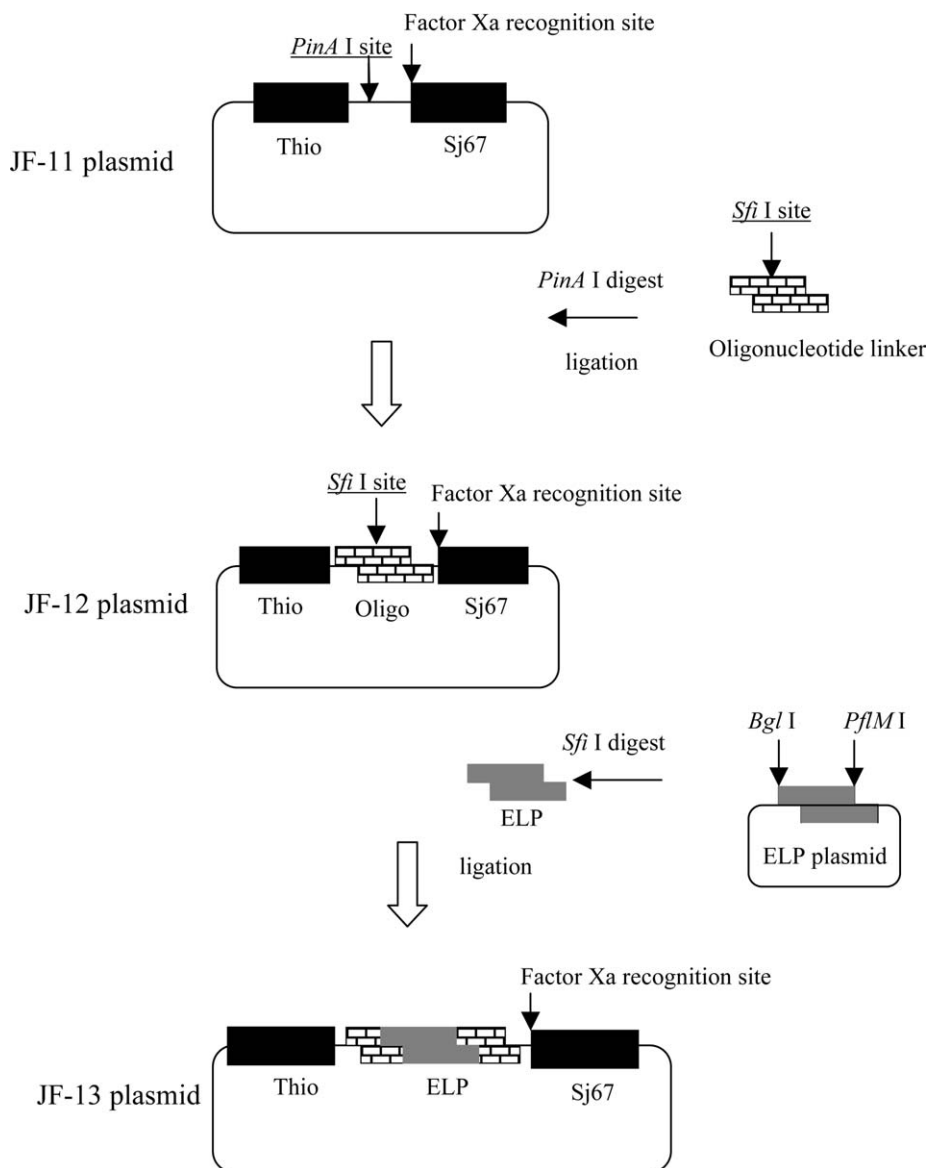


Fig. 1. Construction of Sj67 expression vector. pJF-11 plasmid is linearized by digestion with *PinA*I and a compatible linker encoding an *Sfi*I site is ligated to the *PinA*I ends to make pJF-12 plasmid. pJF-12 plasmid is linearized with *Sfi*I and *ELP* is excised from its parent plasmid by digestion with *Bgl*I and *Pfl*MI, leaving compatible ends for the *Sfi*I site in pJF-12. pJF-13 is produced by ligating *Sfi*I linearized pJF-12 with the compatible *ELP* fragment.

Centricon Plus-20 centrifugal filter devices (Millipore, Bedford, MA) at 1664g for 10 min. Concentrated material was applied to a 600 ml (35/600) BioPilot size exclusion column packed with SuperDex 200 Prep matrix (Pharmacia). This column was equilibrated with two column volumes of TE 150 mM NaCl, pH 8.0, and baseline resolution of Thio-ELP-Sj67 was achieved with a flow rate of 0.5 ml/min. Purified Thio-ELP-Sj67 was then digested with Factor Xa, 1 mM CaCl₂, and 0.005% Triton X-100 for 7 h, with slow shaking at 4°C to cleave the fusion partner from rSj67. The reaction was stopped by addition of AEBSF-HCl (Calbiochem, San Diego, CA) to a final concentration of 200 nM. The cut material was filtered through a 0.45 µm filter and loaded onto a

Bioscale type I ceramic hydroxyapatite column CHT-10-1 (Bio-Rad). The column was equilibrated in 4 column volumes of 10 mM potassium phosphate, 0.005% Triton X-100, pH 7.0, and developed in a linear gradient of 10–500 mM potassium phosphate over 15 column volumes. The purified rSj67 eluted between 214 and 267 mM potassium phosphate with baseline resolution. Fractions containing rSj67 were pooled and buffer exchanged on a 53 ml Desalting HiPrep 26/10 column packed with Sephadex G-25 Fine resin (Pharmacia) into TE, 150 mM NaCl, and 0.005% Triton X-100, pH 8.0. Contaminating endotoxin was removed by application of the purified material to a 2 ml column (HR 10/5, Pharmacia) packed with Affi-Prep Polymyxin resin (Bio-Rad). The column

was equilibrated in 6 column volumes of TE, 150 mM NaCl, and 0.005% Triton X-100, pH 8.0, at a flow rate of 3 ml/min. Sample was applied and developed with 2 column volumes of TE, 150 mM NaCl, and 0.005% Triton X-100, pH 8.0, at a flow rate of 0.25 ml/min. All buffers for the polymyxin chromatography were prepared from endotoxin-free water.

Multiple applications of purified rSj67 to the polymyxin support were required to reduce the endotoxin concentration to below 150 EU/mg protein. Purified rSj67 was flash-frozen in a dry ice/ethanol bath at protein concentrations below 125 µg/ml and stored at -80°C .

All protein samples were analyzed on 10% SDS–PAGE gels, at 200 V for 0.75 h (Mini-Protean, Bio-Rad). The protein gels were stained with Gel-Code Blue (Pierce, Rockford, IL) for 1 h and destained with deionized water. The Western transfer to nitrocellulose was performed for 1 h at 100 V according to manufacturer's instructions (Bio-Rad).

The Western blot was blocked in 1% milk/TBS for 1 h at 37°C , probed with mAb A6 for 1 h at 37°C , washed 3×5 min in TBS, probed with alkaline phosphatase conjugated goat anti-mouse IgG, IgA, and IgM (Sigma, St. Louis, MO) diluted 1:1000 in milk/TBS, washed 3×5 min in TBS, and finally exposed to substrate (NBT/BCIP fast tab, Sigma).

Study volunteers

Sera samples were obtained from 13 *S. japonicum* infected adult volunteers, residing in an *S. japonicum* endemic village in the Philippines. Seven milliliters of venous blood was extracted into serum separator tubes (Vaccunainer, Becton–Dickinson, Franklin Lakes, NJ) and volunteers were offered treatment for schistosomiasis with Praziquantel. Serum samples were also obtained from five North American adults with no history of exposure to schistosomiasis. All volunteers provided written informed consent and this protocol was approved by the Ethical Review Boards of Brown University and the Research Institute of Tropical Medicine, The Philippines.

Bead-based ELISA

One hundred micrograms of rSj67 was coupled to 1.25×10^6 polystyrene beads (Luminex, Austin, TX) according to manufacturer's protocols. Ninety-six-well filter-bottomed plates were prewetted with 100 µl/well assay buffer (PBS, pH 7.4, 10 mg/ml BSA, and 0.05% sodium azide) and vacuum dried on a vacuum manifold. Approximately 200 beads were dispensed into each well of the plate. Serum samples were diluted 1:100 in assay buffer and added to individual wells of the plate. The plate was incubated for 30 min on a microplate shaker at 300 RPM in the dark at 25°C . The plate was vacuum

dried and washed three times with 100 µl/well wash buffer (PBS, pH 7.4, and 0.05% Tween 20). Twenty-five microliters of 1 µg/ml of biotinylated mouse anti-human IgG (PharMingen, San Diego, CA) in assay buffer was added to each well. The plate was incubated for 30 min on a microplate shaker at 300 RPM in the dark at 25°C . The plate was vacuum dried and washed three times with 100 µl/well of wash buffer (PBS, pH 7.4, and 0.05% Tween 20) and 50 µl of 1 µg/ml of streptavidin-PE (PharMingen) was added to each well. The plate was incubated for 10 min on a microplate shaker at 300 RPM in the dark at 25°C . The plate was vacuum dried and washed three times with 100 µl/well wash buffer (PBS, pH 7.4, and 0.05% Tween 20). The beads were resuspended in assay buffer and read on a Bio-Plex Protein Array System (Bio-Rad). A Mann–Whitney *U* test compared the fluorescence results for samples from uninfected and infected volunteers.

Results and discussion

Here, we report the successful cloning, expression, and purification of rSj67, using an ELP fusion tag to maintain protein solubility. The cloning scheme is shown in Fig. 1. Optimized induction conditions produced 18.7 g of protein per 9 L of induced *E. coli* culture. The column chromatography sequence produced 1.5 mg of purified rSj67 (Table 1) from this starting material. The induction and purification sequence are summarized in Figs. 2A and B.

We originally included ELP in our fusion construct to permit rapid purification via inverse transition cycling. ELPs tend to reversibly precipitate when the temperature and salt concentration exceed a threshold level [4]. Meyer and Chilkoti [3] purified recombinant proteins fused to ELPs by exploiting reversible precipitation and centrifugation. Unfortunately, the addition of an ELP fusion tag to rSj67 did not allow for rapid protein purification through transition cycling. Addition of ELP did, however, increase the solubility of rSj67 and facilitated its purification using standard chromatographic techniques. Once the ELP protein was removed from Sj67 by Factor Xa digest, it was necessary to include a detergent, 0.005% Triton X-100, to prevent irreversible precipitation of rSj67.

Table 1
Purification of recombinant Sj67 from *E. coli*

Purification step	Total protein (mg)	Recovery (%)
Crude lysate ^a	18,700	100
Anion exchange	155.5	0.83
Size exclusion	5.10	0.027
Hydroxyapatite	2.00	0.011
Buffer exchange	1.50	0.0080

^a From 9 L of induced culture.

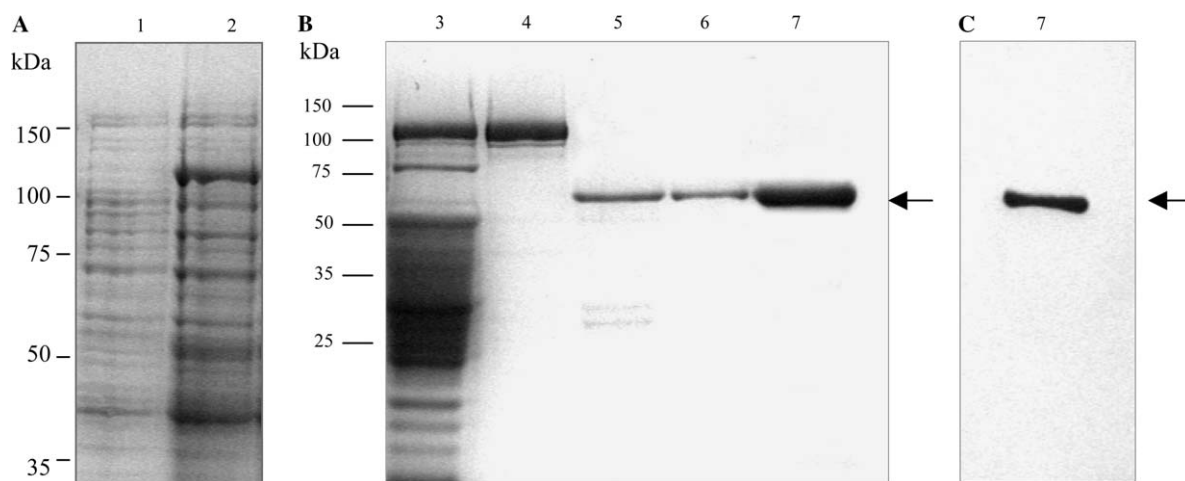


Fig. 2. SDS-PAGE of fractions from the purification of Sj67. (A and B) Coomassie; (C) Western blot probed with Sj67 specific mAb 6. Lane 1, uninduced lysate; lane 2, induced lysate; lane 3, fusion protein containing peak from anion-exchange column; lane 4, fusion protein containing peak from size exclusion column; lane 5, digestion products from Factor Xa digest of purified Thio-ELP-Sj67; lane 6, purified Sj67 containing peak from hydroxyapatite column; and lane 7, concentrated Sj67, postbuffer exchange column. Intermediate thioredoxin-ELP-Sj67 fusion product is 120 kDa. Final Sj67 product is 67 kDa.

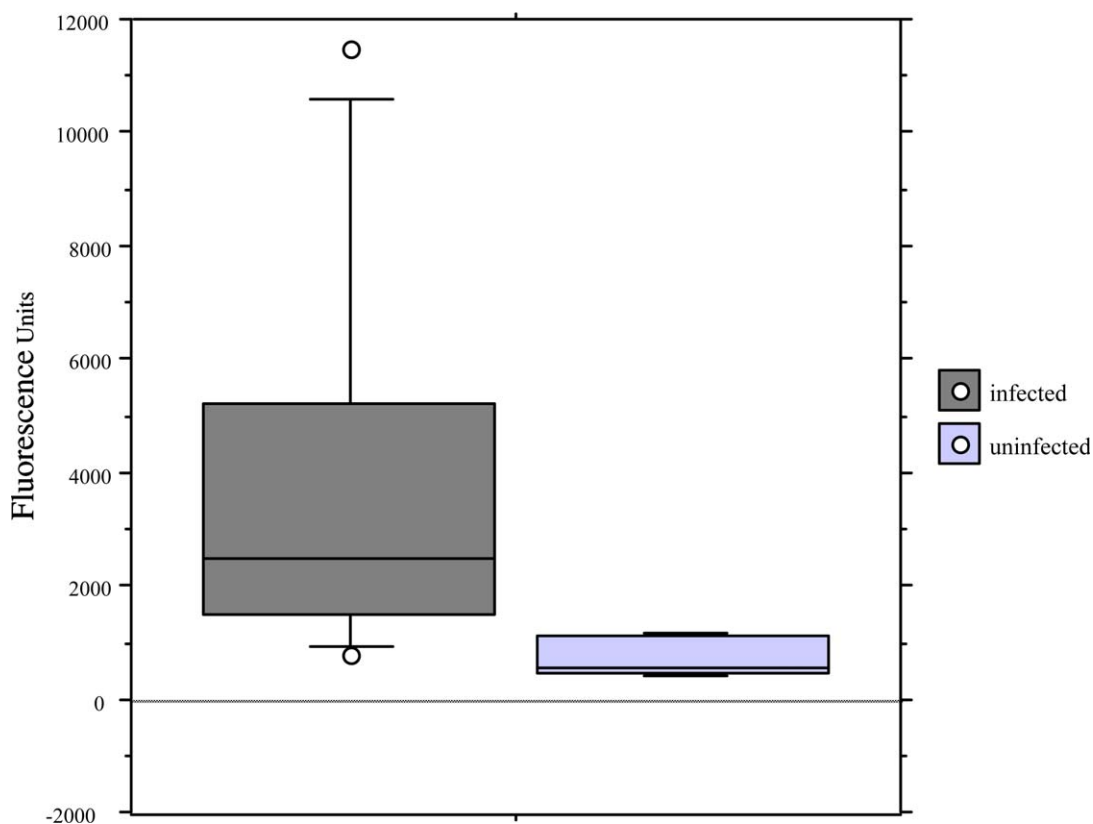


Fig. 3. Sj67 is preferentially recognized by IgG antibodies in the sera of infected ($n = 13$) versus uninfected individuals ($n = 5$). Box plots depict median (central line) 25–75th percentile (box), 10–90th percentile (whiskers), and < 10th and > 90th percentile (individual circles) of fluorescence values from antigen specific antibody assay (see Materials and methods). Recognition of Sj67 was greater in infected versus uninfected individuals (Mann–Whitney U test, $p < 0.009$). Infected individuals recognized Sj67 with a 4.7-fold higher median fluorescence than uninfected individuals.

Protein sequencing of the first 5 amino acids from the N-terminus of rSj67 indicated that the sequence of the purified recombinant protein matched the predicted amino acid sequence for the Sj67 gene. A Western blot

with mAb 6, the murine antibody initially used to clone the gene for Sj67 [2], indicated that the purified protein was immuno-reactive (Fig. 2C). Another indication of the antigenicity of rSj67 was the differential immune

response in 13 schistosomiasis japonica infected volunteers compared to five uninfected controls. Recognition of Sj67 was greater in infected versus uninfected individuals (Mann–Whitney U test, $p < 0.009$); infected individuals recognized Sj67 with a 4.7-fold higher median fluorescence than uninfected individuals (Fig. 3). Together, these data confirm that rSj67 displays epitopes that are immunogenic during the course of natural human infection—an attribute of paramount importance for a potential vaccine candidate.

Sj67 is homologous to a family of actin-binding proteins, showing up to 71% homology and 43% identity with members of the Band 4.1 gene family [2]. The Band 4.1 gene family contains Band 4.1, ezrin, radixin, and moesin. These proteins are involved in linking transmembrane and cytoskeletal proteins [5–9] and family members have been implicated in a variety of processes including membrane stability, locomotion, and receptor signaling [10–12]. The importance of membrane/cytoskeletal interactions makes Sj67 a promising target for an anti-parasitic immune response.

Conclusion

In conclusion, we have described a method for expression and purification of Sj67, a putative vaccine candidate against schistosomiasis japonica. We describe a cloning protocol, using an ELP fusion tag, which allowed for the successful purification of an otherwise insoluble protein.

In these initial studies, rSj67 was specifically recognized by IgG in the sera of infected individuals. We are now exploring the relationship between cytokine and antibody responses to rSj67 and resistance to re-infection after treatment with Praziquantel in a cohort of 624 infected volunteers in an endemic region of the Philippines.

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