

Characterization of a profilin-like protein from *Schistosoma japonicum*, a potential new vaccine candidate

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Abstract The tegumental membrane of platyhelminth parasites is of crucial importance for modulation of the host response and parasite survival. A complementary deoxyribonucleic acid (cDNA) containing an open reading frame of 390 bp, which encodes a profilin-like tegumental protein with a theoretical isoelectric point of 6.02 and a molecular weight of 14.42 kDa, had been identified by bioinformatic analysis. The coding region of the cDNA was cloned into the prokaryotic expression vector pGEX-4T-1 and expressed in *Escherichia coli*. The purified recombinant protein named rSj15 was immunogenic and could elicit a high titer of antibody in mice. Western blot analysis revealed that the protein was differentially expressed during the different growth stages of *Schistosoma japonicum*. Immunohistochemical analysis localized the protein to the tegument and underlying tissue of the *S. japonicum* adult worm. The rSj15 could induce the expressions of IL-12 in the cultured mouse dendritic cell.

Introduction

Schistosomiasis is a widely spread infectious disease and constitutes a serious and worldwide public health problem. Despite major advances in its control that have lead to substantial decreases in morbidity and mortality, Schistosomiasis continues to spread to new geographic areas (Patz et al. 2000). Although significant progress has been made in chemotherapy with safer and more effective drugs, these cannot prevent the high re-infection rates of schistosomes, and there have been dramatic recurrences in both its prevalence and associated morbidity (McManus 2005). For this reason, vaccine development is seen as a complementary approach.

The pathogens of human schistosomiasis primarily include *Schistosoma mansoni*, *Schistosoma hematobium*, and *Schistosoma japonicum*. The current *S. japonicum* vaccine candidates being investigated do not show effective protection against *S. japonicum* infection (Wilson and Coulson 2006; Pacífico et al. 2006). It is important to identify new target antigens and a rational approach to the development of efficient vaccine through understanding the mechanism of host–parasite interaction. Excretory–secretory products and molecules exposed on epithelial surfaces (including receptors) warrant especial attention. The tegumental outer surface of parasites is a unique, double-membrane structure that is very important for modulation of the host response and parasite survival (Van Hellemond et al. 2006). Tegumental proteins are structural proteins expressed in the teguments at different developmental stages and are potential vaccine candidates. In an attempt to isolate the more efficient vaccine antigen from tegumental proteins, the tegumental protein data of *S. japonicum* has been downloaded from the National Center for Biotechnology Information (NCBI) website (<http://www.ncbi.nlm.nih.gov/sites/entrez?db=Protein>) and their similarities with four local protein

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databases of *S. mansoni*, *H. sapiens*, *Rattus norvegicus*, and *Mus musculus* were analyzed. At last, 28 theoretic tegumental proteins, specific for *S. japonicum*, were obtained, of which one was the profilin-like protein (AAP06493).

Infections with schistosomes are chronic and are characterized immunologically by an initial T helper type 1 (Th1) response that switches to a highly Th2-polarized response following the onset of egg production at approximately week 6 (Grzych et al. 1991; Pearce et al. 2002; Stadecker et al. 2004). How schistosome eggs induce Th2 responses is unclear but appears to be linked to their inherent ability to avoid directly inducing dendritic cell (DC) maturation and interleukin (IL)-12 production (La Flamme et al. 2001; Pearce et al. 2002). This is directly the result of unregulated IL-12 production, since mice deficient for IL-12 and IL-10 fail to polarize in this fashion and develop excessively Th2-skewed responses (Hoffmann et al. 2000), and a profilin-like molecule (AAX33672) from the protozoan parasite *Toxoplasma gondii* generates a potent IL-12 response in murine DCs activated through TLR11 (Yarovinsky et al. 2005). However, the profilin-like protein (AAX33672) of *T. gondii* and the profilin-like protein (AAP06493) of *S. japonicum* share a similar conserved domain, respectively, cd00148 and smart00392, both of which can bind actin monomers, membrane polyphosphoinositides such as PI(4,5)P2, and poly-L-proline. The key role of profilin is regulating the dynamics of actin polymerization in vivo during cell motility, but there is growing evidence that profilins function as hubs that control a complex network of molecular interactions and cellular processes such as membrane trafficking, small-GTPase signaling, and nuclear activities, in addition to neurological diseases and tumor formation (Witke 2004).

What then is the relationship between the production of IL-12 and the profilin-like protein (we have named this as Sj15 by the molecular weight large as 15,000 kDa) of *S. japonicum*? To address this, we report the cloning and expression of the Sj15 protein, its localization in adult worm and cercaria, its immunogenicity, and the effect on IL12 expression in DCs.

Materials and methods

Screening and bioinformatics analysis of target protein

Protein sequences of the tegumental proteins were extracted from the NCBI database (<http://ncbi.nlm.nih.gov>) using the key words “*S. japonicum*” and “membrane or tegument.” These protein sequences were searched for the homologous sequences in the protein databases of *S. mansoni*, *H. sapiens*, *R. norvegicus*, and *M. musculus*. The related sequences were aligned with a multialign program. Twenty-eight protein

sequences of *S. japonicum* with similarity less than 20% to the related proteins of *S. mansoni*, *H. sapiens*, *R. norvegicus*, and *M. musculus* were identified. These 28 protein sequences of the potential tegumental proteins were analyzed by the proteomics and sequences analysis tools in the websites: <http://www.cbs.dtu.dk/services/> and <http://ca.expasy.org/> tools.

Amplification and cloning the gene of the target protein

The specific primers (sense primer: 5'-TAG GAA TTC AGC GCT GAT AGT TGG GAT AAT C-3' and antisense primer: 5'-AAT CTC GAG GTA ACC CAT TCG CTC GTA ATG-3' including a *EcoRI* site and a *XhoI* site, respectively) were designed for the amplification of the coding sequence of the selected gene (GenBank no. AY223457). Total ribonucleic acid (RNA) of the adult worm of *S. japonicum* was extracted and used to perform reverse transcriptase (RT) polymerase chain reaction (PCR) using the above primers and an amplification profile: 94°C for 5 min, followed by 30 cycles at 94°C/1 min, 60°C/1 min, and 72°C/1 min and 72°C/7 min (final extension). PCR experiments were performed on a programmable Perkin-Elmer GeneAmp PCR system 2400 (Applied Biosystems) using *Taq* polymerase (Takara Biotechnology, Dalian, China), and the PCR products were separated on 1.0% agarose gels, visualized under UV light by ethidium bromide staining. The PCR products were extracted from the agarose gel, double-digested with *EcoRI* and *XhoI* endonucleases, and cloned into the prokaryotic expression vector pGEX-4T-1 (Pharmacia). Recombinant plasmid, pGEX-4T-1-sj15, was isolated and confirmed by DNA sequencing.

Prokaryotic expression and purification of the target protein

pGEX-4T-1-sj15 was transformed into *Escherichia coli* BL21 (DE3). Sj15 was expressed as a glutathione-S-transferase-Sj15 (GST-Sj15) fusion protein upon isopropyl-β-D-thiogalactopyranoside induction (1 mg/ml for 6 h). The recombinant protein was purified using a GStrap™ FF resin column (Pharmacia) followed by thrombin (Novagen) cleavage. Protein samples were fractionated using 15% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and visualized by Coomassie brilliant blue G-250 staining. The cleaved protein was excised from an SDS-PAGE gel, and its identity was confirmed by mass spectrometry. The protein concentration was determined by the method of Bradford (1976).

Preparations of mouse anti-rSj15-specific antiserum

Six female BALB/c mice were prebled (0.2 ml, day 0). On day 0, all mice were injected subcutaneously with 200 µl of

50 µg purified thrombin-cleaved rSj15 recombinant protein, which was emulsified with equal volume of complete Freund's adjuvant (Sigma) and followed by two boosting with the same amount of antigen but was emulsified with incomplete Freund's adjuvant (Sigma) at 2-week intervals. On day 45, serum samples were collected from the mice, and the Sj15-specific antibodies were detected by the enzyme-linked immunosorbent assay (ELISA) using purified thrombin-cleaved protein Sj15 as antigen. The antisera titer of the thrombin-cleaved protein Sj15 was 1:12,800 detected by ELISA.

Western blotting analysis

The purified recombinant GST-Sj15 fusion protein, thrombin-cleaved Sj15 protein, and the natural crude antigen prepared from different growth stages of *S. japonicum* were subjected to 15% SDS-PAGE, electrotransferred to nitrocellulose membrane (Osmonics), and probed with mouse anti-Sj15 sera (1:1,500 dilution in phosphate-buffered saline [PBS]) as the primary antibody and horseradish peroxidase-conjugated rabbit anti-mouse immunoglobulin G (Sigma) as the secondary antibody. The signal was detected by an electrochemiluminescence Western blotting system (GE Healthcare/Amersham Biosciences). In addition, the purified recombinant thrombin-cleaved Sj15 protein was probed with mouse and rabbit sera (1:500) infected with the cercariae of *S. japonicum*.

Histologic immunolocalization of Sj15 on adult worm

Freshly collected adult worms were washed in PBS and fixed overnight in 4.5% neutral paraformaldehyde. The parasites were washed with PBS, dehydrated, embedded in paraffin, and cut into 5-µm-thick sections. Slides were deparaffinized, hydrated, and soaked in PBS for 5 min and subjected to the antigen retrieval by immersion in 50 mM Tris-HCl (pH 7.6) and autoclaving at 95.8°C for 10 min. Endogenous peroxidase was inactivated with 1% H₂O₂ in methanol for 20 min at room temperature. Afterward, the slides were incubated overnight with mouse anti-Sj15 sera diluted at 1:500 in PBST (PBS containing 5% non-fat milk and 0.1% Triton X-100) or with preimmune sera diluted at 1:500 in PBST at room temperature in a humidity chamber. After four washings in PBST (5 min each), the slides were incubated for 1 h at room temperature with a fluorescein isothiocyanate (FITC)-conjugated anti-mouse IgG and then washed and examined with a Leica fluorescence microscope.

Immunofluorescence staining of cercariae

A whole-worm staining was done to locate Sj15 expression in cercariae. The cercariae were incubated in 50 µl PBS in

an Eppendorf tube for 5 min. It was then centrifuged at 3,000×g for 5 min, and the supernatant was removed. Cercariae were resuspended in 50 µl of blocking solution (0.5% [w/v] nonfat dried milk in PBS) and incubated for 30 min. The sample was centrifuged at 3,000×g for 5 min, and the supernatant was removed. Cercariae were resuspended and incubated with anti-Sj15 serum or normal mouse serum diluted in 1:50 in blocking solution for 1 h at room temperature. Cercariae were collected by centrifugation and washed with PBS for three times. Cercariae were then incubated with the secondary antibody Cy3-linked goat anti-mouse IgG diluted 1:50 in blocking solution for 1 h. Finally, the cercariae were collected, washed as above, and resuspended in 20 µl PBS. The suspension was transferred to a clean glass slide and investigated with a fluorescence microscope.

IL-12 expression in DCs that incubated with thrombin-cleaved Sj15

The solution of thrombin-cleaved Sj15 protein was filtered through a 0.22-µm syringe filter (Acrodisc, Pall, Auckland, USA) and then applied to the Affinity Park™ Detoxi-gel™ Endotoxin Removing Gel column (Pierce, USA) to remove the endotoxin in the protein solution according to the manufacturer's instructions; at last, the concentrations of Sj15 and the endotoxin in the elution were determined. After being cultured for 8 days, the DCs derived from mouse bone marrow was incubated with 0.05, 0.1, 1, or 10 µg/ml of rSj15 for 24 h. Lipopolysaccharide (LPS; 0.1 µg/ml) and Roswell Park Memorial Institute (RPMI) 1640 culture medium were used to replace the protein as the positive and negative control, respectively. Total RNA samples were then prepared from the treated cells for analyzing the expression of the p35 and p40 subunits of IL-12 by RT-PCR using primer pairs MF208/MF209 (5'-TGC CAG GTG TCT TAG CCA GTC-3'/5'-ATT TTC ACT CTG TAA GGG TCT GC-3', for p35) and MF211/MF212 (5'-CAG CTT CTT CAT CAG GGA CAT C-3'/5'-TTT CCT TTC CAA CGT TGC ATC CT-3', for p40), and β-actin as the control using primer pair m-actin F/m-actin R (5'-GTG GGG CGC CCC AGG CAC CA-3'/5'-CTT CCT TAA TGT CAC GCA CGA TTT C-3'), with an amplification profile: 94°C for 5 min, followed by 30 cycles at 94°C/30 s, 66°C (for p35 and p40) or 60°C (for mouse β-actin)/30 s, 72°C/45 s, and a final extension at 72°C for 7 min. In all cases, 5 µl of PCR products were resolved in a 1.5% agarose gel with ethidium bromide and photographed under UV light. The intensities of the amplified DNA bands were analyzed by the Bio-rad Quantity One 1-D Analysis Software (Version 4.4), and the data were analyzed by SPSS (version 13.0) software.

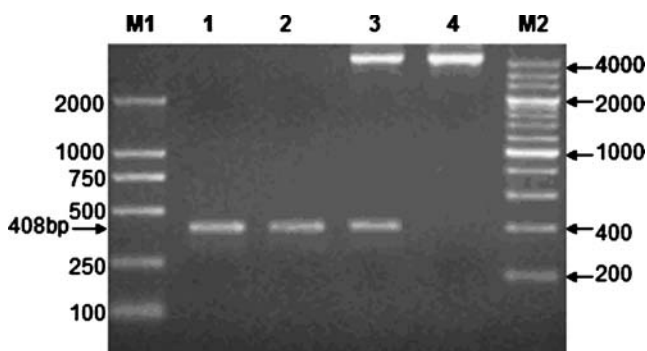


Fig. 1 Identification of recombinant pGEX-4T-1-sj15 PCR product and double-digestion of pGEX-4T-1-sj15 were analyzed in 1% agarose gel electrophoresis. *M1*, DL2,000 DNA marker (Takara Biotechnology); *lanes 1* and *2*, PCR product of gene *sj15* using *S. japonicum* adult worm cDNA and pGEX-4T-1-sj15 as the template, respectively; *lanes 3* and *4*, double digestion of pGEX-4T-1-sj15 and pGEX-4T-1 with *EcoRI* and *XhoI* endonucleases; *M2*, 200-bp DNA Ladder marker (Takara Biotechnology)

Results

Identification of Sj15 by screening the databases

One hundred fifty-three protein sequences were extracted from the NCBI database (<http://ncbi.nlm.nih.gov>) using the keywords “*S. japonicum*” and “membrane or tegument” and searched for the homologous sequences in the four protein databases. To screen the target sequences that should have the lowest homology with related proteins in other four databases, the limit of the *E* value was set to no less than 10^{-6} . At last, 28 tegumental proteins that have low

similarity with the related proteins of the other four protein databases were selected (data not shown), and of which, AAP06493 was a putative profilin-like protein. It was reported that profilin-like protein (AAX33672) from the protozoan parasite *T. gondii* could generate a potent IL-12 response in murine DCs (Yarovinsky et al. 2005), and the two profilin-like proteins from *S. japonicum* and *T. gondii* belonged to the same superfamily. We wondered whether the profilin-like protein of *S. japonicum* had the same effect, so the profilin-like protein was chosen as the target molecular for further studies.

Amplification and cloning of the gene encoding Sj15

The *Sj15*-coding sequence was PCR amplified from the *S. japonicum* complementary DNA (cDNA) using the designed primers. Agarose gel electrophoresis showed a single band about 400 bp (Fig. 1, lane 1). The PCR product was cloned into the pGEX-4T-1 vector to generate the pGEX-4T-1-sj15 recombinant plasmid. The recombinant plasmid was confirmed by DNA sequencing and double digestion with *EcoRI* and *XhoI* (Fig. 1, lane 3).

Bioinformatics analysis of the target protein Sj15

Using a public analysis software in the website CBS (<http://www.cbs.dtu.dk/services/>), bioinformatical analysis of *Sj15* protein showed that it carried GlcNAc glycosylation sites and protein phosphorylation sites. The theoretical *pI* and molecular weight (MW) were predicted as 6.02 and

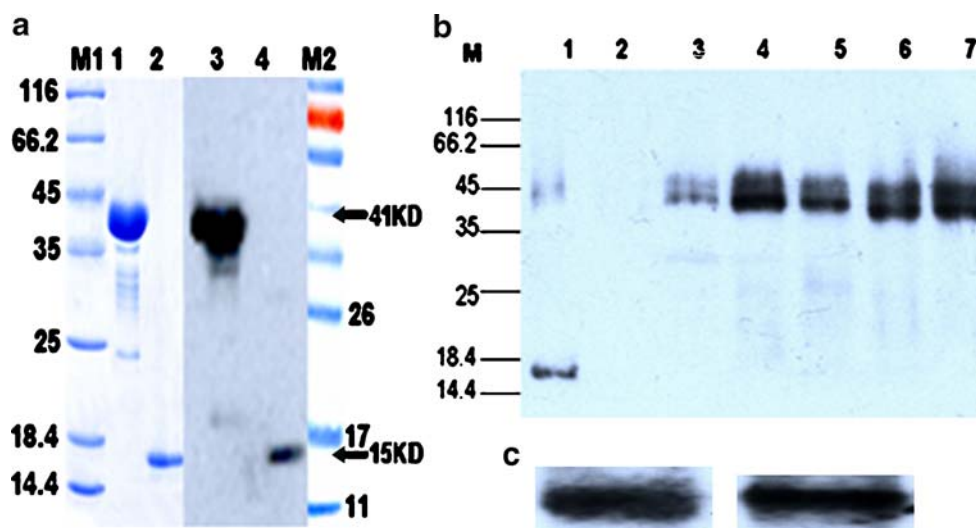


Fig. 2 Western blotting analysis. **a** Left: 15% SDS-PAGE analysis of the purified rGST-Sj15 (lane 1) and the purified rSj15 cleaved from rGST-Sj15 by thrombin (lane 2); *M1*: unstained protein molecular weight marker (Fermentas); right: Western blot analysis of rGST-Sj15 (lane 3) and rSj15 (lane 4) probed with mouse anti-rSj15 serum; *M2*: prestained protein molecular weight marker (Fermentas). **b** Western blot of the purified rSj15 (lane 1), purified rGST (lane 2), crude

antigen of mixed-sex adult worm (lane 3), crude antigen of male adult worm (lane 4), crude extract of female adult worm (lane 5), crude extract of egg (lane 6), and crude extract of cercaria (lane 7) of *S. japonicum* probed with mouse anti-rSj15 serum; *M*, showed the molecular size. **c** Western blot of the purified rSj15 probed with the *S. japonicum* infected mouse (left) and rabbit (right) sera

14.42 kDa, respectively. TMpred (http://www.ch.embnet.org/software/TMPRED_form.html) analysis showed Sj15 had a transmembrane helix length between residues 14 and 33. BepiPred 1.0 Server analysis (<http://www.cbs.dtu.dk/services/BepiPred/>) predicted three linear B cell epitopes between residues 1–7, 31–42, and 102–112. Analysis with NetCTL-1.2 identified four T cell epitopes between residues 5–13, 25–33, 57–65, 61–69, and 116–124.

Expression and Western blotting analysis

Recombinant GST-Sj15 fusion protein was expressed in *E. coli* BL21 (DE3) and purified by affinity column chromatography. After dialysis, about 30 mg of purified protein was obtained from 2,000 ml of culture. The final concentration of the protein was 0.5 mg/ml in PBS. SDS-PAGE showed that the size of the purified fusion and thrombin-cleaved recombinant Sj15 were 41 and 15 kDa, respectively (Fig. 2a, lanes 1 and 2).

Western blotting showed that the sera from mouse immunized with the recombinant thrombin-cleaved Sj15 had a specific reaction with the purified recombinant GST-Sj15 protein (Fig. 2a, lane 3) showing a band of approximately 41 kDa and with the thrombin-cleaved rSj15 showing a band of approximately 15 kDa (Fig. 2a, lane 4). The total proteins obtained from *S. japonicum* at different stages were also probed with the mouse anti-rSj15 serum (Fig. 2b). The result showed that a double band about 41 to 45 kDa was probed with the serum at all the stages instead of a single band of 15 kDa. Moreover, the recombinant thrombin-cleaved rSj15 could also be probed with sera from mouse or rabbit that was infected with cercaria of *S. japonicum* (Fig. 2c).

Sj15 is localized in the tegument of adult worm and cercaria of *S. japonicum*

Immunostaining of the adult worm sections and whole cercaria detected where the Sj15 was expressed in the adult

Fig. 3 Immunolocalization of Sj15 on *S. japonicum* tegument. Fluorescence microscopy images (right) and corresponding optical images (left) of tegument and underlying tissues of adult worm are shown, O and T indicated the coelom and tegument of *S. japonicum*. **a** Serum from naive mice was used as negative control; **b** Anti-rSj15 serum and secondary FITC-conjoined antibody were used for fluorescence detection of Sj15 in adult worm sections

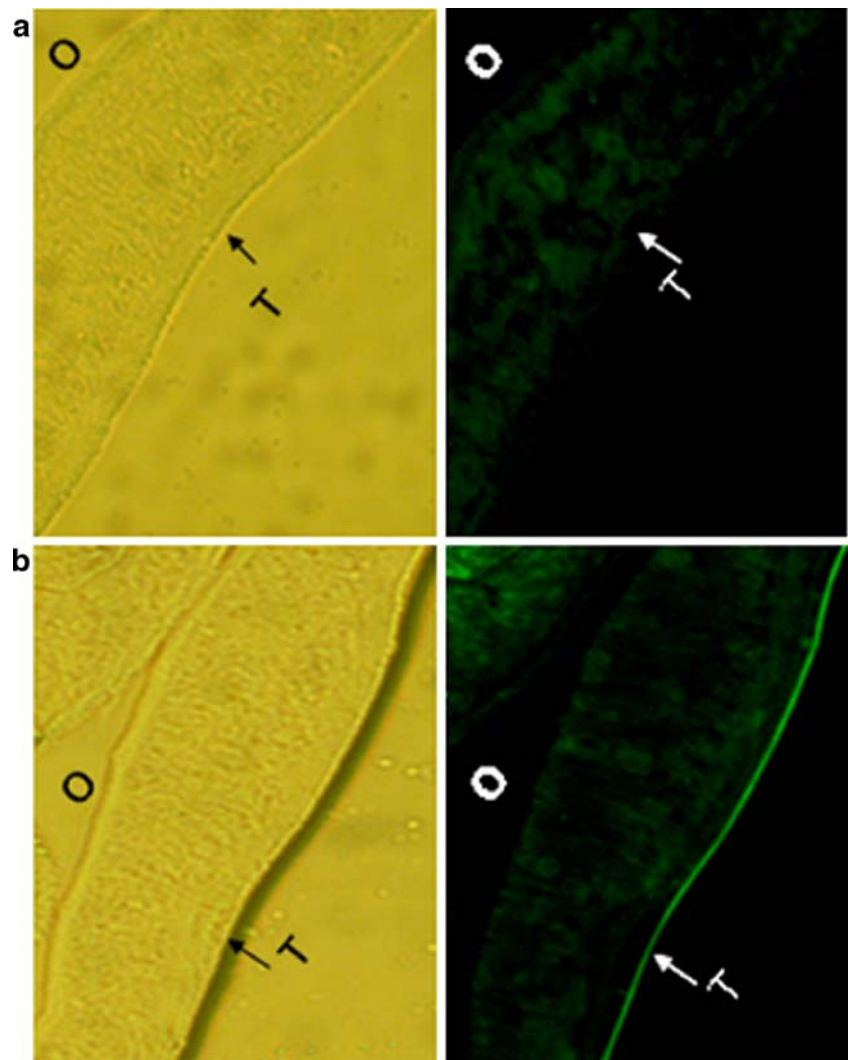
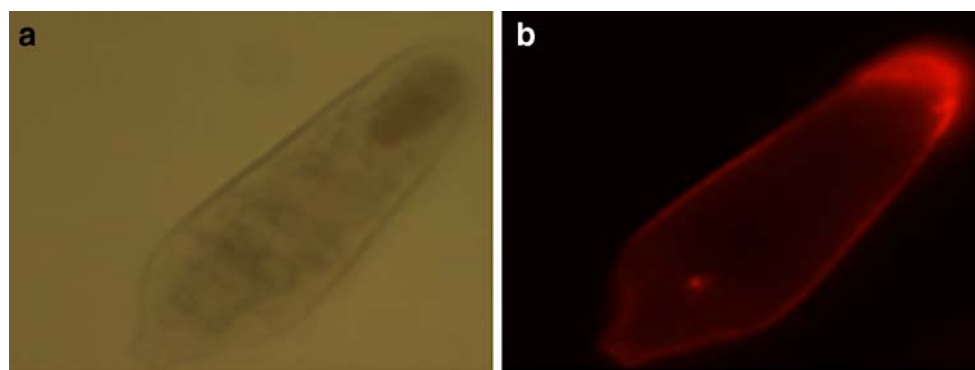


Fig. 4 Immunolocalization of Sj15 on cercaria. Optical images (a) and corresponding fluorescence microscopy images (b) of tegument and underlying tissues of cercaria are shown. Anti-rSj15 serum and secondary antibody coupled to Cy3 were used for fluorescence detection of Sj15 on cercaria



worm (Fig. 3) and cercaria (Fig. 4). The result showed that Sj15 was expressed in the tegument (Fig. 3b). In addition, Sj15 was expressed in the tegument and higher in at the forehead of the cercaria (Fig. 4b).

Expression of IL-12 in mouse DCs incubated with recombinant Sj15

DCs are the most potent antigen-presenting cells and play a major role in the initiation and regulation of the adaptive immune response to antigens (Banchereau and Steinman 1998; Cella et al. 1997; Steinman 1991). IL-12 produced by DCs during antigen presentation promotes Th1 differentiation, which results in enhancement of the cell-mediated immune response against various pathogens (Scott 1993; Trinchieri 1995). LPS could induce DCs expressing IL-12 effectively (Usluoglu et al. 2007; Martínez et al. 2007) and was used as the positive stimulation to evaluate the expression of IL-12 in cultured mouse DCs incubating with thrombin-cleaved protein Sj15. Total RNA were extracted from each treated DC culture to evaluate the expression of IL-12 by amplifying the partial gene fragments of its two subunits p35 and p40 with RT-PCR; the

amplification of a partial fragment (540 bp) of mouse actin was used as positive control (Fig. 5a). The relative expression level of IL-12 in each treated DC culture was normalized by beta-actin and displayed as the ratio of p35/p40 to beta-actin (Fig. 5b). rSj15 did not significantly alter the expression level of p40 in the DCs; however, it significantly upregulated the expression of p35 and peaked at 1 $\mu\text{g/ml}$.

Discussion

In China, recurrent epidemics of schistosomiasis have been reported in endemic communities that have been exposed to flood (Liu et al. 2005, Lu et al. 2007). Vaccination is an effective alternative to current methods used in the control of schistosomiasis, and the tegument of this helminth is an ideal target to look for protective antigens. The surface tegument that covers the schistosomulum and adult stages of schistosomes is of critical importance to host–parasite interactions, being essential for nutrient uptake, parasite growth, development, and as a protective barrier against host immune responses. In light of their importance for

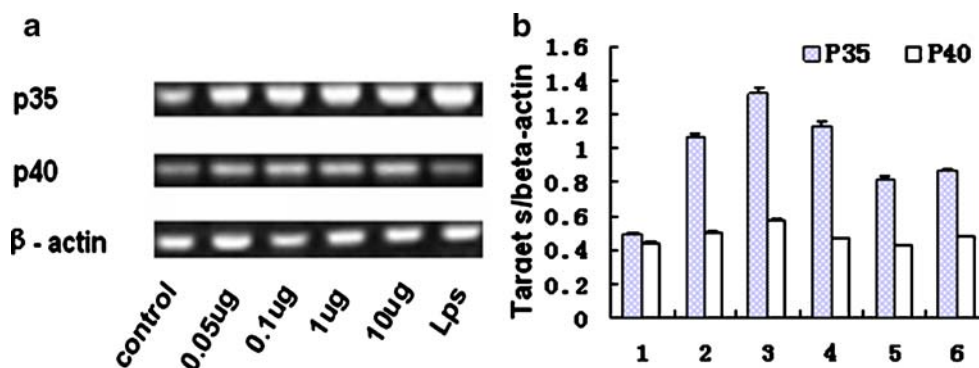


Fig. 5 Relative expression of P40 and P35 in DCs incubated with different concentration of thrombin-cleaved recombinant Sj15. Total RNA from DC, which incubated with different doses of the thrombin-cleaved Sj15, were used for the detection of p35 and p40 of IL12 by RT-PCR. **a** Agarose gel analysis of PCR product of p35, p40, and β -

actin. **b** Images of the DNA bands were scanned, and the ratio of the signal of the target density to β -actin was calculated and plotted against the different dosage of rSj15. Columns 1 to 4: 0.05, 0.1, 1, and 10 μg of rSj15, respectively; columns 5 and 6: RPMI 1640-negative and LPS-positive control, respectively

parasite survival, tegumental proteins are recognized as prime candidate targets for chemotherapy and immunotherapy of schistosomiasis (Jones et al. 2004). We screened 28 tegumental proteins of *S. japonicum* using bioinformatics and a profilin-like protein, one of which was chosen for further study in this work.

Recently, there was no standard on the *E* value by which two sequences could be determined to be homologous or not. To excavate more candidate proteins to screen for the target molecule, the limit of the *E* value was set at $E < 10^{-6}$ in this study. Based on this screening, a profilin-like protein of *S. japonicum*, which showed the lowest similarity with the related proteins of rat, mouse, and human was chosen for further studies. The profilin-like gene, Sj15, was cloned and expressed revealing a polypeptide with a MW of 15 kDa and agrees with the predicted MW 14.42 kDa.

The purified recombinant Sj15 protein could induce high titer of anti-Sj15 antibody that showed the recombinant Sj15 protein has perfect immunogenicity. Instead of 15 kDa, Western blot probing with anti-rSj15 serum identified double-protein bands 41 and 45 kDa in size at different growth stages of *S. japonicum*. The discrepancy in size may be due to the posttranslational modification such as glycosylation and phosphorylation. Fluorescence immunolocalization on the adult worm and cercaria revealed that the protein was expressed in the tegument of the adult worm and mainly confined in the tegument of the body of cercaria but not on the tail.

It is well known that the cytokine IL-12 is a critical inducer for the development of Th1-type immune responses (Heufler et al. 1996; Albert et al. 1998). The biologically active form of this cytokine is a secreted 70-kDa heterodimer composed of disulfide-linked p40 and p35 subunits, which are transcribed from separate genes (Trencher 1998). In this pilot study, rSj15 showed a similar property of the profilin-like protein of *T. gondii*, which can induce the production of the specific subunits p35 of IL-12.

In conclusion, the tegument protein Sj15 of *S. japonicum* had been characterized. The work showed that the protein expressed in the tegument at different growth stages. Its membrane affiliation, its immunogenicity and its inducibility of IL-12 could make Sj15 a potential antigen candidate and adjuvant for the development of a new vaccine. Studies are in process to further define the potential of Sj15 as a vaccine candidate.

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