

Molecular characterization of a calcium-binding protein SjCa8 from *Schistosoma japonicum*

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Abstract A full-length cDNA encoding a cercarial stage-specifically expressed 8-kDa calcium-binding protein (SjCa8) was isolated from *Schistosoma japonicum* cercarial cDNA library using microarray screen. The putative gene coding for SjCa8 is of 371 bp with an open reading frame of 69 amino acid (aa). The deduced aa sequence showed 83% identity with the *Schistosoma mansoni* 8-kDa CaBP, 47% identity with *Clonorchis sinensis* calcium-binding protein, and 38% identity with *Fasciola hepatica* putative calcium-binding protein. Also, it shares more than 30% identity with the calmodulin of many different species, while the most significant similarity between them lies around the two calcium-binding loop regions. There are two potential sites for phosphorylation and one potential site for N-myristoylation in the sequence. The SjCa8 has also been predicted to contain a single pair of EF-hand Ca^{2+} -binding domain. The recombinant SjCa8 (rSjCa8) protein expressed and purified from *E. coli* has been demonstrated to possess the calcium-binding activity. Immune serum from UV-attenuated *S. japonicum* cercariae-immunized rabbit detected rSjCa8 by Western blot assay, while the sera from *S. japonicum* naturally infected rabbit and normal rabbit could not. These findings may contribute to the development of an effective vaccine against schistosomiasis.

Introduction

Schistosomiasis is the second most prevalent tropical disease caused by trematode flatworms of the genus *Schistosoma*. Schistosomes have a complex life cycle involving two hosts (human and aquatic snail) and two short-lived free-swimming larval forms (Pearce and MacDonald 2002). The successful infection involves profound structural, biochemical, and physiological changes which are vital for schistosomes' adaptation of drastic temperature and osmolarity variations (Ram et al. 1999). Some of these changes should be determined to a large content of the expression of certain stage-specific genes. We have previously reported the analyses of *Schistosoma japonicum* cercarial stage-specific genes by cDNA library-based microarray (Hu et al. 2008, submitted for publication). In that study, a cDNA clone SjCa8 (GenBank accession number: EU046088), which was most abundant among the 19 identified cercarial stage-specific clones, had been found to be similar to the *Schistosoma mansoni* 8-kDa calcium-binding protein (SmCaBP). The SmCaBP has been reported to be preferentially expressed at the cercarial stage during the life cycle of the *S. mansoni* (Ram et al. 1989). It contains two typical calcium-binding loops and shows Ca^{2+} -dependent electrophoretic mobility. Further studies indicated that the SmCaBP is associated with organ/tissue remodeling via modulating some proteasome activities during transformation of cercariae to schistosomulum (Ram et al. 1994; Ram et al. 2003). Due to its similarity with SmCaBP and its cercarial stage-specific property, it is reasonable to propose that SjCa8 possesses calcium-binding activity and plays important roles during the *S. japonicum* cercariae transformation.

In this paper, we describe the cloning and characterization of the *S. japonicum* calcium-binding protein SjCa8. Results from the present study show that SjCa8 exhibits a

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calcium-binding property. Furthermore, SjCa8 has been shown to be able to probe with the serum from rabbit immunized with UV-attenuated cercariae, but not normal *S. japonicum*-infected rabbit sera.

Materials and methods

Cloning, expression, and purification of SjCa8

The full-length open reading frame (ORF) of SjCa8 was amplified from the *S. japonicum* cercaria cDNA library by PCR using specific primers (upstream primer: 5'-AAA AAG CTT CGA TGG TCA ATC GAG CAG AGG CTG-3' with an in-frame *Hind*III restriction site; downstream primer: 5'-CCG CTC GAG AAG AAA TTA TTG GCA CAA AAC TTT AAG-3' with an in-frame *Xho*I restriction site). The ORF of SjCa8 was then cloned into the *E. coli* expression vector pET32a(+). Recombinant plasmid was isolated, and the insert was sequenced to ensure direction and authenticity of the cloned gene. For expression of the protein, the recombinant construct was transformed into *E. coli* BL21 (DE3) and induced for 3 h using 1 mM IPTG after the culture OD₆₀₀ has reached 0.6. Recombinant SjCa8 (rSjCa8) protein was purified from the bacterial culture with Novagen His•Bind purification kit (EMD Biosciences, Inc., USA) under non-denaturing condition according to the manufacturer's protocol. The purified recombinant protein was confirmed by mass spectrometry with ABI 4700 Proteomics Analyzer TOF/TOF (Applied Biosystems, USA).

Sera

For production of antiserum, rSjCa8 protein bands were cut from sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) gels, ground, and mixed with equal volume of complete Freund's adjuvant (Sigma, USA). Each mouse was injected intradermally with about 0.1 ml of this mixture which contained 25 µg recombinant protein, followed by two additional injections of the same antigens mixed with incomplete Freund's adjuvant (Sigma, USA) at intervals of 2 weeks. The antiserum was collected 2 weeks after the last immunization and stored at -70°C until use.

The normal rabbit serum, serum from rabbit infected with *S. japonicum*, and serum from rabbit immunized with UV-attenuated *S. japonicum* cercariae are gifts from Dr. Chen, XG (Department of Etio-biology, Southern Medical University, Guangzhou, China).

Ruthenium red binding assay

Two micrograms of rSjCa8 or bovine serum albumin was dotted on a Milli-Q water-wetted nitrocellulose membrane

with Bio-Rad blotting manifold (Bio-Rad, USA). After transferring the protein, the membrane was incubated in the ruthenium red assay buffer (Fluka, USA) for 10 min. Membranes incubated with ruthenium red assay buffer containing 1 mM CaCl₂ or 1 mM ethylenediamine tetraacetic acid (EDTA) were used as controls. The membranes were then briefly rinsed with Milli-Q water, and the images were scanned when the membranes were still wet.

Electrophoretic mobility shift assay

rSjCa8 protein was electrophoretically separated on a 10% non-denaturing polyacrylamide gel (Sauter et al. 1995), in the presence of (a) 1 mM CaCl₂, (b) 1 mM EDTA, or (c) without CaCl₂ and EDTA. Bovine serum albumin was run alongside with rSjCa8 as the control.

Results

Isolation and sequence analysis of SjCa8

The full-length ORF of SjCa8 was amplified from the cercaria cDNA library. After sequencing, a complete ORF consisting 210 nucleotides, which encoded for 69 amino acid residues, was identified (Fig. 1a). After searching with TBLASTN at the NCBI database, the SjCa8 protein showed 83% homology with the SmCaBP (J04641) and 100% the same as the *S. japonicum* 8-kDa calcium-binding protein (AY274581). Besides, SjCa8 displays 47% identity with *Clonorchis sinensis* calcium-binding protein (AF527456), 38% identity with *Fasciola hepatica* putative calcium-binding protein (AF213970), and 30% with calmodulin from several species. The 8-kDa calcium-binding protein of *S. japonicum* and *S. mansoni*, calcium-binding protein of *C. sinensis* and *F. hepatica*, and calmodulin proteins from many species show many conserve domains in the alignment of the sequences using the ClustalW alignment (<http://www.ebi.ac.uk/Tools/clustalw/>; Fig. 1b). The phylogram tree shown in Fig. 1c indicates that the *S. mansoni* SmCaBP is most related with SjCa8, while the calcium-binding proteins of *C. sinensis* and *F. hepatica* are more related to each other and distinct from SjCa8 and SmCaBP.

An analysis of the amino acid sequence of SjCa8 with ScanProsite (<http://au.expasy.org/tools/scanprosite/>) revealed that SjCa8 contains a pair of putative EF-hand calcium-binding domains at amino acid positions 2–37 and 38–69 (de Castro et al. 2006). The amino acid sequences of the two calcium-binding loops were compared with the 12 residues of EF-hand loop consensus sequence, and the results indicated that they fulfill the requirements of a typical EF-hand (Fig. 1d; Moncrief et al. 1990). In addition,

a

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gggtatttctaatggtaaggctttgtgacttgcttgtaatttaataatcctagtggt
tttacgtattgagtaaaaaatgggtcaatcgagcagaggctgcacaacttctaaaacatctt
      M V N R A E A A Q L L K H L
gacaagataacagtggtggaaatcagcacacaagagctaattggaatttttgagcagcggt
D K D N S G K I S T Q E L M E F L S S V
aactgcccattcaacaaagcacaggttgagaagtttattaaagtacatgacgcggatgga
N C P F F N K A Q V E K F I K V H D A D G
gatggacaactaaataactgatgaacttcttaagttttgtgccaataatttcttgaattt
D G Q L N T D E L L K V L C Q -
tcagtaatgttactctcataataaataactattttaatttcaaaaaaaaaaaaaaaaaaa
aaaaaaaaaa

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b

Dd_calmodulin	-MASQESLTEEQIAEFKEAFSLFDKDGDSITTKELGTVMRSLGQNPTAEELQDMINEVD	59
Pi_calmodulin	-MADQ--LSEEQISEFKEAFSLFDKDGDTITTKELGTVMRSLGQNPTEGELQDMINEVD	57
Bb_calmodulin	-MADQ--LSEEQIAEFKEAFSLFDRDGDGSIITTKELGTVMRSLGQNPTAEELADMINDID	57
At_calmodulin	-MEET-ALTKDQITEFKEAFCLFDKDGDCITVEELATVIRSLDQNPTQELHDIITEID	58
Nt_calmodulin	-MGD--ILNQDQIVELQEAFLFDRDGDGCIITVEELATVIRSLDQNPTHEELQDMITEVD	57
Py_calmodulin	MRSATQDLSEETIREFKEAFALFDKDGDTITSTELGAVMRSLGQQPTAALKQMISEVD	60
SjCa8	-----	
Sm_CaBP	-----	
Cs_CaBP	-----	
Fh_CaBP	-----	
Dd_calmodulin	ADGNGNIDFPEFLIMARKMQDITDEEEIREAFKVFDDKNGYISAAELRHVMTSLGEKL	119
Pi_calmodulin	ADGNGTIDFPEFLIMARKMRDITSEEEIKEAFKVFDDKNGYISAAELRHVMTNLGEKL	117
Bb_calmodulin	TSGTGAIDFPEFLIMARKMKEGDTSEELVQAFKVFDRDNGFISAQELRHVMTNLGEKL	117
At_calmodulin	SDSNGTIEFAEFLNIMAKKLQESDAEEELKEAFKVFDDKNGYISASELSHVMTNLGEKL	118
Nt_calmodulin	SDGNGTIEFTEFLNIMAKKMKDITAEELKEAFKVFDDKNGYISANELRHVMTNLGEKL	117
Py_calmodulin	ADGSGTIDFAEFLTIMSRKMKADSQAELIEAFKVFDDKSGKISADELRQVMNMLGEKL	120
SjCa8	-----MVNRAEAQQLLKHLDKDNISGKISTQELMEFLSSVNCPP	38
Sm_CaBP	-----MVNRTAAQQLLKHLDKSGKISSQELMEFLHTVNCPP	38
Cs_CaBP	-----MPTKSEVKAVLECLDSNKDGKISVEELKSFLDSANCNL	38
Fh_CaBP	-----MPSVQVEKLLHVLDRNGDGKVSAAELKAFADDSKCPL	38
	* :. : * : . * : * * . :	
Dd_calmodulin	TNEEVDEMIREADLDGQVNYDEFVKMMIVRN	152
Pi_calmodulin	TDTEVDEMIREADVDDGQINNYDEFVKMMLSK-	149
Bb_calmodulin	TNEEVEMLREADVDGDKINYYEFVKLMISK-	149
At_calmodulin	TDEEVEQMIKEADLDGQVNYDEFVKMMINID	151
Nt_calmodulin	TDEEVEQMIKEADLDGQVNFDEFVKMMNVG	150
Py_calmodulin	SDEEVSEMIREADTINGDGEIDVKEFVKMMRL--	151
SjCa8	NKAQVEKFIKVHDAADGDGQLNTDELLKVLCC--	69
Sm_CaBP	KKEQVEKFIKQHDKDGQQLNTDELLDVLCSS--	69
Cs_CaBP	KREDVEAFIKTHDADKDGKLDLDELVACLSCS--	70
Fh_CaBP	DSNKIKAFIKHDKNKDGKLDLDELVSILSS--	69
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Fig. 1 a Complete cDNA sequence and the deduced amino acid sequence of SjCa8. The ORF of SjCa8 comprised of 210 nucleotides which encoded for 69 amino acid residues. **b** ClustalW alignment of SjCa8 with the deduced amino acid sequences of *S. mansoni* SmCaBP (Sm_CaBP, accession no. J04641), *Clonorchis sinensis* calcium-binding protein (Cs_CaBP, accession no. AF527456), *Fasciola hepatica* putative calcium-binding protein (Fh_CaBP, accession no. AF213970), and calmodulin from *Arabidopsis thaliana* (At_calmodulin, accession no. NM_117546), *Dictyostelium discoideum* (Dd_calmodulin, accession

no. XM_636603), *Nicotiana tabacum* (Nt_calmodulin, accession no. AB050849), *Paxillus involutus* (Pi_calmodulin, accession no. AY585925), *Babesia bovis* (Bb_calmodulin, accession no. XM_001609007), *Porphyra yezoensis* (Py_calmodulin, accession no. EF375999). The EF-hand calcium-binding loop regions of SjCa8 are underlined. **c** The phylogram tree analysis of SjCa8 with the homologues. **d** Comparison of the residues of calcium-binding loops of SjCa8 with that of EF-hand calcium-binding loop consensus sequences

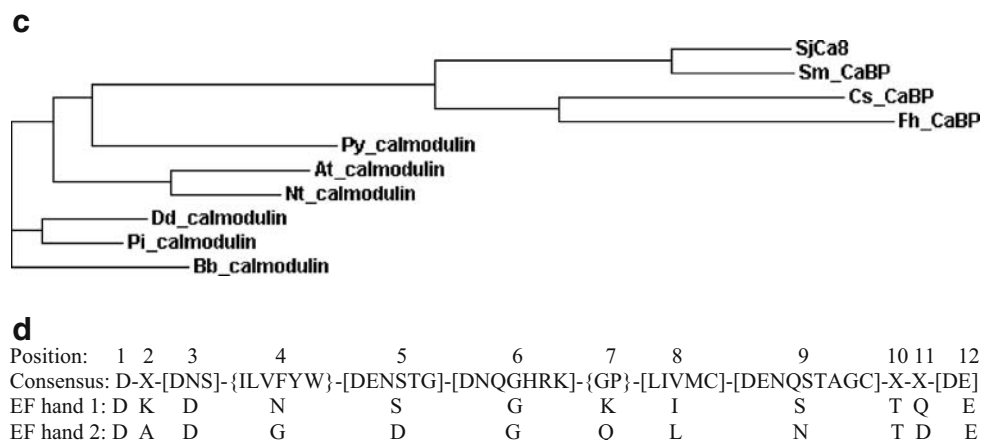


Fig. 1 (continued)

SjCa8 contains one potential protein kinase C phosphorylation site (amino acid positions 19–21), one potential casein kinase II phosphorylation site (amino acid positions 23–26), and one potential N-myristoylation site (amino acid positions 56–61).

Expression and purification of rSjCa8

The ORF of SjCa8 was cloned into pET-32a(+) expression vector, and the recombinant fusion protein expressed was purified by affinity chromatography using a Ni-NTA

agarose gel column under native conditions. The purified protein was analyzed with SDS-PAGE (Fig. 2) and confirmed by mass spectrometry (data not shown). The yielded protein was determined by Bradford assay (Bio-Rad, USA) and kept at -70°C for future use.

Evaluation of antigenicity of rSjCa8 protein

As shown in Fig. 3, rSjCa8 was specifically recognized only by serum from rabbit immunized with UV-attenuated cercaria. Normal uninfected rabbit serum and serum from *S.*

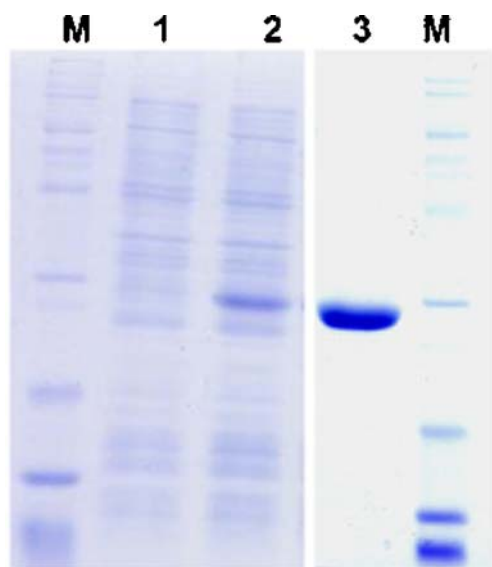


Fig. 2 Sodium dodecyl sulfate polyacrylamide gel electrophoresis (12.5%) analysis of rSjCa8. Lane 1 Total cell lysate of *E. coli* BL21 (DE3) cells before isopropylthiogalactoside (IPTG) induction, lane 2 total cell lysate of *E. coli* BL21(DE3) cells after IPTG induction, lane 3 recombinant fusion protein purified by affinity purification, lane 4 molecular mass standards (myosin, β -galactosidase, phosphorylase b, serum albumin, ovalbumin, carbonic anhydrase, trypsin inhibitor, lysozyme, aprotinin)

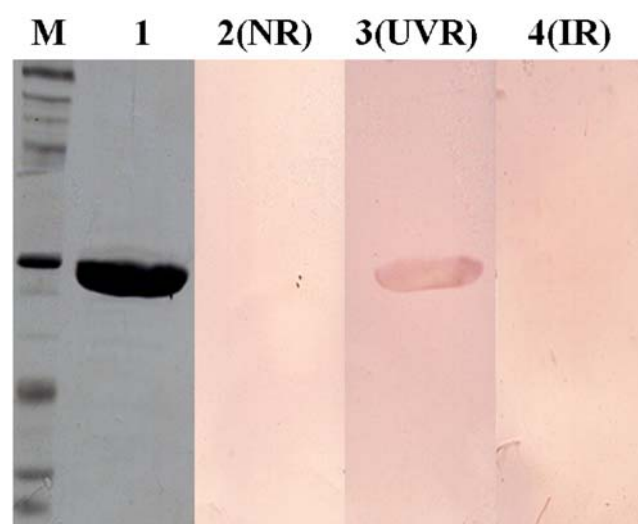


Fig. 3 Western blot for evaluating rSjCa8 antigenicity. Lane M Molecular mass standards (myosin, β -galactosidase, phosphorylase b, serum albumin, ovalbumin, carbonic anhydrase, trypsin inhibitor, lysozyme, aprotinin), lanes 1–4 purified rSjCa8 protein. Lanes M and 1 were stained with amido black. Lane 2 was probed with normal rabbit serum (NR), 1:500. Lane 3 was probed with serum from rabbit immunized with UV-attenuated cercariae (UVR), 1:500. Lane 4 was probed with serum from rabbit infected by normal *S. japonicum* (IR), 1:500

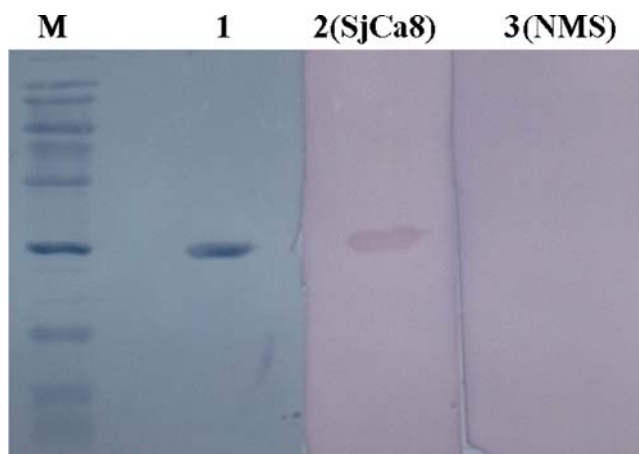


Fig. 4 Evaluation of immunogenicity of rSjCa8 protein by Western blot. *Lane M* Molecular mass standards (myosin, β -galactosidase, phosphorylase b, serum albumin, ovalbumin, carbonic anhydrase, trypsin inhibitor, lysozyme, aprotinin), *lanes 1–3* purified rSjCa8 protein. *Lanes M* and *1* were stained with amido black. *Lane 2* was probed with anti-SjCa8 serum (*SjCa8*), 1:500 as primary antibody. *Lane 3* was probed with normal mouse serum (*NMS*), 1:500 as primary antibody

japonicum naturally infected rabbit did not recognize the protein.

Immunogenicity of SjCa8 in mice

Mice immunized with the protein developed specific IgG antibodies to titers $>10^6$ as determined by enzyme-linked immunosorbent assay (ELISA; data not shown). These antibodies were able to recognize the recombinant protein SjCa8 (Fig. 4) by immunoblot. Sera from unimmunized control mice did not recognize the recombinant protein either by ELISA or immunoblot.

Demonstration of calcium-binding property of SjCa8

The electrophoretic motility of SjCa8 in native protein gel was compared with bovine serum albumin (BSA) and protein marker in either the presence or absence of added CaCl_2 or EDTA. In the presence of CaCl_2 , SjCa8 migrated more slowly while BSA and protein marker migrated in the same rate as in the normal native gel (Fig. 5a). In the gel containing EDTA instead of CaCl_2 , there were no such changes in electrophoretic mobility.

For ruthenium red binding assay, SjCa8 and BSA were dotted onto nitrocellulose membrane. The membrane was then stained with ruthenium red solution in the presence/absence of CaCl_2 or EDTA. As shown in Fig. 5b, in the absence of CaCl_2 and EDTA, ruthenium red stained SjCa8 red but not BSA. Neither SjCa8 nor BSA was stained red in the presence of CaCl_2 or EDTA.

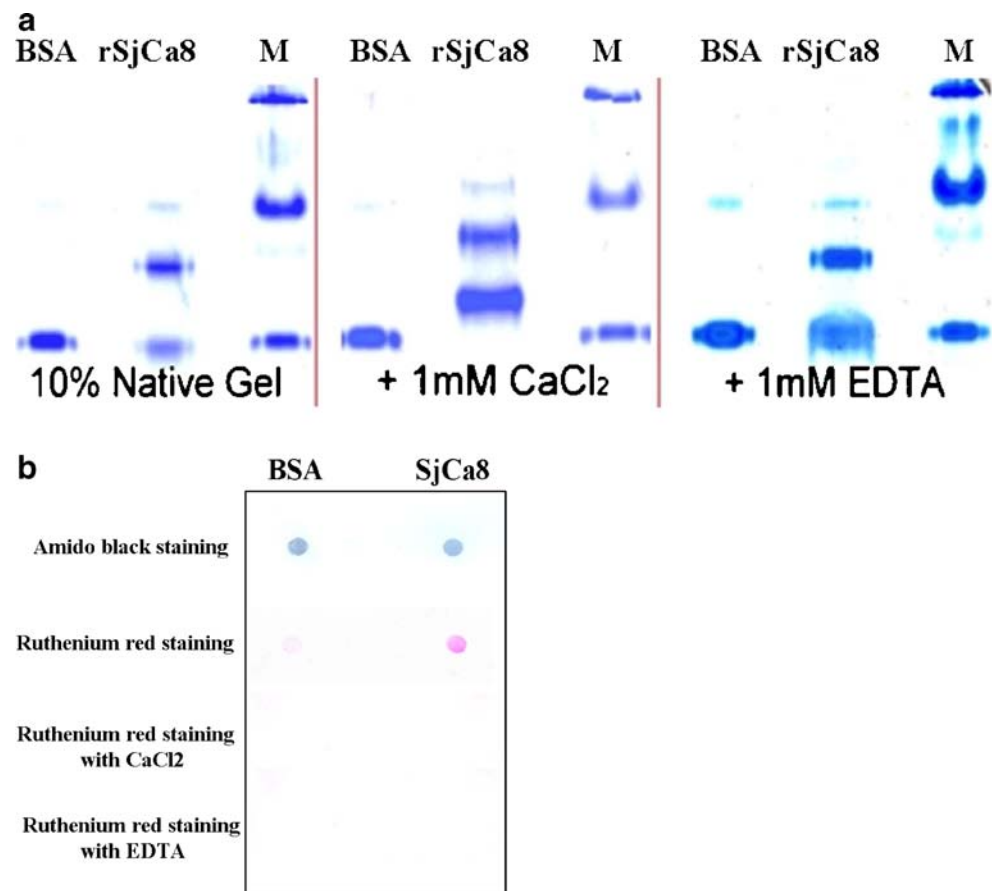
Discussion

Calcium (Ca^{2+}) is a ubiquitous intracellular signal responsible for an array of cellular functions involving fertilization, contraction, cell differentiation and proliferation, development, apoptosis, and even cancer (Berridge et al. 2000). At the fundamental level, the action of calcium in these events is very simple: cells are activated when Ca^{2+} concentration in cytoplasm rises from ~ 100 to $\sim 1,000$ nM (Berridge et al. 2003). These increased Ca^{2+} result either from the influx of extracellular Ca^{2+} or the release of this cation from internal stores. Once inside the cytoplasm, the calcium will not retain free, but bind to a wide variety of calcium-binding proteins (CaBPs). These CaBPs play key roles in modulating the Ca^{2+} signal both spatially and temporally (known as Ca^{2+} buffers), or in translating the Ca^{2+} signal to the diverse cellular responses (known as Ca^{2+} sensors; Gifford et al. 2007).

In this study, we have cloned a *S. japonicum* calcium-binding gene SjCa8 (Fig. 1a). Sequence analysis by tblastn in NCBI revealed that SjCa8 is 83% identical to a *S. mansoni* 8-kDa protein CaBP. The SmCaBP was first cloned by Ram et al. and had been demonstrated to interact with S5a/Rnp10, regulate the proteosome function, and may play a role on organ/tissue remodeling during the life cycle of schistosome (Ram et al. 1994, 2003). Since the SjCa8 has been shown to share similarities with this SmCaBP in several aspects such as protein sequences, cercarial stage-specific expression, calcium-binding activity, and possessing a single pair of EF-hand motifs, this gene is likely to be the homologue of SmCaBP.

EF-hand calcium-binding proteins represent the most important homologous calcium-binding protein superfamily which binds Ca^{2+} using a characteristic helix–loop–helix structural motif termed the ‘EF-hand’. The motif ‘EF-hand’ was first discovered and named by Kretsinger in his study of the structure of a carp muscle Ca^{2+} -binding protein parvalbumin (Kretsinger and Nockolds 1973). The EF-hand motifs were then identified in a lot of other Ca^{2+} -binding proteins from different species (Gifford et al. 2007). Today, it is estimated that there are more than 3,000 EF-hand related entries in the NCBI Reference Sequences Data Bank (Grabarek 2006). These proteins can be ordered into at least 45 distinct subfamilies according to Kawasaki et al. (1998). The calcium-binding domains within the proteins from the same subfamily are evolutionarily conserved and originated from a common precursor (Nakayama et al. 1992). In this study, we discovered that SjCa8 displays high similarities (more than 30%) with calmodulins from a lot of different species, and it is obvious that the most significant homology between SjCa8 and those calmodulins is displayed around the two calcium-binding loops (Fig. 1b). Thus, these results suggest that SjCa8 may be ordered into

Fig. 5 a Electrophoretic mobility shift of SjCa8. Two micrograms of SjCa8 (*SjCa8*), bovine serum albumin (*BSA*), and Bio-Rad molecular weight standards (*M*, from top to bottom, myosin, β -galactosidase, and bovine serum albumin for the three major bands) were resolved in 10% native gel (left diagram), 10% native gel with 1 mM CaCl_2 (middle diagram), and 10% native gel with 1 mM EDTA (right diagram), respectively. In the presence of CaCl_2 , migration of SjCa8 was retarded while that of BSA and marker was unaffected. In the presence of EDTA, the migration of SjCa8, BSA, and marker was unaffected. **b** Ruthenium red binding assay of SjCa8. Two micrograms of SjCa8 (right spot) and BSA (left spot) were dotted onto nitrocellulose membranes. The membranes were then stained with amido black, ruthenium red, or ruthenium red staining in the presence of CaCl_2 or EDTA, respectively



calmodulin subfamily. However, SjCa8 is distinct from calmodulin in the amount of Ca^{2+} -binding motifs: it just contains a pair of Ca^{2+} -binding motifs while the latter contains two. The phylogram tree analysis of SjCa8 showed that it is closely related to the *S. mansoni* SmCaBP, and the calcium-binding proteins of the parasites are more closely related than the calmodulins from other species (Fig. 1c). Besides, SjCa8 shows a surprisingly high degree of conservation within the framework of calcium-binding domains (Fig. 1d). Similar to the *S. mansoni* SmCaBP, functional assay showed that rSjCa8 has calcium-binding property. The calcium-binding activity of rSjCa8 was verified by an electrophoretic motility shift assay and a ruthenium red binding assay (Fig. 5). It should be noted that in the electrophoretic motility shift assay, the rSjCa8 fusion protein has been displayed as three bands instead of one band as shown in SDS-PAGE, and each of the bands exhibits calcium-binding property. This may be due to homo-dimerization of the rSjCa8 in native condition, which is a common phenomenon of many calcium-binding proteins (Grabarek 2006).

We have also obtained the purified rSjCa8 protein from *E. coli* (Fig. 2). Previous studies showed that animals vaccinated with attenuated cercariae could get 70–90% protection against cercariae challenge infection (Ruppel et

al. 1990). Passive transfer of the immunized sera also showed certain degree of protection (Moloney et al. 1987). It is assumed that some antibodies in the immunized sera can protect against *S. japonicum* infection. Screening the cercariae antigens that can be probed with UV-attenuated *S. japonicum* cercariae-immunized sera but not with naturally infected sera is of great interest. The result of our antigenicity assay showed that rSjCa8 may be a potential vaccine candidate molecule since it can be probed by UV-attenuated *S. japonicum* cercariae-immunized rabbit serum but not with naturally infected rabbit serum (Fig. 3). This is reasonable as UV irritation may affect the development and migration of the cercariae; thus, some antigens may have more chance to stimulate host immune cells to produce corresponding antibodies (Cardoso Gde and Coelho 1989; Moloney et al. 1985). Besides, UV irritation may also suppress the synthesis and release of certain kinds of immunomodulatory molecules by the cercariae (Jenkins et al. 2005) and arouse humoral response against the cercariae stage-specific antigens.

We have identified this *S. japonicum* 8-kDa calcium-binding gene (SjCa8) by microarray analysis. It is a cercarial stage-specific gene and the encoded protein possessing a single pair of EF-hand motif and shows a calcium-binding activity. This SjCa8 gene may play a role

in cercariae-to-schistosomula transformation. It should also be mentioned that Xu et al., working independently, have recently cloned a gene SjCBP from *S. japonicum* cercarial cDNA library by expression sequence tag strategy (Xu et al. 2003). A comparison of the nucleotide sequence of SjCBP showed 100% identity with SjCa8; therefore, SjCa8 and SjCBP should be the same. However, Xu reported that the SjCBP was not reactive with the serum for UV-attenuated cercariae-immunized rabbit. In our study, we have demonstrated that SjCa8 could be probed with UV-attenuated cercariae-immunized rabbit serum but not with the normally infected serum. Our next study will be the test of the immunoprotective effect of SjCa8 against *S. japonicum*.

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