SUPPLEMENTARY TEXT ADDITIONAL MATERIALS AND METHODS

Parasites and experimental animals

Naturally infected cyprinoid fish were collected in northeast Thailand for isolation of metacercariae as described by Srisawangwong et al. [1]. To prepare newly excysted juveniles (NEJ), around 1,500 metacercariae were excysted by alcohol-enhancement [2]. Adults, 4- and 2-week-old juveniles were obtained by infection of Syrian golden hamsters (Mesocricetus auratus) with metacercariae as described by Geadkaew et al. [3]. All parasites were kept in liquid nitrogen until use. All animals experiments in this study were approved by the Thammasat University Animal Ethics Committees (Project No. 014/2557, 28 October 2014). Animals were maintained under strict hygienic conventional conditions on corncob bedding (B&C Pulaski Corporation Ltd., Bangkok Thailand) in solid bottom cages (Tecniplast, Milan, Italy) for group housing (3 mice per cage) at temperature of 22±1˚C, humidity of 30-70%, 10-15 air change per hr and 12:12 hr light/dark cycle controlled automatically. Animals were fed ad libitum with commercial diet (Charoen Pokphand, Bangkok, Thailand) and RO filtered drinking water in bottles with sipper tube (Tecniplast).

Molecular cloning and sequence analysis

NCBI-BLAST screens of *Opisthorchis viverrini* and *Clonorchis sinensis* [4-7] protein/nucleotide data were performed to identify genes that encode calcium-binding proteins containing EF hand motifs and dynein light chain-like domain (CaBPs). Four undescribed genes of *O. viverrini* were selected for molecular analysis and their complete coding sequences (CDS) were cloned from adult stage total RNA by reverse transcriptase PCR with the primers detailed in Table 1. The cDNAs were inserted into pGEM-T Easy (Promega, Wisconsin) by ligation and sequenced (SolGent, Daejeon, Korea). Standard sequence analysis was performed using EMBOSS version 6.5 [8] including pairwise alignment (EMBOSS needle with parameters: EBLOSUM62, gap penalty 10.0, extend penalty 1.0). Phylogenetic analysis was performed starting with the ETE 3 metaligner workflow [9] that comprises Clustal Omega, MAAFT, MUSCLE, and M-Coffee [10-13] trimAl 1.4 [14] with the gappyout setting. Finally, PhyML 3.0 [15] was used to construct a maximum likelihood phylogeny using the LG model and 100 bootstraps.

Reverse transcriptase PCR analysis

Total RNA was isolated with TRIzol (Ambion, California, USA) from newly excysted juveniles (NEJ), 2-and 4-week-old juveniles and mature parasites. The extracted RNAs were treated with DNase I (Promega, Madison, Wisconsin, USA) and 100 ng of each treated RNA sample was reverse transcribed (RevertAid, Thermo Fisher Scientific, Waltham, Massachusetts, USA) to cDNAs by using specific reverse primers (Table 1) for

Table 1. Primers and introduced restriction sites used for cloning the complete coding sequence of OvCaBP1-4

OvCaBP	Primer sequence[a]	Restriction site	Size (bp)
1	Fwd: ggatccATGACACAACAAGCAGCACA Rev: aagcttCTATGCGCGGTTAGTACG	BamH I Hind II	588
2	Fwd: ggatccATGGAAGGCATTGAATCAATG Rev: aagcttTTAACACTGAGGGGTGCG	BamH I Hind III	555
3	Fwd: catatgGCACAGGTTCAAACG Rev: ctcgagCGTCCGGTTCGTACGCCA	Nde I Xho I	606
4	Fwd: ggatccATGGGTGAACAAGGATCG Rev: aagcttTTAGTTGATGGTGGTACG	BamH I Hind III	597

[a]restriction sites indicated by underlined small letters.

Table 2. Amino acid identity (bold) and similarity values (%) of OvCaBP1-4 and OvCaBP22.8

	OvCaBP1	OvCaBP2	OvCaBP3	OvCaBP4	OvCaBP22.8
OvCaBP1	-	37.9	52.2	37.4	37.2
OvCaBP2	57.4	-	35.8	41.9	42.1
OvCaBP3	71.1	63.2	-	32.6	38.2
OvCaBP4	60.7	58.6	55.8	-	41.2
OvCaBP22.8	56.0	61.5	58.5	58.8	-

Table 3. Ion-binding patterns of rOvCaBP1-4 with Ca^{2+}, Mg^{2+}, Zn^{2+} and Cu^{2+} by mobility shift assay

	Ca^{2+}	Mg^{2+}	Zn^{2+}	Cu^{2+}
OvCaBP1	+	+	+	+
OvCaBP2	+	-	+	-
OvCaBP3	+	-	+	-
OvCaBP4	+	-	+	-

+, bound; -, not bound.

each OvCaBP. The following standard PCR was performed by using Taq DNA polymerase (Thermo Fisher Scientific) and specific primers for each isoform as shown in Table 1. The RT-PCR products were analyzed by 1% agarose gel electrophoresis and subsequently inserted into pGEM T- Easy by ligation.

Expression and purification of rOvCaBPs

Sequence-verified OvCaBP cDNAs were subcloned from pGEM T-Easy into the expression vectors pQE30 (for OvCaBP1, 2, and 4) or pET21b (for OvCaBP3). The expression strains *Escherichia coli* M15 (for pQE30) and *E. coli* BL21 (for pET21b) were transformed with the recombinant vectors. Following bacterial culture and induction with 1 mM IPTG the expressed soluble recombinant proteins were purified under native conditions by Ni-NTA affinity chromatography (QIAGEN, Hilden, Germany). The purified recombinant proteins were dialyzed against different buffers depending on their solubility. OvCaBP1: 10 mM PBS, pH 7.2; OvCaBP2 and OvCaBP3: 50 mM Tris-HCl, pH 8.0; OvCaBP4: elution buffer. The purified soluble OvCaBPs were used for functional analysis and antibody production.

Preparation of crude worm extracts (CWEs) and excretory secretory product (ES product) from adult *O. viverrini*

Adult *O. viverrini* worms were homogenized in lysis buffer (150 mM NaCl, 10 mM Tris-HCl pH 7.2, 1 mM EDTA pH 8.0, 1 mM PMSF and 0.5% [v/v] Triton-X 100) by using an Ultra-Turrax T25 tissue homogenizer (IKA, Staufen, Germany). The homogenate was rotated at 4˚C for 1 hr and centrifuged at 12,000×g and 4˚C for 15 min. The supernatant was collected as soluble CWE and the pellet was solubilized in 50 mM Tris-HCl, pH 8.0 and 3% SDS at 37˚C for 1 hr. The lysate was centrifuged at 12,000×g for 15 min and the solubilized CWE was collected. To prepare ES product, fresh adult parasites were washed several times with 0.85% NaCl and then incubated in 0.01 M PBS, pH 7.2 in 5% CO at 37˚C for 12 hr. The buffer was collected and centrifuged at 5,000×g at 4˚C for 20 min to eliminate parasite eggs and insoluble material. The supernatant was concentrated using a centrifugal concentrator (3 kDa cut off, GE Healthcare, Buckinghamshire, UK). The supernatant was collected as ES product. Protein concentrations were measured by a Bradford assay (Bio-Rad, Hercules, California) for soluble CWE and ES product, and by a BCA assay (Thermo Fisher Scientific) for insoluble CWE. All proteins were stored at -20˚C for 20 min prior to further experiments.

Western blot analysis

O. viverrini soluble and insoluble CWEs (20 μg each), ES products (20 μg) and rOvCaBPs (100 ng each) were size-separated by 12.5% SDS-PAGE and transferred onto a nitrocellulose membrane (Bio-Rad) by semi-dry transfer using a Fastblot B33 instrument (Whatman, Biometra, Germany). The membrane-bound proteins were probed separately with the 4 specific rOvCaBP antisera at a dilution of 1:2,000. Pre-immune sera were used as negative controls at the same dilution. Alkaline phosphatase-goat anti-mouse IgG (Sigma, Saint Louis, Missouri) was used as secondary antibody (1:30,000). Colorimetric signals were developed with BCIP/NBT phosphatase substrate (Amresco, Solon, Ohio, USA). Each recombinant OvCaBP was also detected with pooled *O. viverrini*- infected hamster sera (n=10) by Western blot analysis. The 4 rOvCaBPs (500 ng each) and soluble CWE (5 μg) were size-separated by 12.5% SDS-PAGE and transferred onto a nitrocellulose membrane as described above. Pooled pre-infection sera were used as a negative control (n=10). The membranes were incubated with 12-week-infected sera at a dilution of 1:200 or pre-infection sera at the same dilution. Goat anti-hamster IgG conjugated HRP (Life technologies, Maryland) and Metal enhanced DAB (3,3′-diaminobenzidine) substrate kit (Thermo Scientific, Chicago, Illinois) were used as secondary antibody and colorimetric substrate, respectively.

Immunohistochemistry

The distribution of each OvCaBP in mature (8-week-old) parasites was analyzed in paraffin- embedded tissue-sections as described in our previous experiments (15). All mouse anti-rOvCaBP antisera and mouse pre-immune sera were used at a dilution of 1:1,000. A biotinylated rabbit anti-mouse antibody (Dako, Carpinteria, California) was used as secondary antibody (1:200). Colorimetric detection was performed by using an ABC peroxidase detection kit (Thermo Scientifi). The sig-

nals were developed using AEC (3-amino-9-ethylcarbazole) substrate (Thermo Fisher Scientific).

Analysis of ion binding properties by mobility shift assay

Native polyacrylamide gel electrophoresis without SDS was used to study the ion-binding properties of OvCaBPs by changes in their migration patterns. Five micrograms of each rOvCaBP and rGST (glutathione S-transferase, negative control, expressed by pGEX-5X-1, GE Healthcare, Illinois) was pre-incubated with 5 mM EDTA to chelate contaminating metal ions. EDTA-treated proteins were incubated with 25 mM CaCl or without CaCl . Electrophoresis buffer (120 mM Tris-HCl, 0.05% [w/v] Bromothymol blue, 1% [w/v] DTT, 20% [v/v] glycerol) was then added to the samples and thoroughly mixed. The samples were resolved in 8.5-12.5% continuous native gels in SDS-free electrophoresis buffer. The recombinant proteins were visualized by Coomassie blue staining. In addition to Ca^{2+}, the divalent cations Mg^{2+}, Zn^{2+} and Cu^{2+} were also tested at the same concentration.

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ADDITIONAL RESULTS AND DISCUSSION

As described in the main publication the 4 anti-rOvCaBP antisera showed cross reactivity. This is also demonstrated in Supplementary Fig. 4. Therefore, the results of the immunohistochemical analysis are underdetermined, i.e. it cannot be excluded that the observed staining obtained with each antiserum originates from the detection of more than one isoform. A common problem if conserved isoforms have comparable expression profiles.

The analysis of paraffin-embedded tissue from adult *O. viverrini* with anti-rOvCaBP1-3 antisera detected the antigens in the tegument layer and at a lesser staining intensity in the tegumental cell bodies (Supplementary Fig. 5). OvCaBP4, surprisingly, could not be detected. Although the detection might not be isoform-specific it supports the largely tegumental distribution reported for other family members. Staining in the tegu-

mental cell bodies was often weak or absent suggesting fast transport of the proteins to the syncytial tegument after translation in the cell bodies. Unexpectedly, anti-rOvCaBP4 antisera, while positive with CW extracts and ES product in western analysis, could not detect OvCaBP4 in the parasite tissue. We tried antibody dilutions as low as 1:100 and several epitope-retrieval conditions without success and concluded that the antisera from the 3 mice did not detect the set of epitopes available in the tissue sections. The orthologous CsTegu21.6 was detected in the tegument but seemingly weakly [1]. The results are also comparable with CsTegu20.6 [2]. In F. gigantica, FgCaBP1, 3 and 4 were found in the tegument, the epithelial lining of the excretory system, and in the intestinal tract [3,4]. OvCaBP22.8, as mentioned above, was detected in the intestinal tract and parenchyma, but not in the tegument [5]. This indicates that distinct family members can have roles in different tissue types and that it is important to analyze the basic properties of all family members.

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