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Spatiotemporal Expression Patterns and Antibody Reactivity of Taeniidae Endophilin B1

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Larval Taeniidae, such as metacestodes of *Taenia solium*, *Echinococcus granulosus*, and *Echinococcus multilocularis*, produce chronic and fatal helminthic diseases. Proper identification of these zoonotic cestodiases is often challenging and is hampered in some clinical settings. Endophilin B1 plays critical roles in the maintenance of membrane contours and endocytosis. We isolated proteins homologous to endophilin B1 from *T. solium*, *Taenia saginata*, and *Taenia asiatica*. The three Taeniidae endophilin B1 proteins shared 92.9 to 96.6% sequence identity. They harbored a Bin1/amphiphysin/Rvs (BAR) domain and residues for a dimeric interface but lacked a SRC homology 3 (SH3) domain. Endophilin B1 showed a unique immunological profile and was abundantly expressed in the tegumental syncytium of Taeniidae metacestodes and adults. Bacterially expressed recombinant *T. solium* endophilin B1 (rTsMEndoB1) demonstrated a sensitivity of 79.7% (345/433 cases) for serodiagnosis of larval Taeniidae infections. The protein showed strong immune recognition patterns against sera from patients with chronic neurocysticercosis, cystic echinococcosis, or advanced-stage alveolar echinococcosis. Adult Taeniidae infections exhibited moderate degrees of positive antibody responses (65.7% [23/35 samples]). rTsMEndoB1 showed some cross-reactivity with sera from patients infected with Diphyllobothriidae (23.6% [25/106 samples]) but not with sera from patients with other parasitic diseases or normal controls. The specificity was 91.7% (256/301 samples). The positive and negative predictive values were 93.6% and 73.4%, respectively. Our results demonstrate that Taeniidae endophilin B1 may be involved in the control of membrane dynamics, thus contributing to shaping and maintaining the tegumental curvature. rTsMEndoB1 may be useful for large-scale screening, as well as for individual diagnosis and follow-up surveillance of Taeniidae infections.

Tapeworms that infect humans (class Cestoda) can be divided into two different orders, Pseudophyllidea and Cyclophyllidea (1). Among cyclophyllidian tapeworms, larval Taeniidae forms, such as metacestodes of *Taenia solium* and *Echinococcus* spp., cause formidable public health problems worldwide. Humans can serve as intermediate hosts of these parasites and are infected with larval worms (metacestodes). When humans ingest parasite eggs, oncospheres are activated in the small intestine and are released into the circulation. The metacestodes can end up lodged anywhere in the body, resulting in granulomatous lesions in the affected organs and tissues (2, 3).

Taenia solium metacestodes usually infect subcutaneous tissues but also may invade the central nervous system and cause neurocysticercosis (NC). Metacestodes of *Echinococcus granulosus* and *Echinococcus multilocularis* mostly infect the liver and result in space-occupying cystic echinococcosis (CE) and tumor-like alveolar echinococcosis (AE). Although the favored infection sites differ according to parasite species, infections of vital organs and tissues are frequently associated with fatalities. NC is a leading cause of adult-onset seizures in areas in which the disease is endemic, such as Latin America, sub-Saharan Africa, China, and India (3–5). CE and AE have resulted in chronic morbidity and significant mortality rates in several regions in central Asia, Europe, North America, Latin America, and northwestern China (2, 6, 7). Those enzootic diseases have a great impact on disability-adjusted life years and are regarded as major neglected tropical diseases by the World Health Organization (WHO) due to substantial disease burden and social stigmatization along with economic losses (http://who.int/neglected_diseases/diseases/en).

One of the characteristic clinical features of tissue-invasive larval cestodiasis is slow progression with minimal symptoms unless infected parasites provoke acute debilitating symptoms in critical foci (8, 9). Therefore, proper diagnosis is often challenging and is hampered in some clinical settings. The equivocal nature of imaging findings and the similarity to other granulomatous or cystic lesions make diagnosis more difficult. In such intricate situations, suspicion of parasitic diseases and the results of serological tests may provide supporting evidence.

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Endophilin is a highly conserved cytosolic protein that contains an N-terminal Bin1/amphiphysin/Rvs (BAR) domain and a C-terminal SRC homology 3 (SH3) domain. The N-BAR domain constitutes a major site of dimerization and is involved in the formation of membrane curvature, as it creates dynamic mobility (10–12). The SH3 domain has critical functions in recruitment of proteins and in protein-protein interactions via recognition of proline-rich motifs in its binding partners (12). The SH3 domain interacts with synaptojanin and dynamin during clathrin-mediated endocytosis (13, 14).

Parasitic endophilin was initially identified in *E. granulosus*. The protein, designated P29 antigen, was detected in the protoscolex and the germinal layer but not in hydatid fluid. Expression of P29 was shown to be developmentally regulated and was observable only in the metacestode stage (15). The protein appeared to be a reliable serodiagnostic antigen for detection of homologous infection (15, 16). Moreover, it was reported to be a potential biomarker for posttreatment monitoring of CE patients. Serum levels of specific antibodies against P29 antigen gradually decreased after successful treatments (17).

In this study, we isolated cDNA putatively encoding endophilin B1 from *Taenia saginata* and *Taenia asiatica*. We also retrieved data for *T. solium* endophilin B1 (TsMEndoB1) from the established GeneDB database (http://www.genedb.org/featureSeq/TsM_000719500). Analysis of the molecular properties of the gene sequences revealed that they share significant sequence identity and have a tightly conserved N-terminal BAR domain, as well as amino acid residues responsible for dimeric interfaces, thus being orthologs of the *E. granulosus* P29 antigen. The endophilin B1 proteins expressed in Taeniidae parasites are immunologically quite similar to one another but not to those found in other cestodes, including Hymenolepidae and Diphyllobothriidae. We demonstrate that endophilin B1 is localized to the tegumental syncytium beneath the microtriches of the metacestodes and adults of Taeniidae species. Bacterially expressed recombinant *T. solium* endophilin B1 (rTsMEndoB1) protein exhibited fairly high sensitivity and specificity in diagnosing larval Taeniidae infections. Interestingly, the protein showed strong immune recognition patterns against sera from patients with chronic NC and CE and from those with advanced-stage AE. Adult worm infections also produced a moderate degree of positive antibody response.

MATERIALS AND METHODS

Parasites. *Taenia solium* metacestodes and *T. solium*, *T. saginata*, and *T. asiatica* adults were obtained from naturally infected pigs and humans in regions in which the parasites are endemic (Tiandong County and Sanjiang Dong Autonomous County, Guangxi Province, China). The species were identified by observing specific bands for *T. solium* (474 bp), *T. saginata* (629 bp), and *T. asiatica* (706 bp) by multiplex PCR (18). CE2 cysts and AE masses were collected from naturally infected sheep and experimentally infected mice in Xining (Qinghai Province, China) (19, 20). *E. granulosus* and *E. multilocularis* adults were collected during necropsies of stray dogs (Chengdu, Qinghai Province, China). Species were identified on the basis of the *cox1* gene sequence (21). Other parasite samples were obtained from the Parasite Resource Bank (Chungbuk National University, Cheongju, South Korea). The study protocol was approved by the Institutional Review Committees of the Guangxi Center for Disease Prevention and Control (protocol no. GX 2010-7-21), the Qinghai Institute for Endemic Disease Prevention and Control (protocol no. QH 2013-7-22), and Sungkyunkwan University (protocol no. SKKU 2013-17).

TABLE 1 Serodiagnostic evaluation of recombinant TsMEndoB1

Disease category[a]	No. positive/ no. tested	Sensitivity (%)	Specificity (%)
NC	216/271	79.7	
Active NC	142/169	84	
Chronic NC	74/102	72.5	
CE	81/101	80.2	
CE1	14/20	70	
CE2	14/16	87.5	
CE3	21/22	95.5	
CE4	18/25	72	
CE5	14/18	77.8	
AE	48/61	78.7	
Early AE	22/32	68.8	
Advanced AE	26/29	89.7	
Adult *T. solium* infection	7/11	63.6	
Adult *T. saginata* infection	8/12	66.7	
Adult *T. asiatica* infection	8/12	66.7	
Sparganosis	22/92		76.1
Diphyllobothriasis	3/14		78.6
Other parasitic infections[b]	0/120		100
Normal control	0/75		100
Overall[c]	368/468	78.6	
	25/301		91.7

[a] NC, neurocysticercosis; CE, cystic echinococcosis; AE, alveolar echinococcosis.
[b] Other parasitic infections included *Schistosoma japonicum* schistosomiasis ($n = 20$), paragonimiasis ($n = 20$), clonorchiasis ($n = 20$), anisakiasis ($n = 20$), trichuriasis ($n = 20$), toxoplasmosis ($n = 10$), and amoebiasis ($n = 10$).
[c] Overall sensitivity and specificity were calculated by employing 468 Taeniidae-infected serum samples and 301 other serum samples.

Sera from patients and experimentally infected animals. A total of 468 serum specimens from patients infected with Taeniidae spp., including 271 NC patients, 101 CE patients, and 61 AE patients, and samples from 35 patients infected with *T. solium*, *T. saginata*, and *T. asiatica* adults were used in this study. Sera from 106 Diphyllobothriidae-infected patients and 120 patients infected with other parasites were tested. Sera from 75 healthy individuals who denied possible exposure to helminthic and/or protozoan infections were used as controls (see Table 1 for a detailed description). CE cases were diagnosed and classified by typical ultrasonographic findings combined with immunoproteomic analysis using ovine CE2 hydatid fluid as an antigen (19). AE cases were ultrasonographically diagnosed and grouped into early invasive versus advanced stages (22). The diagnoses were serologically confirmed by immunoblotting with recombinant EmAgB3 (20). NC cases were diagnosed by neuroimaging scans with concomitant positive antibody responses against a recombinant chimeric antigen, assessed by immunoblotting and an enzyme-linked immunosorbent assay (ELISA) (23). Chronic cases had marginal levels of specific antibodies in sera but showed positive reactions in cerebrospinal fluid. Active-stage NC cases were differentially classified by pathognomonic computed tomography/magnetic resonance imaging findings of multiple low-density lesions. Chronic inactive cases mostly showed multiple small round calcifications (24). Adult worms expelled from the patients were identified on the basis of morphological features and the presence of specific DNA bands characteristic of each species, as described above. All samples were collected as part of a public health promotion campaign; the patients were anonymized in this study. Informed consent was obtained from individual patients, parents/teachers, and/or local health authorities. In cases of illiterate patients, verbal consent was obtained (protocols GX 2010-7-21 and QH 2013-7-22).

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Sera from mice that had been experimentally infected with 1,000 viable AE protoscoleces were analyzed. Mice (5 to 7 per group) were sacrificed serially at 1, 3, 6, 9, and 14 months after infection (20). Sera from swine ($n = 3$) that had been experimentally infected with 100,000 viable *T. solium* eggs were serially collected at 1, 2, 3, 4, 6, 8, and 12 months postinfection (25).

Cloning of Taeniidae endophilin B1. Total RNA was extracted and purified from whole *Taenia solium* metacestode worms using the R&A-BLUE total RNA extraction kit (iNtRON, Seongnam, South Korea). The *T. solium* endophilin B1 (TsMendoB1) gene (TsM_000719500) was amplified by reverse transcription (RT)-PCR with gene-specific primers containing forward EcoRI and reverse XhoI restriction sites (forward, 5′-CG CGAATTCATGTCCGGATTTGACGTT-3′; reverse, 5′-CGCGAGCTCC TACTCGCCCAACATCAC-3′), using total RNA (200 ng). The thermal cycling reaction consisted of 30 min at 45°C followed by 35 cycles of 95°C for 1 min, 55°C for 45 s, and 72°C for 2 min, with a final extension at 72°C for 5 min. Endophilin B1 sequences from *T. saginata* and *T. asiatica* were isolated from adult cDNA libraries, which were constructed in a Uni-ZAP XR vector using poly(A)$^+$ RNA (1 μg) with Moloney leukemia virus reverse transcriptase (Stratagene, La Jolla, CA). The TsMEnodB1 cDNA fragment was used in colony hybridization by dot blotting. Endophilin B1-related clones were purified from positive colonies and identified by sequencing analysis using the T3 or T7 primer. The nucleotide sequences of both strands were automatically determined using a BigDye Terminator v3.0 cycle sequencing core kit (PerkinElmer, Foster City, CA) and an automated ABI Prism 377A DNA sequencer (Applied Biosystems, Foster City, CA).

Structural and phylogenetic analyses. Platyhelminth proteins homologous to TsMEndoB1 were retrieved from the GenBank proteomic database (http://www.ncbi.nlm.nih.gov/protein/?term) and the Sanger Institute GeneDB database (http://www.genedb.org/Homepage). Domains were analyzed using the NCBI conserved domain search program (http://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi). Representative human BAR-domain-containing proteins were similarly isolated from a publicly available database (26). The sequences were aligned using MUSCLE (27), and the amino acid positions corresponding to the BAR domains were extracted from the alignment (42 entries; approximately 228 amino acids). The alignment was examined with ProtTest v2.4 to produce a best-fit model of protein sequence evolution, and then a maximum likelihood tree was constructed using MEGA v6.0 (LG + F + G model, with eight gamma categories) (28). The tree was visualized with TreeView, and the statistical significance of each branching node was tested by a bootstrap analysis of 1,000 replicates.

Recombinant protein and specific antibodies. Full-length TsMEndoB1 cDNA was amplified by PCR as described above. PCR products were digested and ligated into the pET-28a(+) vector (Novagen, Madison, WI). The plasmid was transformed into *Escherichia coli* BL21(DE3) and cultured in LB medium supplemented with antibiotics. Recombinant protein (rTsMEndoB1) was expressed with 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) for 4 h at 37°C, after which rTsMEndoB1 was purified with a Ni-nitrilotriacetic acid (NTA) column (Qiagen, Valencia, CA).

Polyclonal monospecific antibodies were generated in BALB/c mice. rTsMEndoB1 (100 μg) emulsified with 2% ammonium hydroxide gel adjuvant (InvivoGen, San Diego, CA) was subcutaneously immunized. Starting 2 weeks later, booster shots of the proteins (100 μg) mixed with the same emulsifier were administered three times, at 1-week intervals. One week after the final boosting, sera were collected and IgG fractions were purified using a Protein G affinity column.

Immunoblotting. Proteins separated by 12% reducing SDS-PAGE were electrotransferred to nitrocellulose membranes (Santa Cruz Biotechnology, Santa Cruz, CA). The membranes were blocked for 1 h with Tris-buffered saline (pH 8.0) containing 0.05% Tween 20 and 3% skim milk. Membranes were then incubated overnight at 4°C with anti-rTsMEndoB1 antibodies (1:2,000 dilution), followed by incubation for 2 h with horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG antibodies (1:4,000 dilution; MP Biochemicals, Santa Ana, CA). The blots were developed with a West-Q Pico ECL kit (GenDEPOT, Dallas, TX) after 1 min of exposure. Images were digitalized with an Epson Perfection V700 photo scanner (Epson, Long Beach, CA).

rTsMEndoB1 was separated by 12% reducing SDS-PAGE and transferred to polyvinylidene difluoride (PVDF) membranes (Santa Cruz Biotechnology). The membranes were cut into strips and incubated overnight at 4°C with individual serum samples (1:200 dilution in casein buffer). The strips were further incubated for 4 h with HRP-conjugated goat anti-human IgG antibodies (1:1,000 dilution; MP Biochemicals). Blots were developed for 10 min with 4-chloro-1-naphthol chromogen (4C1N) (Sigma-Aldrich, St. Louis, MO). Immunoblotting results were analyzed for diagnostic sensitivity [sensitivity = number of true-positive samples/(number of true-positive samples + number of false-negative samples) × 100], specificity [specificity = number of true-negative samples/(number of false-positive samples + number of true-negative samples) × 100], positive predictive value [positive predictive value = number of true-positive samples/(number of true-positive samples + number of false-positive samples) × 100], and negative predictive values [negative predictive value = number of true-negative samples/(number of true-negative samples + number of false-negative samples) × 100].

Immunohistochemical staining and immunogold labeling. Worm sections (4-μm thick) were permeabilized for 15 min with 0.5% Triton X-100 in 100 mM phosphate-buffered saline (PBS) (pH 7.2) and were incubated at 37°C for 15 min in 10 mM Tris-HCl (pH 8.0) containing proteinase K (20 ng/ml). The sections were blocked for 1 h with PBS containing 0.05% Tween 20 and 3% bovine serum albumin (PBS/T-BSA), incubated overnight at 4°C with anti-rTsMEndoB1 (1:500 dilution in PBS/T-BSA), and then treated for 2 h at 4°C with FITC-conjugated goat anti-mouse IgG antibodies (1:500 dilution; Abcam, Cambridge, CA). The sections were counterstained with 10 mg/ml 4′,6-diamidino-2-phenolindole (DAPI) (ThermoFisher Scientific, Waltham, MA) for 5 min at 4°C. Fluorescent images were visualized using an IX71 inverted microscope (Olympus, Tokyo, Japan). Preimmune mouse serum diluted at the same ratio was used as a control.

For immunogold labeling, fresh worms were prefixed in 2% paraformaldehyde-0.4% glutaraldehyde, embedded in LR white resin, and polymerized for 72 h at −20°C under UV illumination. Ultrathin sections (60-nm thick) were mounted on nickel grids. The sections were incubated in PBS/T-BSA for 10 min and further incubated with anti-rTsMEndoB1 antibodies (1:500 dilution) for 2 h at room temperature. The sections were incubated overnight at 4°C with 5-nm gold-conjugated goat anti-rabbit IgG. Silver enhancement was performed using a commercial kit (GE Healthcare, Little Chalfont, United Kingdom), followed by uranyl acetate and lead citrate staining. The tissue sections were observed using a JEM-2100F transmission electron microscope (Jeol, Tokyo, Japan).

Immunoprecipitation and mass spectrometry. Proteins extracted from whole metacestodes of *T. solium*, *E. granulosus*, and *E. multilocularis* (50 μg each) and rTsMEndoB1 (10 μg) were reacted with anti-rTsMEndoB1 antibodies (5 μl) overnight at 4°C, after which they were further incubated for 4 h at 4°C with 30 μl of protein G-coupled agarose 4B (Peptron, Seoul, South Korea). The immune complex was recovered in the pellet following centrifugation at 20,000 × *g* for 5 min. Bound proteins were eluted with 50 mM glycine buffer (pH 3.0). The pH of the eluent was immediately adjusted to 7.2 with 1 M Tris. The purified proteins were incubated for 2 h in PBS at room temperature to promote oligomerization. The proteins (5 μg) were mixed with native-PAGE sample buffer (62.5 mM Tris-glycine, 25% glycerol, and 0.001% bromphenol blue [pH 6.8]). Proteins were separated on 4 to 20% Mini-Protean TGX gels (Bio-Rad, Hercules, CA) using a constant current of 20 mA. The proteins were visualized by Coomassie brilliant blue G-250 (CBB) staining.

Nano-liquid chromatography-electrospray ionization-multistage mass spectrometry (nano-LC-ESI-MS/MS) was performed using a model 1200 nanoflow system (Agilent Technologies, Paul Alto, CA) connected to a linear ion trap mass spectrometer at *m/z* 300 to 2,000 (LTQ; Thermo

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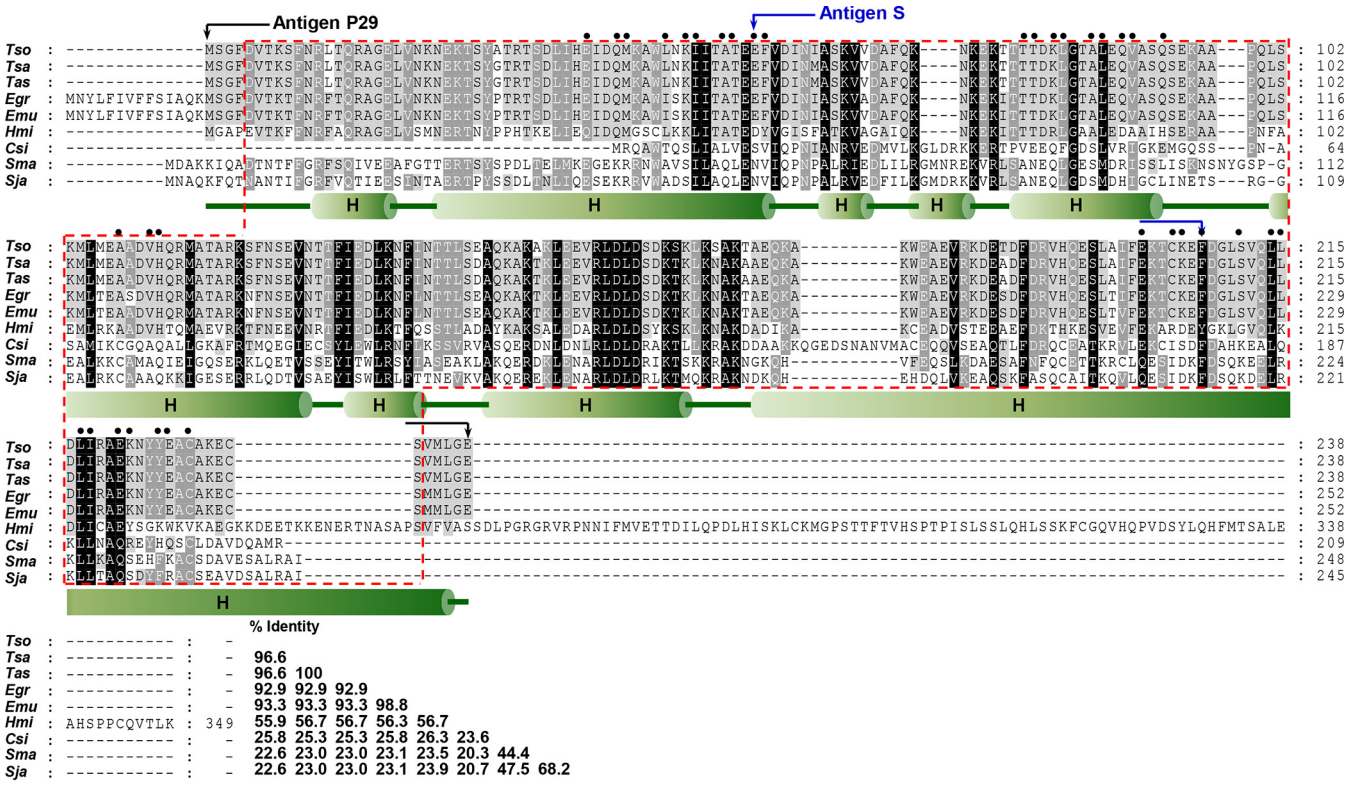

FIG 1 Structural analysis of Taeniidae endophilin B1-like molecules. The amino acid sequences of endophilin B1 proteins from Taeniidae, Hymenolepidae, and other trematode parasites were aligned using Clustal Omega (http://www.ebi.ac.uk/Tools/msa/clustalo). Functionally and/or structurally conserved domains were identified using the NCBI conserved domain search (http://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi). Dashed red box, N-BAR domain. Helical structures are indicated below the alignment. Circles, residues responsible for dimer interfaces. Black arrow, *E. granulosus* antigen P29 (15); blue arrow, *E. granulosus* antigen S (30), a partial fragment of endophilin B1. Gaps were introduced into the sequences to maximize identity values. Completely conserved amino acids in all species are marked in black, identical amino acids in dark gray, similar amino acids in light gray, and different amino acids in white. *Tso*, *T. solium* (GeneDB accession no. TsM_000719500); *Tsa*, *T. saginata* (GenBank accession no. KU341059); *Tas*, *T. asiatica* (GenBank accession no. KU341060); *Egr*, *E. granulosus* (GenBank accession no. CDS21096); *Emu*, *E. multilocularis* (GenBank accession no. CDS38179); *Hmi*, *Hymenolepis microstoma* (GenBank accession no. CDS27696); *Csi*, *Clonorchis sinensis* (GenBank accession no. GAA56152); *Sma*, *Schistosoma mansoni* (GenBank accession no. CCD74824); *Sja*, *Schistosoma japonicum* (GenBank accession no. CAX70483).

Electron, San Jose, CA). Three data-dependent MS/MS scans of isolation width (*m/z* 1.5), normalized collision energy (25%), and dynamic exclusion duration (180 s) were applied. Mass spectrometry (MS) data were generated in a RAW file format (Thermo Scientific) with Xcalibur 1.4 (Tune 1.0). The peptide peaks were identified with a MS/MS ion search in the Mascot server. The mass values were selected with monoisotopic masses. Peptide and MS/MS tolerances were ±1.2 and ±0.6 Da, respectively. Ions score is $-10\log(P)$, where P is the probability that the observed match is a random event. Individual ions scores of >56 were considered to indicate significant identity or extensive homology ($P < 0.05$). Two modifications, i.e., cysteine carbamidomethylation and methionine oxidation, were considered during the analyses. A MS database search was performed with merged files from the Sanger Institute *T. solium* GeneDB database (http://www.genedb.org/Homepage/Tsolium). Independent triplicate samples were analyzed.

Accession number(s). The *T. saginata* and *T. asiatica* endophilin B1 sequences were deposited in GenBank under accession numbers KU341059 (*T. saginata*) and KU341060 (*T. asiatica*).

RESULTS

Isolation and sequence analysis of Taeniidae endophilin B1-like molecules. Alignment of the isolated Taeniidae proteins revealed considerable similarities in length, with conserved residues in the dimeric interface and α-helix-enriched N-BAR domains. The proteins shared 92.9 to 96.6% sequence identity (Fig. 1). *Echinococcus granulosus* P29 (15, 29) and antigen S (30), both of which were shown to be effective serodiagnostic antigens for CE, were found to be a protein identical to and a partial fragment (amino acids 70 to 221) of these molecules, respectively. The sequence identity values of the Taeniidae proteins were markedly decreased, compared to homologs isolated from other Cestoda (Hymenolepidae) and Trematoda species, i.e., 22.6 to 56.7%, but all of those proteins harbored amino acid residues responsible for dimerization (Fig. 1, circles) and the BAR domain (Fig. 1, dashed red box). We designated these molecules endophilin B1 protein of *T. solium* (TsMEndoB1), *T. saginata*, and *T. asiatica*.

The phylogenetic tree clearly distinguished Taeniidae endophilin B1 proteins from other platyhelminth orthologs/homologs and suggested that at least two common intermediates have emerged during the evolution of endophilin B1-like proteins in cestodes. Taeniidae endophilin B1 contained an N-BAR domain but did not have a SH3 domain in the C-terminal region. These molecules appeared to have expanded along with their respective donor organisms (see Fig. S1, gray box, in the supplemental material). Taeniidae parasites also possessed two additional paralogous endophilin B proteins (endophilin B2 and B3), which har-

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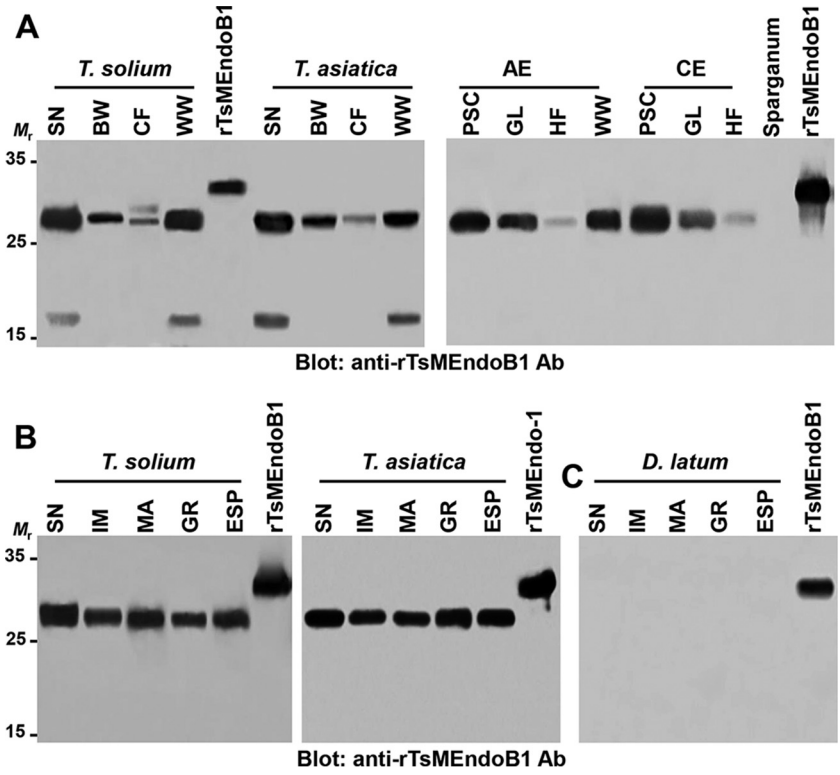

FIG 2 Expression patterns of Taeniidae-specific endophilin B1. Proteins (10 μg each) extracted from individual compartments of larval (A) or adult (B) Taeniidae or Diphyllobothriidae (C) were separated by 12% reducing SDS-PAGE and transferred to nitrocellulose membranes. Blots were incubated with anti-rTsMEndoB1 antibodies (1:2,000 dilution) and then with HRP-conjugated goat anti-mouse IgG antibodies (1:4,000 dilution). The images were developed with an enhanced chemiluminescence kit. AE, *E. multilocularis* metacestode; CE, *E. granulosus* metacestode; SN, scolex and neck; BW, bladder wall; CF, cyst fluid; WW, whole worm; PSC, protoscolex; GL, germinal layer; HF, hydatid fluid; IM, immature proglottid; MA, mature proglottid; GR, gravid proglottid; ESP, excretory-secretory products; M_r, molecular masses (in kilodaltons).

bored the SH3 domain in their genomes and were clustered into different clades (see Fig. S1, dashed box).

Taeniidae parasites express immunologically closely related endophilin B1 proteins. The high degree of sequence identity shared by endophilin B1 proteins isolated from human-infecting *Taenia* spp. and *Echinococcus* spp. (Fig. 1) suggested an intimate immunological similarity. We generated specific antibodies against rTsMEndoB1 and observed their immunological relationships. When blots containing proteins extracted from different compartments of the metacestodes and adults of Taeniidae and Diphyllobothriidae were probed with anti-rTsMEndoB1 antibodies, specific immunoreactive signals at approximately 29 kDa were observed with Taeniidae proteins, regardless of metacestode or adult origin. rTsMEndoB1 showed an apparent molecular mass of 33 kDa, owing to the His tag sequence (Fig. 2A and B). Conversely, proteins from Diphyllobothriidae parasites (*Diphyllobothrium latum* and spargana) showed no positive reaction. Endophilin B1 was ubiquitously detected in the whole metacestodes and the entire strobila of adults. In the metacestodes, expression of the protein was augmented in the scolex/protoscolex but the protein was not secreted into the cyst fluid or hydatid fluid in large amounts. A protein band observable at 16 kDa represented a partial fragment of TsMEndoB1 by LC-MS/MS analysis (see below). Proteins extracted from other parasites examined (trematodes, nematodes, and protozoa) did not exhibit any detectable antibody response against anti-rTsMEndoB1 antibodies (data not shown). These results demonstrated that Taeniidae parasites express immunologically distinct endophilin B1 proteins during the metacestode and adult stages, which are different from other types of endophilin (endophilin B2 and B3) and also different from endophilin B1 proteins from other parasites examined.

Tissue localization of endophilin B1. We assessed the tissue distribution of endophilin B1 using metacestode and adult worm sections. In *T. solium* metacestodes, endophilin B1 was specifically localized to the lining of the trabeculae in the scolex/neck and tegumental region of the bladder wall (Fig. 3A). Immunogold labeling showed that endophilin B1 was strongly and exclusively associated with the tegumental syncytium beneath the microtriches in metacestodes (Fig. 3B). When tissue sections of *E. granulosus* and *E. multilocularis* metacestodes were probed with anti-rTsMEndoB1 antibodies, positive signals were detected in the tegument of the protoscolex and the germinal layer (Fig. 3C, upper). In coronally sectioned adult worm specimens, more-characteristic localization patterns were observable. Endophilin B1 was abundantly localized to the tegumental syncytium throughout the strobila (Fig. 3C, lower). Worm sections incubated with preimmune mouse serum showed no immunoreactive signal (Fig. 3D). These results collectively suggested that endophilin B1 might be a crucial component of the tegument and might play a role in creating and maintaining the morphological contours of Taeniidae parasites.

TsMEndoB1 is oligomerized by self-assembly. We analyzed

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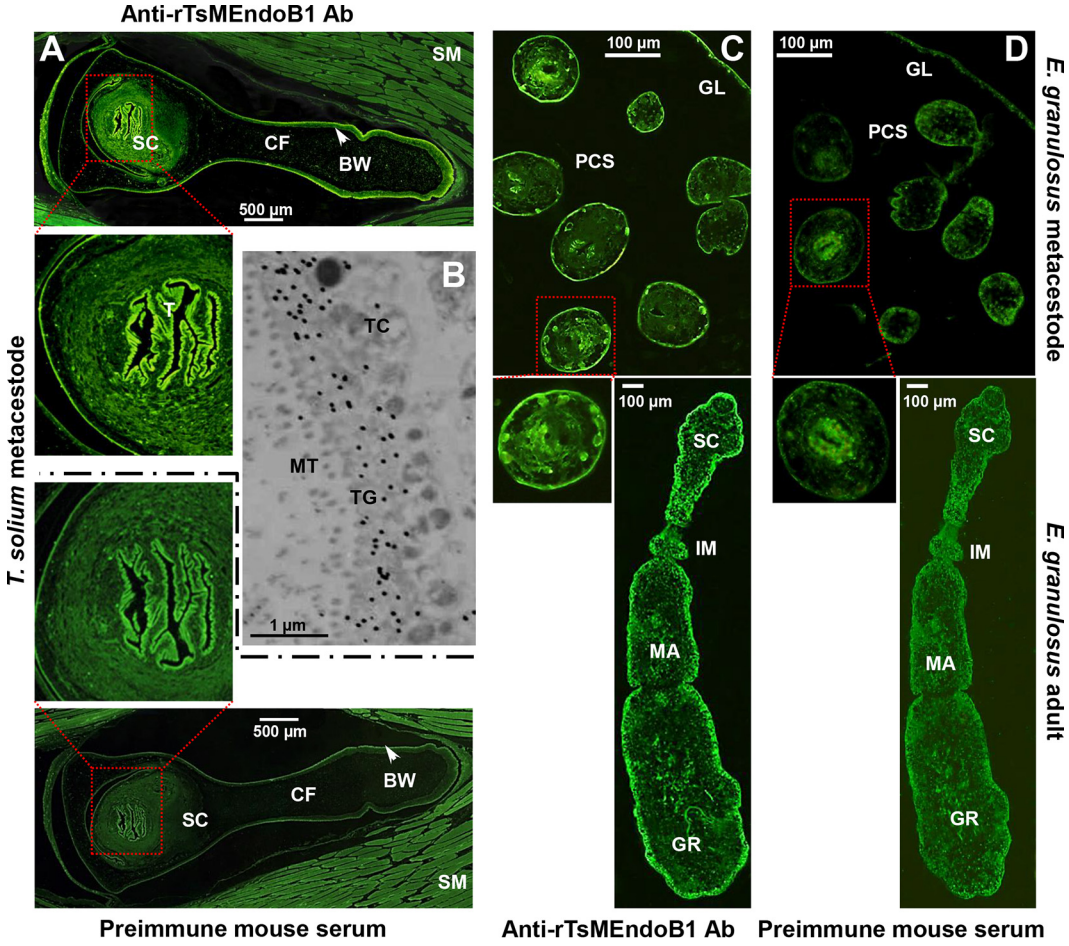

FIG 3 Immunohistochemical localization of endophilin B1 in *T. solium* and *E. granulosus*. (A) *T. solium* metacestode sections (4-μm thick) were permeabilized, incubated with anti-rTsMEndoB1 antibodies (1:500 dilution), and then incubated in the dark with fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse IgG antibodies (1:500 dilution). The sections were counterstained with DAPI. Preimmune mouse serum diluted at the same ratio was used as a control. BW, bladder wall; CF, cyst fluid; SC, scolex; SM, swine muscle; T, trabeculae. (B) For immunoelectron microscopic observation, *T. solium* metacestode sections (60-nm thick) were incubated with anti-rTsMEndoB1 antibodies and further incubated with 5-nm gold-conjugated goat anti-rabbit IgG antibodies. MT, microtriches; TG, tegument; TC, tegumental cell. (C and D) *E. granulosus* metacestode and adult worm sections (4-μm thick) were probed with anti-rTsMEndoB1 antibodies (C) or preimmune mouse serum (D), as described above. PSC, protoscolex; GL, germinal layer; SC, scolex; IM, immature proglottid; MA, mature proglottid; GR, gravid proglottid.

whether TsMEndoB1 could oligomerize through self-aggregation, since oligomerization of endophilin B1 through its BAR domain is responsible for membrane sensing (31). Native endophilin B1 proteins were purified from *T. solium*, *E. granulosus*, and *E. multilocularis* metacestodes by immunoprecipitation, after which they were oligomerized. When we determined their behavioral patterns, the proteins migrated to 29 kDa under reducing conditions (Fig. 4A). A faint band at 16 kDa, which represented a partial fragment of endophilin B1, was also observable (Fig. 2). Oligomerized macromolecular endophilin B1 proteins were seen as a broad band at >240 kDa in native PAGE (Fig. 4B). We analyzed the proteins resolved by SDS-PAGE and native PAGE by LC-MS/MS. All of those bands (bands 1 to 3) contained TsMEndoB1-specific peptide fragments (Fig. 4C; also see Table S1 in the supplemental material). This result suggested that endophilin B1 might function in a macromolecular homooligomeric form under physiological conditions, for the maintenance and control of the structural integrity and flexibility of the tegument.

Serodiagnostic performance of rTsMEndoB1. To evaluate the diagnostic reliability of rTsMEnodoB1, we observed the immunoreactivity profile of rTsMEndoB1 using large-scale immunoblotting with individual serum samples taken from patients with various parasitic diseases caused by cestode (Diphyllobothriidae and Taeniidae), trematode, nematode, and protozoan infections. Figure 5 presents a typical immunoblotting result. Strong antibody responses against rTsMEndoB1 were evident in 79.7% of samples (216/271 samples) from NC patients. Of the 101 and 61 serum samples from patients with CE and AE, respectively, that were tested, 81 samples (80.2%) and 48 samples (78.7%) exhibited positive reactions. Interestingly, rTsMEndoB1 revealed strong antibody responses against sera from patients with chronic infections, including 74/102 samples (72.5%) from patients with chronic NC, 32/43 samples (74.9%) from patients with stage CE4 or CE5 disease, and 26/29 samples (89.7%) from patients with advanced AE (Fig. 5A). Sera from patients infected with adult *T. solium*, *T. saginata*, or *T. asiatica* worms showed positive antibody responses in 63.6% to 66.7% of cases (Fig. 5B). Serum samples from patients with sparganosis and diphyllobothriasis exhibited

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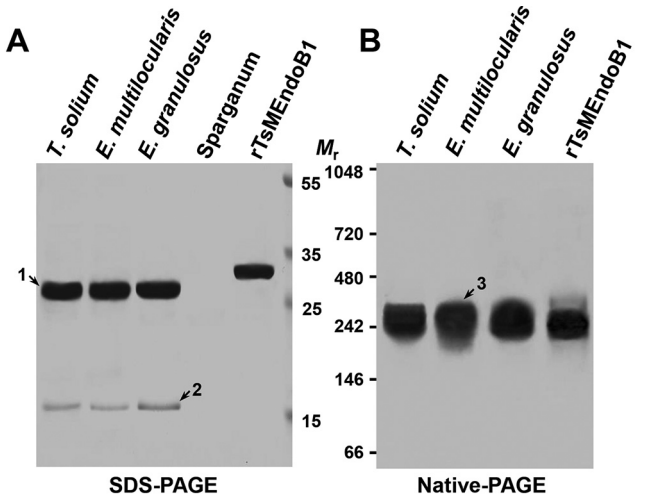

C

No.	DB no.[a]	Protein name	MS[b]	MP[c]	SC[d]	Theoretical M_r/pI	emPAI
1	TsM_000719500	endophilin B1	384	28	64	27199/5.63	11.5
2	TsM_000719500	endophilin B1	183	23	38	27199/5.63	0.41
3	TsM_000719500	endophilin B1	823	106	91	27199/5.63	13.3

[a] *T. solium* GeneDB (http://www.genedb.org/Homepage/Tsolium)
[b] Mascot score (MS) >25 indicates identity or extensive homology (p <0.05)
[c] Matched peptides
[d] Sequence coverage (%)

FIG 4 Multimeric states of native Taeniidae endophilin B1 proteins. Native endophilin B1 proteins from *T. solium*, *E. granulosus*, and *E. multilocularis* metacestodes were purified through immunoprecipitation. The purified proteins were oligomerized by incubation in PBS for 2 h at room temperature. The oligomerized proteins (5 μg each) were electrophoresed on either 12% reducing SDS-PAGE gels (A) or 4 to 20% native PAGE gels (B) and visualized by CBB staining. M_r, molecular masses (in kilodaltons). The monomeric and multimeric forms of Taeniidae endophilin B1 were identified by mass spectrometry (C). emPAI, exponentially modified protein abundance index.

some degree of cross-reaction (22/92 cases [23.9%] and 3/14 cases [21.4%], respectively) (Fig. 5C). No cross-reaction was observed against sera from patients with other parasitic infections (0/120 samples) or samples from normal controls (0/75 samples) (Fig. 5D and E). The overall sensitivity and specificity of rTsMEndoB1 were determined to be 78.6% and 91.7%, respectively (Table 1). The positive and negative predictive values were 93.6% and 73.4%, respectively.

We next evaluated the time point at which specific antibody levels against rTsMEndoB1 began to be elevated. Sera from swine that had been experimentally challenged with 100,000 *T. solium* eggs revealed positive antibody responses at 2 months postinfection. Murine sera collected 1 month after secondary infection with 1,000 AE protoscolices showed positive reactivity. The responses became stronger and continued over time until termination at 12 months for cysticercosis and at 14 months for AE (Fig. 5F and G).

DISCUSSION

In this study, we demonstrate that endophilin B1 is a major component of tegumental protein and might be a crucial regulator of the outermost contour of Taeniidae parasites. The protein was found to be a reliable biomarker for serological diagnosis of larval Taeniidae infections, irrespective of species. The protein was particularly effective in detecting chronic cases, which are frequently associated with fatal outcomes.

Most platyhelminths utilize endocytosis to take up nutrients and other essential resources from the host environments, due to their limited capacity for metabolism (32, 33). In addition, the tegument plays multifunctional roles in osmoregulation and excretion of waste products (34). However, the nature of the tegumental proteins and the molecules involved in creating the outermost morphological architecture have not been addressed. This study shows, for the first time, that TsMEndoB1 is abundantly and specifically associated with the tegumental syncytium. The protein can self-oligomerize and thus might function as a macromolecule under physiological conditions. TsMEndoB1 (and *Echinococcus* endophilin B1) did not interact with other molecules, such as dynamin and synaptojanin (Fig. 4). We attempted to identify interacting proteins by pulldown and gel overlay assays but could not detect any binding proteins except TsMEndoB1 (data not shown). These results suggest that the biological role of TsMEndoB1 might be mainly confined to sensing of the membrane curvature, probably because of the absence of a SH3 domain (12, 35). Taeniidae parasites might exploit another endophilin system to recruit essential proteins during endocytosis; endophilin B2 and B3, which harbor SH3 domains, may participate in the process. More-comprehensive studies regarding the molecular and cell biological network of endophilin proteins within the tegument are warranted, to gain insights into the functional relevance of the Taeniidae tegument.

Echinococcus granulosus P29 antigen has proved useful for diagnosis and posttreatment monitoring of CE patients (15–17, 29). This study provides evidence that TsMEndoB1 may be applicable for serodiagnosis of NC and other Taeniidae infections, such as CE and AE. In the course of our experiments, we cloned and expressed *E. granulosus* endophilin B1 and observed that the protein exhibited comparable diagnostic sensitivity and specificity, compared with rTsMEndoB1, in diagnosing NC, CE, and AE (data not shown). Several different strains of *E. granulosus* complex and *E. multilocularis* that were examined harbored endophilin B1 orthologs that shared 97.9 to 99.6% sequence identity (29). We surmise that these endophilin B1 proteins may also be useful as biomarkers for the detection of specific antibodies in patients with NC, CE, and AE.

In cases of larval Taeniidae infections, antigens specific to the active stage appear to originate from the cyst fluid of *T. solium* metacestodes (low-molecular-weight proteins) (23, 36) or the hydatid fluid of larval *Echinococcus* spp. (several antigen B isoforms) (19, 20, 37, 38), except for a few entities in the case of AE (37). Those proteins are rapidly drained and absorbed with parasite involution and result in minimal antibody responses in chronic cases (19, 20, 23, 39, 40). In contrast, parenchymal cellular components, such as the scolex/protoscolex and the bladder wall/germinal layer, may be relatively resistant to degenerative changes. rTsMEndoB1 showed specific antibody responses from the early stage of infection to the later chronic stage, which indicates that expression of endophilin B1 begins in the early stage of metacestode development, to create the morphological features. Moreover, rTsMEndoB1 potently reacted with serum samples from patients with chronic infections (Fig. 5 and Table 1).

The characteristic patterns of TsMEndoB1 expression may be related to the antibody responses observed in chronic cases. In our previous work, scolex-derived *T. solium* fasciclin potently reacted with sera from patients with chronic NC, whose imaging scans revealed only multiple calcifications (41). Significantly grown CE4

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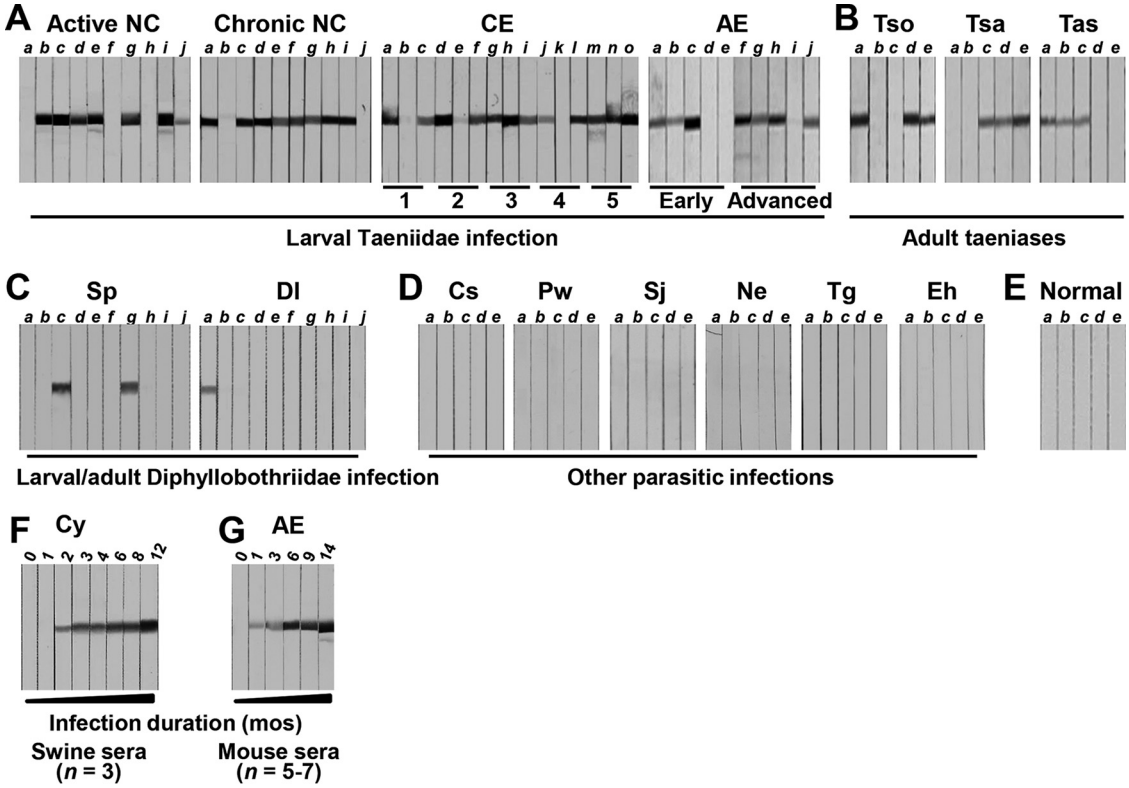

FIG 5 Immunoblotting assessment of the diagnostic applicability of rTsMEndoB1 in sera from patients with diverse parasitic infections. Strips were incubated with individual serum samples from patients with larval Taeniidae infections, including neurocysticercosis (NC), cystic echinococcosis (CE), and alveolar echinococcosis (AE) (A), or serum samples from patients with adult *T. solium* (Tso), *T. saginata* (Tsa), or *T. asiatica* (Tas) infections (B). Sera from patients with Diphyllobothriidae infections, such as sparganosis (Sp) and diphyllobothriasis (Dl), were also incubated with rTsMEndoB1 (C). Serum samples from patients with other parasitic infections, including clonorchiasis (Cs), paragonimiasis (Pw), *S. japonicum* schistosomiasis (Sj), nematodiasis (Ne), toxoplasmosis (Tg), and amoebiasis (Eh) (D), and from normal controls (Normal) (E) are shown. Blots containing rTsMEndonB1 were also probed with swine sera (Cy) (F) and murine sera (AE) (G) serially collected after experimental infections. All sera used were diluted 1:200, and HRP-conjugated goat host-specific anti-IgG antibodies were used at a dilution of 1:1,000. The blots were developed with 4C1N chromogen for 10 min. Lanes a to o, different patients.

and CE5 masses contained large amounts of the germinal layer. AE masses mostly showed morphological features typical of peripheral proliferation of the germinal layer and central necrosis, at the same time, for a considerable period of time. Endophilin B1, as a major constituent of the tegument, may be continuously expressed during the involution process, although its expression levels decline over time. It may continually stimulate the host immune systems to produce specific antibodies until the masses are completely regressed or calcified.

In clinical practice, several different antigens specific to the active stages of the respective diseases might be superior for initial diagnosis, because some regimens can differentially diagnose NC, CE, and AE with high sensitivity and specificity (19, 20, 23, 36–38). TsMEnodoB1 and/or *Echinococcus* orthologs may be beneficial for follow-up monitoring of patients to evaluate treatment efficacy (16, 17, 42). The protein might also be applicable for detection of chronic infections in cases in which imaging tests yield ambiguous findings and/or serum shows marginal negative or positive responses. Pathological specimens obtained from biopsies in chronic eosinophilic or noneosinophilic granulomatosis cases often demonstrate morphological features that cannot be differentially diagnosed, due to severe degenerative changes. Endophilin B1 would be valuable for etiological identification of biopsy specimens (Taeniidae or not) using *in situ* hybridization and/or immunohistochemical staining.

rTsMEndoB1 showed approximately 66% sensitivity in diagnosing adult Taeniidae infections. Adult worms thrive in the lumen of the small intestine; therefore, the protein may be hardly recognized by the host immune system operating in the intestinal mucosa, including intraepithelial lymphocytes. Accordingly, the host may produce few antibodies, although the tegument continuously expressed endophilin B1 and released the protein during tegumental shedding and metabolic turnover. It seems reasonable to consider that the antibody responses to adult infections might be weak, compared to the responses to more-invasive larval infections.

Proteins extracted from Diphyllobothriidae (*D. latum* and spargana) did not show any reactivity to anti-rTaMEndoB1 antibodies. This result suggests that Diphyllobothriidae parasites might not express endophilin B1-like molecules immunologically related to Taeniidae endophilin B1 (Fig. 2). However, sera from patients infected with those parasites revealed some cross-reaction against rTsMEndoB1, regardless of adult (*D. latum*) or plerocercoid (sparganum) infection (Fig. 5 and Table 1). This unexpected observation is currently difficult to explain. Since Diphyllobothriidae are relatively close to Taeniidae phylogenetically (1), some as-yet-unknown Diphyllobothriidae protein(s) bearing partial epitopes that mimic Taeniidae endophilin B1 might preferentially present this molecule to $CD4^+$ cells. $CD4^+$ cells with different specificities might then stimulate B cells to produce antibodies

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reactive to Taeniidae endophilin B1. Further epitope mapping may provide clues to elucidate this hypothesis and to facilitate understanding of the serological cross-reactivity observed for those parasitic diseases.

Echinococcus granulosus P29 was reported to be expressed only in the metacestode stage (15). In this study, however, endophilin B1 was detected in both the metacestode and adult stages. This apparent discrepancy may be attributable to the particular antibodies used. Previous study utilized a monoclonal antibody (47H.PS) with an epitope sequence confined to a relatively short amino acid stretch (DAEQKNKE; positions 68 to 75). The monoclonal antibody might have a low antibody titer, compared to the polyclonal antibodies used in this study. Indeed, proteins from *E. granulosus* hydatid fluid, which did not show a response to the 47H.PS monoclonal antibody (15), exhibited a weak antibody response to our polyclonal antibodies. *T. solium* cyst fluid and *E. multilocularis* hydatid fluid also showed responsiveness similar to that of *E. granulosus* hydatid fluid (Fig. 2).

In conclusion, we demonstrate that Taeniidae parasites express an endophilin B1 protein that is immunologically distinct from proteins found in other cestode parasites. The protein is localized mainly to the tegumental syncytium, which suggests a biological function involving the maintenance of architectural contours. rTsMEndoB1 showed specific antibody responses to serum samples from patients with larval Taeniidae infections, especially patients with chronic infections. rTsMEndoB1 might prove useful for large-scale serological surveys of patients in the field and for diagnosis and follow-up surveillance of larval Taeniidae infections in several regions where the parasitic diseases are prevalent. Elucidation of the biological significance of tegumental proteins in Taeniidae parasites may greatly reinforce our understanding of these parasitic infections; it will also be helpful in establishing novel strategies for patient management.

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