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Biochemical Characterization and Differential Expression of a 16.5-Kilodalton Tegument-Associated Antigen from the Liver Fluke *Fasciola hepatica*

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A cDNA encoding a 16.5-kDa protein termed FhTP16.5 was identified by immunoscreening of a cDNA library from *Fasciola hepatica* adult flukes using pooled sera from rabbits infected with *F. hepatica* for 4 weeks. Quantitative reverse transcriptase PCR (qPCR) analysis revealed that FhTP16.5 is not expressed in unembryonated eggs. It is poorly expressed in miracidia and highly expressed at the juvenile and adult stages; however, significant differences were found between the expression levels of FhTP16.5 in juveniles versus adult flukes. Recombinant FhTP16.5 was expressed at high levels in *Escherichia coli*, purified by affinity chromatography, and used to raise anti-FhTP16.5 polyclonal antibodies in rabbits. Immunoblot analysis using the anti-FhTP16.5 IgG antibody identified FhTP16.5 in crude and tegumental extracts and in excretory-secretory products of *F. hepatica*. The protein was not detected in crude extracts of *Schistosoma mansoni* or *Schistosoma japonicum*. Antibodies to FhTP16.5 were detected in the sera of rabbits at 3 to 12 weeks of *F. hepatica* infection as well as in the sera of humans with chronic fascioliasis; these findings suggest that FhTP16.5 could be a good antigen for serodiagnosis of fascioliasis. Immunohistochemistry demonstrated that FhTP16.5 localizes to the surface of the tegument of various developmental stages and in parenchymal tissues of the adult fluke. Such specific localization makes FhTP16.5 an attractive target for immunoprophylaxis or chemotherapy.

Fascioliasis ranks as one of the most serious parasitic diseases of livestock worldwide and as a major zoonotic disease (48–50). The control of fascioliasis is difficult because many mammals, including all herbivores, humans, rodents, and many others, can serve as definitive reservoirs of infection. In addition, triclabendazole, the only existing drug that is effective against juvenile and mature forms of *Fasciola hepatica*, is ineffective in preventing reinfection and has induced drug resistance in sheep (30, 58). The life cycle of *F. hepatica* in the mammalian host is complex and includes defined stages, such as excystment of ingested metacercariae, penetration of the intestinal wall, migration through the abdominal cavity toward the liver, migration through the liver parenchyma, and finally, lodgment within the bile ducts as a mature egg-laying parasite. *Fasciola* parasites in the bile ducts are able to survive for many years despite being exposed to the immune system. *Fasciola* worms present a large interface with the host, which means that they must possess an evasion mechanism(s) to overcome antiworm immune strategies. One major interface between the parasite and the host is the tegument. The liver fluke's tegument is the outermost surface that covers the parasite; it is rich in secretory inclusions and bounded externally by a plasma membrane (22, 72). The tegument carries out several functions, including osmoregulation, secretion, uptake of nutrients, and evasion from host immune responses (22, 23). Tegumental proteins of trematodes have been shown to be targets of several anthelmintic drugs (51, 52) and vaccine candidates (46, 57, 65). The importance of the tegumental surface for parasite functions is clear, and due to their accessibility in worms, tegumental proteins are potential targets not only for vaccines but also for new immunodiagnostic tools.

At present, diagnosis of fascioliasis is normally based on the observation of *Fasciola* eggs in feces (13). However, early diagnosis by coprological examination cannot be performed because parasite eggs are not found in feces until 10 to 12 weeks of infection, when the flukes reach maturity and hepatic injury has occurred (68). Moreover, during the patent period, diagnosis by coprological examination is often insensitive due to intermittent shedding of parasite eggs into the feces (35). To improve diagnosis during both early and chronic infection, a large number of enzyme-linked immunosorbent assay (ELISA) techniques have been described. Most of these assays rely on antibody detection using crude somatic extracts or excretory-secretory (ES) products of *F. hepatica* (16, 25, 61, 71). However, because of the high complexity of these antigenic extracts, cross-reactions with other parasites have been reported, diminishing the specificity of the techniques (14, 20, 38). Several *F. hepatica* recombinant antigens have been purified to enhance the specificity of the diagnostic assays (8, 15, 32, 62). Some of these antigens have also been found to be excellent immunogens capable of inducing partial protection against a challenge *F. hepatica* infection in a variety of experimental models (1, 3, 9, 21, 28). Most notable are fatty acid binding proteins (FABPs) (36), cathepsin proteases (60), and saposin like-proteins (FhSAP2) (32), which have been documented as useful immunodiagnostic molecules for fascioliasis. The tegument is another source of immunodiagnostic antigens. Many studies have shown that tegument antigens from trematodes are highly species specific and could represent good molecules in immunodiagnosis (17). There-

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fore, the study of the tegument and its associated proteins could help in the discovery and validation of new molecules not only as vaccine candidates but also as reagents for diagnosis of trematode infections at early stages.

This paper describes the cloning and biochemical characterization of a 16.5-kDa *F. hepatica* tegument-associated protein termed FhTP16.5 that has potential for serodiagnosis of human fascioliasis. As mentioned earlier, the tegument of *F. hepatica* serves as the interface between the parasite and the host, and tegumental proteins are easily released to stimulate host responses. Thus, FhTP16.5 as tegument-associated protein is a potential antigen for developing diagnostic kits and vaccines.

MATERIALS AND METHODS

Adult parasite collection. *F. hepatica* adult flukes were collected from the gallbladders and intrahepatic bile ducts of infected cattle killed at local abattoirs and transported to the laboratory in RPMI 1640 medium (Sigma-Aldrich, St. Louis, MO). Flukes were removed from the RPMI medium and washed three times with sterile 0.1 M phosphate-buffered saline (PBS; pH 7.4), snap-frozen in liquid nitrogen, and stored at −80°C until use. For cross-reactivity studies adult *Schistosoma mansoni* and *Schistosoma japonicum* were obtained by perfusing mice, 8 weeks after infection, with schistosome cercariae. All parasites were washed several times with PBS to remove all traces of the host blood, bile, and contaminating microorganisms before being processed for further experiments.

***F. hepatica* eggs and miracidia.** During transportation to the laboratory, *F. hepatica* adult flukes released a large number of eggs into the RPMI medium, which were allowed to settle. The eggs were removed from the medium, resuspended in fresh double-distilled water, and then allowed to settle. After several washes, the eggs were examined at ×100 magnification to confirm the absence of visible contaminants. Eggs were snap-frozen in liquid nitrogen and stored at −80°C until use. Another batch of eggs was allowed to mature by incubation in the dark at 22°C for 9 to 12 days and then stimulated to hatch by exposure to light for 2 h at 25°C. Free-swimming miracidia were collected using a transfer pipette, immediately snap-frozen, and stored as described above.

NEJs. *F. hepatica* metacercariae (Baldwin Aquatic, Inc., OR) were stored in fresh sterile water at 4°C until use (within 4 months of shedding from snails). Metacercariae were transferred to watch glasses and preincubated in 1.2% sodium bicarbonate, 0.9% sodium chloride, and 0.8% sodium tauroglycolate for 30 min at 37°C. They were then allowed to excyst for up to 3 h at 37°C in excystment medium freshly prepared by diluting the preincubation solution 1:1 with 0.33% HCl and 0.8% L-cysteine. Newly excysted juveniles (NEJs) were removed from the excystment medium and maintained in fresh *Fasciola* saline (FS; Dulbecco's modified Eagle's medium [DMEM] plus 0.5 ml distilled water per milliliter of DMEM, 2.2 mM calcium acetate, 2.7 mM $MgSO_4$, 61.1 mM glucose, 1 μM serotonin, 5 μg/ml gentamicin, 15 mM HEPES [pH 7.4]) at 37°C. NEJs were snap-frozen and stored at −80°C until use.

Preparation of soluble whole-adult extracts. *F. hepatica*, *S. mansoni*, and *S. japonicum* adult worms were homogenized with a Teflon homogenizer in PBS in the presence of a proteases inhibitor cocktail and then centrifuged at 30,000 × *g* for 30 min at 4°C as previously described (29). The supernatants containing the soluble whole-worm extract (FhWE, SjWE, or SmWE) were aliquoted and stored at −20°C until use.

FhTA. *F. hepatica* tegument extract (FhTA) was prepared according to the method described by Hillyer (37). Live flukes were incubated in PBS containing 1% of the nonionic detergent Nonidet P-40 (NP-40; Sigma) for 1 h at 4°C with gentle shaking, and the supernatant was collected. NP-40 was removed from the supernatant using an Extracti Gel-D kit (Pierce).

FhES. *F. hepatica* excretory-secretory (FhES) products were prepared by culturing live adult *F. hepatica* in RPMI medium for 24 h at 4°C as previously described (25). The medium was centrifuged at 6,000 × *g* at 4°C and stored at −20°C.

Sera. A total of 15 human serum samples were obtained from serum banks of the National University of Cajamarca, Peru, and stored at −20°C until use. Ten serum samples were from patients with chronic fascioliasis, which was confirmed by coprological examination, and five serum samples from healthy subjects; these were used as negative controls. Four New Zealand White (NZW) rabbits were infected orally with 60 *F. hepatica* metacercariae each. Blood was obtained for the collection of serum before infection and then at weekly intervals for 12 weeks.

cDNA library screening. An unamplified cDNA expression library of *F. hepatica* constructed in λgt11 (26) was screened using a pool of sera from rabbits with 4 weeks of *F. hepatica* infection using standard procedures (73). After three rounds of low-density rescreening, clones that remained positive were selected, amplified, subcloned into pCR-Blunt vector (Invitrogen, Carlsbad, CA) and sequenced in both directions using an ABI PRISM BigDye Terminator sequencing kit (Perkin-Elmer, MA). One clone was selected for further DNA and peptide analysis because of sequence similarity (*E* value ≈ $2e^{-30}$) to a tegumental protein from *S. japonicum* (44). For the purpose of this study, this protein was designated FhTP16.5.

DNA and peptide sequence analyses. FhTP16.5 sequence was examined for similarity against the GenBank nonredundant protein and nucleic acid databases using the BLAST algorithm (4). Secondary structure predictions were made using the SOPMA server (33). All complete FhTP16.5 cDNA and predicted protein sequences were compared by pairwise alignment to determine percentages of sequence identity using the Genetics Computer Group GAP program (version 10.0, for Unix) with default settings. The predicted amino acid sequence was analyzed to identify conserved motifs using the Conserved Domain Search service (47). Phosphorylation of Ser, Thr, and Tyr residues was predicted using the NetPhos 2.0 server (12).

RNA isolation and quantitative reverse transcriptase PCR (qPCR). Total RNA was extracted from *F. hepatica* adults, unembryonated eggs, miracidia, and NEJs using a PureLink RNA minikit (Invitrogen) followed by treatment with Turbo DNA-free endonuclease (Ambion, Austin, TX) to remove contaminating genomic DNA. The RNA was quantified using a Nanodrop-1000 spectrophotometer (Thermo-Scientific, Wilmington, DE). One hundred nanograms of RNA from each life cycle stage was used to synthesize cDNA using a high-capacity RNA-to-cDNA kit (Applied Biosystems, Carlsbad, CA). qPCR experiments were conducted in triplicate using a StepOne Plus real-time PCR system (Applied Biosystems) with cDNA equivalent to 5 ng of total RNA and SYBR green PCR Master Mix (Applied Biosystems). The $2^{-\Delta\Delta CT}$ (threshold cycle) method (45) was used to quantify relative FhTP16.5 expression using the *F. hepatica* glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) gene (GenBank accession no. AY005475) as an internal control. The primers used to amplify approximately 100 bp of FhTP16.5 were 5′-GGGCCGAGAACATG AATATC-3′ (sense) and 5′-GTATTTTGGAACATGGCCAC-3′ (antisense). Primers used to amplify ~176 bp of Fh-*GAPDH* were 5′-GCGCC AATGTTCGTGTTCGG-3′ (sense) and 5′-TGGCCGTGTACGAATGCA C-3′ (antisense). The cycling conditions were as follows: 95°C for 15 min followed by 40 cycles of 95°C for 15 s, 55°C for 30 s, and 72°C for 30 s. The primer concentration was optimized, and dissociation curves were generated for each FhTP16.5 and Fh-*GAPDH* primer set to verify the amplification of a single PCR product. To compare the FhTP16.5 transcript levels between life stages, we used the ΔC_T of the miracidium stage as a calibrator. Thus, FhTP16.5 expression levels in the various life cycle stages were expressed as fold changes relative to the expression in the miracidium stage.

Construction of the FhTP16.5-pGEXT4T-1 expression vector. The coding region of FhTP16.5-cDNA was amplified by PCR using the primers 5′-GGTGGCGACGACTCCTGGAGCCCG-3′ (sense) and 5′-TTGAC ACCAGACCAACTGGTAATG-3′ (antisense). The cycling parameters were as follows: 95°C for 5 min, followed by 25 cycles of denaturing at

95°C for 1 min, annealing at 65°C for 1.5 min, extension at 72°C for 2 min, and a final extension at 72°C for 5 min. The 554-bp insert was gel purified, digested with EcoRI, and ligated into the prokaryotic expression vector pGEXT-4T-1, which contains an isopropyl-β-D-thiogalactoside (IPTG)-inducible promoter and a glutathione *S*-transferase (GST) coding sequence. The resulting plasmid (named pGEXT-FhTP16.5) was transformed into competent *Escherichia coli* BL-21 (DE3) cells (Stratagene, Santa Clara, CA).

Expression and purification of recombinant FhTP16.5. Recombinant FhTP16.5 was induced by adding IPTG (Promega) to a final concentration of 0.2 mM to the culture medium and incubated for 3.5 h at 37°C. After induction, bacteria were harvested by centrifugation and cell pellets were suspended in 50 ml of cold PBS buffer containing 5 mM phenylmethylsulfonyl fluoride (PMSF), 5 mM dithiothreitol (DTT), 20 μg/ml lysozyme, 5 mM iodoacetamide, and 2 mM EDTA and incubated for 30 min on ice. Lysis was achieved by three cycles of sonication, 20 s each. Triton X-100 (final concentration, 0.1%) was added to the bacterial lysate, and the mixture was incubated for 40 min at room temperature (RT) with gentle agitation. Cell debris and insoluble proteins were removed by centrifugation at 6,000 $\times$ *g* for 30 min at 4°C. The soluble cell extract was filtered through a 0.45-μm filter (Millipore, Billerica, MA) and loaded onto a GSTrap HP column (GE Healthcare, Piscataway, NJ). The fusion protein was recovered by elution with 50 mM Tris-HCl (pH 8.1) containing 50 mM reduced glutathione (GSH). After elution, the GST-FhTP16.5 was desalted by gel filtration using a PD-10 column (GE Healthcare). To obtain the fusion protein free from the GST tag, protein was incubated with 150 units of thrombin (GE Healthcare) in PBS overnight at RT. Sample purity was evaluated by 12.5% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gels stained with silver stain.

Rabbit anti-FhTP16.5 immune serum. An NZW rabbit was immunized with 250 μg recombinant FhTP16.5 protein mixed with an equal amount of complete Freund's adjuvant by subcutaneous injection. The rabbit was boosted twice with equal amounts of protein mixed with incomplete Freund's adjuvant at 2-week intervals. Blood was collected 2 weeks after the last immunization and evaluated by ELISA for specific antibody production. The rabbit IgG fraction was purified by affinity chromatography using protein-A affinity chromatography (GE Healthcare).

Immunolocalization of FhTP16.5 at different stages of the *F. hepatica* life cycle. Adults, miracidia, and NEJs of *F. hepatica* were fixed with 4% paraformaldehyde in PBS for 4 h at 4°C. Transversal sections of adult fluke (20 μm thick) were mounted on microscope slides (Fisher), washed three times for 10 min each time with PBS, and incubated at RT with 100 mM ammonium chloride in PBS for 10 min. After three washes, the slides were incubated with 0.1% Triton X-100/PBS for 20 min at RT and blocked first with 0.1% glycine in PBS (30 min, at RT) followed by 2 h of incubation at RT with 3% bovine serum albumin (BSA) in PBS. The slides were then incubated overnight at 4°C with rabbit anti-FhTP16.5 IgG diluted 1:100 in 3% BSA-PBS. Some slides were incubated with anti-FhTP16.5 IgG antibody previously absorbed for 2 h at 37°C with 200 μg/ml of recombinant FhTP16.5. Preimmune rabbit serum was employed as a negative control. The slides were washed twice with PBS and subsequently incubated in fluorescein isothiocyanate (FITC)-conjugated anti-rabbit IgG (1:100; Bio-Rad) followed by incubation in phalloidin-tetramethylrhodamine isothiocyanate (TRITC) and 4′,6′-diamidino-2-phenylindole (DAPI; 1:1,000) to counterstain muscle and nuclei, respectively. For whole-mount preparation of miracidia and NEJs, parasites were blocked in antibody diluent solution (AbD; 0.1 M PBS, pH 7.4, containing 0.1% [vol/vol] Triton X-100, 0.1% [wt/vol] BSA, and 0.1% [wt/vol] NaN_3), incubated overnight at 4°C with rabbit anti-FhTP16.5 IgG diluted 1:500 (in AbD), washed twice with AbD, and incubated in secondary antibody (1:10,000 Bio-Rad) followed by incubation in phalloidin-TRITC. All specimens were examined using confocal laser scanning microscopy (CLSM; Leica AOBS SP2).

Immunoblot analysis. FhWE, FhES, FhTA, and FhTP16.5 were separated by 12.5% SDS-PAGE according to the method of Laemmli (41) and transferred onto nitrocellulose (NC) membranes, which were probed for 2 h at RT with anti-FhTP16.5 IgG antibody (1:100 diluted in PBS containing 0.05% Tween 20 [PBST]). After three washes with PBST, membranes were incubated with horseradish peroxidase (HRP)-labeled anti-rabbit IgG (1:3,000 dilution) (Bio-Rad). After another three washes with PBST, antibody binding was visualized by the addition of diaminobenzidine as a chromogenic substrate. In the cross-reactivity study, SmWE and SjWE were separated and analyzed with anti-FhTP16.5 IgG antibody as described above. FhTP16.5 was also probed with sera from rabbits at 3 and 12 weeks of infection as well as with sera from humans with chronic fascioliasis (diluted 1:100). Negative rabbit and human sera were used as negative controls. In this experiment, HRP-labeled anti-rabbit or anti-human IgG (1:3,000 dilution; Bio-Rad) was used as the secondary antibody, and the reaction was revealed as described above.

Antibody response against FhTP16.5 by indirect ELISA. To determine the kinetics of the IgG antibody responses elicited against FhTP16.5 during an active *F. hepatica* infection in rabbits, we developed an ELISA protocol that was optimized by checkerboard titration. Antigen, serum, and conjugate were assayed at different concentrations to maximize the sensitivity of the assay. The optimal concentration of FhTP16.5 was determined to be 10 μg/ml, and the optimal dilution of serum and conjugate was determined to be 1:200 and 1:7,500, respectively (data not shown). The ELISA was performed following a basic protocol as previously reported (27).

Statistical analysis. All ELISA determinations were performed in triplicate in three independent experiments, and the results were expressed as the mean absorbance at 492 nm (A_{492}) for each determination. A sample was considered positive when its corresponding A_{492} exceeded the cutoff value calculated as the mean $A_{492} \pm 2$ standard deviations (SD) of results for the negative-control sera. Statistical analysis was performed weekly after infection. The infected group was compared with the control one by the nonparametric Krustal-Wallis test to determine significant differences between the mean A_{492} values of serum samples taken weekly for 12 weeks. qPCR amplifications were also performed in triplicate, and the experiments were replicated three times using different batches of RNA. Results were expressed as the mean value for each determination. Statistical analysis was performed using GraphPad Prism software.

Nucleotide sequence accession number. *F. hepatica* FhTP16.5 cDNA has been assigned EMBL/GenBank accession no. AY851159.

RESULTS

FhTP16.5 sequence analysis. Screening of 5×10^7 plaques of the λgt11 cDNA library with sera from rabbits with 4 weeks of *F. hepatica* infection revealed 13 positive clones. The cDNA fragment inserts of all positive clones were amplified by PCR, subcloned into pCR-Blunt vector, and sequenced from both ends. A clone (FhTP16.5) with significant similarity (71%) and identity (50%) to the *Schistosoma japonicum* tegumental protein SJCHGC09059 (44) was selected for further analysis. The complete sequence of FhTP16.5 was determined to be 554 bp, with an open reading frame (ORF) of 454 bp. The resulting ORF has a coding capacity of 150 amino acids with a calculated molecular mass of 16.5 kDa and a predicted isoelectric point (pI) of 6.6. The deduced polypeptide contains 48% hydrophobic amino acids and 31.3% polar amino acids. It also contains 2.7% acidic and 18% basic amino acids. The secondary structure predicted for FhTP16.5 is 6.67% α-helix, 51.33% random coil, 30.67% extended strand, and 11.33% β-turn. FhTP16.5 has seven potential phosphorylated serine residues (Ser27, -64, -109, -111, -124, -141, -142), five potential phosphorylated tyrosine residues (Tyr7, -62, -93, -107, -143), and one threonine residue (Thr76), as predicted by NetPhos 2.0 (Fig. 1A). The amino acid sequence of FhTP16.5

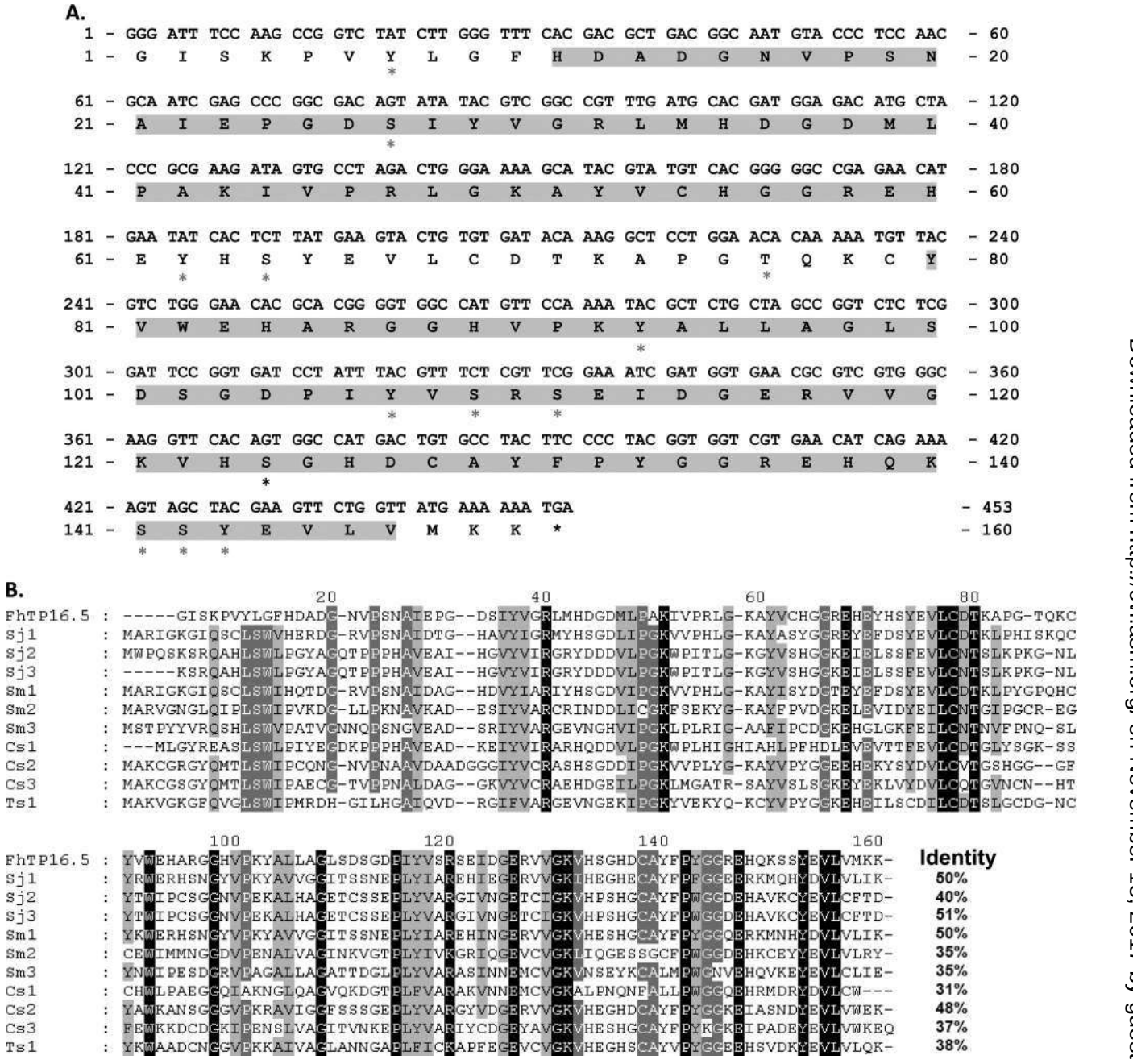

FIG 1 cDNA and deduced amino acid sequences of the FhTP16.5. (A) Nucleotide and amino acid sequences of FhTP16.5, potential phosphorylation sites (denoted by asterisks), and the two DM9 domains (highlighted in gray) are shown. (B) CLUSTAL alignment of the deduced FhTP16.5 protein (GenBank accession no. AY851159), the *Schistosoma japonicum* (Sj) tegumental protein SJCHGC0959, and uncharacterized proteins of *S. japonicum*, *S. mansoni* (Sm), *Clonorchis sinensis* (Cs), and *Taenia solium* (Ts) that contain DM9 domains (Sj1, GenBank accession no. AAW26133.1; Sj2, AAX25885.1; Sm1, CCD74958.1; Sm2, CCD74957.1; Sm3, CCD74787.1; Cs1, GAA34696.2; Cs2, GAA30369.2; Cs3, GAA52059.1; Ts, CAD21525.1).

exhibits two conserved repeats belonging to the DM9 superfamily, which are also present in the *S. japonicum* tegumental protein SJCHGC09059. A search of the GenBank database revealed that several uncharacterized proteins from *S. japonicum*, *S. mansoni*, *Clonorchis sinensis*, and *Taenia solium* also contain DM9 motifs. These uncharacterized proteins share 31.0% to 51.0% amino acid sequence identity with FhTP16.5 (Fig. 1B).

qPCR analysis of FhTP16.5 mRNA expression. To investigate whether FhTP16.5 is developmentally expressed in *F. hepatica* stages other than the adult, expression of FhTP16.5 at the mRNA level was evaluated for adults, unembryonated eggs, miracidia, and NEJs by using qPCR. The data were standardized relative to *F. hepatica* GADPH, and the $2^{-\Delta\Delta CT}$ method was used to quantify relative FhTP16.5 expression. Primer concentration was optimized to a final concentration of 200 nM for every primer. The presence of a single peak confirmed the specificity of the primers

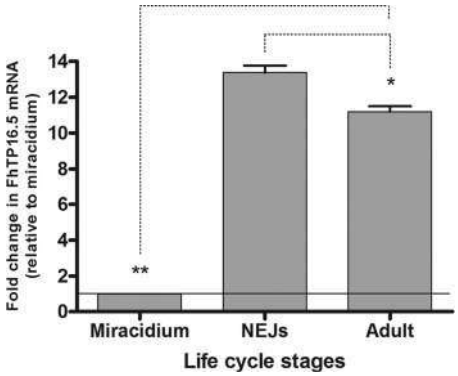

FIG 2 Developmental expression of FhTP16.5 mRNA in *F. hepatica*. Expression of FhTP16.5 at the mRNA level was determined by quantitative reverse transcriptase PCR (qPCR) at different stages of the *F. hepatica* life cycle. Results are shown as fold changes in expression relative to that of the miracidium stage. Bars represent the mean fold change ± standard error of the mean (SEM) from a minimum of three experiments, each performed in triplicate. The single asterisk represents a significant difference ($P < 0.05$) between FhTP16.5-mRNA expression in newly excysted juveniles (NEJs) and that in adult flukes. The double asterisks represent a significant difference ($P < 0.001$) between FhTP16.5-mRNA expression in NEJs or adults and that at the miracidium stage.

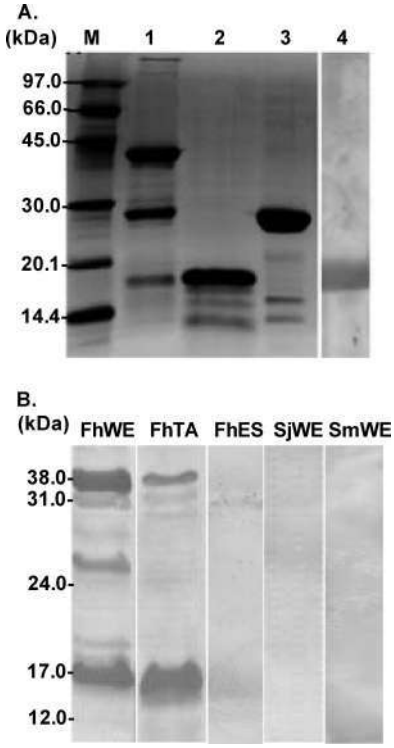

FIG 3 Recombinant FhTP16.5 preparation and Western blot analysis. (A) Twenty micrograms of protein was analyzed by SDS-PAGE under reducing conditions (β-mercaptoethanol), including purified FhTP16.5-GST fusion protein (lane 1), FhTP16.5 (lane 2), a GST tag (lane 3), and a specific rabbit anti-FhTP16.5 IgG antibody probe (lane 4). (B) The anti-FhTP16.5 IgG antibody recognized the native form of FhTP16.5 in *F. hepatica* crude extract (FhWE; lane 1), tegument extract (FhTA; lane 2), and excretory/secretory products (FhES; lane 3). The antibody did not recognize the major band of FhTP16.5 in crude extracts of *Schistosoma japonicum* (SjWE; lane 4) or *S. mansoni* (SmWE; lane 5). M, molecular mass markers (in kilodaltons).

for the target DNA, which was confirmed by the presence of single bands of the expected length when the PCR products were examined by agarose gel electrophoresis (data not shown). No expression of FhTP16.5 mRNA (C_T values at a total of 40 cycles) was detected in unembryonated eggs. Expression of FhTP16.5 mRNA became detectable at the miracidium stage and reached its maximal level at the NEJ stage before diminishing at the adult stage. By using miracidium as a calibrator, FhTP16.5 transcript was found to be expressed at a 13-fold higher level in NEJs and at an 11-fold higher level in adults than in miracidia ($P < 0.001$). FhTP16.5 was expressed at a significantly higher level in NEJs than in adults ($P < 0.05$) (Fig. 2). qPCR products were sequenced and found to have >98% identity with the expected sequence (data not shown).

Expression, purification, and identification of FhTP16.5. The recombinant plasmid pGEXT-FhTP16.5 expressed a fusion protein with an N-terminal GST tag in *E. coli* (BL21) under IPTG induction. GST-FhTP16.5 fusion protein was purified to near homogeneity by a single round of GSH affinity column chromatography and then digested with thrombin to cleave off the GST tag. Silver staining of the purified FhTP16.5 showed a strong signal visible at ~17 kDa, which corresponds to the expected mass of the protein according to the amino acid sequence (Fig. 3A). Anti-FhTP16.5 IgG antibody identified major polypeptides of ~17 kDa and 38 kDa in the FhTA and FhWE extracts. These bands were lightly stained in the FhES products. In FhWE, an additional band of ~28 kDa was also revealed. The 17-kDa band was not detected in SmWE or SjWE (Fig. 3B). The presence of a polypeptide band of 17 kDa was also revealed when recombinant FhTP16.5 was tested with sera obtained from humans with chronic fascioliasis (Fig. 4). In contrast, this polypeptide band was not observed in immunoblots probed with negative sera.

ELISA with rabbit sera. ELISA plates were sensitized by coating them with recombinant FhTP16.5 protein and then probed with rabbit sera. The mean absorbance value for sera collected prior to infection ranged between 0.05 and 0.15, with a mean value of 0.1 ± 0.037. Thus, the cutoff value established was 0.175 (mean A_{492} + 2 SD). Antibodies to FhTP16.5 protein were detected as early as 3 weeks after infection, peaked between 6 to 8 weeks of infection, and dropped slightly after 10 weeks of infection (Fig. 5A), and the presence of the 17-kDa band was also demonstrated in these sera (Fig. 5B). Significant differences were observed between the absorbance values of sera from 3 to 12 weeks of infection compared to those of control sera ($P < 0.006$).

Immunohistochemical detection of FhTP16.5 in *F. hepatica* developmental stages. Fluorescence analysis using rabbit anti-

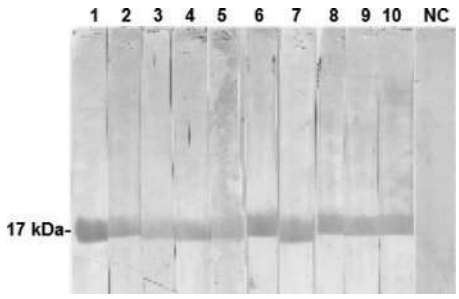

FIG 4 Detection of anti-FhTP16.5 antibodies in human sera. FhTP16.5 was identified by Western blotting using sera from 10 human patients with chronic fascioliasis (lanes 1 to 10) but not in sera from healthy subjects (lane NC).

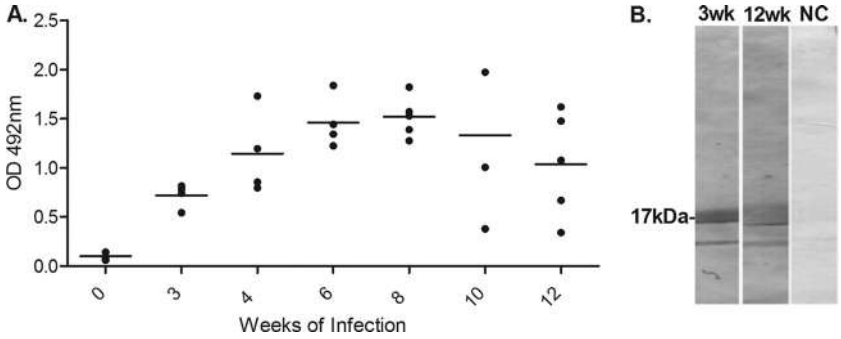

FIG 5 Detection of FhTP16.5 antibodies in rabbit sera. (A) Dynamics of the antibody response to FhTP16.5 protein determined by ELISA in rabbits infected with *F. hepatica*. The experiment was repeated three times, and each determination was performed in triplicate. The results are expressed as the mean value for each determination. Significant differences ($P < 0.001$) were found between the mean optical density (OD; A_{492}) values from 3 to 12 weeks of infection compared to that of the control sera on week 0. (B) Recombinant FhTP16.5 was identified by Western blotting when using sera from rabbits at 3 weeks (lane 1) and 12 weeks (lane 2) of *F. hepatica* infection but not in the negative-control sera (lane 3).

FhTP16.5 IgG antibody revealed strong fluorescence in the outer region of the tegument and parenchymal tissue of the adult fluke, whereas the syncytium was stained moderately (Fig. 6A). This staining was totally eliminated from the surface of the tegument and significantly diminished in the parenchymal cells when the antibody was absorbed with recombinant FhTP16.5 protein (Fig. 6B). No staining was observed when the anti-FhTP16.5 IgG antibody was replaced with negative rabbit serum (Fig. 6C). In NEJs, also, the antibody bound to the surface of the tegument. In a miracidium, staining was observed at the ciliated epithelial cells surrounding the superficial muscle layer that constitutes the primitive tegument of this larva. FhTP16.5 was not detected in NEJs or miracidia incubated with preimmune rabbit serum (Fig. 7).

DISCUSSION

In the present study, we report a novel *F. hepatica* protein, FhTP16.5, which in spite of its relatively small molecular weight is highly immunogenic and able to stimulate potent antibody responses in rabbits. Immunohistochemistry studies demonstrated that in the adult fluke, FhTP16.5 is strongly expressed at the outer surface of the tegument as well as in parenchymal tissue associated with the tegument, which is a pattern similar to those of other proteins of trematodes, such as *F. gigantica* fatty acid binding protein (FgFABP) (59), *S. mansoni* alkaline phosphatase (SmAP) (11), superoxide dismutase (Sm-SOD) (53), and histamine-responsive receptor (SmGPR) (24). However, the observation that it is moderately expressed in the syncytium could indicate that FhTP16.5 is not actually part of the architecture of the tegument but is a protein that is produced in parenchymal cells, is delivered to the tegument, and accumulates there.

The finding of FhTP16.5 in the tegument and in parenchymal tissues is consistent with the appearance of a major component of 17 kDa in both the crude and tegumental extracts of *F. hepatica*. A number of different methods have been described for the preparation of tegument extract. The protocols range from simple ones, such as freezing/thawing/vortexing, to harsher and more sophisticated ones that enrich for tegumental membrane proteins (37, 66, 67, 72). In one study, monitoring of the extraction process by using scanning electron microscopy of the tegument surface revealed that, in addition to the extracting surface antigens, leakage of cytosolic proteins from internal tissues such as the parenchyma

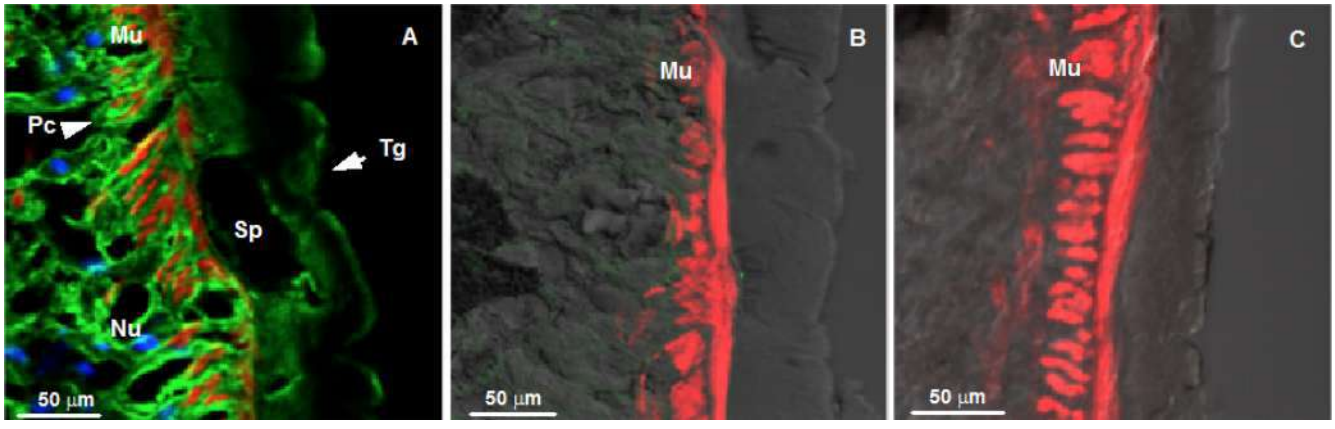

FIG 6 Immunolocalization of native FhTP16.5 in the *Fasciola hepatica* adult worm. (A) Cross section of the middle part of the body of an adult fluke (magnification, ×20) stained with 1:100 diluted anti-FhTP16.5 IgG antibody and showing intense staining in the outer surface of the tegument (Tg; arrow) and parenchymal cells (Pc; filled arrowhead). Phalloidin-TRITC was used to counterstain muscle (Mu) and DAPI to stain nuclei (Nu). (B) Staining was eliminated from the surface or significantly reduced from parenchymal cells when anti-FhTP16.5 IgG antibody was absorbed with 200 μg/ml of recombinant FhTP16.5. (C) No staining was observed when the anti-FhTP16.5 IgG antibody was replaced with negative rabbit serum.

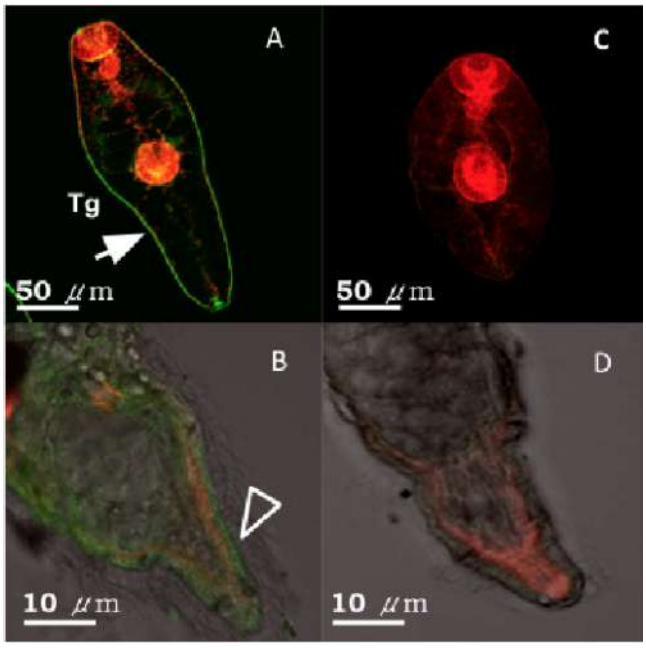

FIG 7 Immunolocalization of native FhTP16.5 in *Fasciola hepatica* newly excysted juveniles and miracidium stages. (A) Whole-worm mount of an NEJ (magnification, ×40) stained with anti-FhTP16.5 IgG antibody diluted 1:500 and showing intense staining in the tegument. (B) Whole-worm mount of a miracidium (magnification, ×60) stained with anti-FhTP16.5 IgG antibody (1:500) and showing staining toward the ciliated epithelium of the miracidium (empty arrowhead), which is the primitive tegument of this larva. Phalloidin-TRITC and DAPI were used to counterstain muscle (Mu) and nuclei (Nu), respectively. (C and D) No staining was observed for controls in which the anti-FhTP16.5 IgG antibody was replaced with negative rabbit serum.

can occur (72). In the present study, we extracted the tegumental antigens by incubating the parasites for 1 h at 4°C with NP-40, and although we did not monitor the extraction by electron microscopy, we are confident that the extraction was harsh enough to extract not only the outer surface antigens but also those localized more deeply within the parenchymal tissue of the parasite (37). Based on its specific localization and the absence of a classic secretory signal peptide or transmembrane region as determined by bioinformatics analysis, we hypothesize that FhTP16.5 can be shed from the worm during tegument renewal and stimulate the immune response. This could also explain the presence of FhTP16.5 in the excretory-secretory (ES) products of *F. hepatica.*

The finding that FhTP16.5 is not expressed in unembryonated eggs indicates a lack of requirement for FhTP16.5 during egg formation in the adult fluke. However, the presence of FhTP16.5 mRNA in the ciliated epithelium of the miracidium suggests that FhTP16.5 protein is likely required for nutrition of the larva during its passage through the snail intermediate host. The miracidium must penetrate the appropriate snail intermediate host to continue its life cycle. Following penetration into the intermediate host, the miracidium transforms sequentially into a sporocyst, a redia, and then a cercaria, which finally emerges from the snail and encysts on aquatic plants as a metacercaria. After excystment, the juvenile larva and adult fluke exhibit high levels of FhTP16.5 mRNA expression and protein localization in the outermost region of the tegument. This finding reinforces the theory that FhTP16.5 could be a protein essential for survival of these developmental stages within the mammalian host.

The presence of two DM9 repeats that extend practically throughout the protein moiety of FhTP16.5 is another interesting finding. About 280 types of DM9 domains have been reported for 89 proteins from a large variety of organisms, including humans, insects, rodents, viruses, bacteria, cyanobacteria, fungi, plants, nematodes, and other metazoans (43, 63). To date, the function of these domains remains unknown. However, three *Drosophila* proteins carrying these motifs have been shown to interact with various proteins involved in transcriptional regulation, intracellular trafficking, cytoskeleton rearrangement, and immune responses (19), suggesting that they could be involved in regulatory protein-protein networks. A search of the proteomic and genomic databases of trematodes and cestodes revealed that a number of uncharacterized proteins from *S. japonicum* (44), *S. mansoni* (10), *C. sinensis*, and *T. solium* possess DM9 domains, and all of them share significant identity with FhTP16.5. This indicates that proteins homologous to FhTP16.5 exist in other platyhelminths. However, the possible roles of the proteins that contain DM9 motifs in *Platyhelminthes* remain unknown. Interestingly, some of these proteins have been localized at the tegument of *S. japonicum* (44), and a recent proteomic study of the *F. hepatica* tegument revealed uncharacterized proteins with DM9 motifs to be associated with the tegumental membrane of the parasite (72), which supports our findings. On the other hand, numerous tegumental proteins lacking the DM9 motif have been identified and purified from *F. gigantica* (5–7, 64), *S. japonicum* (69, 70), *S. mansoni* (31, 39, 55), *C. sinensis* (18, 74), *Echinococcus granulosus* (56), and *Taenia* spp. (40, 42, 54), and none of these proteins shares homology with FhTP16.5. Proteins of 50 kDa and 25 to 40 kDa have also been identified in the tegumental syncytium of various *F. hepatica* stages using monoclonal antibodies (2, 34), but these proteins have not yet been purified and characterized. We do not know the biological function of FhTP16.5 protein, but based on its specific localization in the tegument of various developmental stages of the parasite, we speculate that it could have roles such as nutrition, transport of materials to the tegument, or evasion of the host immune system.

The common characteristic of all characterized tegumental and tegument-associated proteins, including FhTP16.5, is that are highly immunogenic and useful for serodiagnosis of infections. The strong reactivity of FhTP16.5 with sera from rabbits with 3 to 12 weeks of infection and with sera from humans with chronic fascioliasis indicates that, during active infection, specific antibodies against FhTP16.5 are elicited at early stages and remain at relatively high levels during the chronic phase of infection. Only the 17-kDa component was identified from these sera when the recombinant protein was used as an antigen; however, when the anti-FhTP16.5 antibody was tested against the *F. hepatica* crude extract, tegument extract and ES products bands of 28 or 38 kDa were also detected, which may be suggestive of dimer or protein complex formation. This finding suggests that the 17-kDa polypeptide might be used as a marker of active infection. Unfortunately, at the time of this study, sera from persons infected with other parasites were not available. Moreover, we do not know if the persons with chronic fascioliasis whom we studied also carried other infections. For these reasons, we could not determine if the 17-kDa polypeptide is able to induce cross-reactive antibodies. Therefore, we cannot be certain that the FhTP16.5 ELISA is specific for human fascioliasis. To rule out possible cross-reactions between FhTP16.5 and molecules from other trematodes, we

tested the anti-FhTP16.5 IgG antibody against crude extracts of adult *S. mansoni* and *S. japonicum* and demonstrated that although FhTP16.5 shares 35.0 to 51.0% identity with proteins from these parasites, the anti-FhTP16.5 polyclonal antibody did not cross-react with these crude extracts. These results suggest that anti-FhTP16.5 IgG antibody could be a useful reagent to detect the presence of the FhTP16.5 protein in fecal samples from individuals with fascioliasis, as has been reported for other characterized tegumental proteins of *F. hepatica* (2). Unfortunately, *F. gigantica* antigens were not available during this study, and the possibility of finding FhTP16.5 in this species was not investigated. Therefore, it is necessary to expand the serological studies by testing a larger number of sera from patients with fascioliasis and with other parasitic infections or nonparasitic infections to confirm the diagnostic value of this antigen. In addition, more studies are needed to ascertain whether a homolog of FhTP16.5 exists in *F. gigantica*, which could be favorable to develop serological application of this protein to the diagnosis of infections caused by both species.

In conclusion, we identified and characterized a tegument-associated protein of *F. hepatica*. Although this protein shares some identity with proteins of *Schistosoma* spp., anti-FhTP16.5 IgG antibody purified by affinity chromatography did not cross-react with a crude extract of *S. mansoni* or *S. japonicum*. Experiments are under way to test FhTP16.5 against a large panel of sera from patients with fascioliasis, schistosomiasis, and other helminthiases to confirm its potential as an antigen for serodiagnosis. Moreover, because tegumental proteins are important targets for immunological intervention, further studies will also be performed to test this molecule as a vaccine candidate in the control of fascioliasis.

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