

## A Recombinant 10-kDa Protein of *Taenia solium* Metacestodes Specific to Active Neurocysticercosis

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Neurocysticercosis (NCC) is an important cause of neurological disease worldwide. A 10-kDa antigen of *Taenia solium* metacestodes (TsMs) has been shown to be specific for immunodiagnosis of NCC. Screening of a TsM complementary DNA (cDNA) library isolated a cDNA encoding this protein. The cloned cDNA contained a 258-bp complete open-reading frame that encodes an 86-amino acid polypeptide with a calculated molecular weight of 9582 Da. It showed 73% homology with a 10-kDa antigen of *T. crassiceps*. The recombinant protein was expressed bacterially as a fusion protein at a high level. In immunoblot with recombinant protein, 97% (184/190) of sera from patients with active NCC showed strong reactivity, whereas 14% (4/29) of those from patients with chronic calcified NCC reacted weakly. In 180 sera from other patients with parasitic infections and from normal controls, it showed 98% specificity. A single recombinant TsM antigen has a high potential for serological differentiation of active NCC.

Neurocysticercosis (NCC), which is caused by infection of the central nervous system with *Taenia solium* metacestodes (TsMs), is a major cause of neurological diseases in Asia, Africa, and Latin America [1–3]. Surveys in areas where it is endemic show that it is of public health concern, since it causes considerable mortality, chronic morbidity, and economic losses [3, 4]. Substantial evidence has shown that up to 50% of late-onset epilepsy is due to NCC [3–6]. In the United States, >1000 cases of NCC are encountered each year, mostly in immigrants [3, 7].

Diagnosis of NCC can be achieved with a high degree of accuracy by brain computed tomography/magnetic resonance imaging (CT/MRI) [8–10]. These methods are, however, expensive and inaccessible in most areas where NCC is endemic. Moreover, because the number, size, and location of NCC lesions vary and the stage of infection is often unknown, imaging diagnosis of NCC is ambiguous in many patients. The development of immunological tests that detect specific antibodies

either in sera or in cerebrospinal fluid (CSF) would provide a simple and reliable adjuvant for the diagnosis of NCC. Unfortunately, most of the tests that employ crude TsM antigens lack both sensitivity and specificity; cross-reactions occur frequently with other parasitic infections, especially with cystic echinococcosis (CE) and alveolar echinococcosis (AE), which are caused by larval *Echinococcus granulosus* and *E. multilocularis*, respectively [11, 12]. Over the past 2 decades, many efforts have been directed toward characterizing specific antigens of TsMs, either from whole worm or from cyst fluid (CF) [11–16]. The low-molecular-weight antigenic components of TsMs (8–50 kDa) have attracted particular attention due to their high specificity. Immunoblot studies with TsMs found that crude soluble extracts (2 polypeptides at 8 and 26 kDa) were recognized strongly by antibodies in the serum and CSF of patients with NCC and sera from TsM-infected pigs [11, 17]. Tsang et al. [13] purified a group of glycoprotein antigens of 13–50 kDa from TsMs and undertook an immunoblot, which has been reported to be as high as 98% sensitive and 100% specific for NCC. This assay has widely been used for immunodiagnosis of individual patients and for seroepidemiological surveys [4, 18, 19].

Our research interest has been focused on the identification and isolation of specific antigens from CF of TsMs. We have previously shown that a 10-kDa antigen of TsM CF allowed high reliability in detecting the specific anti-TsM antibodies in patients with NCC [12, 15, 16]. Biochemical studies with monoclonal antibody (mAb) revealed the 10-kDa antigen is a subunit of a thermostable 150-kDa protein complex [12, 20]. In an experiment to isolate the 10-kDa protein from TsM CF either by mAb-ligand immunoaffinity chromatography [12, 20]

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or by isoelectric focusing [16], we found that the 10-kDa protein was always linked to 2 other proteins (15 and 7 kDa) and could not be separated from them. Nevertheless, immunological evaluation of a fraction containing the 3 components by both immunoblot and ELISA with sera/CSF from NCC and other helminthic infections, including AE and CE, showed high sensitivity and specificity, both >90% [12, 15, 16].

In the present study, we describe the cloning and sequencing of a complementary DNA (cDNA) that encodes a TsM 10-kDa protein and its expression in *Escherichia coli* cells. We evaluate its diagnostic value and provide evidence that this recombinant antigen is highly useful in differentiating active NCC from chronic NCC and from other parasitic infections.

## Materials and Methods

**Serum samples.** A total of 219 individual serum samples from patients with NCC were selected from our sera bank. Patients were diagnosed on the basis of their clinical manifestations, brain CT/MRI findings, and positive antibody reactions in their serum or CSF upon ELISA done with crude TsM CF antigen [9, 21]. On the basis of neuroimaging findings only, patients were categorized into 2 groups: 190 with active NCC and 29 with chronic inactive NCC. The patients who had lesions with multiple low-density regions (MLDs) or hydrocephalus compatible with NCC were classified as active cases [8, 10, 21, 22]. Patients who exhibited only multiple calcifications in their CT/MRI scan were classified as chronic inactive cases [8, 10, 22] (table 1 and figure 1). In addition, to determine possible cross-reactivity, 130 serum samples were examined from patients with AE ( $n = 11$ ), CE ( $n = 9$ ), sparganosis ( $n = 30$ ), paragonimiasis ( $n = 30$ ), clonorchiasis ( $n = 30$ ), fascioliasis ( $n = 10$ ), and schistosomiasis japonicum ( $n = 10$ ). Normal controls ( $n = 50$ ) who denied any possible exposure to helminth infection were also investigated. All serum samples were stored at  $-70^{\circ}\text{C}$  until use.

**CF antigen of metacestodes from *T. solium* and other taeniid cestodes.** TsMs were obtained from naturally infected pigs in Korea and China. After the cysts were washed, CF was collected from each with a sterile syringe [15]. CF from metacestodes of *T. saginata*, *T. taeniaeformis*, and *T. crassiceps* were collected from experimentally infected calves, rats, and mice, respectively. CF from metacestodes of *E. granulosus* and *E. multilocularis* were collected from a human patient with CE and an experimentally infected rat, respectively. The crude CF samples were centrifuged at 20,000 g for 1 h; the supernatants were used as CF antigens and stored at  $-70^{\circ}\text{C}$  until use. All procedures were done at  $4^{\circ}\text{C}$ .

**Isolation of RNA and construction of cDNA library.** Fresh intact TsMs were ground in liquid nitrogen. Total RNA was isolated by CsCl gradient ultracentrifugation. Poly(A)<sup>+</sup> RNA was prepared by oligo(dT)-affinity chromatography (Qiagen, Valencia, CA). cDNA was synthesized from 1  $\mu\text{g}$  poly(A)<sup>+</sup> RNA with the Capfinder cDNA Library Synthesis Kit (Clontech, Palo Alto, CA). The resulting cDNA fragments were ligated into *EcoRI* linker DNA, then digested with *EcoRI*, and finally ligated with *EcoRI*-cleaved  $\lambda\text{gt} 11$  phage (all included in the Clontech Capfinder

**Table 1.** Summary of clinical and neuroimaging profiles of the patients.

Category	Active NCC ( $n = 190$ )	Inactive NCC ( $n = 29$ )
Age in years (mean $\pm$ SD)	49 $\pm$ 15	54 $\pm$ 15
Sex (% male)	65	79
Symptoms		
Headache	122 (64)	15 (52)
Seizure	91 (48)	20 (70)
Symptoms due to hydrocephalus <sup>a</sup>	41 (22)	0
Symptoms due to IICP <sup>b</sup>	31 (16)	0
Other neurological deficits <sup>c</sup>	41 (22)	9 (11)
CT/MRI findings		
MLDs	78 (41)	0
Hydrocephalus	41 (22)	0
MLDs with calcifications	29 (15)	0
Cystic mass(es)	20 (10)	0
MLDs with hydrocephalus	14 (7)	0
Cystic mass with calcifications	3 (2)	0
Hydrocephalus with calcifications	2 (1)	0
Cystic mass with hydrocephalus	2 (1)	0
Spinal cord cysticercosis (MLDs)	1 (1)	0
Multiple calcifications	0	29 (100)

NOTE. Table values are no. (%) unless otherwise indicated. NCC, neurocysticercosis; CT/MRI, computed tomography/magnetic resonance imaging; IICP, increased intracranial pressure; MLDs, multiple low-density regions.

<sup>a</sup> Including nausea, vomiting, gait disturbance, difficulty voiding, memory loss, and dizziness.

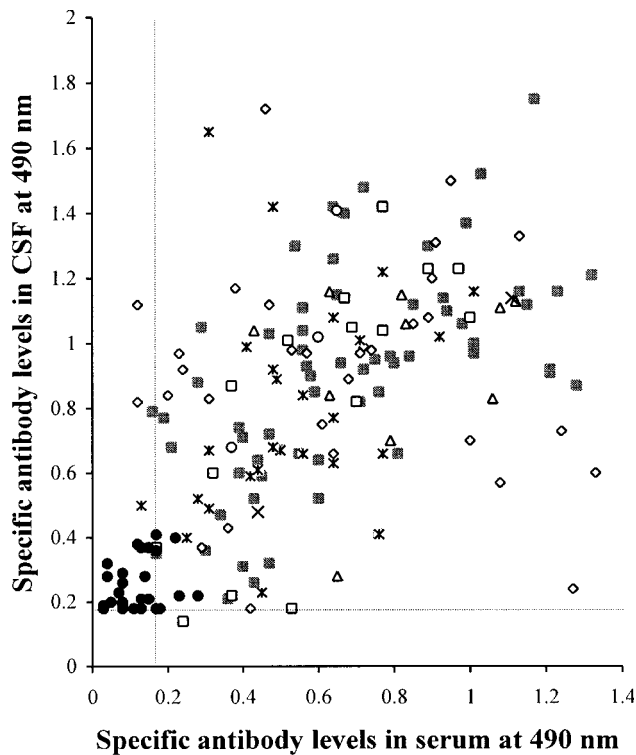
<sup>b</sup> Including nausea, vomiting, stiff neck, and blurred vision; these patients' CT/MRI scans exhibited no evidence of hydrocephalus.

<sup>c</sup> Including dysphasia, hemi- or monoparesis, dysarthria, paresthesia, stupor, coma, and spinal cord symptoms.

cDNA Library kit). The recombinant DNA was packaged in Gigapack III gold packaging extract (Stratagene, La Jolla, CA).

**Reverse transcription polymerase chain reaction and cloning of a cDNA encoding 10-kDa protein.** Two oligonucleotide primers were synthesized, designed on the basis of a published cDNA sequence encoding the 10-kDa immunodiagnostic antigen of *T. crassiceps* (TcA5.5) [23]. The sense primer was 5'-GCGAAAACAAAG-ATGAGGG-3' and antisense primer was 5'-CTATTCAT-TTTCAAGACCC-3'. One microgram of total RNA was reverse-transcribed into cDNA by using an oligo(dT)<sub>18</sub> primer and Moloney murine leukemia virus reverse transcriptase (Gibco-BRL, Grand Islands, NY). The resulting cDNA was subjected to polymerase chain reaction (PCR) amplification in a 50  $\mu\text{L}$  reaction mixture containing 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 2.5 mM MgCl<sub>2</sub>, 0.2 mM dNTPs, 25 pM each primer, and 2.5 units of AmpliTaq DNA polymerase (Perkin Elmer, Foster City, CA). Amplification was done in a DNA thermal cycler (9600; Perkin Elmer) for 35 cycles with denaturation at  $94^{\circ}\text{C}$  for 30 s, primer annealing at  $56^{\circ}\text{C}$  for 30 s, and extension at  $72^{\circ}\text{C}$  for 30 s with a final extension at  $72^{\circ}\text{C}$  for 5 min. The PCR product was analyzed by 1.5% agarose gel electrophoresis. A fragment of 270 bp was isolated and sequenced (described in later section). The PCR product was, finally, subcloned into T7 Blue T-vector (Novagen, Madison, WI).

The phage cDNA library was screened by plaque hybridization. The labeling of the probe and detection of hybridization signal were performed by using the ECL Direct Nucleic Acid Labelling and Detection System (Amersham, Buckinghamshire, UK). Both prehybridization and hybridization were done at  $42^{\circ}\text{C}$ . After sec-



**Figure 1.** Specific antibody levels in serum and cerebrospinal fluid (CSF) by ELISA in patients with neurocysticercosis (NCC) plotted with findings determined by neuroimaging (computed tomography or magnetic resonance imaging). The specific antibody levels in patients with chronic inactive NCC were differentiated. Of 190 patients with active NCC, 152 were examined for their specific antibody levels in both sera and CSF. Horizontal and vertical dashed lines indicate cut-off absorbance (abs.) at 0.18 in both sera and CSF. ■, multiple low-density regions (MLDs); ◇, hydrocephalus; \*, MLDs with calcifications; □, cystic mass(es); △, MLDs with hydrocephalus; ○, cystic mass with calcification; ×, cystic mass with hydrocephalus; and ●, multiple calcifications. Not plotted are the neuroimaging findings in 38 active NCC patients whose sera but not CSF were tested. These neuroimages showed MLDs ( $n = 15$  patients, abs. 0.16–1.31), hydrocephalus ( $n = 10$ , abs. 0.32–1.34), cystic mass ( $n = 5$ , abs. 0.27–0.96), MLDs mixed with hydrocephalus ( $n = 3$ , abs. 0.37–1.19), MLDs or hydrocephalus combined with calcifications ( $n = 2$  each, abs. 0.54, 0.64, 0.80, and 0.96, respectively), and spinal cord cysticercosis ( $n = 1$ , abs. 1.0).

ondary wash with high stringency, the membrane was autographed after a 1-min exposure. Positive plaques were isolated and the lambda DNA was purified by use of a Qiagen  $\lambda$  Kit (Qiagen). The insert was cloned into pGEM-T easy vector (Promega, Madison, WI) by PCR, using gt 11 universal primer (Promega) and advanced *Taq* polymerase with proofreading capacity (Clontech). Recombinant plasmid in bacterial cultures was purified with the Qiagen plasmid midikit (Qiagen).

**DNA sequencing and sequence analysis.** We determined the nucleotide sequence by the dideoxynucleotide chain termination method, using the ABI Prism Dye Terminator Cycle Sequencing Core Kit (Perkin Elmer) and an automated DNA sequencer (Ap-

plied Biosystems model 373A; Perkin Elmer). We analyzed the nucleotide and amino-acid information using the DNA Strider (version 3.0) and the BLAST programs of the NCBI databases (Bethesda, MD).

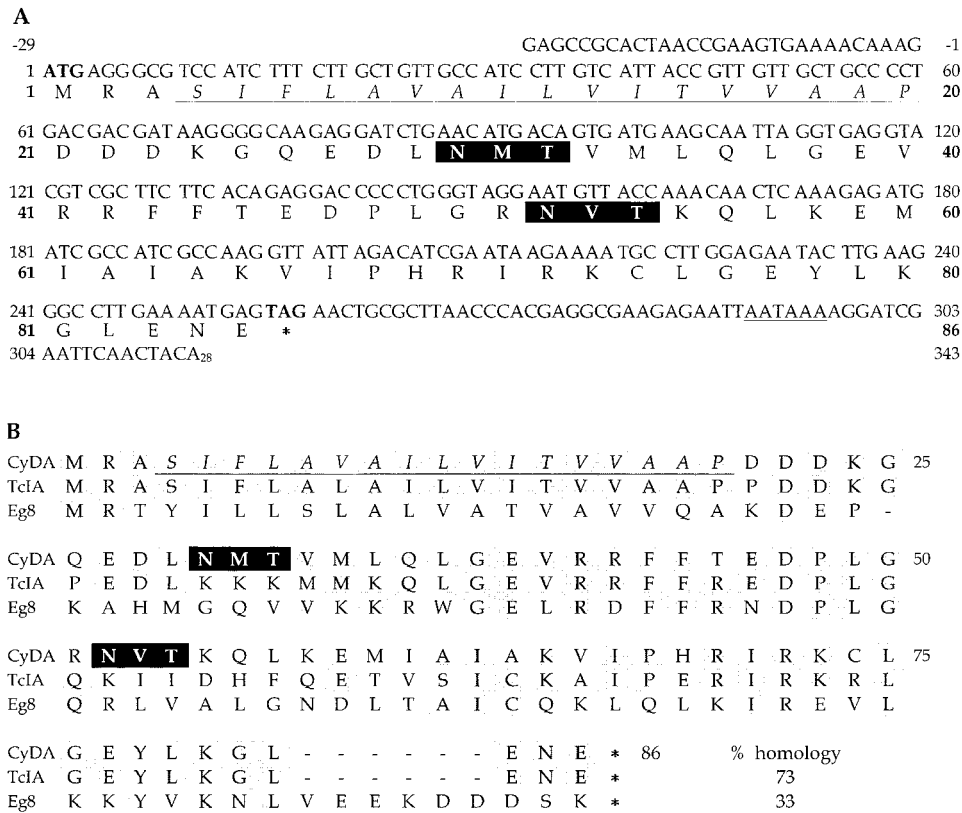
**Expression and purification of the recombinant protein.** The coding region from a cDNA clone was amplified by PCR with a specific primer pair that contained a restriction site added to the 5' end to facilitate cloning of the PCR product. The forward primer was 5'-GTTGGATCCCCTGACGACGATAAG-3' and the reverse primer was 5'-ACTAAAGCTTCTACTCATTTC AAGG-3' (underlined sequences indicate each *Bam*HI and *Hin*dIII site). The PCR was done again as described previously, and the product was subcloned into pGEM T easy-vector (Promega). An insert isolated from 1 clone harboring the expected coding sequence was excised by *Bam*HI and *Sal*I double digestion and ligated into the *Bam*HI/*Sal*I-cleaved pGEX 4T-2 expression vector (Pharmacia) and then introduced into *E. coli* BL21 cells carrying the DE3 bacteriophage. The fidelity of the expression construction was confirmed by DNA sequencing. Upon induction with isopropyl- $\beta$ -D-thiogalactoside, the recombinant protein was expressed as a fusion with glutathione S-transferase (GST). Induced cells were harvested by centrifugation and lysed by sonication. The supernatants were adsorbed to the glutathione-Sepharose 4B resin (Pharmacia) after clearing, and the fusion protein was eluted with reduced glutathione. The GST carrier domain in fusion protein was further removed by thrombin cleavage.

**Preparation of anti-recombinant TsM antibodies.** Polyclonal antisera against the recombinant TsM protein were obtained from BALB/c mice immunized with purified recombinant protein. A monospecific antibody to the recombinant protein was affinity-purified from sera pooled from 10 patients with NCC by means of the SulfoLink Kit (Pierce, Rockford, IL). In brief, 10 mg of the recombinant protein was dialyzed against sodium acetate buffer (100 mM, pH 6.0), after which 6 mg of 2-mercaptoethanol was added. The mixture was then packed into a column preequilibrated with Tris buffer (50 mM, pH 8.5). A total of 5 mL of serum was eluted by glycine buffer (100 mM, pH 2.5). The monospecific antibody was dialyzed against PBS (100 mM, pH 7.2) overnight and stored at  $-70^{\circ}\text{C}$  until use.

**Sodium dodecyl sulfate-polyacrylamide gel electrophoresis and immunoblot.** CF antigen preparations and recombinant protein were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to polyvinylidene difluoride membrane (Millipore, Bedford, MA). Blots were incubated overnight, either with the patient sera or with a monospecific antibody diluted at 1 : 200. The murine antisera were used in appropriate dilution. Peroxidase conjugated anti-human IgG (heavy- and light-chain specific; Cappel, West Chester, PA) or anti-mouse IgG (whole molecule; Cappel) was diluted at 1 : 1000. The blots were developed with 0.03% (w/v) 4-chloro-L-naphthol (4C1N; Sigma, St. Louis).

## Results

**Summary of patient characteristics.** Table 1 and figure 1 show the clinical, serological, and radiological profiles of 219



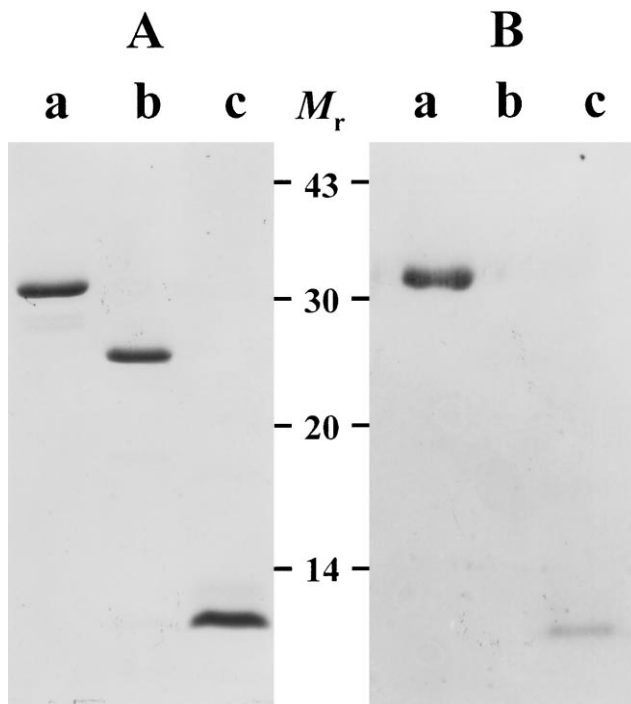
**Figure 2.** *A*, Nucleotide and deduced amino acid sequence of a cDNA encoding a *Taenia solium* metacestode (TsM) 10-kDa antigen. Note the complete nucleotide sequence of 372 bp with an open-reading frame of 258 bp. The start and stop codons (ATG and TAG, respectively) are boldface; asterisk (\*) indicates stop codon. A putative polyadenylation signal (AATAAA) is underlined. A highly hydrophobic polypeptide sequence is italicized and underlined. Potential N-glycosylation sites are indicated in black boxes. *B*, Alignment of deduced amino acid sequence of TsM 10-kDa protein with 2 known taeniid antigens. Gaps indicated by dots are introduced into the sequence to optimize the alignment. Other markings are same as in *A*. The percentage homologies are shown at the end of each polypeptide. The 10-kDa immunodiagnostic antigen of *T. crassiceps* [23] and 8-kDa antigen of *Echinococcus granulosus* (subunit of antigen B) [36] are shown for comparison.

patients with NCC enrolled in this study. The 190 patients in the active NCC group (123 male and 67 female patients) had a mean age of  $49 \pm 15$  years (9–77 years). The common manifestations were headache, seizure, and symptoms due to hydrocephalus or increased intracranial pressure (IICP). In addition, some patients presented other neurological deficits such as dysphasia, hemi- or monoparesis, dysarthria, paresthesia, stupor or coma, and spinal cord syndrome. The majority of this group showed neuroimaging findings of vesicular or colloidal vesicular stage according to Escobar classification [22] and hydrocephalus with or without signs of meningeal irritation [8]. They included MLDs, hydrocephalus, cystic mass(es), MLDs mixed with calcifications or with hydrocephalus, cystic mass(es) combined with calcifications or with hydrocephalus, and hydrocephalus mixed with calcifications. Spinal cysticercosis together with MLDs was also observed in CT/MRI scans. Specific anti-TsM antibody levels, determined by ELISA employing crude CF antigen, were  $0.66 \pm 0.29$  in sera of 190 patients and  $0.89 \pm 0.38$  in CSF of 152 patients examined (the

other 38 were not tested). Cut-off absorbance for positive reaction was 0.18 in both serum and CSF [21].

The chronic calcified inactive group consisted with 29 patients whose antibody titer in serum was marginal or negative ( $0.11 \pm 0.07$ ) and positive in CSF ( $0.25 \pm 0.07$ ) (figure 1). Chronic NCC tended to show positive reactions only in CSF but not in the sera [14, 21, 24]. These cases included 23 male and 6 female patients. Their mean age was  $54 \pm 15$  years (19–83 years). Clinical symptoms included seizure, headache, and other neurological deficits such as hemiparesis, dysphasia, motor weakness, dizziness, and dementia. All the patients exhibited multiple calcifications in their neuroimages.

*Cloning and molecular properties of a cDNA encoding 10-kDa protein.* The antigenic similarity of the 10-kDa protein among genus *Taenia* shown in studies published elsewhere [23, 25] suggested a sequence homology of these related genes. We succeeded in amplifying a TsM cDNA fragment using a degenerate primer derived from a published cDNA sequence of a 10-kDa immunodiagnostic antigen of *T. crassiceps* (TcA5.5) [23]. We



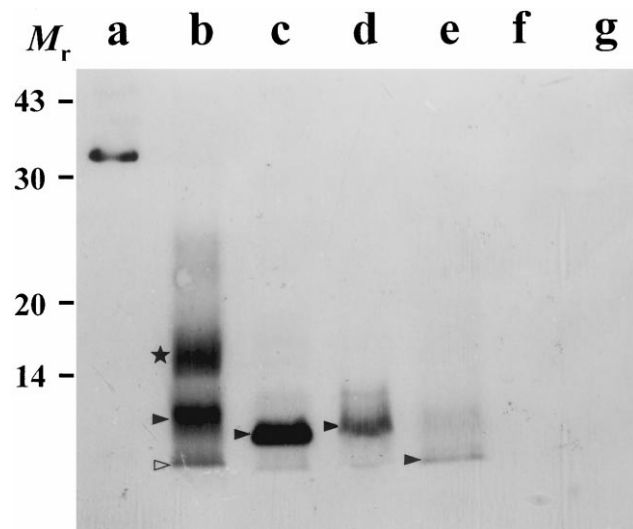
**Figure 3.** Analysis of purified recombinant protein by sodium dodecyl sulfate–polyacrylamide gel electrophoresis stained with Coomassie blue (A) and by immunoblot probed with a pooled serum from 10 patients with neurocysticercosis. (B). Lane a, 33-kDa glutathione S-transferase–fusion protein; lane b, glutathione S-transferase carrier only; lane c, thrombin-treated 7-kDa *Taenia solium* metacestode protein.  $M_r$ , molecular weight in kilodaltons.

obtained a 270-bp cDNA fragment, and its deduced amino acid sequence revealed significant homology to the 10 kDa of *T. crassiceps* (data not shown). This fragment was used as a probe to screen a TsM cDNA library, a search that yielded 30 positive clones.

Figure 2A shows the nucleotide sequence of the longest insert (372 bp; GenBank accession number AF076609). Sequence analysis revealed that a single complete open-reading frame (ORF) of 258 bp contained both translation initiation and stop codons, and a poly (A)<sup>+</sup> tail. A putative polyadenylation signal (AATAAA) was identified at 32 bp downstream from the stop codon. Translation of the ORF gave rise to an 86-amino acid polypeptide with a calculated molecular weight of 9582 Da. The N-terminal region was shown to possess a potential hydrophobic domain encompassing residues 4–20. Two putative N-linked glycosylation sites (N-X-S/T) were identified in the middle region. A sequence-similarity search revealed that the deduced protein sequence was most closely related to the 10-kDa antigen of *T. crassiceps* with the highest degree by 73% and to the 8-kDa subunit of antigen B of *E. granulosus* by 33%, respectively (figure 2B). Northern blot analysis with a full-length cDNA revealed a single RNA transcript ~500 base in size (data not shown).

*In vitro* expression and characterization of a recombinant protein. In a preliminary observation, we tried to express the full-length coding domain (258 bp), although only a small amount of recombinant protein was expressed as insoluble aggregates. We prepared a truncated fragment of 198 bp without the N-terminal hydrophobic sequence. In this case, the recombinant protein was expressed at a high level in a soluble form and could be efficiently purified by glutathione-affinity chromatography. As shown in figure 3A, GST-fusion protein migrated to 33 kDa in SDS-PAGE analysis. After the GST carrier was removed by thrombin cleavage, the purified protein moved as a single band at ~7 kDa, which is in good agreement with what we calculated from the cDNA sequence. In immunoblot analysis (figure 3B), both the 33-kDa GST-TsM fusion protein and the 7-kDa TsM protein reacted strongly with serum pooled from 10 patients with NCC as well as with the antibodies generated (immunoblots probed by monospecific antibody and mouse antisera not shown). On the other hand, no reactivity was observed at the 26-kDa GST band, which suggests that the presence of GST carrier domain in the recombinant protein did not affect the antigenicity (figure 3B, lane b). We have designated recombinant 33-kDa GST-fusion TsM protein as cysticercosis diagnostic antigen (CyDA) and directly used it in further serological evaluation.

Figure 4 shows the immunoblot outcome probed with a mon-



**Figure 4.** Immunoblot analysis of cyst fluid (CF) antigens from different *Taenia* metacestodes and cysticercosis diagnostic antigen (CyDA) against monospecific antibody. A recombinant 33-kDa protein and 10-kDa proteins in CFs from taeniid metacestodes including *Taenia solium* metacestodes (TsMs) show a specific reaction (solid arrowheads). The 15-kDa (star) and 7-kDa proteins (open arrowhead) of TsM CF also reveal a positive reaction. Lane a, CyDA; lanes b–g, CF antigens from, respectively, metacestodes of *T. solium*, *T. crassiceps*, *T. taeniaeformis*, *T. saginata*, *Echinococcus granulosus*, and *E. multilocularis*.  $M_r$ , molecular weight in kilodaltons.

ospecific antibody purified from patients with NCC by using CyDA. The proteins at ~10 kDa in CFs from all *Taenia* species, including *T. solium*, *T. saginata*, *T. crassiceps*, and *T. taeniaeformis*, were recognized by the monospecific antibody despite a minor difference in electrophoretic mobility, possibly due to their different degree of, or lack of, glycosylation [20, 23, 25]. The CF antigens from *E. granulosus* and *E. multilocularis* did not show a positive reaction at 10 kDa. This comparative immunoblot analysis confirms the common presence of the 10-kDa protein in genus *Taenia*. Notably, the 15- and 7-kDa bands in TsM CF were also reactive with the monospecific antibody, which further supports the presumption that the 10-kDa protein is a subunit of the 150-kDa complex of TsM CF [12, 20].

**Evaluation of the diagnostic value of CyDA.** To assess the diagnostic value of CyDA, we further tested its immunoreactivity by a large-scale immunoblot assay using individual sera from patients with various helminthic infections. A typical result of the immunoblot analysis is shown in figure 5. A strong recognition of CyDA was observed in 97% (184/190 cases) of sera from patients with active NCC who were examined, whereas only 14% (4/29 cases) of those from patients with chronic NCC showed a weak reaction. Weak cross-reactivity was observed in only 1 of each serum sample from patients with AE, sparganosis, and clonorchiasis cases (figure 5, panels C, E, and G, respectively). None of the sera from patients with other parasitic infections or from normal controls showed any antibody reactivity. Overall sensitivity and specificity of CyDA were determined to be 97% and 98%, respectively (table 2).

The serum antibody reactivity in patients with NCC, determined by immunoblot assay using CyDA, appeared to be relatively consistent with those determined by ELISA that employed the crude CF (figure 6).

## Discussion

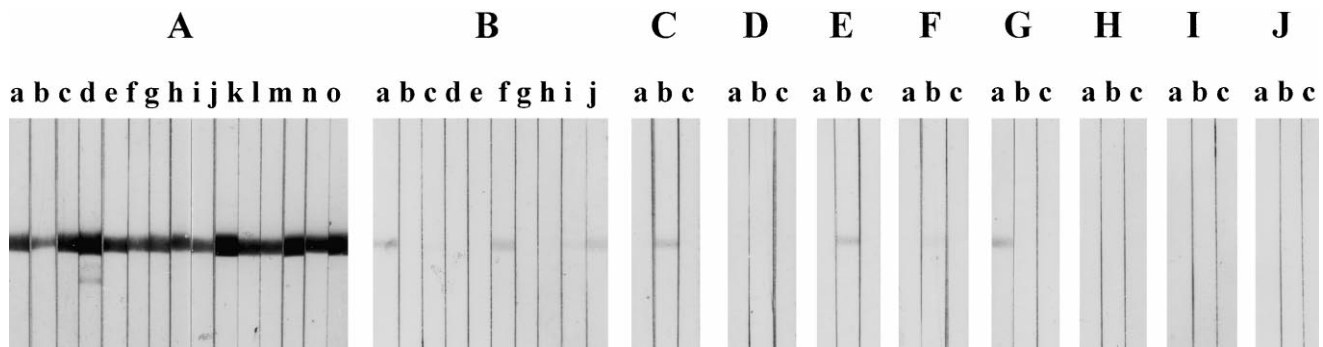
In this study, we have sequenced a cDNA encoding TsM 10-kDa protein and expressed it as a GST-fusion protein in *E.*

**Table 2.** Sensitivity and specificity of immunoblot analysis in sera from patients with neurocysticercosis and other parasitic infections, and normal controls, by use of the recombinant 10-kDa protein of *Taenia solium* metacestodes.

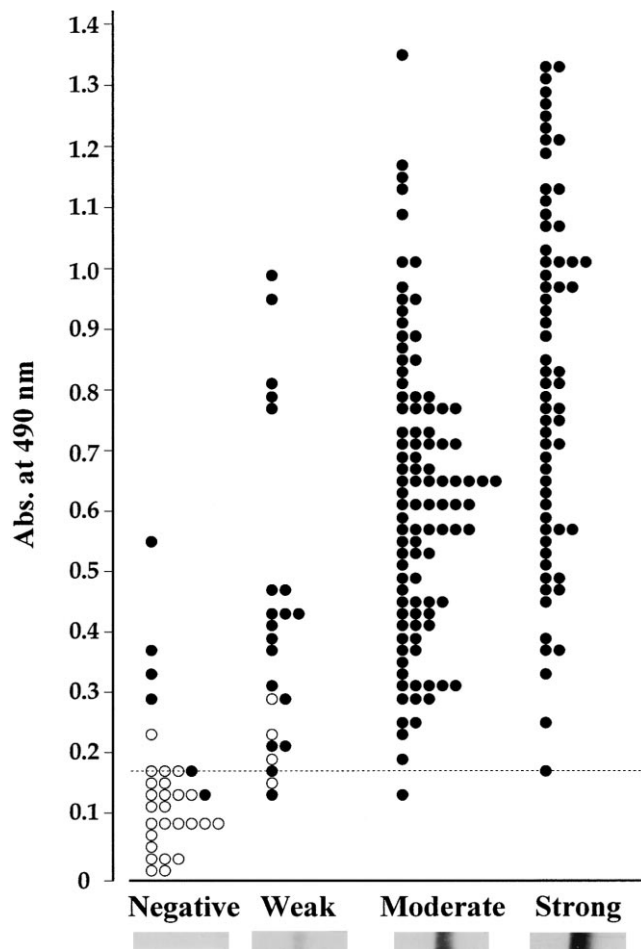
Parasitic infection or control group	No. of cases	No. (%) of positive reactions
Neurocysticercosis	213	188 (88)
Active neurocysticercosis	190	184 (97)
Chronic neurocysticercosis	29	4 (14)
Alveolar echinococcosis	11	1 (9)
Cystic echinococcosis	9	0 (0)
Sparganosis	30	1 (3)
Paragonimiasis	30	0 (0)
Clonorchiasis	30	1 (3)
Fascioliasis	10	0 (0)
Schistosomiasis	10	0 (0)
Normal controls	50	0 (0)

*coli*. Immunoblot analysis of the recombinant protein showed that it has a high potential for serological differentiation of active NCC not only from other parasitic infections but also from chronic inactive NCC.

Effective immunodiagnosis of parasitic diseases depends largely on the availability of well-defined specific antigens. In the case of human and swine cysticercosis as well as bovine cysticercosis (caused by *T. saginata* metacestodes), the difficulties experienced in collecting the metacestodes led researchers to attempt to employ cross-reactive antigenic components from more readily available taeniid cestodes as substitute antigens [23, 25–30]. Several studies have shown that a 10-kDa protein in CF of *T. hydatigena* metacestodes is useful for serological diagnosis of human NCC [25] and for bovine cysticercosis [27, 30]. The 10-kDa protein was subsequently found to be shared among metacestodes of *T. saginata* and *T. crassiceps* [23, 25, 31]. This is also true for TsMs [12, 15, 16] and is confirmed by our comparative immunoblot analysis, in which we employed CF antigens of various taeniid metacestodes, described in this study.



**Figure 5.** Immunoblot analysis of cysticercosis diagnostic antigen with sera from patients with various parasitic infections. Each strip was incubated with an individual serum sample from patients with active neurocysticercosis (NCC) (panel A), chronic calcified NCC (panel B), alveolar echinococcosis (panel C), cystic echinococcosis (panel D), sparganosis (panel E), paragonimiasis (panel F), clonorchiasis (panel G), fascioliasis (panel H), schistosomiasis japonicum (panel I), and normal controls (panel J). Strong positive reactions are shown only in cases with active NCC. Lanes a–o each represent a different patient.



**Figure 6.** Correlation between reactivity of serum in immunoblot assay with cysticercosis diagnostic antigen and serum in ELISA with the crude cyst fluid antigen. Horizontal dashed line indicates cut-off absorbance (abs.) at 0.18. The typical immunoblot responses of weak, moderate, and strong reactions are shown below the figure. ●, active neurocysticercosis (NCC); ○, inactive NCC.

The finding that the 10-kDa antigen of TsMs is highly useful for immunodiagnosis of NCC [12, 15, 16] has stimulated our interest in cloning the gene that encodes the protein. The common epitope shared between metacestodes of different *Taenia* species suggests that it might be possible to exploit the inherent antigenic similarity of a 10-kDa protein to clone the corresponding genes. We designed oligonucleotides on the basis of a published cDNA sequence of 10-kDa antigen of *T. crassiceps* and used them as PCR primers to amplify the homologous cDNA fragment. We obtained a 270-bp cDNA sequence and, by screening a TsM cDNA library with this fragment, isolated a 372-bp cDNA encoding a 10-kDa TsM protein.

Sequence analysis revealed that the 372-bp cDNA sequence was closely related to, but clearly different from, the cDNA sequence encoding the 10-kDa antigen of *T. crassiceps* metacestodes. The N-terminus of the deduced protein was shown

to contain a highly hydrophobic region, which might be the transmembrane domain that is possibly finished by signal peptidase [32] or might be a site for hydrophobic interaction with other molecules that have a biological reactivity other than specific antigenicity [25]. Cloning and characterization of the gene encoding a TsM 10-kDa protein may be an important contribution to the further definition of the genetic relationship among 10-kDa proteins shared in different *Taenia* species.

The major symptoms of active NCC were headache, seizure, and the symptom complex due to hydrocephalus and IICP, whereas those of chronic cases were seizure and headache. Presumably, persistent inflammatory reactions mounted by active immune surveillance of the host cause several symptoms associated with active NCC [33–35]. In chronic NCC, on the other hand, the long-standing inflammation caused by mummified worms served as an abnormal epileptogenic focus, stimulating seizures by intensive gliosis, or produced migraine-type headaches [8, 10, 24]. Differential diagnosis of active NCC from patients with chronic inactive cases is important to establish the appropriate treatment for affected patients, because symptoms due to chronic calcified NCC are not caused by the parasites per se, but by the sequelae of granuloma and residual calcifications, which did not respond to the anticysticercal drug [8, 10]. However, there are few serological methods currently available to assess worm viability in patients. The CyDA described in the present study showed high sensitivity and specificity in differentiating active from chronic NCC. Expression of a recombinant protein that provides an abundant source of high-quality antigen for immunoblot analysis has a definite merit [28].

In the present study, we have shown that CyDA appears to have a potential for the discrimination of active NCC from chronic inactive NCC. However, there were many patients in whom brain CT/MRI revealed both MLDs and calcifications ( $n = 29$ ), hydrocephalus with calcifications ( $n = 2$ ), or cystic mass with calcifications ( $n = 3$ ). These patients were difficult to identify as active or not active. In this study, we classified them as active cases according to criteria given elsewhere [8, 22]. Indeed, they all showed the positive reactions found in active cases, judged by immunoblot outcome with CyDA. There were 6 patients whose brain CT/MRI exhibited pathognomonic findings of the vesicular stage but who showed no reactions to CyDA (figure 6). Sera and CSF from these patients also showed relatively weak positive reactions to crude CF antigen by ELISA ( $0.32 \pm 0.18$ ; figure 1). This discrepancy between neuroimaging findings in patients with active-stage NCC and immunoblot analysis of sera, which shows low levels of specific antibody with no reactivity against CyDA, may argue against the high reliability of CyDA as a specific antigen for NCC. However, many studies have shown that some of NCC patients may undergo immune tolerance or decreased immunity [10, 36], which suggests that nonresponsiveness is not due to antigenic properties but to the immunological status of the infected in-

dividuals. Specific antibodies in these patients may react with other antigenic molecules of TsMs. Further elucidation of the natural course of the parasite infection according to infection stage and clinical course, and of immunological responses against the parasite, is required.

It will be of particular interest to investigate whether CyDA is useful for serological screening of TsM-infected pigs. Because *T. solium* splits its life cycle between humans and pigs, the control of porcine cysticercosis is crucial for control of human NCC [2, 4, 36]. We are currently analyzing the serum antibody responses against CyDA in TsM-infected pigs. Pilot experiments with several serum samples have shown encouraging results.

In conclusion, CyDA, as characterized in this study, offers promises of effective serological differentiation not only between NCC and other parasitic infections, but also between active and chronic inactive NCC. Its potential for serodiagnosis of TsM-infected pigs is proposed. We expect that our results shown here and ongoing studies may contribute ultimately to the successful control and prevention of NCC.

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