Identification of a new C-type lectin, TES-70, secreted by infective larvae of *Toxocara canis*, which binds to host ligands

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SUMMARY

Infective larvae of the dog roundworm *Toxocara canis* survive in the tissues of their hosts for extended periods in a state of developmental arrest, successfully evading immune destruction. This survival strategy is thought to be mediated by *T. canis* excretory/secretory (TES) products which downregulate or divert the immune response. We purified one of the major TES products, TES-70 and gained amino acid sequence from 4 tryptic peptides. These peptides were matched to a predicted protein from a cDNA that was isolated by expression screening a *T. canis* cDNA library with mouse anti-TES serum. The predicted protein (*Tc*-CTL-4) is similar to, but larger than, *Tc*-CTL-1, a 32-kDa C-type lectin secreted by *T. canis* larvae. *Tc*-CTL-4 has a signal peptide, 2 Cys-rich domains and a C-terminal calcium-dependent C-type lectin domain that shares sequence similarity with host immune cell receptors such as macrophage mannose receptor and CD23. The lectin domain was expressed in bacteria and antiserum to the purified recombinant protein was used to confirm that *Tc-ctl-4* did encode the native TES-70 glycoprotein. TES-70 selectively bound to ligands on the surface of Madin–Darby Canine Kidney cells *in vitro* in a calcium-dependent manner, inhibitable by mammalian serum, indicating that a host glycan is the native ligand for this new parasite lectin.

Key words: *Toxocara canis*, C-type lectin, secretory, ligand, immune evasion.

INTRODUCTION

Tissue-dwelling larvae of the ascarid nematode *Toxocara canis* infect a vast range of paratenic hosts including man, in which they are the aetiological agent of visceral and ocular *larva migrans* (Gillespie, 1993; Glickman, 1993). The seroprevalence of anti-*Toxocara* antibodies varies between 2 and 7% in Europe and North America (de Savigny, Voller & Woodruff, 1979; Van Knapen *et al*. 1983; Marmor *et al*. 1987), reaching much higher levels in children in developing countries (Thompson *et al*. 1986; Lokman Hakim *et al*. 1997). Infection is contracted by ingestion of infective eggs, which hatch in the stomach, releasing larvae capable of penetrating the intestinal mucosa. Larvae mature to adult worms only in the definitive, canid host. In other species, larvae survive for many years in the tissues (Lloyd, 1993) in a state of developmental arrest, remaining metabolically active and secreting antigens which are thought to assist the parasite in evading immune destruction (Smith *et al*. 1981; Badley *et al*. 1987*a*; Maizels, Gems & Page, 1993). This stage of the life-cycle can be exploited in the laboratory by maintaining parasites *in vitro* in serum-free medium for extended periods, where they release a defined set of excretory/secretory (TES) products (Maizels, de Savigny & Ogilvie, 1984; Meghji & Maizels, 1986; Badley *et al*. 1987*b*; Page, Hamilton & Maizels, 1992).

We have recently cloned and characterized a cDNA encoding an abundantly expressed TES protein, termed TES-32 or CTL-1 (Loukas *et al*. 1999) and shown that it possesses a C-type (calcium dependent) lectin domain at its C-terminus. C-type lectins (C-TLs) have a functional carbohydrate recognition domain (CRD) consisting of at least 4 (short-form) and sometimes 6 (long-form) conserved cysteine residues in a 110–130 amino acid motif (Drickamer, 1999). Most characterized proteins containing C-TL domains are cell surface receptors involved in immune cell signalling, activation and overall orchestration of immune processes (Weis, Taylor & Drickamer, 1998). As a result, pathogen-derived CTLs are of interest as potential mediators of interference or evasion of the host immune response.

The free-living nematode *Caenorhabditis elegans* has at least 125 C-TL-like domains encoded in its genome (The *C. elegans* Genome Sequence Consortium, 1999), making this protein domain the

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seventh most abundant motif found in *C. elegans*. However, many of these predicted proteins may have divergent functions, and sequence analysis indicates that only a minority are functional lectins (Drickamer & Dodd, 1999). Consequently, experimental confirmation of lectin-like activity is an essential step in characterization of new members of the gene family.

TES-32/CTL-1 is a confirmed functional lectin from *T. canis* and, interestingly, its CRD is more similar at the sequence level to mammalian C-TLs such as macrophage mannose receptor and the low-affinity IgE receptor CD23, than to its closest *C. elegans* homologue (Loukas *et al*. 1999). Structural modelling of the CRD of TES-32 based on the known structure of rat serum mannose-binding protein A (MBP-A) revealed a remarkable similarity of fold between the two domains, suggesting that TES-32 may have convergently evolved to bind host glycans, possibly ones involved in immunity, and thus allow the parasite to interfere with host immune cell signalling and trafficking.

Here we report on the cloning of a cDNA (*Tc-ctl-4*) encoding a 70-kDa TES protein with a C-TL domain that shares sequence similarity with TES-32 and contains an extra N-terminal cysteine-rich domain. In addition, we show that certain lectin-like proteins in TES products, notably that encoded by *Tc-ctl-4,* are capable of binding to mammalian ligands in a calcium-dependent manner, indicating that host glycans are indeed functional ligands for these parasite lectins.

MATERIALS AND METHODS

Parasites and parasite extracts

Live adult *T. canis* were recovered from naturally infected dogs and female worms were maintained in RPMI-1640 supplemented with penicillin (100 U/ml), streptomycin (100 μg/ml), gentamycin (25 μg/ml) and amphotericin B (2·5 μg/ml) for 5 days. Culture medium was replaced daily and eggs liberated into the medium were collected by centrifugation at 1000 ***g***. Eggs were hatched and infective larvae were maintained *in vitro* as described previously (Page *et al*. 1991). TES products were collected and concentrated using an Amicon 10 ultraconcentrator (Amicon) and stored at −80 °C. Protein concentrations were determined using the Bradford method.

Protein separation, digestion, mass spectrometry and sequencing

About 0·6 mg of TES products were radiocarboxymethylated (^{14}C-iodo[2-^{14}C] acetamide) yielding ca. 10^4 cpm per μg of protein, determined by amino acid analysis. After centrifugation (30 min, 10000 ***g***) and filtration (0·1 μm) the sample was separated on a SAX (strong anion exchange) column (2×20 mm, filled with source Q 15 μm, Pharmacia, Germany) using a gradient for elution [100% 20 mmol Tris–HCl, pH 8·0, 20% acetonitrile (ACN) for 10 min, rising over 40 min to 1 M NaCl in the same buffer] at a flow rate of 200 μl/min and detection was carried out at 220 nm and tryptophan-fluorescence (excitation 295 nm/emission ⩾ 310 nm).

The fraction containing TES 70 was concentrated on a 10 kDa ultrafiltration unit and run on a 10% polyacrylamide gel. After Coomassie brilliant blue staining a single band corresponding to TES 70 was excised and as control an empty gel piece was used. Gel pieces were washed in several changes of water for about 6 h, and dried in a Speedvac evaporator. Dried gel pieces were incubated with 0·01 μg/μl trypsin (modified, sequencing grade; EC 3.4.21.4; Boehringer, Germany) in 100 mmol/l Tris–HCl, pH 8·5, 10% ACN for 18 h at 37 °C and resulting peptides desalted on a short C18 column (1·6×20 mm) using 0·1% trifluoric acid (TFA)/water/ACN. After lyophilization and resolubilization with 0·1% TFA in water, the samples were analysed by matrix-assisted laser desorption/ionization time of flight mass spectrometry (MALDI-TOF-MS) and were separated on a C18 column (1×250 mm; Kromasil 10 nm, 5 μm; MZ-Analysentechnik, Mainz, Germany). Elution was carried out with a gradient from 100% 0·1% TFA in water, then 2–12% of 0·08% TFA/ACN in 10 min and to 62% in 50 min and to 100% 0·08% TFA/ACN in 10 min at a flow rate of 50 μl/min. Peak-fractions were analysed by MALDI-TOF-MS (Voyager-RP: PerSeptive Biosystems, Framingham, MA, USA) equipped with a nitrogen laser emitting at 337 nm. One μl of peptide solution was mixed with 1 μl of matrix solution [15 mg/ml 2,5-dihydroxybenzoic acid, 15 mg/ml methoxy-hydroxybenzoic acid in 0·1% (v/v) TFA, 60% ACN, 9+1 (v/v)] and allowed to air-dry. Peptides were amino-terminally sequenced by automated Edman degradation on an Applied Biosystems (Foster City, CA, USA) pulsed-liquid-phase sequencer, Model 477A or 471A, under standard conditions. Phenylthiohydantoin derivates of amino acids were identified by an on-line analyser, model 120A or 140B (Applied Biosystems).

Expression screening of a larval T. canis cDNA library

A cDNA library made from mRNA of *T. canis* infective larvae cloned into the λ-phage vector Uni-ZAP XR (Stratagene) was a generous gift from Drs C. Tripp and R. Grieve (Heska Inc., Fort Collins, Colorado, USA). The library was used to infect *E. coli* strain XL-1 blue and plated at semi-confluency onto 9 cm agar plates containing tetracycline

(12·5 μg/ml) according to the manufacturer's instructions (Stratagene PicoBlue Immunoscreening protocol). Bacteria were grown at 42 °C until plaques were just visible, then plates were overlaid with nitrocellulose filters pre-soaked in 10 mM IPTG and incubated at 37 °C for 3 h to induce production of recombinant proteins. Filters were then removed, rinsed in phosphate-buffered saline (PBS) and blocked overnight in 5% skimmed milk powder in PBS at 4 °C. Filters were rinsed twice with PBS/0·05% Tween-20 (PBS-T) and probed with mouse antiserum raised to TES products. To raise anti-TES antiserum, BALB/c mice were immunized with 10 μg of TES products emulsified in Freund's complete adjuvant followed by 2 boosts at days 28 and 42 with 10 μg of TES in Freund's incomplete adjuvant, administered via the subcutaneous route; sera were collected at day 49 and pooled. Primary antiserum was pre-cleared of antibodies to *E. coli* according to the protocol recommended by Stratagene and diluted 1:1000 in PBS-T/3% skimmed milk before use. Filters were incubated in primary serum at room temperature for 2 h with gentle rocking then washed 3 times in PBS-T for 15 min before being incubated with horseradish peroxidase-conjugated rabbit anti-mouse IgG (BioRad; diluted 1:2000 in PBS-T/3% skimmed milk) for 1 h at room temperature. Filters were washed as before and developed using tetramethylbenzidine (TMB) substrate (Kirkegaard and Perry Laboratories).

Isolation of positive clones and in vivo *excision of phagemids*

Putative positive plaques were cored from the agar plate, phage eluted from the agar plug and replated for secondary screening according to the manufacturer's instructions (Stratagene). Individual plaques that were positive by secondary screen were cored out and phage DNA rescued *in vivo* using ExAssist™ helper phage and non-suppressing *E. coli* strain SOLR™ (Stratagene). Plasmid containing the insert was prepared from bacterial cultures using the Qiagen spin miniprep kit (Qiagen).

Gene sequencing and analysis

Double-stranded *Tc-ctl-4* cDNA was sequenced using universal forward and reverse primers and internal gene-specific primers. Sequencing was performed using the AmpliTaq DyeDeoxy Terminator Cycle Sequencing System (Applied Biosystems) and an automated ABI Prism 377 DNA sequencer (Applied Biosystems). Analyses of nucleotide and deduced amino acid sequences were performed using the MacVector 6.0 software program (Oxford Molecular). Database searches were performed using the BLAST server to search a non-redundant set of databases (Altschul *et al*. 1990). Sequence alignments were performed using the GCG PILEUP program (Genetics Computer Group) and Boxshade server. The translated protein sequence was analysed using the Analyse Signalase program (Eastman Kodak Company) in order to identify a putative signal peptide and corresponding cleavage point (von Heijne, 1986). The nucleotide sequence of *Tc-ctl-4* has been deposited in GenBank, accession no. AF126830.

Expression of recombinant protein

Primers were designed to clone the entire coding region of *Tc-ctl-4* minus the putative signal peptide (Gly-19–Ala-288) into the pET-15b vector (Novagen; contains an N-terminal hexahistidine tag) using the restriction enzyme sites for *Nde*I and *Bam*HI. The CRD alone was also cloned into the vector (Cys-150–Ala-288) using the same restriction sites. Amplicons were generated by PCR and purified using Microcon 100 spin microconcentrators (Amicon), cloned into the vector and the ligated products were transformed into competent *E. coli* strain XL1-blue which lacks the T7 RNA polymerase gene; plasmids from cells carrying the insert were then transformed into *E. coli* strain BL21 (Novagen) carrying the DE3 episome, which expresses T7 RNA polymerase. Protocols followed were as outlined by the manufacturer (Novagen). Recombinant proteins (rCTL-4 – aa 19–288, full length mature protein; rCRD-4 – aa 150–288, CRD only) were soluble only in the presence of 6 M urea in binding buffer (20 mM Tris, pH 7·9/0·5 M NaCl/5 mM imidazole). Bacterial lysates containing the proteins were applied to a nickel chelate resin (His Bind Resin; Novagen) and washed with the same buffer containing 60 mM imidazole. Bound protein was renatured on the column by washing with 20 ml of Tris-buffered saline (TBS) before eluting in non-denaturing elution buffer (same as above but 1 M imidazole). Eluted proteins were dialysed against 2 changes of TBS/5 mM $CaCl_2$ over 24 h. Recombinant proteins precipitated out of solution despite numerous attempts at refolding after the removal of urea. Additional parameters were manipulated in an attempt to maximize expression including induction temperature (30 °C), growth times post-induction (1, 2 and 3 h) and the use of different bacterial strains (BL21-DE3 and DE3-pLys), but none resulted in increased protein expression. Further attempts at expression of this construct were not pursued due to the successful production of recombinant CRD (rCRD-4) alone. In addition, antiserum raised to rCRD-4 proved to be highly specific for the native TES protein (see Results section).

Immunization of mice

BALB/c mice were immunized with precipitated rCRD-4 (aa 150–288, approx. 10 μg) emulsified in

Table 1. Mass and sequence analysis of TES-70 tryptic peptides and comparison with the corresponding predicted masses from *Tc*-CTL-4 protein

(Pep, amino acid sequence determined by Edman degradation. Residues in parentheses were uncertain assignations; dashes indicate no residue could be identified. Bold typeface denotes identity with sequence deduced from cDNA. DNA, amino acid sequence deduced from nucleotide sequence of cDNA. Bold typeface denotes identity with sequence deduced from Edman degradation.)

Peptide	Sequence		Corresponding amino acids	Actual mass (Da)	Predicted mass (Da)
1	Pep:	**GVQ** (N) **YWLGLN**	Gly 211–Arg 221	1307·7	1307·5
	DNA:	**GVQ** T **YWLGLN**			
2	Pep:	**QFGQWVWT-G**	Gln 222–Arg 240	2325·5	2324·6
	DNA:	**QFGQWVWT**N**G**			
3	Pep:	**GSEPDG** (GV) **GG** (V) **VT-ALV** (**N**) **YA**	Gly 241–Lys 284*	4873·7	4927·3
	DNA:	**GSEPDG** CC **GG** N **VT**C**ALV N YA**			
4	Pep:	**NPQGFICK**	Asn 277–Lys284	964·2	964·5
	DNA:	**NPQGFICK**			

* Mass data suggest that peptide 3 terminates at Lys-284 rather than the expected tryptic cleavage site at Arg-276. The discrepancy of 53·6 between predicted and actual masses, and the loss of tryptic cleavage, can be accounted for by a Arg → Thr substitution at position 276 (predicted difference 55·1). The presence of peptide 4, resulting from tryptic cleavage at position 276, indicates that a polymorphism may exist at this residue. Note that the cDNA library and the purified TES-70 protein are derived from different isolates of *T. canis*.

Freund's complete adjuvant followed by 2 boosts at days 28 and 42 with approx. 10 μg of rCRD-4 in Freund's incomplete adjuvant, administered via the subcutaneous route. Sera were collected at day 49. rCTL-4 (full-length protein) was produced at extremely low levels by bacteria and the amount of protein purified was insufficient for immunization purposes.

Western blots

rCTL-4 (0·2 μg), rCRD-4 (0·2 μg), recombinant *Tc*-CTL-1 (TES-32; 0·2 μg, (Loukas *et al*. 1999)) and TES products (2·5 μg) were probed with sera from mice immunized with either TES, rCRD-4 or rCTL-1, as well as normal mouse serum (diluted 1:1000) followed by horseradish peroxidase-conjugated rabbit anti-mouse IgG (diluted 1:2000; BioRad). All incubations were carried out at room temperature for 1 h interspersed with 3 washes using PBS-T. Blots were developed using the chemiluminescent substrate ECL (Amersham).

Incubation of TES products with canine kidney epithelial cells

Thirty μg of TES products were labelled with ^{125}Iodine (IMS.30; Amersham) using the Iodogen method according to established protocols (Maizels *et al*. 1991). Madin–Darby canine kidney (MDCK) cells were maintained in Dulbecco's medium containing penicillin (100 U/ml), streptomycin (100 μg/ml), 2 mM L-glutamine and 10% foetal calf serum (FCS) at 37 °C with 5% CO_2. Cells were grown in 12-well tissue culture plates in a volume of 1 ml/well and were depleted of FCS 18 h before addition of TES products by incubating in 2 changes of serum-free medium. ^{125}I-TES products (9×10^7 cpm) were added to each well containing confluent, serum-depleted cells in the presence of 20 mM $CaCl_2$ or 20 mM EDTA (final concentrations). In some cases, ^{125}I-TES products were pre-incubated for 1 h with either FCS (final concentration of 10% v/v) or an equivalent amount of haemoglobin (non-glycosylated control protein). Cells were incubated with ^{125}I-TES products at 37 °C overnight. Culture supernatants were collected the following day and all cells were recovered (adherent cells by scraping and non-adherent cells by centrifugation) and washed 3 times with media containing either $CaCl_2$ or EDTA, then resuspended in 50 μl of reducing SDS–PAGE loading buffer, boiled and electrophoresed. Gels were dried and exposed to autoradiography film overnight before developing.

RESULTS

Purification and peptide sequencing of TES-70

TES-70 is a major glycoprotein secreted by the long-lived tissue phase infective larvae of *T. canis*, with an apparent M_r of 70 kDa on gradient SDS–PAGE. To identify the molecular nature of this product, we excised the band corresponding to TES-70 from SDS–PAGE and subjected it to tryptic digestion for amino acid sequence and peptide mass analyses. Four peptides were isolated, partial sequences determined by Edman degradation, and their mass values identified as presented in Table 1. None of the peptides matched exactly any known *T. canis*

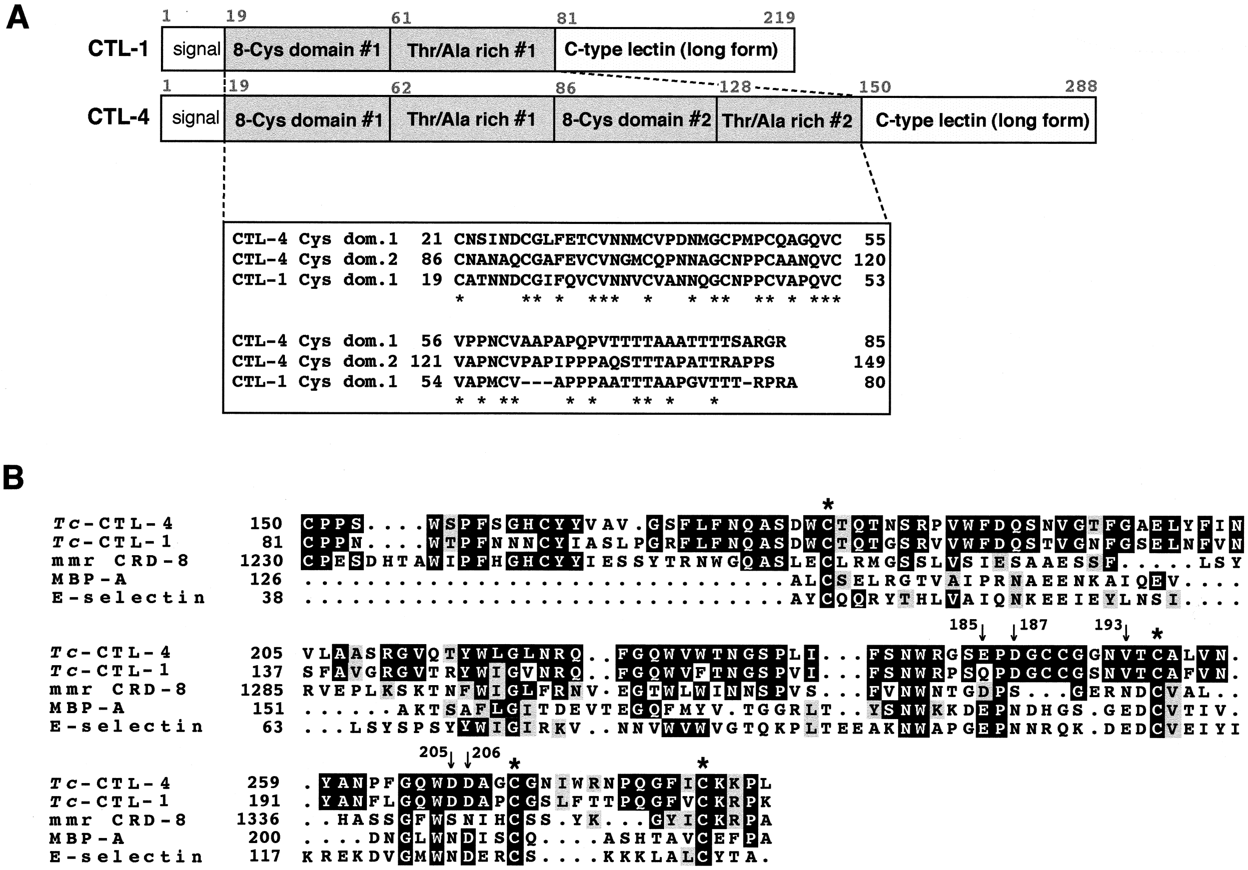

Fig. 1. Sequence similarity of Tc-CTL-4 with other C-type lectins. (A) Schematic representation comparing the predicted *Tc*-CTL-4 and *Tc*-CTL-1 proteins and alignment of the cysteine-rich and threonine rich domains of the 2 proteins. Residues identical to all 3 domains are highlighted with asterisks, and deletions with dots. (B) Alignment of the carbohydrate recognition domains (CRDs) of *Tc*-CTL-4 with *Tc*-CTL-1 and mammalian homologues. Residues shared by CTL-4 and any other sequence are boxed (black for identical, grey for conservative changes). The 4 cysteines found in all C-type lectin CRDs are marked with an asterisk and the 5 residues involved in ligating calcium and saccharides (Weis *et al.* 1992) are highlighted with arrows and numbered according to MBP-A. GenBank accession numbers are as follows: *Tc*-CTL-4 – AF126830; *Tc*-CTL-1 – AF041023; human macrophage mannose receptor – M93221; rat MBP-A – M14105; human E-selectin – M24736.

sequence, or any of the Expressed Sequence Tag deposits made from this parasite (Tetteh *et al.* 1999). However, the TES-70 peptides were similar to sequences from TES-32, identified as the C-type lectin CTL-1 (Loukas *et al.* 1999), and to 2 partial C-type lectin sequences revealed by EST analysis which are variants of CTL-1, named CTL-2 and -3 (Tetteh *et al.* 1999). The sequence information from TES-70 suggested that it is encoded by a distinct gene belonging to the C-type lectin superfamily.

Tc-ctl-4 *cDNA encodes the secreted TES-70 glycoprotein*

A cDNA library derived from *T. canis* larvae was then screened with anti-TES serum to identify additional TES proteins not discovered by the EST approach. This screen revealed an immunopositive clone with an open reading frame that exactly matched 3 of the 4 peptides from TES-70, and showed one discrepancy with the fourth. The discrepancy would be explained by a substitution of Arg → Thr at residue 271 in some, but not all, of the native protein sample, abolishing a tryptic cleavage site and altering the peptide mass (Table 1). The full sequence of the cDNA clone, *Tc-ctl-4*, was then determined.

Tc-ctl-4 is 1044 nucleotides including 21 bp of 5′ and 138 bp of 3′ UTR from the TGA stop codon to the polyA tail (GenBank accession no. AF126830). The cDNA encodes a protein of 288 amino acids including a putative signal peptide of 18 residues, 2 cysteine-rich domains each with threonine-rich tails, and a long-form C-TL C-terminal domain starting at residue 150. The predicted M_r of the protein (excluding the signal peptide and any post-translational modifications) is 28·4 kDa.

The predicted protein (*Tc*-CTL-4) shares significant sequence similarity with *Tc*-CTL-1 but differs most markedly in the doubling of the cysteine-rich domain, of which *Tc*-CTL-1 has only 1 (Fig. 1A). Each 40 amino-acid domain contains 8 cysteine residues, but with no reported homologues outside of *T. canis*; its structure and function remain

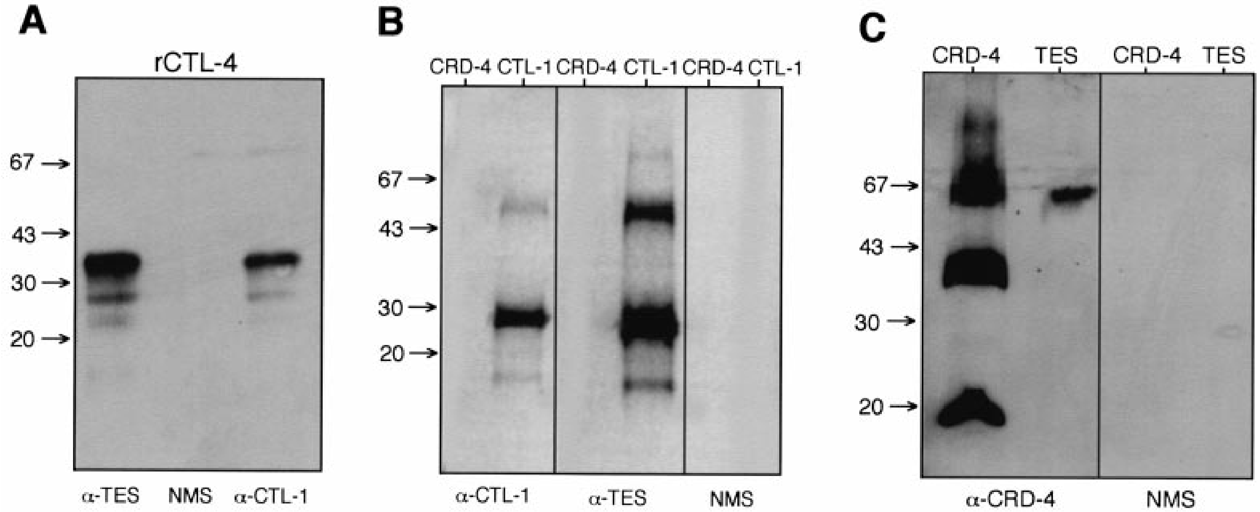

Fig. 2. Western blot confirmation of identity of *Tc*-CTL-4 and TES-70. Purified proteins/extracts in each lane are listed at the top of the panels, and the antiserum used to probe each sample is identified beneath the panels. (A) Recognition of rCTL-4 (aa 19–288, full-length protein minus signal peptide) by antiserum to *Toxocara canis* excretory/secretory (TES) antigens and anti-rCTL-1 (TES-32) serum. NMS, normal mouse serum. (B) Absence of recognition of rCRD-4 (carbohydrate recognition domain of CTL-4, aa 150–288) by anti-TES serum and anti-rCTL-1 serum, indicating that the immunodominant epitopes are in the N-terminal region of the protein. NMS, normal mouse serum. (C) Recognition of rCRD-4 and TES-70 (from native TES products) by antiserum raised to rCRD-4, and non-reactivity of normal mouse serum (NMS).

unknown. The short threonine-rich tracts may act as scaffolds for *O*-glycosylation and there are 2 potential *N*-glycosylation sites at Asn-230 and -251. The C-terminal portion of CTL-4 contains a carbohydrate-recognition domain (CRD) with close similarity to the previously reported CTL-1 (TES-32). Over this part of the molecule (Fig. 1B) the 2 *T. canis* ES proteins share 69% identity (96/139 residues), including the unusual double-cysteine loop adjacent to the predicted sugar-ligating site of CTL-1 (Loukas *et al*. 1999). However, the contact residues Glu-Pro-Asp (EPD, 185–187 according to MBP-A numbering) are not identical to CTL-1 (which has Gln-Pro-Asp, QPD at this site), indicating that the specificity of CTL-4 is likely to be quite different.

Further confirmation that *Tc-ctl-4* encodes TES-70 was sought by testing antibodies to the recombinant lectins and CRDs. Antibodies to TES and to rCTL-1 (TES-32) were cross-reactive with the recombinant CTL-4 fusion protein (Fig. 2A), suggesting that an anti-rCTL-4 serum was unlikely to uniquely recognize that protein. However, anti-rCTL-1 and anti-TES did not react with the separately expressed rCRD of CTL-4 (aa 150–288, named rCRD-4) as shown in Fig. 2B. Antiserum was therefore raised to rCRD-4, which reacted with rCTL-4 (data not shown), rCRD-4 and to native TES-70 on Western blots (Fig. 2C). rCRD-4 migrated with an M_r of 20 kDa on SDS–PAGE, however, additional bands were seen at 40 and 60–70 kDA (Fig. 2C); these probably represent dimers and trimers of the overexpressed and incorrectly folded recombinant protein. As the CRDs of CTL-1 and CTL-4 share greater amino acid identity than the cysteine-rich regions (69% vs. 56%), these results suggest that the latter contains an immunodominant determinant, a conclusion supported by recognition of the N-terminal Cys-rich region but not the CRD of CTL-1 by an anti-TES-32 mAb Tcn-3 (A. Loukas and R. Maizels, unpublished observations). An alternative explanation for the lack of reactivity by anti-TES and anti-CTL-1 sera to rCRD-4 is that epitopes on the latter are destroyed by denaturation or become unavailable on binding to nitrocellulose.

Multiple TES products bind to host epithelial cells

An important question to test for parasite lectins is whether their ligands are host- or parasite-derived. In the absence of correctly folded and functional recombinant lectin (rCRD-4 precipitated from solution when denaturants are removed), we addressed this by testing ^{125}I-TES binding to the canine epithelial cell line, MDCK, in the presence or absence of exogenous calcium or chelating agent. Products with an M_r of 35 and 70 kDa notably bound to MDCK cells in Ca^{2+}-dependent manner, and additional weaker bands could also be discerned (Fig. 3). All binding was abolished in the presence of EDTA, when FCS or normal canine serum were included in the culture medium (results not shown) or when TES products were pre-incubated with an equal amount of FCS before being added to cells.

Other abundant TES proteins with confirmed identities such as TES-120, a family of mucin-like

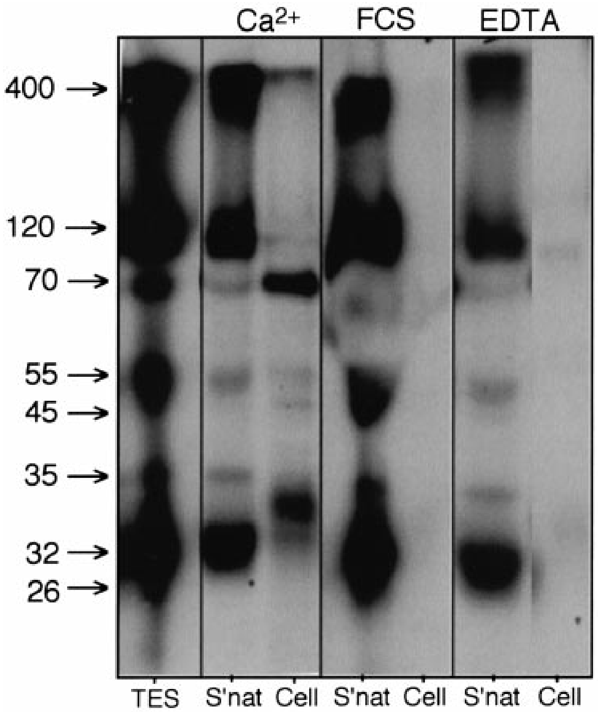

Fig. 3. Binding of TES-70 and other TES products to ligands on MDCK cells and in foetal calf serum in a calcium-dependent fashion. Whole iodinated *Toxocara canis* excretory/secretory products (TES), shown in the left hand lane, incubated with MDCK cells in the presence of 20 mM calcium (Ca^{2+}), 20 mM EDTA or pre-treated with foetal calf serum (FCS) in the presence of 20 mM calcium. In each of the panels, SDS–PAGE analysis is presented of TES proteins remaining in the supernatant after incubation with cells (S'nat) and of the TES proteins that remained attached to cells after washing (Cell).

glycoproteins (Page & Maizels, 1992; Gems & Maizels, 1996; Loukas *et al*. 1999), and TES-26, a phosphatidylethanolamine-binding protein (Gems *et al*. 1995) did not bind to MDCK cells, providing an internal control. Pre-incubation of TES products with haemoglobin as a control non-glycosylated protein did not significantly inhibit binding to cells (data not shown). FCS that had been dialysed (10 kDa cut-off membrane) to remove free sugars still inhibited binding of TES products to MDCK cells but the dialysate did not (data not shown), indicating that TES lectins bind to glycoproteins rather than free simple sugars in FCS. Cells fixed with glutaraldehyde were also probed with ^{125}I-TES products, resulting in an identical pattern of binding (data not shown), indicating that TES proteins bind to the surface of the cells and are not internalized by metabolizing cells.

DISCUSSION

C-type lectins are a well-documented family of proteins in mammals, and play diverse roles in physiological and immunological processes. By binding to defined glycans usually expressed on cell surface glycoproteins, C-TLs play a pivotal role in orchestrating the immune response (Weis *et al*. 1998). The crystal structures and mechanisms of ligand binding have been determined for some lectins including MBP-A (Weis, Drickamer & Hendrickson, 1992), E-selectin (Graves *et al*. 1994) and the C-TL-like domain of CD94 from NK cells (Boyington *et al*. 1999). C-TLs have, however, only recently been reported from parasitic helminths, the first mRNA being *Tc-ctl-1* from *T. canis* (Loukas *et al*. 1999). Here we show that larval *T. canis* express at least 1 more member of the C-type lectin family, *Tc-ctl-4*, and that the protein product of this mRNA encodes the excretory/secretory glycoprotein TES-70. In addition, some TES products, notably TES-70, bind to host proteins in a calcium-dependent manner, indicating that host serum and/or cell surface glycans are ligands for these lectins.

There is a significant discrepancy in M_r between the predicted secreted CTL-4 protein (28·4 kDa) and the actual M_r of TES-70 as determined by SDS–PAGE. Previous work suggests both *N*- and *O*-linked sugars are indeed present on TES-70 (Page & Maizels, 1992) and these could account for the discrepancy between predicted and actual, post-translational molecular weights. It is also possible that post-translational modifications of native TES-70 might result in the formation of an irreversible dimer.

While TES-32 was shown to bind weakly to monosaccharides in a calcium-dependent manner (Loukas *et al*. 1999), TES-70 was not found to bind these simple sugars, suggesting that the ligand bound by CTL-4 is different from that of CTL-1/TES-32. Moreover, in neither case is the native ligand known, although evidence presented here shows selective binding of TES-70/CTL-4 to serum and/or cell surface ligands. These differences are likely to reflect substitutions at key substrate binding/determining residues. As a general rule, Gln-Pro-Asp (QPD) is found in galactose/GalNAc-binding C-TLs, while Glu-Pro-Asn (EPN) is found in mannose/GlcNAc-binding members (Weis *et al*. 1992; Kolatkar & Weis, 1996). CTL-1 has QPD, supporting its ability to bind to galactose-type sugars but CTL-4 has EPD, with acidic residues at both variable positions. Although this motif gives no clue as to the likely ligand for CTL-4, the ability of this lectin to bind to presumably complex mammalian glycans provides an experimental route to define a host ligand recognized by a *T. canis* secreted protein.

Since the discovery of TES-32/CTL-1 as a C-type lectin, we have found that *T. canis* larvae may be secreting multiple carbohydrate-binding proteins. An EST analysis recorded 2 further sequences designated CTL-2 and -3 (Tetteh *et al*. 1999), and peptide sequencing of TES-70 revealed a similar but novel protein we have now characterized as

CTL-4. Preliminary peptide sequence data indicates that TES-55 represents yet another related lectin (A. Loukas and M. Hintz, unpublished observations), while binding assays to monosaccharide columns (Loukas *et al*. 1999) and serum and/or canine cells (reported here) indicate that additional carbohydrate-binding proteins remain to be identified. It would therefore seem that *T. canis* secretes a barrage of C-TLs, some at least of which are capable of binding to mammalian ligands.

C-TLs secreted by tissue-dwelling nematode larvae could perform a diverse range of functions. Much of the existing literature on C-TLs deals with proteins involved in immunity, and it is not illogical to suggest that parasite homologues might combat the immune response by competitively inhibiting host immune cell lectins from recognizing their ligands, thus interfering with adhesion and/or signalling pathways.

Some bacteria use surface proteins containing CRDs to bind to host cells subsequent to invasion (Ofek & Sharon, 1988; Maganti *et al*. 1998), and the protozoan parasites *Giardia intestinalis* (Ward *et al*. 1987) and *Entamoeba histolytica* (Talamas-Rohana *et al*. 1995) both have surface lectins involved in adhesion to intestinal brush border cells, the former being calcium dependent. Interestingly, T cells from mice immunized with the L220 lectin from *E. histolytica* were unable to proliferate but selectively secreted T helper 2 (Th2) cytokines in response to parasite antigen (Talamas-Rohana *et al*. 1995). This finding is typical of helminth infections where Th2 responses and (in cases such as filariasis) proliferative suppression accompany chronic infections (Allen & Maizels, 1996; Finkelman *et al*. 1997; Maizels, Allen & Yazdanbakhsh, 2000). Host-derived CTLs can selectively drive the immune response; at sites of tissue inflammation, P- and E-selectin bind to Th1 and not to Th2 cells, imposing selective recruitment and thus skewing the type of T helper response generated (Austrup *et al*. 1997). Based on these findings, parasite-derived C-TLs might well be instrumental in driving the Th2 response seen in helminth infections.

The explosion of nematode EST projects recently has seen the identification of genes encoding protein families previously unrecognized in this phylum. A number of ESTs encoding C-TLs from parasitic nematodes have now been identified in *Necator americanus* (Daub *et al*. 1999) as well as *T. canis* (Loukas *et al*. 1999; Tetteh *et al*. 1999). In addition, an unexpectedly large range of C-TLs have been predicted within the *C. elegans* genome (The *C. elegans* Genome Sequence Consortium, 1998; Drickamer & Dodd, 1999). All of these data indicate that this protein family is highly utilized by both free-living and parasitic helminths. The studies presented here will provide a platform from which to investigate the biological role of *T. canis* C-TLs and determine if and how they enable the parasite to survive for such long periods of time in the host without being destroyed by the immune system.

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