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Molecular and functional characterization of a recombinant protein of *Trichuris trichiura*

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The pore-forming protein of the human whipworm, *Trichuris trichiura*, has been postulated to facilitate invasion of the host gut and enable the parasite to maintain its syncytial environment. The data presented here describe the first, to our knowledge, molecular characterization of a pore-forming protein in any helminth and provide a unique demonstration of the functional interaction between a parasite antigen and host molecules. Immunological screening of a *T. trichiura* cDNA library with *T. trichiura* infection sera identified a clone of 1.4 kB, the cDNA consisting of 1495 base pairs encoding a protein of 50 kDa. The sequence has a highly repetitive nature containing nine four-disulphide-bonded core domains. Structural prediction analyses reveals an amphipathic nature. TT50 induced pore formation in bilayers in a manner identical to that of the native protein. IgG antibody isolated from *T. trichiura* infection serum was observed to abolish channel activity.

Keywords: pore-forming protein; syncytium; *Trichuris*

1. INTRODUCTION

Intestinal nematodiases are among the most prevalent of all chronic human infections with the major species, *Trichuris trichiura*, *Ascaris lumbricoides* and the hookworms, *Necator americanus* and *Ancylostoma duodenale*, currently estimated to infect over 1000 million individuals (Bundy *et al*. 1997). Given the global prevalence of these parasitic infections and their impact upon world health (Bundy & Cooper 1989; Bundy *et al*. 1997), surprisingly little is known of the molecular interaction between parasite and host. Here we describe the characterization of a recombinant antigen of the human whipworm, *Trichuris trichiura*, and report upon the effect of the action of host immune molecules on protein function.

Previously, we hypothesized that TT47, the secreted pore-forming protein of *T. trichiura*, is of functional significance in the establishment and subsequent maintenance of the parasitic habit within the host (Drake *et al*. 1994), the anterior portion of the adult worm remaining embedded within a syncytial tunnel derived from host caecal epithelium. We suggest that this tunnel is formed first by intracellular secretion of TT47 by the larval stage and then by intra-syncytial secretion of the protein by the adult worm. The activity of TT47 may also contribute to the pathological consequences associated with severe trichuriasis: studies indicate the importance of plasma protein leakage across the gut mucosal surface in disease pathogenesis (Cooper *et al*. 1992).

A pore-forming protein (TM43) has also been identified in the secretions of congeneric *T. muris* from the mouse (Drake *et al*. 1994). TM43 and TT47 are not only biochemically similar, but also possess highly comparable ionic permeability characteristics. Antibody raised against TM43 was shown to abolish channel activity in lipid bilayers. Furthermore, HPLC-purified TM43 elicited a degree of protective immunity comparable to that induced by whole worm homogenate in susceptible mice. These data suggest that an immune response generated against TM43 is central to the rapid expulsion of the parasite from resistant murine hosts (Wakelin 1967; Drake *et al*. 1994).

To expand our understanding of the function of TT47 in human infection, the present study adopted a recombinant DNA approach, enabling us to study the gene encoding the protein and to analyse the relationship between structure and function. We present data on the molecular characterization of TT47 and upon the electrophysiological and immunological activity of the expressed gene product, TT50.

Nucleotide sequence data reported in this paper have been submitted to the GenBank/EMBL database with the accession no. S47040.

2. MATERIALS AND METHODS

(a) *SDS-PAGE analysis*

Electrophoresis was performed according to Laemmli (1970). A BioRad Mini Protean II system and 10% acrylamide gels were used. Samples to be electrophoresed were added to an equal volume of sample buffer (125 mM Tris, pH 6.8, 40 g l^{-1}

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SDS, 200 g l^{-1} glycerol, 0.42 g l^{-1} Bromophenol blue). When reducing conditions were desired, 100 mM β-mercaptoethanol was added before incubation at 100 °C for 5 min before loading.

(b) *Western blotting*

After electrophoresis, proteins were transferred to nitrocellulose paper (Schleicher & Schuell Inc., catalogue no. BA85) using a discontinuous buffer system and semi-dry blotting apparatus for 2 h at 194 mA. The efficiency of the transfer was confirmed using Ponceau S solution (Sigma, UK), and the blotted membrane blocked overnight at 4 °C in 5% foetal calf serum (FCS) in Tris saline/Tween (TBS; 10 mM Tris, 14 mM NaCl, 0.05% Tween 20, pH 8.5). The membrane was then incubated with pooled sera taken from *T. trichiura*-infected individuals diluted in 5% FCS/TBS at 1/200 for 2 h. For each blot, negative reference sera was included, together with controls to assess the specificity of the reagents. Membranes were washed with TBS for 3 × 20 min, and incubated with peroxidase-labelled anti-human IgG (Dako, UK) diluted in 5% FCS/TBS at 1/1000 overnight at 4 °C. Membranes were washed extensively as before, with a final wash in PBS alone. Peroxidase labelling was detected by chemoluminescence using ECL (Amersham).

(c) *Construction of planar lipid bilayers*

Phospholipid bilayers (diphytanoylphosphatidylcholine, Avanti Polar Lipids) were constructed according to the techniques described by Montal & Muller (1972) and that described by Schindler (1980). Two Teflon chambers (0.1 ml capacity) were connected by an aperture (10–20 μm thick Teflon film. KCl at 0.1 M concentration containing 0.005 M HEPES, pH 5.2, was added to both chambers. Currents were measured at room temperature (21–27 °C) with Ag/AgCl electrodes monitored with a virtual grounded operational amplifier with a feedback resistor of 10^9 Ω. The electrode connected to virtual ground was in the chamber (*cis*) to which protein was added, and voltage signs refer to this chamber; at positive potentials cations flow form *cis* to *trans*. Currents were displayed on an oscilloscope and on a physiograph recorder; they were recorded by using a Biologic DTR-1202 digital audio tape recorder and subsequently analysed by using Patch-clamp software written in LabView, National Instrument®.

(d) *cDNA library screen*

The adult *T. trichiura* cDNA library was constructed in Lambda Zap II by Professor A. E. Bianco (Imperial College, London). The library was grown in *Escherichia coli* XL1 Blue cells (according to the Stratagene cDNA Synthesis Kit, catalogue no. 200 400) and overlaid with IPTG (isopropyl-β-D-thio-galactosidase)-impregnated nitrocellulose membranes (Schleicher & Schuell Inc.). Filters were screened with pooled human sera containing *T. trichiura*-specific antibodies diluted 1:200 in 5% FCS, TBS and *E. coli* lysate (BioRad, catalogue no. 170-3205) according to the method described by Huynh *et al.* (1985). Reactivity was detected by horseradish peroxidase (HRP)-conjugated rabbit anti-human IgG secondary antibody (Dako, catalogue no. P214) diluted 1:2000. Positive clones were rescreened to purity. Purified plaques were excised using the Stratagene protocol (catalogue no. 200 400), the resulting phagemid was grown up and DNA extracted using standard methods (Sambrook *et al.* 1989). The DNA was subsequently sequenced by the dideoxynucleotide termination method described by Sanger *et al.* (1977) using the Sequanase version 2 protocols and reagents (United States Biochemical Co.). The nucleotide and derived amino acid sequences were analysed using the Lasergene Software programme (DNAstar) and Seqnet programmes (Daresbury). The EMBL/GenBank database (version 1997) was screened for DNA and amino acid sequence similarity.

(e) *Production and purification of recombinant TT50*

AD494 (DE3) pLysS cells (Novagen) were transformed with the pET-23a vector containing the full coding sequence according to the manufacturer's instruction. Positive clones were grown in 1 l cultures (10 g tryptone, 5 g yeast extract, 10 g sodium chloride, 100 mg ampicillin, 100 mg chloramphenicol per litre). Cultured cells were induced with 0.4 mM IPTG for 2 h, harvested by centrifugation at 5000 × *g* for 5 min and resuspended in 40 ml ice-cold Binding Buffer (His-Bind Buffer Kit component, Novagen, catalogue no. 69755-1). The cells were lysed by freeze-thawing and sonication for 1 min (6 × 10 s pulses). The lysate was centrifuged at 39 000 × *g* for 20 min. The supernatant was passed over a His-Bind metal chelation resin (Novagen, catalogue no. 69670). TT50 bound to the metal chelation resin. The column was washed extensively with Wash Buffer (Buffer Kit component). Bound proteins were released using Elute Buffer (Buffer Kit component). Protein concentration in the eluate was determined with a Bio-Rad Protein Assay Kit.

(f) *Isolation of IgG antibody from human infection serum*

Human sera previously identified as eliciting a strong IgG antibody response to TT47 by ELISA (Needham *et al.* 1993) were pooled together. The IgG fraction was isolated by affinity chromatography using HiTrap® Protein G (Pharmacia Biotech) according to the manufacturer's instructions.

3. RESULTS

A *T. trichiura* cDNA library was screened using human sera previously identified as eliciting a strong antibody response to TT47 (Needham *et al.* 1993; Brooks 1996). This rational strategy allowed the identification of a cDNA clone which was subsequently sequenced. The nucleotide sequence of the TT50 cDNA was determined directly from a Bluescript excision clone by the dideoxynucleotide chain termination method (Sanger *et al.* 1977). The sequence was found to have an open reading frame of 1425 base pairs encoding a 475-amino acid protein having a calculated mass of 50 kDa (figure 1). The open reading frame ends at nucleotide 1435 with a TGA stop codon.

Analysis of the translated sequence identified a number of internal repeat regions (figure 1, box highlighted). Spacing of the cysteine residues within these internal repeat regions was found to be highly conserved (figure 1, bold type). A comparison of the protein sequence with the Prosite Database of patterns (DNAstar) identified homology with the four-disulphide-bonded core domain (as illustrated in table 1). This motif homology was additionally identified after comparison of the protein with the NBRF-PIR Database using the BLAST programme. This was the only homology identified. In addition, cross-species DNA hybridization was not evident by Southern blot analysis using various nematode species (data not shown; Brooks 1996). These results indicate TT50 to be a novel protein sequence.

MTDPSPLLIGLAICLLQGIQDAESAKVGR**C**PSPPFGAGRARY**C**YTDRQ**C**PGRMK**CC**LTKRGYA
CTAPDISYPDADKQKPGT**C**PPSNMFTGTAPF**C**NSDTD**C**KGTEK**CC**LTKAGHQ
CVQPNQTPRPIAKKGS**C**PPKPIGAVGLANF**C**QNDYD**C**DGSMK**CC**LTNVGYD
CKAPVQESEEVQKPGS**C**PAVPAVTGKALF**C**RSDKD**C**DRSEK**CC**LTKVGKE
CVQPTDKPKPIAKKGS**C**PPTPVGPVGLANF**C**QTDYD**C**NGSMK**CC**LTTIGYD
CKAPVQESEEVQKPGS**C**PAAPAITGKALF**C**RNDND**C**DGREK**CC**STKVGKE
CVQPVRWPGPNAKPGS**C**PPSPVGAVGLASF**C**QSDFD**C**MGYQK**CC**ITTAGYE
CTHPVDENMRQSKRGI**C**PWPSSVQGPVVL**C**NTDDD**C**EGSQK**CC**PTKAGSG
CVNAVSEEMASGRVS**C**PPAAYVMFLQGS**C**RRDED**C**DDGKK**CC**DGNGRKS
CSVLVLRS

Figure 1. Primary translation sequence of recombinant TT50. Amino acids are given by their one letter code. The polypeptide sequence is 475 amino acids in size. The putative N-terminal signal sequence is displayed underlined and in bold type. Internal repeat regions are box encased. The conserved nature of the cysteine residue spacing is highlighted by bold-type C. Consensus asparagine-linked glycosylation sites are indicated by asterisks.

Table 1. *Comparison of the gross structure of the nine four-disulphide core domains of TT50 and other proteins carrying this structural motif*

((Garczynski & Goetz 1996 (unpublished: GenBank accession number U67854); Drenth *et al*. 1980; Stetler *et al*. 1986; Kirchhoff *et al*. 1991). The regions are aligned according to the spacing of the cysteine residues, which are illustrated in bold-type C. The amino acid spacing between the cysteines is also given.)

four disulphide core domain	**C**xx (**D**/**N**) xx**C**xxxxx**CC**
domain 1	
TT50	**C**xx**D**xx**C**Pxxx**K****CC**xxxxGxxC
trout ovulatory protein (Top2)	**C**xx**D**xD**C**Pxxx**K****CC**xxxCGxxC
anti-leukoproteinase (ALP-1)	**C**xx**D**xD**C**Pxxx**K****CC**xxxCGxxC
whey acidic protein (WAP)	**C**xx**N**xx**C**xxxxx**CC**xxxCGxxC
epidymis specific protein (HE4)	**C**xx**D**xx**C**xxxxK**CC**xxxCGxxC
other domains	
TT50 (2–8)	**C**xx**D**xD**C**xxxxK**CC**xxxxGxxC
TT50 (9)	**C**xx**D**xD**C**xxxxK**CC**xxxxxxxC
Top2 (2–4)	**C**xx**D**xD**C**Pxxx**K****CC**xxxCxxxC
ALP-1 (2)	**C**xx**D**xD**C**Pxxx**K****CC**xxxCxxxC
WAP (2)	**C**xx**D**xD**C**xxxxK**CC**xxxCxxxC
HE4 (2)	**C**xx**D**xx**C**Pxxx**K****CC**xxxCGxxC

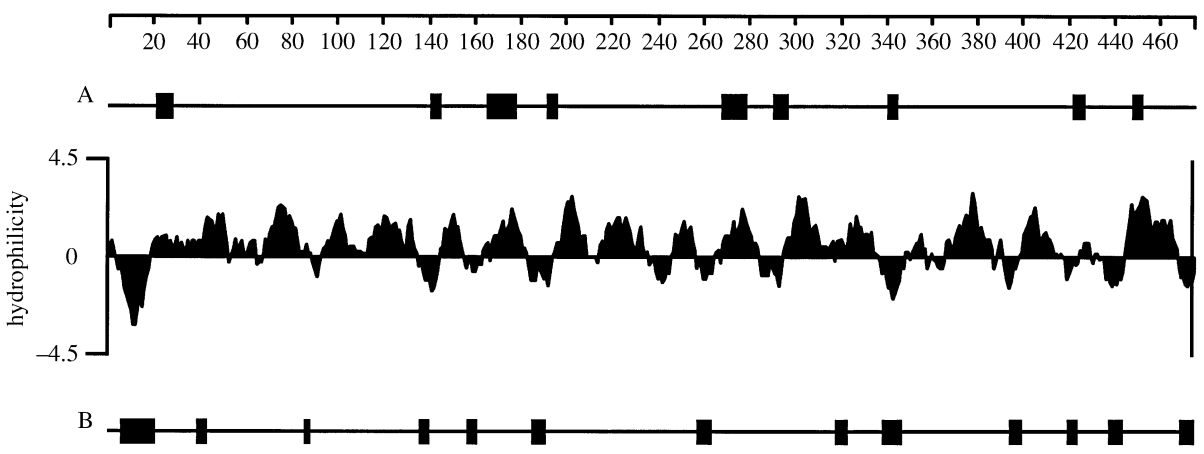

Figure 2. A Kyte–Dolittle hydropathicity plot is shown. Regional hydrophilicity is represented graphically by shaded areas positioned above the zero line and regional hydrophobicity by areas positioned below the zero line. Hydropathy values were averaged over an 11-residue window. Results of a Chou–Fasman structural prediction analysis are also illustrated. Filled boxes positioned along line A represent distinct α-helical domains. Filled boxes positioned along line B represent distinct β-sheet domains. The amino acid residues corresponding to these regions are given in numerical form at the top of the illustration.

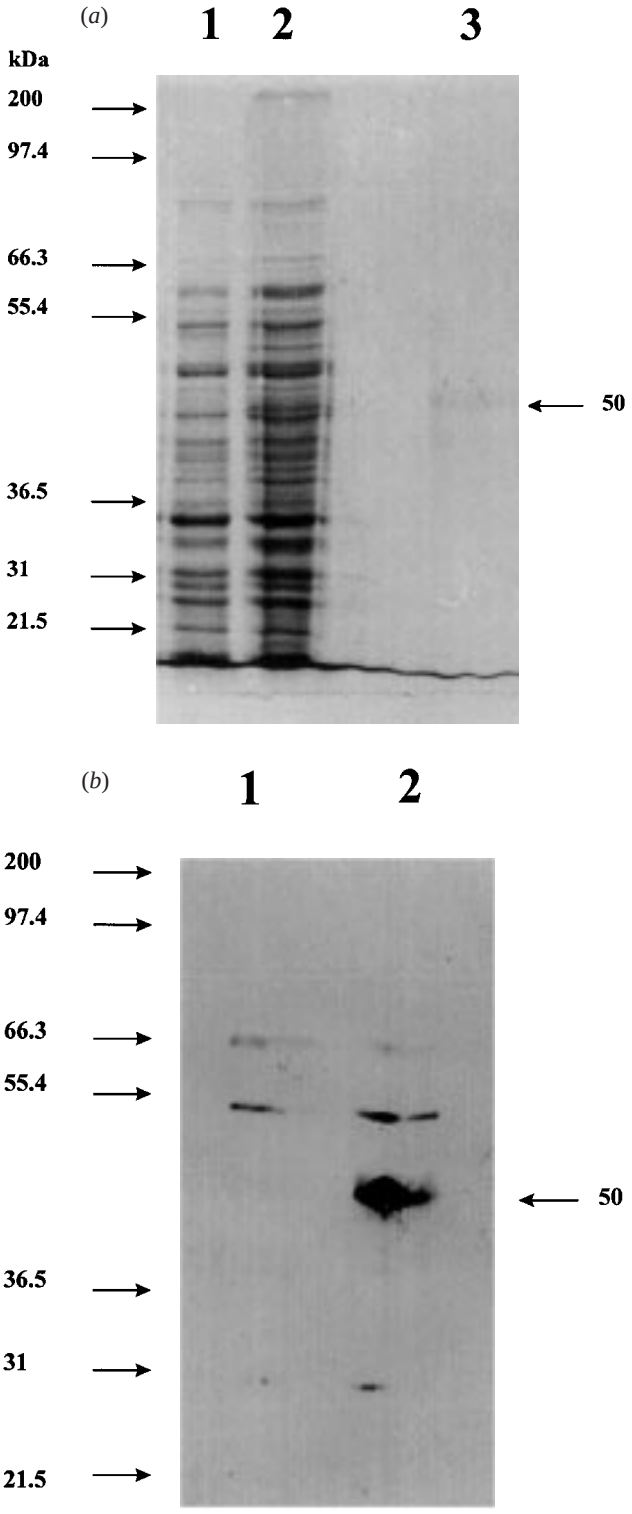

Figure 3. (*a*) SDS-PAGE analysis of AD494 (DE3) pLysS cell lysates containing the pET-23a vector before induction (lane 1) and after induction with 0.4 mM IPTG (lane 2). Lane 3 contains the protein species purified from induced cell lysates by nickel chelate affinity chromatography. Proteins were visualized using Brilliant Blue G-Colloidal stain (Sigma). (*b*) Western blot of AD494 (DE3) pLysS cell lysates containing the pET-23a vector before induction (lane 1) and after induction with 0.4 mM IPTG (lane 2), separated on SDS-PAGE, transferred to nitrocellulose and probed with *T. trichiura* infection sera.

Hydropathicity analysis suggests a distinct amphipathic configuration commencing with an 18-amino acid hydrophobic stretch (figure 2). This is consistent with the presence of an N-terminal signal sequence, as predicted by the Von Heijne method (1983). The Chou–Fasman secondary structure prediction method indicates that TT50 is a member of the Alpha+Beta class of proteins (according to the Chou classification method) containing both α-helix and β-sheet structures (figure 2). The repetitive nature of the primary translation sequence and the predicted secondary molecular structure give an indication as to the highly ordered nature of this molecule, which is suggestive of specific function.

There is little difference between the electrophoretic M_r of the native and recombinant proteins, suggesting that TT47 undergoes little post-translational modification. Previous studies have shown that TT47 is not heavily N-glycosylated (Drake 1993). In concurrence, there are only two potential N-glycosylation sites at asparagines 120 and 254 of the deduced amino acid sequence, as indicated by asterisks in figure 1 (Bause 1983).

Induction of transformed AD494 (DE3) pLysS cells with IPTG resulted in the production of a novel protein of 50 kDa. This protein was the only species in induced cell lysates that could be purified by nickel chelate affinity chromatography (figure 3*a*, lane 3). Antisera collected from a number of *T. trichiura*-infected individuals strongly recognized this recombinant protein (figure 3*b*, lane 2). By contrast, sera from uninfected UK individuals did not recognize TT50 (data not shown).

To ascertain whether TT50 possessed pore-forming activity, the recombinant protein was added to a planar lipid bilayer system. The formation of TT50-induced ionic channels is illustrated in figure 4*a*. Single-channel conductance ranged between 10 pS and 100 pS; small channels appeared before larger channels on most occasions. Although single-channel conductance remained similar at both positive and negative voltages, the probability of channels being in a conducting state at negative voltages was lower. Therefore, to quantify the observed rectification, current was only measured after steady state was obtained at each voltage point tested (figure 4*b*). The insert represents the switch in polarity at 120 mV, illustrating the voltage dependence of opening and closure of TT50-induced channels. These characteristics are highly similar to those of native protein-induced channels (Drake *et al*. 1994).

Addition of heat-inactivated human infection serum to the *cis* chamber of bilayers treated with recombinant antigen was observed to partly reduce TT50-induced current (figure 5, trace A). A subsequent addition of sera to the *trans* chamber resulted in a further decrease in current (figure 5, trace A). The membrane rapidly progressed to a non-conducting state and no further channels were formed. A similar effect was observed by addition of sera to the *trans* chamber alone (figure 5, trace B). Addition of purified IgG antibody alone was shown to have a similar effect on induced current (figure 5, trace C), indicating that the observed inhibition was due to specific binding of antibody to TT50 and not to the action or presence of other serum components. Addition of pooled UK negative sera to either chamber did not

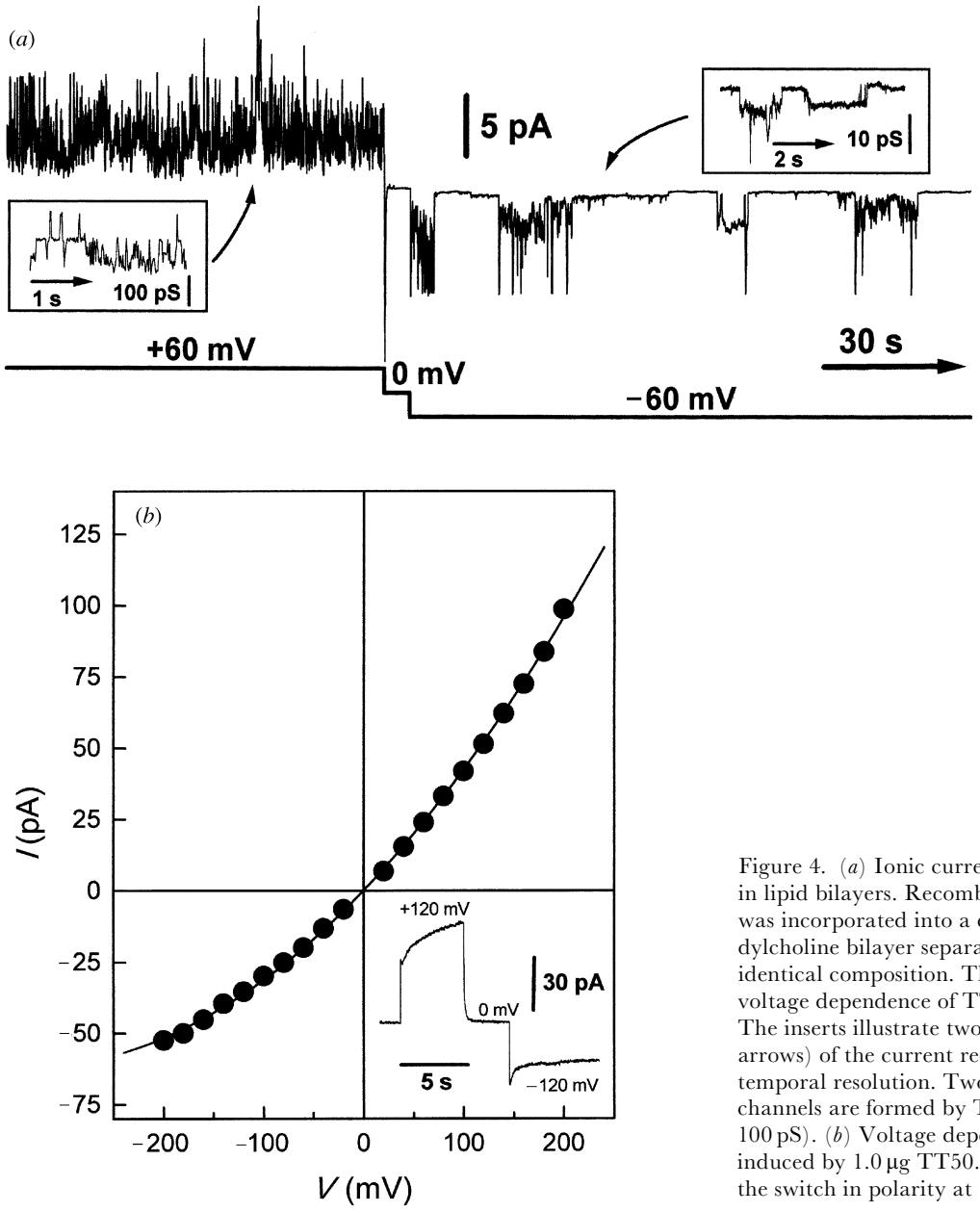

Figure 4. (*a*) Ionic currents induced by TT50 in lipid bilayers. Recombinant protein (1.0 µg) was incorporated into a diphytanoylphosphatidylcholine bilayer separating two chambers of identical composition. The figure shows the voltage dependence of TT50-induced channels. The inserts illustrate two sections (indicated by arrows) of the current record with higher temporal resolution. Two different conductance channels are formed by TT50 (*ca.* 10 and 100 pS). (*b*) Voltage dependence of currents induced by 1.0 µg TT50. The insert represents the switch in polarity at 120 mV.

result in any alteration in channel activity (data not shown).

4. DISCUSSION

Although we provide no direct evidence that TT50 is a recombinant protein of TT47, the body of indirect evidence is substantial and does suggest that this is indeed the case: TT50 was demonstrated to form pores in lipid bilayers qualitatively and quantitatively similar to those induced by TT47. This has been shown to such an extent that both proteins induce channels with identical anion selectivity with a calculated *t*-value of 0.64 (Drake *et al.* 1998). TT50 is also recognized by *T. trichiura*-infection sera, but not by UK negative sera. In addition, native and recombinant proteins also share a number of distinct biochemical characteristics: (i) both proteins are of a similar molecular weight and display reduction sensitivity under electrophoretic conditions, increasing in apparent M_r by approximately 10 kDa; this migration anomaly is due to the presence of intramolecular disulphide bonds, a biochemical feature common to other pore-forming proteins (Bernheimer & Rudy 1986; Leippe *et al.* 1992); (ii) the amino acid composition and isoelectric point of TM43 (Drake 1993) and TT50 are very similar; and (iii) the presence of an N-terminal signal sequence (as predicted by the Von Heijne method) confers the capacity for TT50 to be translocated through cellular export systems and thus may be subsequently secreted. Attempts to produce direct evidence by probing with anti-TT50 serum have been hindered by the high degree of cross-reactivity between this molecule and other *T. trichiura* proteins. We now have evidence that TT50 is part of a multigene family, the members of which possess similar epitopes (Barker & Bundy 1998). This could explain the observed cross-reactivity.

Structural prediction analyses indicate that the secondary structure of TT50 has a highly ordered amphipathic nature with both α+β regions and nine four-disulphide-bonded core domains. These structural motifs are common to many proteins of widely differing functionality, including a number of proteinase inhibitors

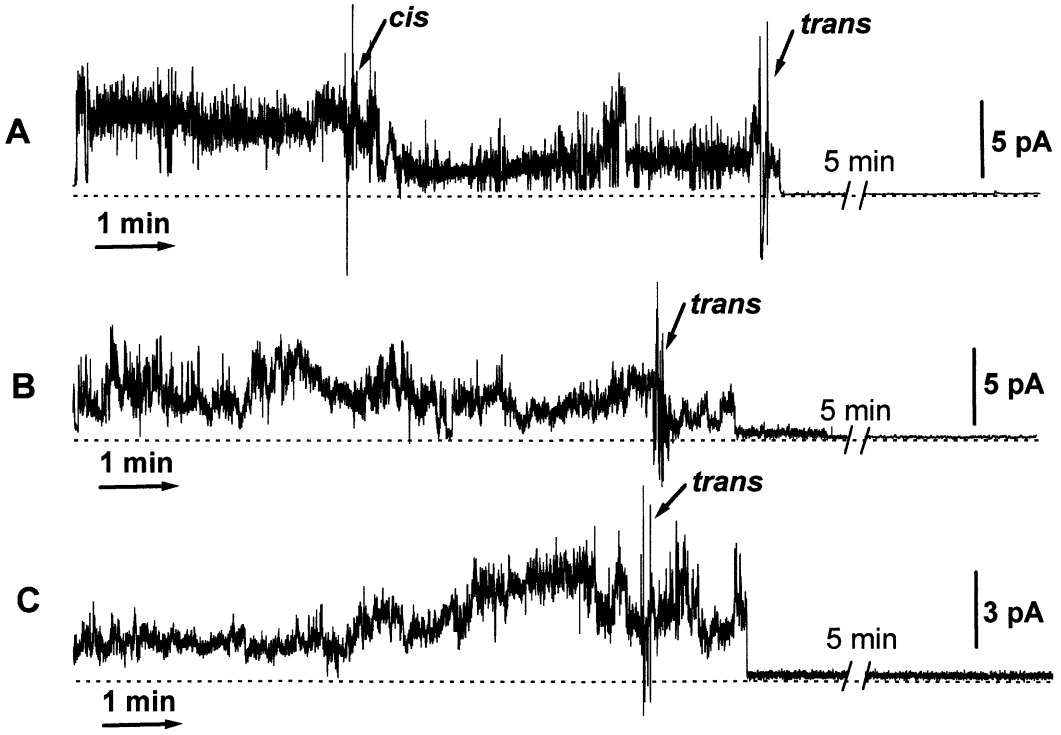

Figure 5. Effect of antibody on TT50-induced channels. Addition of 10 µl of heat-inactivated *T. trichiura*-infection sera to the *cis* chamber (indicated by the arrow, trace A). Addition of heat-inactivated sera to the *trans* chamber (indicated by the arrows, traces A and B) and, addition of purified IgG antibody to the *trans* chamber alone (trace C). Current was measured with respect to applied potential of +50 mV. Dotted line indicates zero current.

(Seemuller *et al*. 1986). Such linkages are known to confer stability and structural integrity upon favourable conformational arrangements (Drenth *et al*. 1980). TT50 is unusual in possessing nine core domains: most proteins so far identified with this structural motif possess only one or two such domains (refer to table 1). This is perhaps an indication of the importance of structural integrity and the maintenance of correct molecular orientation with regards to TT50 function.

The predicted structure of TT50 is consistent with the secondary structure of other known pore-forming molecules (Lakey *et al*. 1991; Li *et al*. 1991; Bontems *et al*. 1991; Leippe *et al*. 1992). An amphipathic configuration (either an α-helix or a β-sheet) appears to be an essential prerequisite to pore formation, even small alterations in critical regions can markedly alter membranolytic capacity (Steiner *et al*. 1988; Leippe *et al*. 1992). The observation that many pore-forming proteins are water soluble suggests that these proteins must undergo conformational changes to expose potential membrane spanning regions. It is hypothesized that these changes may be initiated by a specific receptor-binding event or by electrostatic interactions with charged phospholipids in the membrane (Massotte *et al*. 1989; Li *et al*. 1991, 1996). Alternatively, the membrane spanning regions may be masked in solution by an oligomerization event, dissociation occurring upon membrane binding thus exposing the hydrophobic domains to the membrane. Interestingly, under non-reducing conditions, Western blot analyses detect an additional seroreactive body (>60 kDa) when TT50 is incubated in the presence of *T. trichiura*-infection sera, indicating a degree of protein aggregation (data not shown).

Previous work has demonstrated antigenic cross-reactivity using sera from either human or mouse host species (Roach *et al*. 1988; Else *et al*. 1989). We have shown previously that anti-TM43 antibody was able to close TM43-induced channels but not TT47-induced channels, suggesting a highly worm- or host species-specific interaction (Drake *et al*. 1994). These observations led us to hypothesize that although the proteins are related on a structural basis, the antibody–antigen interaction has a high level of specificity, which is essential to correct antibody functioning. The present results confirm that human IgG antibody purified from *T. trichiura*-infection serum is able to abolish TT50-induced channel activity. This may indicate a mechanism by which the human immune response can regulate infection intensity.

Many characteristics of TT50-induced channels, including voltage dependency, rectification properties, conductance states and their fast, 'noisy' transitional nature, are all highly comparable to those of channels induced by native-state proteins (Drake *et al*. 1994). A detailed electrophysiological characterization of TT50-induced channel activity has now been completed (Drake *et al*. 1998), providing further evidence of the similar nature of these *Trichuris* channel-forming proteins.

The apparent paradox that channel activity was markedly reduced by addition of antibody to the *trans* side of the lipid bilayer may simply reflect the unusual *in vivo* situation of the adult worm. *Trichuris* larvae penetrate epithelial cells and undergo intracellular moults during a growth phase which creates a host-cell derived syncytial tunnel around the thread-like anterior end of the worm (Bundy & Cooper 1989). We hypothesize that this tunnel is formed by first intracellular then intra-syncytial secretion of TT47,

and that maintenance of the parasitic habit requires continuous secretion and tunnel formation owing to the rapid turnover of the mucosal epithelium. TT47 will be orientated through the membrane during pore formation, but it is the binding epitopes on the *trans* side of the membrane that are exposed to host antibodies in serum or gut secretions. In addition to our electrophysiological investigations, we have now demonstrated that TT50 is able to lyse caecal epithelial cells *in vitro* (L. J. Drake, Y. Korchev, A. Shevchuk, M. Lab and D. Bundy, unpublished observations).

This paper describes, to our knowledge, the first molecular and functional characterization of a recombinant pore-forming protein in any helminth parasite and provides a unique demonstration of the functional interaction between parasite and host immune molecules. Given the apparent importance of TT47 to parasite survival, it is possible that recombinant TT50 may serve as a potential vaccination target.

We gratefully acknowledge the support of the Wellcome Trust in the laboratory investigations. Y.K. and M.L. were supported by the Garfield Weston Trust.

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As this paper exceeds the maximum length normally permitted, the authors have agreed to contribute to production costs.