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A putative serine protease among the excretory–secretory glycoproteins of L1 *Trichinella spiralis*

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Abstract

Trichinella spiralis first-stage larvae infect susceptible hosts by invading epithelial cells that line the small intestine. During this process the larva disgorges several glycoproteins that bear an unusual, highly antigenic sugar moiety, tyvelose (3,6-dideoxy arabinohexose). Monoclonal antibodies specific for tyvelose protect the intestine against infection, implicating tyvelose-bearing glycoproteins as mediators of invasion and niche establishment in the intestinal epithelium. In order to investigate these glycoproteins at the molecular level, we first prepared monoclonal anti-peptide antibodies. The antibodies bind a family of glycoproteins that are present in excretory–secretory products of first-stage larvae and are delivered to epithelial cells during invasion by *T. spiralis*. The major species present in an affinity purified fraction of crude *T. spiralis* antigens were subjected to tryptic peptide digestion. De novo amino acid sequencing of the peptides using Q-TOF tandem mass spectrometry, in combination with database searches and antibody screening of an L1 cDNA library, showed that the glycoproteins are variably glycosylated homologues of the serine protease family.

Keywords: *Trichinella*; Nematode; Mass spectrometry; Serine protease; cDNA; Monoclonal antibodies; Peptides

1. Introduction

The parasitic nematode, *Trichinella spiralis*, demonstrates an unusual life cycle that is completed in a single host and is largely intracellular. Infection is initiated with the consumption of meat contaminated with first-stage larvae. Larvae invade the epithelium of the small intestine where they mature to the adult stage, mate, and produce live-born larvae. Newborn larvae enter blood and migrate to striated muscle, where they invade myotubes. Larvae grow and mature to the infectious L1 stage, completing the life cycle. The L1 excretes and secretes several glycoproteins that bear an unusual, highly immunogenic sugar moiety, tyvelose (3,6-dideoxy arabinohexose) [1]. These glycoproteins are released by mature L1 in muscle [2,3] and also when larvae invade the intestinal epithelium [4,5]. Indirect evidence suggests that these secreted proteins may play important roles in parasitism. For example, monoclonal antibodies specific

Abbreviations: ANOVA, analysis of variance; AO, Albino Oxford; cDNA, complementary deoxyribonucleic acid; dgESP, deglycosylated excretory–secretory products; DPBS, Dulbecco's phosphate buffered saline; EDTA, ethylenediaminetetraacetic acid; ELISA, enzyme-linked immunosorbent assay; ESP, excretory–secretory products; FBS, fetal bovine serum; IPTG, isopropyl-1-thio-β-D-galactopyranoside; mAb, monoclonal antibody; MDCK, Madin Darby Canine Kidney; MEM, minimal essential medium; MS, mass spectrometry; PAGE, polyacrylamide gel electrophoresis; Q-TOF, quadrupole orthogonal acceleration time of flight; RACE, rapid amplification of cDNA ends; SDS, sodium dodecyl sulfate; TFMS, trifluoromethanesulfonic acid; TspSP-1, *Trichinella spiralis* serine protease 1.

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for tyvelose bind to hypertrophic nuclei in parasitized muscle cells [6,7], suggesting a role for the associated glycoproteins in maintaining the muscle niche. These same antibodies protect the intestine against infection, implicating tyvelose-bearing glycoproteins as mediators of invasion and niche establishment by L1 in the intestinal epithelium [8,9].

Although a few excretory–secretory products (ESP) have been identified, defined functions for any of the glycoproteins in the muscle or intestine are unknown. In order to investigate ESP at a molecular level, we prepared monoclonal anti-peptide antibodies and used them to characterize a family of glycoproteins that are delivered to epithelial cells during invasion by *T. spiralis*. De novo peptide sequencing, using a proteomics strategy based on the Q-TOF mass spectrometer, combined with traditional cDNA library screening with antibodies, and database searches for sequence homologies, identified a highly processed member of the serine protease family.

2. Materials and methods

2.1. Rats

Albino Oxford (AO) strain rats were produced and maintained in the James A. Baker Institute vivarium in accordance with the guidelines of the American Association for Accreditation of Laboratory Animal Care.

2.2. Antibodies

Rat mAb 18H is specific for tyvelose on *N*-glycans of ESP [10]. Rat mAb 6G3 is specific for phosphorylcholine on *N*-glycans of L1 proteins not found in ESP [11]. Antibody 10E binds adult *T. spiralis* [12] and was a gift from R.G. Bell (Cornell University).

2.3. Parasites and protein preparations

T. spiralis (pig strain) infectious larvae were recovered from muscles of irradiated, AO strain rats by digestion with 1% pepsin in acidified water [13]. Donor rats had been infected at least 28 days prior to collection. Adult worms were recovered from intestines of AO strain rats 5 days following oral infection with L1. For in vitro experiments, L1 were activated using pig bile or gut contents as described previously [5].

Crude homogenates of larvae, L1 ESP, and crude homogenates of adult worms were prepared as described previously [14]. Larval ESP were deglycosylated by treatment with trifluoromethanesulfonic acid (TFMS) [6].

2.4. Production of monoclonal antibodies against deglycosylated L1 ESP

An AO rat was immunized intraperitoneally (i.p.) with 50 μg of deglycosylated ESP (dgESP) in complete Freund's adjuvant and boosted i.p. after 52 days with 50 μg dgESP in incomplete Freund's adjuvant. About 35 days later 50 μg dgESP was administered intravenously. Splenic lymphocytes were harvested for fusion after 3 days and fused with mouse myeloma cells (SP2/O-Ag14) at a ratio of 2:1 [15]. Hybridomas were screened by ELISA with ESP or dgESP coupled to the plate (5 or 2.5 μg ml^{-1}) according to methods we have described previously [8]. Cells of interest were cloned by limiting dilution. One hybridoma, 1H7, was grown in nude mice, the antibody was recovered from ascites fluid, concentrated, and conjugated to agarose beads for affinity chromatography [14].

2.5. Cell culture

The Madin Darby Canine Kidney (Strain 1) (MDCK) cell line was a gift from William Young (University of Kentucky) [16]. MDCK cells were cultured (5% CO2, 37C) in MEM which contained 10% fetal bovine serum (FBS) and 2 mM L-glutamine. Cells were harvested by trypsinization (0.5% trypsin/0.65 mM EDTA) and passaged no more than 15 times prior to their use in experiments.

2.6. Epithelial cell invasion by T. spiralis

The invasion assay was performed as previously described [5] with modifications. Epithelial cells were grown to confluence on 8-well chamber slides (NUNC). Monolayers were overlaid with activated larvae suspended in MEM (without FBS) with 15 mM HEPES and 1.75% agarose containing the appropriate concentration of antibody or protease inhibitor. Following an incubation of 1–2 h at 37 °C, 5% CO_2, chamber housings, gaskets and media were removed from slides. Dead cells in monolayers were stained with 0.4% trypan blue in saline (SIGMA). Stained monolayers were rinsed in DPBS (with $MgCl_2$ and $CaCl_2$) and fixed in 10% buffered formalin for 20 min. Cover slips were mounted on slides with glycergel (DAKO Corp.). A total of at least 25 microscope fields from each monolayer were captured using a 4× objective on a bright-field microscope (Labophot; Nikon) fitted with a black-and-white video camera (Cohu, Inc.). A frame grabber captured the image and the area of dead or damaged cells was determined with NIH IMAGE 1.58 software. Mean areas of damage for each treatment were analyzed by ANOVA and significant differences determined by Scheffe's mean separation test.

In experiments designed to evaluate the ESP deposited in epithelial cells during invasion, cells were cultured in 6-well plates and inoculated with 1200 larvae in 3 ml agarose per well. Following incubation at 37 °C for 4 h, agarose and larvae were removed, monolayers were washed three times with DPBS, and cells were lysed with sample preparation buffer (0.0625 M Tris/2% SDS/10% glycerol/pH 6.8) for SDS-PAGE [17].

2.7. SDS-PAGE electrophoresis and western blotting

Polyacrylamide gels (10%) with 3 or 4% stacking gels, in standard or mini-format (as noted) were used. Samples were boiled for 3–5 min prior to loading in sample preparation buffer with or without 5% 2-mercaptoethanol. Prestained molecular weight standards (Diversified Biotech) were resolved in parallel. Western transfer to nitrocellulose was performed according to Burnette [18] or Towbin and Gordon [19]. Nitrocellulose was blocked with DPBS/0.2% Tween/7.5% dry milk/2% BSA (Sigma, St. Louis, MO). Antibodies and conjugates (goat anti-rat IgG or anti-mouse IgG conjugated to horseradish peroxidase (0.5 μg ml^{-1}) (ICN Pharmaceuticals, Inc.) were diluted in DPBS/0.2% Tween/2% dry milk/0.25% BSA. Conjugate diluent included 10% normal goat serum. The nitrocellulose was washed in DPBS/0.2% Tween. Antibody binding was detected with a chemiluminescent substrate (ECL reagent, Amersham Pharmacia Biotech) and autoradiography. Films were scanned and images prepared using PHOTOSHOP (Adobe Systems Inc.).

2.8. Peptide sequencing

Glycoproteins isolated by affinity chromatography using mAb 1H7 were separated by SDS-PAGE, stained using GELCODE blue reagent (Pierce & Warriner), and the desired proteins excised, lyophilized and digested with trypsin (E.C. 3.4.21.4, Sigma) overnight. Peptides were extracted from gel pieces and purified using a C-18 microtrap peptide cartridge (Jones Chromatography) in preparation for sequencing by mass spectrometry (MS) and tandem mass spectrometry (MS/MS) using a hybrid quadrupole orthogonal acceleration time of flight (Q-TOF) mass spectrometer (Micromass UK Ltd.). MS and MS/MS spectra were collected in the positive ion mode as described previously [20,21].

2.9. Immunohistochemistry

Staining of sections of *T. spiralis*-infected mouse muscle was performed as described previously [22]. Sections were stained by the indirect immunoperoxidase method, photographed, stained with Wheatley's trichrome stain and then re-photographed.

2.10. Screening of the T. spiralis cDNA library

A UniZAP XR cDNA library prepared from first-stage *T. spiralis* larvae was screened with a polyclonal rat serum raised against ESP [23]. The serum had been pre-adsorbed with an *E. coli*/phage lysate. Antibody binding was detected using a protocol similar to that employed for Western blots. Recombinant phage of interest were plaque purified and DNA inserts amplified by PCR (Amplitron II thermal cycler, Barnstead/Thermolyne) using primers derived from the pBluescript vector (T3 and T7 primers, IDT, Inc.) and Taq polymerase (Gibco BRL). Cycle conditions were: 94 °C, 5 min; (94 °C, 30 s; 50 °C, 30 s; 72 °C, 3 min) × 35 cycles, ending with 10 min extension at 72 °C. The amplified product was purified (QiaQuick kit, Qiagen) and sequenced (Center for Advanced Technology, Cornell University).

A 5′ rapid amplification of cDNA ends (RACE) was performed to obtain the full length cDNA (SMART RACE cDNA amplification kit, Clontech Laboratories, Inc.). Analyses of nucleotide and deduced amino acid sequences and sequence alignments were carried out using the OMIGA™ 2.0 software program (Accelrys Inc.). Database searches were carried out using the BLAST server (ncbi.nlm.nih.gov/BLAST) [24]. The predicted amino acid sequence was analyzed by the SIGNALP server (cbs.dtu.dk/services/SignalP) in order to identify a putative signal peptide and corresponding cleavage site [25].

2.11. Expression of rTspSP-1

A 705 bp fragment of *TspSP-1* (*TspSP-1* (54–289)) was amplified from the recombinant phage using specific primers designed to ensure that the ORF was cloned in frame with the DNA encoding the N-terminal peptide (containing a 6xHis tag) of the pTrcHis-TOPO expression vector (Invitrogen). The primer sequences (5′–3′) were: SP-1 forward GCAAGGCCATATTCATTTCC and SP-1 reverse TCAGGAAGCCATGACAGTTG. The recombinant plasmid was transformed into *E. coli* (One-Shot competent cells, Invitrogen) and isolated (Mini plasmid purification kit, Qiagen) for restriction enzyme digestion and DNA sequence analysis, which confirmed that the insert was in the correct orientation and in frame. Transformants were grown in LB broth with 50 mg ml^{-1} ampicillin, induced with isopropyl-1-thio-β-D-galactopyranoside (IPTG; 1 mM final concentration) and harvested by centrifugation.

2.12. Production of polyclonal antibodies against rTspSP-1 (54–289)

Bacterial pellets containing rTspSP-1 (54–289) were solubilized in 8 M guanidine HCl and fusion proteins

isolated by nickel chelation chromatography (Ni-NTA agarose, Qiagen) in the presence of 8 M urea. The pTrcHis-TOPO vector adds an N-terminal fusion tail of 35 amino acids (3.9 kDa) containing a 6xHis tag, and also five amino acids at the C-terminus. These two modifications contribute 4.4–26.6 kDa of the parasite protein, so that the predicted size of the recombinant protein would be 31 kDa. Anti-ESP sera detected a protein of 31 kDa in Western blots of the insoluble fraction of crude, IPTG-induced, rTspSP-1 (54–289) bacterial lysates (not shown). A 31 kDa protein was eluted with 8M urea at pH 5.8. We found that, in the absence of urea, rTspSP-1 was unstable or insoluble. Therefore, 66 μg protein in urea were emulsified in Freund's complete adjuvant for intraperitoneal immunization of each rat. Blood samples were collected 20–30 days later. A recombinant fragment of *lacZ* was expressed in the same vector and the protein processed by the same procedure for use as a control. Sera were tested on Western blots of ESP.

3. Results

3.1. Characterization of anti-peptide monoclonal antibodies

Five antibodies were selected based on their binding to TFMS-treated (deglycosylated) ESP in ELISA (Table 1). Three of those (1H7, 9E10 and 6C1) bound equally well to native or deglycosylated ESP (dgESP). Using this criterion, we chose antibody 1H7 for affinity purification of native glycoprotein(s) for peptide sequencing. Two antibodies (9H8 and 7H5) bound strongly to dgESP but not to the native glycoproteins (Table 1), indicating that glycans block access of these mAbs to their peptide epitope(s). Further distinction among the antibodies was evident in Western blots of native ESP (Fig. 1). Under non-reducing conditions, mAb 7H5 bound to bands of 125, 70 and 64 kDa. Under reducing conditions, the 125 kDa species disappeared and two bands of 48 and 55 kDa were bound in addition to the 70 and 64 kDa species. Blots of crude larval homogenates developed with 7H5 showed banding patterns distinct from those for ESP (Fig. 1). Under non-reducing conditions 7H5 bound a group of proteins of 94–104 kDa, and also two proteins of approximately 55 and 48 kDa. The other four antibodies bound selectively to members of this group of glycoproteins. Under reducing conditions, the high molecular weight species disappeared and all five antibodies detected several proteins between 70 and 45 kDa, with the 70 and 64 kDa species most prominent. Proteins bound by the anti-peptide antibodies are tyvelose-bearing glycoproteins, as demonstrated by recognition by anti-tyvelose mAb 18H (Fig. 1).

Table 1
Binding in ELISA (A_{495}) by rat monoclonal antibodies prepared against deglycosylated ESP

Antibody[a]	Antigen	
	Native ESP	Deglycosylated ESP
6C1	0.30	0.28
7H5	0.08	0.88
1H7	0.28	0.28
9H8	0.0	0.95
9E10	0.16	0.24
α-Tyvelose 18H	> 2.00	0.0

[a] Hybridoma culture supernates were tested at 1:2 dilutions in duplicate. Native and deglycosylated (TFMS treated) ESP (2.5 μg ml^{-1}) were bound to PVC 96 well plates as described [8].

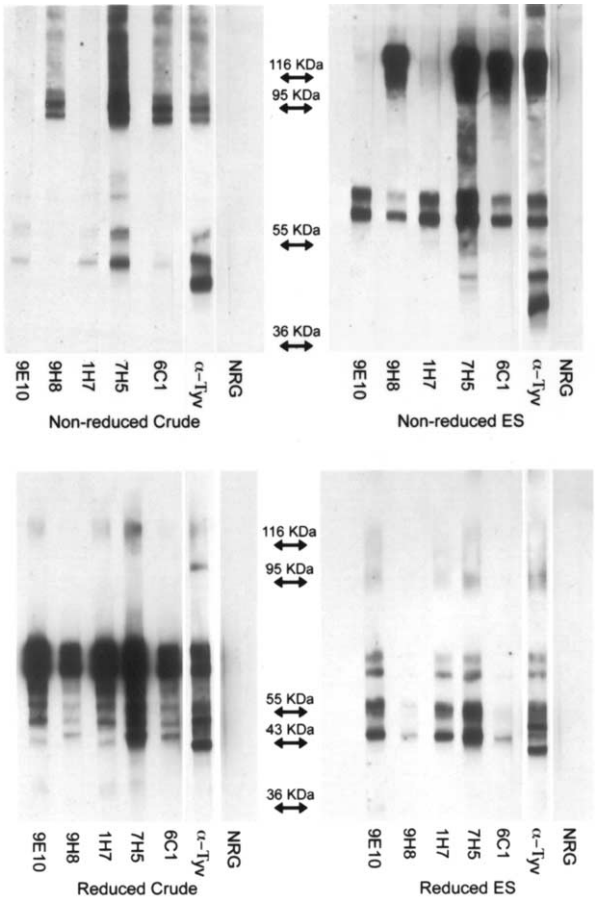

Fig. 1. Specificities of mAbs produced against *T. spiralis* deglycosylated ESP in Western blots. Crude larval homogenates and native ESP were separated in 10% standard size SDS-PAGE under reducing or non-reducing conditions, as indicated. Anti-tyv antibody is mAb 18H (specific for tyvelose-bearing glycans; positive control) and NRG is normal rat globulin (negative control). Migrations of molecular weight standards are indicated.

The recognition of multiple glycoproteins by anti-peptide mAbs may indicate that a common precursor protein undergoes varied post-translational processing, or that different proteins share common peptide epi-

topes. Fig. 2a shows that after removal of *N*- and *O*-glycans from ESP via treatment with TFMS, the mAbs bound a single broad band of protein of 38 kDa. This supports the conclusion that the multiple, antibody reactive native species are differentially processed forms of a single polypeptide.

The anti-peptide antibodies did not bind to Western blots of crude homogenates of 5 day old adult worms, although the adult worm reactive antibody, 10E [12], did bind (Fig. 2b). Female worms in these preparations bear recently hatched L1. Thus, the target peptides were unique to mature, infectious, muscle stage L1.

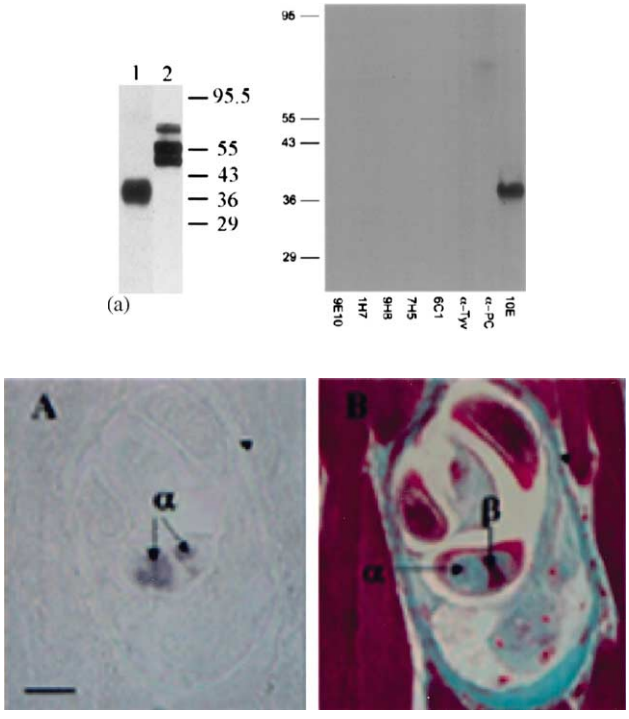

Fig. 2. (a) TFMS treatment preserved the epitope recognized by anti-peptide antibody 1H7. ESP of L1 *T. spiralis* were treated (Lane 1) or untreated (Lane 2) with TFMS, resolved under reducing conditions in 10% SDS-PAGE mini-gels, Western blotted and developed with mAb 1H7. Deglycosylation was confirmed by the failure of anti-tyvelose mAb 18H to bind to treated material (not shown). Mass of deglycosylated protein is estimated to be 38.4 kDa. Migrations of molecular weight standards are indicated. (b) Anti-peptide mAbs are stage-specific. Western blot of crude adult *T. spiralis* homogenate analyzed with the anti-peptide mAbs 9E10, 1H7, 9H8, 7H5 and 6C1. Two larva specific antibodies were included as controls: mAb 18H, specific for tyvelose (α-Tyv) and mAb 6G3, specific for phosphorylcholine (α-PC). Antibody 10E is specific for a 40 kDa adult protein. Migrations of molecular weight standards are indicated. (c) Anti-peptide mAb 1H7 binds to α-stichocytes in muscle stage L1 *T. spiralis*. (A) Immunohistochemical staining of *T. spiralis* infected mouse muscle tissue with anti-peptide mAb 1H7. Antibody 1H7 binds to α-stichocytes in a cross section of a muscle larva in a Nurse cell. (B) The same section after staining with Wheatley's trichrome reveals the alternating α- and β-stichocytes. The collagen capsule surrounding the nurse cell is indicated by an arrow head. Bar = 50 μm. Antibodies 9E10 and 7H5 demonstrated the same specificity (not shown).

3.2. Anatomic location of the epitopes recognized by anti-peptide mAbs

The stichosome of *T. spiralis* is comprised of four classes of stichocytes that can be distinguished by staining with Wheatley's trichrome [22]. Stichocytes are the principal secretory cells of the parasite and are the main source of ESP in *T. spiralis*. Immunohistochemical staining with anti-tyvelose mAbs has shown that alpha, beta and gamma stichocytes synthesize tyvelose-bearing glycoproteins [6]. Immunohistochemical staining followed by Wheatley's trichrome staining showed that antibodies 7H5, 1H7 and 9E10 bound only alpha stichocytes in muscle stage larvae (Fig. 2c).

3.3. In vitro invasion assay

Larvae disgorge glycoproteins into intestinal epithelial cells during invasion [5]. To investigate if the subset of ESP bound by 1H7 was deposited in epithelial cells by larvae during invasion, we used Western blotting to evaluate MDCK cell lysates after removal of invading larvae from monolayers. As shown in Fig. 3, several ESP were detected in cell lysates by anti-tyvelose mAb 18H, and a subset of these were bound by 1H7. These glycoproteins migrated with masses different from those detected in conventional ESP (Fig. 1) or when ESP was mixed with MDCK cell lysates (Fig. 3). Specifically, three 1H7-reactive glycoproteins migrating with estimated masses of greater than 100 kDa were detected with both anti-peptide and anti-glycan antibodies. It is possible that these were minor species in ESP that were selectively retained by the cells, or alternatively that they are the dominant ESP products that have been modified by the host cell. Either selection or alteration have potential significance in invasion and/or migration by larvae and merit further investigation.

Previous results have shown that anti-tyvelose mAbs protect rats against oral infection [8] and prevent entry of larvae into epithelial cells in vitro [9]. Since the anti-peptide antibodies bound a subset of tyvelose-bearing ESP, we tested the inhibitory effects of these mAbs on larvae in vivo and in vitro. When tested in passive immunization protocols that demonstrate high levels of protection for tyvelose-specific antibodies [8], the anti-peptide antibodies were not protective (data not shown). When individual antibodies were tested in vitro for interference with epithelial cell invasion, they were variably, weakly protective (data not shown). Only a pool of anti-peptide antibodies tested at high concentration (0.5 mg ml^{-1}) was moderately effective when compared with anti-tyvelose mAb (damage to cells without larvae: 0.002 ± 0.001; damage to cells by larvae $+0.5$ mg ml^{-1} normal rat globulin: 0.014 ± 0.002: damage to cells by larvae $+0.5$ mg ml^{-1} anti-tyvelose mab18H: 0.003 ± 0.001 ($P < 0.01$ when compared with

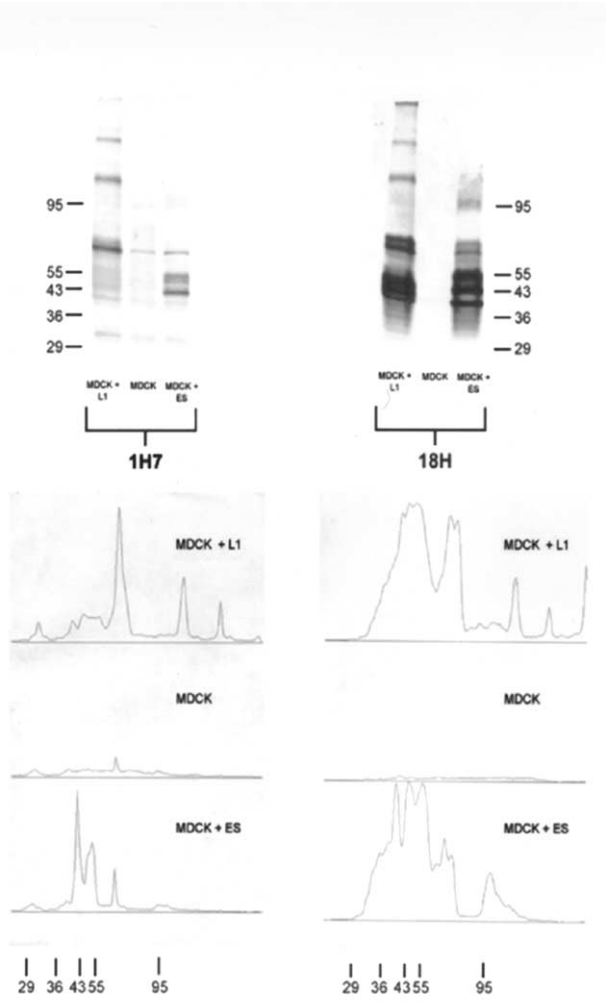

Fig. 3. Glycoproteins released by L1 larvae during invasion are selectively retained or modified by MDCK cells. MDCK cell monolayers were inoculated with L1 larvae, incubated for 4 h and larvae removed. Cell lysates (MDCK+L1) were assayed by Western blot for detection of ESP using anti-tyv mAb 18H and anti-peptide mAb 1H7. MDCK cell lysates (MDCK) and lystes with ESP added to a final concentration of 1.5 ng μl^{-1} (MDCK+ESP) were prepared as controls. Densitometry traces of blots were prepared using NIH IMAGE. Migrations of molecular weight standards are indicated.

normal rat globulin treatment); damage to cells by 0.5 mg ml^{-1} pooled anti-peptide mAbs: 0.008 ± 0.001 ($P <$ 0.01 compared with normal rat globulin treatment)). Thus, the target proteins of anti-peptide mAbs may play a role in epithelial cell parasitism, but do not appear to be a principal target of protective, intestinal immunity.

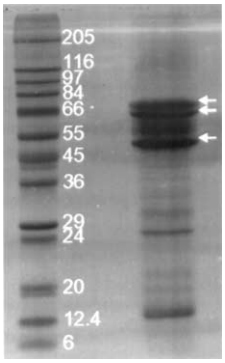

Fig. 4. Glycoproteins purified by affinity chromatography using anti-peptide mAb 1H7 were subjected to 10% SDS-PAGE (right lane). The arrows indicate the proteins that were excised, digested and analyzed by MS/MS sequencing. Migrations of molecular weight standards are indicated (left lane).

3.4. Affinity purification and determination of internal peptide sequences

From 33 mg of crude larval homogenate, approximately 650 μg protein was specifically bound and eluted from an affinity column prepared with anti-peptide mAb 1H7. The eluted proteins were analyzed by SDS-PAGE under reducing conditions (Fig. 4). The major proteins detected had estimated masses of 50, 64 and 70 kDa. The isolated proteins were bound by anti-tyvelose mAb 18H in Western blot, confirming their identities as tyvelose-bearing glycoproteins (not shown).

Peptides derived from tryptic in-gel digestion of the major components in the purified fraction (50, 64 and 70 kDa) were analyzed using a Q-TOF mass spectrometer, a novel instrument that allows ultra-high sensitivity MS/MS sequencing [20,21]. The objective was to provide amino acid sequence data for searching the *Trichinella* EST database or for the design of oligonucleotide primers to amplify cDNAs. Fig. 5a shows a typical nanoelectrospray mass spectrum from the in-gel tryptic digest of the 50 kDa band. Since tryptic peptides are protonated at both the N-terminus and the C-terminal side chain of Lys or Arg, they will typically be doubly charged, showing half-mass unit spacings in the natural ^{13}C isotope satellites [20]. Two such doubly charged ions are shown in the insets (m/z 464.25 and 873.94). Selecting these ions for MS/MS analysis produced the spectra shown in Fig. 5b and d, respectively, where excellent series of N-terminal and C-terminal (b and y″) fragment ions could be readily interpreted [20] to give the sequences GRPI/LSEI/LR and I/LVDANAI/LTVTAGAFDI/LR via the presence of signals shown

Fig. 5. Analysis of tryptic peptides by Q-TOF mass spectrometry. (a) Mass spectrum of in-gel tryptic digest of the 50 kDa band shown in Fig. 4. The insets expand two of the doubly charged ions which were subjected to MS/MS. (b) CAD MS/MS spectrum of the m/z 464.25 ion showing the assignment of fragment ions used to determine the peptide sequence. (c) Interpretation of the MS/MS data showing how the N-terminal (b) and C-terminal (y″) ion masses are assigned to give the sequence GRPI/LSEI/LR. (d) CAD MS/MS spectrum of the m/z 873.94 ion showing the assignment of fragment ions used to determine the peptide sequence. (e) Interpretation of the MS/MS data showing how the N-terminal (b) and C-terminal (y″) ion masses are assigned to give the sequence I/LVDANAI/LTVTAGAFDI/LR.

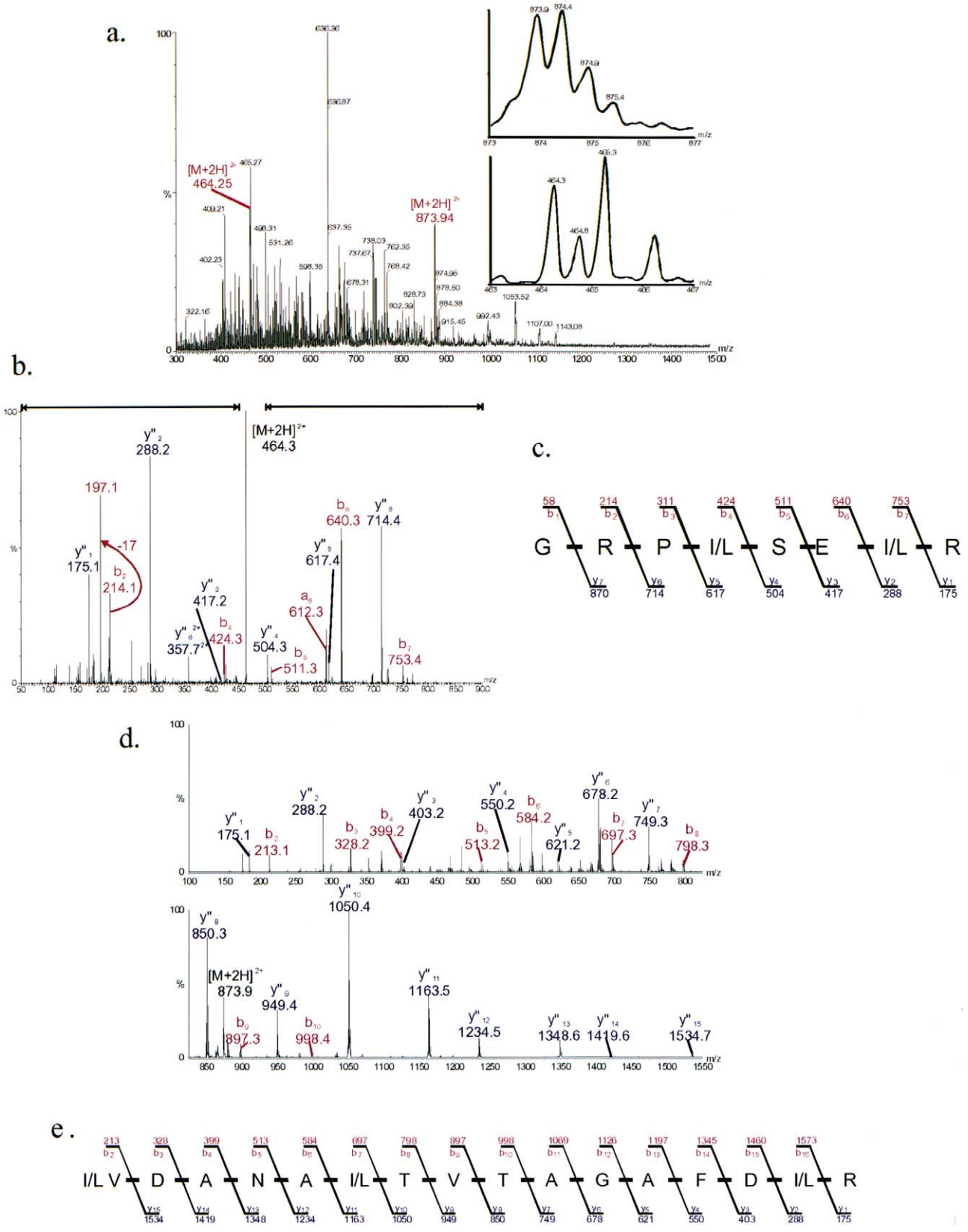
a.
[M+2H]2+ 464.25
[M+2H]2+ 873.94
b.
[M+2H]2+ 464.3
y"2 288.2
197.1
y"1 175.1
b2 214.1
-17
y"3 417.2
y"6 2+ 357.7 2+
b4 424.3
y"4 504.3
b5 511.3
a6 612.3
y"5 617.4
b6 640.3
y"6 714.4
b7 753.4
c.
G R P I/L S E I/L R
58 214 311 424 511 640 753
870 714 617 504 417 288 175
d.
y"1 175.1
b2 213.1
y"2 288.2
b3 328.2
b4 399.2
y"3 403.2
y"4 550.2
b5 513.2
b6 584.2
y"5 621.2
y"6 678.2
b7 697.3
y"7 749.3
b8 798.3
y"10 1050.4
y"8 850.3
[M+2H]2+ 873.9
y"9 949.4
b9 897.3
b10 998.4
y"11 1163.5
y"12 1234.5
y"13 1348.6
y"14 1419.6
y"15 1534.7
e.
I/L V D A N A I/L T V T A G A F D I/L R
213 328 399 513 584 697 798 897 998 1069 1126 1197 1345 1460 1573
1534 1419 1348 1234 1163 1050 949 850 749 678 621 550 403 288 175

Fig. 5

in Fig. 5c and e, respectively, where Ile and Leu are of course indistinguishable in mass (isobaric). Additional sequences were determined as EI/LNEPHR, HHPGI/LYSK, PYSFPWTVH, and GI/LVNQ/KPNDVAMI/LR from MS/MS data derived from doubly charged ions at *m*/*z* 441.0, 469.5, 567.4 and 713.6, respectively (data not shown; the *Q*/*K* ambiguity is due to these residues having the same nominal mass). Similar analysis of the bands at 64 and 70 kDa yielded the following sequences: HYPNQI/LVGE and VI/LAY-M_{ox}SDNFGDVGQ/KPNDVAMI/LR (64 kDa band) and AHGIPI/LI/LR and NRPPYSHSI/LR (70 kDa band) (data not shown).

High confidence portions of the sequences from the 50 kDa band, namely PYSF…, …PI/LSEI/LR and …DANAI/LTVTA… were used to search the *Trichinella* EST database employing the tblastn algorithm. At the time this work was carried out, no matches were found. Subsequently, however, with new entries having been made to the EST database [26], matches were then found with *T. spiralis* EST number PS36H08.Y1, a serine protease homologue. Coincidentally, a clone (*TspSP-1*) with significant levels of sequence homology to the same EST was identified in a *T. spiralis* L1 cDNA library using polyclonal rat antibody to ESP in antibody screening. The predicted amino acid sequence of this clone incorporates five peptides identified by MS/MS in the 50 kDa species, one from the 64 kDa species and two from the 70 kDa species (double underlined in Fig. 6), confirming that the clone encoded the purified protein(s). Blast searches indicated that this clone was almost identical to accession number AAK31787, a sequence from *T. spiralis* reported to GeneBank by Nagano, Wu and Takahashi (unpublished); however, no further information about that sequence was available.

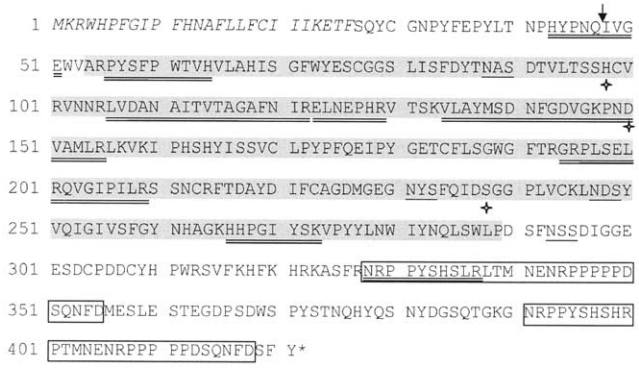

Fig. 6. Deduced amino acid sequence of *TspSP-1*. The signal peptide sequence is shown in italics. *N*-glycosylation motifs are underlined. Peptides deduced by MS sequencing of affinity purified glycoproteins are double underlined. The amino acids that form the catalytic triad in serine proteases are indicated by stars. Proline-rich regions near the C-terminus are boxed. The fragment of TspSP-1 that was cloned for expression is shaded (rTspSP-1 (54–289)).

The peptides AHGIPILR (70 kDa band) and GI/LVNQ/KPNDVAMI/LR (50 kDa band) were partial matches with TspSp-1 (corresponding to residues 202–209 and 143–155, respectively), with two amino acid substitutions in each case. Interestingly, a sequence identical to residues 143–155 in TspSp-1 occurs in the longer peptide VLAYM_{ox}SDNFGDVGKPND-VAMLR observed in the digest of the 64 kDa band. These data suggest that *T. spiralis* may express more than one isoform of the protein.

3.5. Cloning of a cDNA encoding a putative serine protease

The sequence of clone *TspSP-1* is comprised of 1436 bp with an ORF of 1254 bp. The end of the ORF is marked by a stop codon followed by a 3′-untranslated region containing the conserved sequence AATAAA, 18 nucleotides upstream of a poly(A) tail. The sequence lacked an obvious initiator codon. Using RACE-PCR, the ORF was extended 77 bp, to include an in-frame, ATG at position 69. Analysis of the predicted amino acid sequence of *TspSP-1* by SIGNALP predicted that the first 26 amino acids encoded a signal sequence (in italics in Fig. 6), with the cut site predicted to occur between residues F26 and S27. The mature polypeptide would be predicted to be 395 amino acids in length, 44.9 kDa in mass, with four potential *N*-glycosylation sites at positions 88, 231, 247 and 293 (underlined in Fig. 6). The C-terminal region contained two conserved, proline-rich repeats of 28 amino acids (boxed in Fig. 6). The proline content in each repeat is 25% (7/28) while the complete TspSP-1 polypeptide was comprised of 9.3% (39/421) proline.

Sequence comparisons with serine proteases suggested that TspSP-1 was formed as a pro-protein precursor. The pro-region cleavage site can be predicted between Q48 and I49 based on alignment with other serine proteases (Fig. 7, vertical arrow). Other than the aforementioned AAK31787, the closest homologues of TspSP-1 were also from *Trichinella*: newborn larvae-specific serine protease SS2 with 27% identity (AAK16520.1), and a serine protease with 32% identity (AAD09211.1). Bovine chymotrypsinogen A (P00766) shares 28% identity with TspSP-1. Like all members of the serine protease family, the protein encoded by *TspSP-1* contains the conserved residues involved in catalysis (catalytic triad), namely His-98, Asp-150 and Ser-238, together with eight cysteine residues involved in disulfide bond formation (Fig. 7).

3.6. Confirmation that TspSP-1 is secreted by L1 T. spiralis

A fragment of *TspSP-1* cDNA encoding amino acids 54–289 (26.6 kDa) was introduced in frame into the

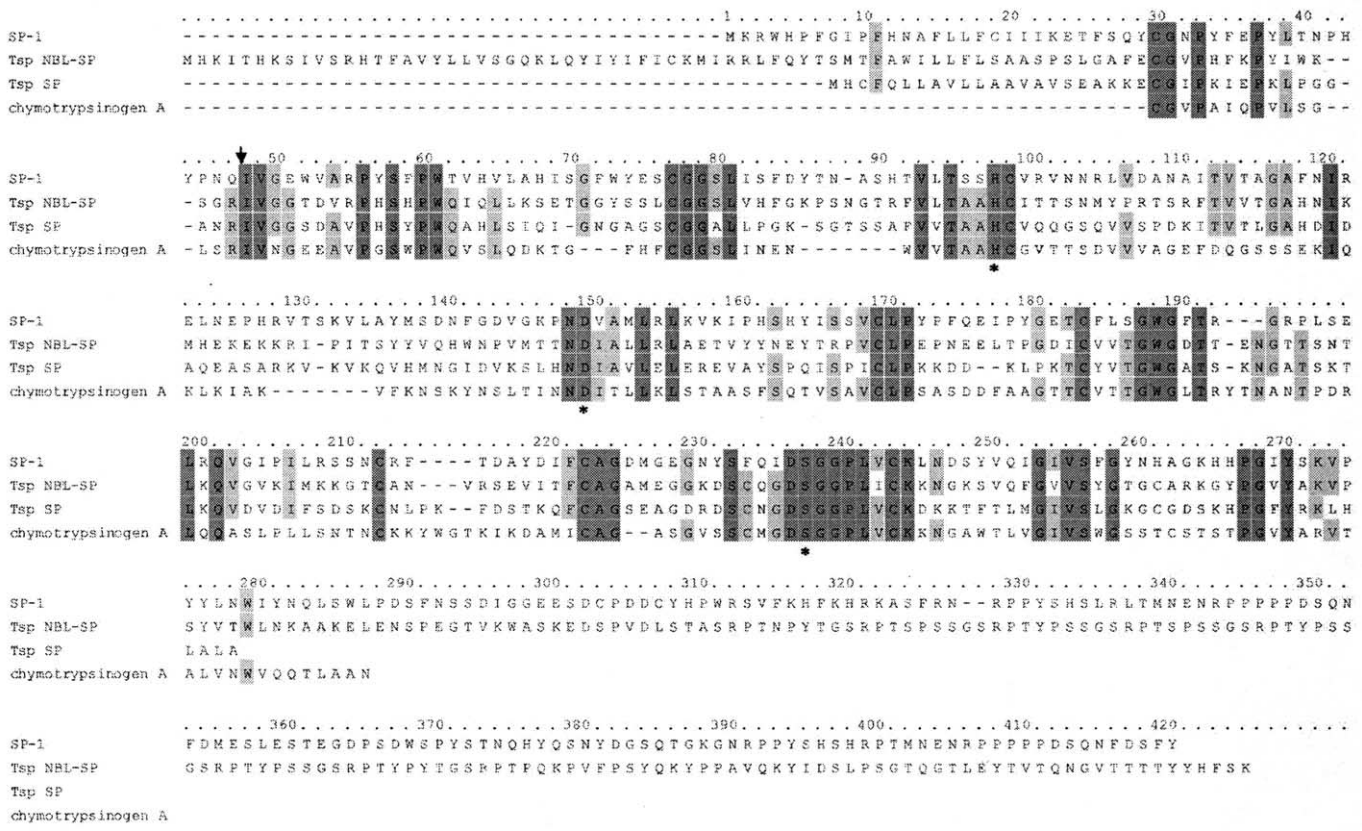

Fig. 7. ClustaLW alignment of the predicted protein encoded by *TspSP-1* with its closest homologues. Residues corresponding to the catalytic triad are marked with asterisks. The putative pro-enzyme cleavage site is indicated by an arrow. Accession numbers are: Tsp NBL-SP: *T. spiralis* newborn larvae-specific serine protease SS2, AAK16520; Tsp SP: *T. spiralis* serine proteinase, AAD09211; chymotrypsinogen A: bovine chymotrypsinogen A, P00766.

bacterial expression vector pTrcHis-TOPO. Rats immunized with rTspSP-1(54-289) produced antibodies that detected a 38.4 kDa protein in Western blots of deglycosylated ESP (not shown). This protein migrated in parallel with the deglycosylated protein detected by anti-peptide mAb 1H7 (not shown; Fig. 2). In blots of native ESP, rat antibodies induced with TspSP-1 (54–289) bound the same group of glycoproteins recognized by mAb 1H7, confirming the identity of the recombinant protein (Fig. 8).

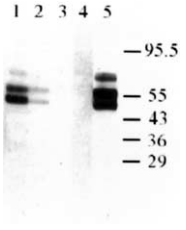

Fig. 8. Rat antibodies induced by rTspSP-1 (54–289) bind to native ESP with specificity similar to anti-peptide mAb 1H7. Western blots were developed with sera from each of two rats immunized with rTspSP-1 (54–289) (Lanes 1 and 2), two rats immunized with rß-galactosidase (Lanes 3 and 4), or with mAb 1H7 (Lane 5).

4. Discussion

Proteases feature prominently in discussions of nematode parasitism and anti-nematode immunity. Blood feeding and tissue migrating nematodes deploy cysteine and aspartic proteases for degradation of hemoglobin and extracellular matrix [27]; reviewed in [28]. Recent work by Salter et al. [29] characterized a serine protease (elastase) secreted by cercariae of *S. mansoni*. Serine protease activity has been reported in ESP of *T. spiralis* adults [30,31] and L1 [32–34] however, the enzymes have not been characterized at the molecular level. In addition, sequences of serine protease homologues from *T. spiralis* have been submitted to GeneBank without further information (Fig. 7). Although we have been unable to confirm enzymatic activity of TspSP-1 using conventional methods (data not shown) the protein has the appropriate structural features to be an active enzyme. In contrast with bovine chymotrypsin, however, TspSP-1 is glycosylated with complex *N*-glycans [6]. There are four *N*-glycosylation sites in the protein; three of these are near histidine and serine residues that comprise the catalytic site. Thus, glycosylation may influence enzymatic activity.

The immune response to *Trichinella* during the muscle phase of infection is dominated by antibodies specific for a group of tyvelose bearing glycoproteins found in ESP [1,2]. Study of tyvelose-bearing glycoproteins has been limited by a shortage of adequate tools (e.g. monoclonal anti-peptide antibodies) for isolation and investigation of individual molecules. Our anti-peptide antibodies bound to a group of related glycoproteins in crude larval extracts and ESP. These proteins are stage-specific and are synthesized in α stichocytes. They are highly processed proteins that form aggregates. The forms of TspSP-1 that the larva releases into ESP are distinct from those recovered from whole worm homogenates, suggesting that modification occurs after degranulation of the stichocytes, or that the species present in ESP are a fraction of the total. Our data support the former conclusion because we did not detect TspSP-1 in body sites other than the stichocyte. The biology of the proteins may be further complicated by nucleotide sequence polymorphism. Comparisons of peptide sequences derived from native proteins with deduced amino acid sequences from cDNAs and ESTs indicate that larvae may synthesize more than one form of the protein (data not shown).

A notable feature of TspSP-1 is a repetitive element of 28 amino acids in the C-terminal region. The functional significance of this domain is unknown, however, proline rich sequences have been shown to mediate protein–protein binding between mediators of the TGF-ß–activin signaling pathway [35]. Similar repeat structures have been described in other parasitic proteases, e.g. subtilisin-like serine proteases from *Neospora caninum* (NC-p65) [36] and *Toxoplasma gondii* (TgSUB1) [37]. The repeats may mediate binding to other parasite proteins, however, the possibility that TspSP-1 may interact with host proteins via proline-rich repeats is an intriguing one.

First-stage larvae of *T. spiralis* reside in two distinct intracellular habitats: intestinal epithelia and the myotube. The presence of TspSP-1 in stichocytes indicates that it has potential to function in either habitat, or both. A specific role for TspSP-1 remains to be elucidated; however, tyvelose bearing glycoproteins have been implicated as mediators of parasitism in both sites. Tyvelose has been detected in hypertrophic nuclei in the muscle stage, Nurse cell complex. The attached glycoproteins (the so-called 'nuclear antigens') are candidates to mediate changes in gene expression associated with the development of the Nurse cell (reviewed by Jasmer [38]). The molecular identity of nuclear antigens is unknown. Although cDNAs encoding tyvelose-bearing glycoproteins and constituents of ESP have been cloned [39–42,23], with the exception of macrophage migration inhibitory factor [42], the biological functions of these molecules are undocumented. With regard to the intestinal stage of infection, it has been postulated that proteases participate in intestinal invasion by *T. spiralis* [43]. The habitat of L1 stage *T. spiralis* is the cytoplasm of epithelial cells; larvae do not cross the basal cell membrane or the basement membrane [44]. Thus, a role for a protease in intestinal occupation is less obvious than for parasites that move through connective tissue. Nevertheless, our results show that TspSP-1 is deposited in and retained by epithelial cells invaded by *T. spiralis*. The form of TspSP-1 recovered from infected cell monolayers is distinct from that found in ESP, suggesting host cell induced modification or selective retention of certain, otherwise minor forms of the protease. A proteolytic enzyme released by the parasite could help to degrade cytoplasmic or intercellular proteins, thereby facilitating movement of the larva. Inhibition of epithelial cell invasion and migration by TspSP-1 mAbs, as we have observed in MDCK cells, supports this conclusion.

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