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Teladorsagia circumcincta: Activation-associated secreted proteins in excretory/secretory products of fourth stage larvae are targets of early IgA responses in infected sheep

A.J. Nisbet [a], S.K. Smith [a], S. Armstrong [b], L.I. Meikle [a], L.A. Wildblood [a], R.J. Beynon [b], J.B. Matthews [a,c,*]

[a] Parasitology Division, Moredun Research Institute, Pentlands Science Park, Bush Loan, Penicuik, Midlothian EH26 0PZ, UK
[b] Proteomics and Functional Genomics Group, University of Liverpool, Liverpool L69 7ZJ, UK
[c] Department of Veterinary Clinical Studies, Royal (Dick) School of Veterinary Studies, University of Edinburgh, Midlothian EH25 9RG, UK

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ABSTRACT

A detailed proteomic analysis of excreted/secretory (ES) proteins derived from fourth stage larvae (L4) of *Teladorsagia circumcincta* identified a number of components, including N-type and C-type single domain activation-associated secreted proteins (ASPs). Immunoblotting of L4 ES extracts with abomasal mucus derived from infected, immune sheep demonstrated the immunogenicity of some of these components, including an N-type single-domain ASP, designated Tci-ASP-1. The full-length cDNA encoding this protein was isolated and sequenced. Homology searches using the inferred amino acid sequence of Tci-ASP-1 showed that it had highest identity (75% over 231 residues) to, a N-type, single-domain ASP from *Ostertagia ostertagi*. Phylogenetic analysis confirmed the relationship of Tci-ASP-1 with other N-type ASPs. Reverse-transcriptase (RT)-PCR experiments demonstrated the presence of transcript encoding Tci-ASP-1 in L4 and adult stage *T. circumcincta* but not in pre-parasitic stages such as eggs and third stage larvae. A recombinant version of Tci-ASP-1 was expressed in *Escherichia coli* and the purified protein was reactive with IgA present in abomasal mucus derived from immune sheep.

1. Introduction

Teladorsagia circumcincta is a pathogenic parasitic nematode that inhabits the abomasum of small ruminants. This parasite is endemic in temperate regions of the world and is currently the major cause of parasitic gastroenteritis (PGE) in the UK. In 2005, the cost of PGE was estimated to cost UK sheep farmers around £84 M annually (Nieuwhof and Bishop, 2005). Worldwide, gastrointestinal parasitic nematodes of sheep are controlled using a combination of pasture management and periodic anthelmintic treatments. Anthelmintic resistance in *T. circumcincta* is now common with increasing reports of isolates found to be resistant to all three available anthelmintic drug classes (Bartley et al., 2004; Wrigley et al., 2006). *T. circumcincta* primarily causes disease in lambs, and this is observed as anorexia, diarrhoea and death; however, the major impact of teladorsagiosis is its effect on lamb productivity via a reduction in weight gain (Gibson and Everett, 1976).

T. circumcincta infective third stage larvae (L3) are ingested from pasture and penetrate the gastric glands of the abomasum within a day of infection. Here, the L3 subsequently moult to fourth stage larvae (L4), which grow rapidly within the gland. The L4 or fifth stage larvae (L5) emerge from the gland into the abomasal lumen at approximately 10 days post-infection (dpi). A combination of parasite-induced damage and host inflammatory/immune response is thought to result in a protein-losing gastropathy (Simpson, 2000). Immunity against *T. circumcincta* challenge takes time to develop and, experimentally, the development of protective immunity requires a trickle infection of several thousand L3 repeatedly over several months (Seaton et al., 1989). Once sheep do mount an effective immune response, this acts to decrease larval establishment in the abomasal mucosa, slow larval development and to diminish female worm fecundity (Seaton et al., 1989; Smith et al., 1985, 1986; Stear et al., 2004). As animals can acquire an effective immune response, vaccination against this parasitic nematode is a possible alternative for control. Despite the relative importance of *T. circumcincta* as a pathogen of sheep, few details have been published with regard to the identification of vaccine candidates. A number of previous studies have focused on assessment of immune responses to the parasite in lambs and ewes responding to experimental challenge infections. This work highlighted that local responses have an important role in regulating parasite development and growth (Smith et al., 1985, 1986; Stear et al., 2004). The importance of early local immune responses

* Corresponding author. Address: Parasitology Division, Moredun Research Institute, Pentlands Science Park, Bush Loan, Penicuik, Midlothian EH26 0PZ, UK. Fax: +44 131 445 6235.
E-mail address: jacqui.matthews@moredun.ac.uk (J.B. Matthews).

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(i.e., within 5 days of challenge in immune sheep) has been indicated in experiments in which antibody and cellular parameters were measured in previously-infected animals (Balic et al., 2003). In these studies, antibody-secreting cells were harvested from lymph nodes and the derived antibodies used to probe *T. circumcincta* antigens by immunoblotting; however, the nature and/or function of the antigens that reacted with the immunoglobulin were not elucidated. Findings by other groups also indicate that parasite-specific IgA plays an important role in restricting larval growth and development (Stear et al., 2004; Smith et al., 2009). On the basis of these, we have chosen to use locally-derived IgA from immune sheep as probes to select larval antigens that are targets of early immune responses in immune sheep responding to a challenge infection. We have chosen to focus on antigens secreted or excreted by mucosal L4 [harvested at 5 days post-infection (dpi)] as these are likely to have essential functions in nematode growth and survival.

2. Materials and methods

2.1. Preparation of L4 ES products

To provide L4 for excretory/secretory (ES) products, helminth-free lambs (<6 months old) were infected with 50,000 *T. circumcincta* L3 *per os* and necropsied at 5 dpi. At post-mortem, abomasa were removed and cut along the greater curvature. Contents were removed and the surface washed with physiological saline. Abomasa were pinned out on polystyrene boards and floated in phosphate buffered saline (PBS) at 37 °C in Baermann funnels. Parasites were allowed to emerge and sediment for between 3 and 5 h at 37 °C. Harvested parasites were then collected and subjected to three washes in sterile PBS followed by a fourth in RPMI-based medium (Redmond et al., 2006; Smith et al., 2009) by centrifugation at 50*g* for 5 min. Parasites were checked for integrity microscopically before being placed in culture flasks. Parasites were characterised as L4 based on observations of L4 larvae at day 5 post-infection and previously described characteristics (Seaton et al., 1989). The ES products were prepared and concentrated as described previously (Smith et al., 2009). Protein concentrations were determined using the Pierce BCA protein assay, with bovine serum albumin (BSA) standards, and 50–100 μl aliquots stored at −80 °C.

2.2. Two-dimensional (2D) polyacrylamide gel analysis and mass spectrometry

Five volumes of ice cold acetone were added to one volume of ES product containing 50 μg of protein, vortexed briefly and incubated at −20 °C for 1 h. The protein pellet was recovered by centrifugation at 5000 rpm for 5 min. The supernatant was discarded and the pellet allowed to air dry. The pellet was solubilised in 8 M urea, 2 M thiourea, 2% w/v CHAPS, 20 mM DTT, 0.2% v/v carrier ampholytes (pH 3–10), with a trace of bromophenol blue dye. The sample was incubated at room temperature for 1 h before centrifugation at 8000 rpm for 5 min. The sample (125 μl) was applied to 7 cm, pH 3–10, IPG strips (Bio-Rad, Hemel Hempsted, UK). Active rehydration of the strips was at 50 V for 500 Vh, followed by a voltage ramping step of 100–8000 V for 24,000 Vh. All focusing steps were at 20 °C in a Protean IEF cell (Bio-Rad). Proteins resolved in the first dimension strips were reduced and alkylated prior to second-dimensional electrophoresis. Strips were incubated for 15 min in a solution containing 50 mM Tris–HCl, pH 8.8, 6 M urea, 30% v/v glycerol, 2% w/v SDS and 2% w/v DTT, followed by 15 min in the same solution containing 2.5% w/v iodoacetamide in place of the DTT. The second-dimensional electrophoresis (SDS–PAGE) was performed on a mini-Protean system (Bio-Rad). After laying the strip on top of a 15% polyacrylamide gel and sealing it with agarose, electrophoresis was carried out for 10 min at constant voltage of 70 V, after which time a constant voltage of 200 V was applied until the dye front reached the lower end of the gel. Proteins were stained overnight with 0.08% w/v Coomassie brilliant blue G-250 in 40% v/v methanol, 10% v/v acetic acid and were visualised by background destaining in a solution of 10% v/v methanol, 10% v/v acetic acid.

Plugs were removed from protein spots on the two DE gel using a thin glass pipette and placed into microcentrifuge tubes. Each gel plug was destained using 100 μl of 50 mM ammonium bicarbonate, 50% (v/v) acetonitrile and was incubated at 37 °C for 30 min. This step was repeated until no stain was visible. The plugs were then washed twice with 100 μl of 50 mM ammonium bicarbonate which was then discarded. The plugs were then incubated with 50 μl of a 10 mM dithiothreitol stock solution. After 1 h at 50 °C the dithiothreitol was discarded and 50 μl of a 55 mM iodoacetamide stock solution was added to each tube and incubated for 1 h at room temperature in the dark. The iodoacetamide was discarded and the plugs washed twice as above. The plugs were dehydrated in 100% acetonitrile. The plug was rehydrated in 19 μl of 50 mM ammonium bicarbonate. Sequencing grade trypsin (1 μl, 0.1 μg/μl, Roche, Basel, Switzerland) was added and the digest was incubated overnight at 37 °C. The reaction was stopped with 1 μl formic acid.

In-gel trypsin digests were analysed using a LTQ ion trap mass spectrometer (Thermo Electron, Hemel Hempstead, UK) coupled online to a U3000 nanoflow HPLC (Dionex, Sunnyvale, CA, USA) equipped with a LC Packings PepMap 100 C18 reverse phase column (75 μm internal diameter × 15 cm length, 3 μm particle size, 100 Å pore size). Tandem mass spectra were searched against publicly available protein database MSDB (Release 20063108, http://proteomics.leeds.ac.uk/bioinf/) and a six-frame translation of the *T. circumcincta* expressed sequence tag (EST) database held in Nembase (Parkinson et al., 2004; http://nema.cap.ed.ac.uk/nematodeESTs/nembase.html) using Mascot software (Perkins et al., 1999). MS/MS ion search parameters were set to tolerate a maximum of one missed enzyme cleavage with carbamidomethyl cysteine as a fixed modification, methionine oxidation as a variable modification; precursor peptide mass tolerance was ±1.2 Da, and product ion mass tolerance was ±0.6 Da.

2.3. Infection protocol for production of mucus for immunoblot analysis and ELISA

To provide material for comparison of abomasal IgA responses in immune ewes vs. lambs given a primary infection, mucus was obtained at post-mortem from seven, previously infected, then challenged, adult ewes (Group 1) and from eight lambs given a single infection (Group 2). The ewes had been naturally infected by grazing a mixed-nematode species infected pasture prior to the study and were then subjected to a *T. circumcincta*-only trickle infection protocol described previously (Smith et al., 2009). These, along with eight helminth-free lambs, were challenged with 50,000 *T. circumcincta* L3 and necropsied three days later. Lumenal and mucosal nematode burdens (adult and larval parasites) were enumerated following standard techniques. The parasitological data has been published previously (Smith et al., 2009). At necropsy, abomasal mucus was obtained and stored at −20 °C (Smith et al., 2009). The experiment was performed under the regulations of a UK Home Office Project Licence.

2.4. Two-dimensional immunoblotting

Antigens (approximately 50 μg L4 ES products per gel), separated by two-dimensional gel electrophoresis as detailed above, were transferred electrophoretically onto Immobilon P membranes