Saposin-like proteins from the intestine of the blood-feeding hookworm, *Ancylostoma caninum*

T. A. DON[1,2], Y. OKSOV[3], S. LUSTIGMAN[3] *and* A. LOUKAS[1]*

[1] *Helminth Biology Laboratory, Division of Infectious Diseases and Immunology, Queensland Institute of Medical Research, Queensland 4006, Australia*
[2] *School of Molecular and Microbial Sciences, The University of Queensland, Queensland 4072, Australia*
[3] *Lindsley F. Kimball Research Institute, The New York Blood Center, NY 10021, USA*

(*Received 21 July 2006; revised 23 August 2006; accepted 23 August 2006; first published online 17 November 2006*)

SUMMARY

Hookworms feed on blood, utilizing haemoglobin for nutrition, growth and reproduction. The haemoglobin digestion cascade has been partially elucidated, but the process immediately preceding this event, haemolysis, has received considerably less attention. We have cloned and expressed *Ancylostoma caninum* mRNAs encoding 2 proteins belonging to the saposin-like protein (SAPLIP) family, termed *Ac-slp-1* and *Ac-slp-2*. The open reading frames of SLP-1 and SLP-2 were used to identify expressed sequence tags encoding SAPLIPs from the 4 major clades of animal parasitic nematodes. Both *Ac-slp-1* and *slp-2* mRNAs were shown to be expressed in all life stages assessed, with *slp-1* predominantly being expressed in third-stage larvae (L3) before and after activation with dog serum. Recombinant SLP-1 and SLP-2 were expressed in insect cells and used to raise specific antisera in mice. These antisera were used as probes in fluorescence microscopy to localize the anatomic expression sites of both proteins to small, punctate organelles or vesicles within the intestinal cells of adult worms; weak staining was detected on the microvillar brush border of the intestine. Using transmission electron microscopy, both proteins were localized to similar vesicles in the intestinal cells of the L3. Recombinant proteins contained C-terminal purification tags that potentially precluded dimerization and possibly interfered with the subsequent detection of haemolytic activity. Their expression in the gut of the L3 and adult stages suggests a role for these hookworm SAPLIPs in the lysis of host cells during tissue migration and/or feeding.

Key words: *Ancylostoma caninum*, hookworm, SAPLIP, saposin.

INTRODUCTION

Ancylostoma duodenale and *Necator americanus* (Nematoda) are human hookworms which infect 0·5–1·0 billion people in developing countries and are a major cause of iron deficiency anaemia (de Silva *et al*. 2003). Hookworms are responsible for the loss of more disability adjusted life years than many of the more well-known tropical diseases, including African trypanosomiasis, dengue, Chagas' disease, schistosomiasis and leprosy (Hotez *et al*. 2004).

Hookworms feed on blood and intestinal tissues (Roche and Layrisse, 1966), utilizing released haemoglobin (Hb) and the subsequent break-down products for nutrition, growth and reproduction. The cascade of proteolytic degradation of Hb in the hookworm gut has been elucidated partially (Williamson *et al*. 2002, 2003*a*, *b*, 2004) but the molecules involved in the process immediately preceding Hb degradation, haemolysis, have yet to be identified. Previous research in our laboratory identified a haemolytic, pore-forming protein from membrane extracts of adult hookworms (Don *et al*. 2004), but the molecular identity of this protein remains elusive.

Recently, proteins identified in 2 liver flukes, *Clonorchis sinensis* and *Fasciola hepatica*, were shown to lyse erythrocytes (Lee *et al*. 2002; Espino and Hillyer, 2003). These fluke cytolysins share sequence identity with amoebapores, pore-forming peptides from the protozoan parasite, *Entamoeba histolytica* (see Leippe *et al*. 1992), and can be grouped within a family of distantly related saposin-like proteins (SAPLIPs) that have 6 conserved cysteine residues forming 3 disulfide bridges (Leippe *et al*. 1994; Andersson *et al*. 1995; Zhai and Saier, 2000; Bruhn, 2005). Native and recombinant SAPLIPs from both of the liver flukes lysed red cells *in vitro* (Lee *et al*. 2002; Espino and Hillyer, 2003), but the exact mechanisms of lysis were not determined – i.e. pore formation was not reported. Clonorin, the cytolysin from *C. sinensis*, is expressed exclusively in the gut of juvenile and adult flukes, where it is thought to enhance digestion (Lee *et al*. 2002). Hookworms ingest and subsequently lyse intact erythrocytes with a pore-forming protein (Don *et al*. 2004), so, we hypothesized that homologues of these fluke

* Corresponding author: Helminth Biology Laboratory, Division of Infectious Diseases and Immunology, Queensland Institute of Medical Research, 300 Herston Road, QLD 4006, Australia. Tel: +61 7 3845 3702. Fax: +61 7 3845 3507. E-mail: alex.loukas@qimr.edu.au

doi:10.1017/S003118200600148X Printed in the United Kingdom

pore-forming proteins might also lyse erythrocytes in the intestine of blood-feeding adult hookworms.

In this study, we describe the identification and characterization of mRNAs encoding 2 SAPLIPs, termed *Ac*-SLP-1 and *Ac*-SLP-2, from the dog hookworm, *Ancylostoma caninum*. Antisera raised to recombinant SLP-1 and SLP-2 (produced in insect cells) were used in immunofluorescence to localize the expression sites of the native proteins to vesicles within the intestines of adult and larval stages of *A. caninum*.

MATERIALS AND METHODS

Sequence identification and analysis

Clonorin from *C. sinensis* (GenBank Accession number AF421960) was used to search by BLASTx the parasitic nematode expressed sequence tag (EST) dataset (WuBlast – http://www.ebi.ac.uk/blast2/parasites.html) to identify hookworm homologues. Full length cDNA sequences were obtained for *Ac-slp-1* and *slp-2* by PCR using gene-specific primers designed from GC-rich regions of the EST sequences in combination with vector-derived primers which flanked the multiple cloning site of an *A. caninum* adult worm cDNA library constructed in λ-ZAP Express (Stratagene). Primers Ac1GCF (5′ CTGGACCAGCTGGAGAAGGAG) and Ac2GCR (5′ CGGTGCAGATCTTCTCCGGTGAG) were used to obtain the 3′-end of *Ac-slp-1* and the 5′-end of *Ac-slp-2*, respectively. The presence of predicted signal peptides was assessed using SignalP 3.0 (http://www.cbs.dtu.dk/services/SignalP/). Homologues of the hookworm SAPLIPs were identified by BLASTp searches against GenBank nr (http://www.ncbi.nlm.nih.gov/BLAST/) and the parasite EST dataset (http://www.ebi.ac.uk/blast2/parasites.html). Multiple sequence alignments were carried out using the program ClustalW. The analyses were restricted to sequences containing a full length SAPLIP domain; only sequence between the first and sixth Cys residues were included.

Transcriptional analysis in different developmental stages

Reverse transcription PCR (RT-PCR) was used to determine the life-cycle stages in which mRNAs for *slp-1* and *slp-2* were transcribed. Total RNAs from adult worms (mixed sexes), first-stage larvae (L1), L3 and serum-stimulated L3 (designated L3SS) (Hawdon and Schad, 1991) were extracted using Trizol (Invitrogen) according to the manufacturer's instructions. The cDNA was generated using a Super SMART™ PCR cDNA synthesis Kit (Clontech). Primers were designed within GC-rich regions of *slp-1* and *slp-2* to yield expected products of ~150 base pairs (bp). The following primers were used for *slp-1*: Ac1RTF – 5′ GCAAGATGGCCGTCAAACTCA; Ac1RTR – 5′ GCCAACGCCTTGCACTCCTTC; and for *slp-2*: Ac2RTF – 5′ GCCGTTGTGCGAGATGTGTGAGG; Ac2RTR – 5′ CGGTGCAGATCTTCTCCGGTGAG. Primers designed to amplify the *A. caninum* beta-tubulin mRNA (GenBank Accession number AF077870) were used as a constitutively expressed control: AcTubF – 5′ GGAACAATGGATTCGGTCC; AcTubR – 5′ CAGGGAAGCGAAGGCAG. One ng of cDNA was used as a template for the PCR using the following cycling parameters: initial denaturation at 94 °C for 15 min, followed by 35 cycles (94 °C for 30 sec, 55 °C for 30 sec, 72 °C for 1 min), followed by a final extension at 72 °C for 10 min. RT-PCR amplicons were subjected to electrophoresis through 1% agarose gels, stained with ethidium bromide and photographed upon ultraviolet transillumination.

Expression and purification of recombinant proteins in baculovirus

Recombinant proteins were expressed in a baculovirus shuttle plasmid (pMelBac – Invitrogen), such that they were fused to an N-terminal melittin signal peptide. Primers were designed to amplify the open reading frames (ORFs) of *Ac-slp-1* and *slp-2* without their predicted signal peptides and containing restriction sites for *Sac* I (5′) and *Nco* I (3′); 3′-primers also contained nt encoding a 6×His tag, followed by a stop codon for downstream purification using metal ion affinity chromatography. The following primers were used for *slp-1*: Ac1pmbF (5′ GCGCGCGAGCTCACTCCAGTAGTGATCAACAAC); Ac1pmbR (5′ GCGCGCCCATGGTTAATGGTGATGGTGATGATGACAAGCATGCAGAGTTGTGCA); and for *slp-2*: Ac2pmbF (5′ GCGCGCGAGCTCGAGAAGAGAAAAAAGCCGTTG); Ac2pmbR (5′ GCGCGCCCATGGTTAATGGTGATGGTGATGATGAACAG CACAAAGATGTATGTCGGT). PCR products were generated from the *A. caninum* cDNA library using *Pfu* polymerase (Stratagene). After gel extraction (Qiagen), amplicons were double-digested with *Sac* I/*Nco* I, gel purified and cloned into double-digested pMelBac, followed by transformation into *Escherichia coli* (Top10 cells) by heat shock at 42 °C. Selected plasmids were sequenced using vector primers to identify inserts and determine their orientation. Sf9 (*Spodoptera frugiperda*) and Hi5 (*Trichoplusia ni*) insect cells were maintained according to standard procedures (Invitrogen). Transfection and expression of recombinant protein were performed following the Bac-N-Blue protocol (Invitrogen) with minor modifications (Williamson *et al*. 2004). Recombinant proteins were purified from culture supernatants by gravity-flow affinity chromatography on Ni-NTA agarose (Qiagen).

https://doi.org/10.1017/S003118200600148X Published online by Cambridge University Press

Supernatants were buffer-exchanged into nickel binding buffer (5 mM imidazole, 0·5 M NaCl, 40 mM Tris-HCl pH 7·9) (Williamson *et al*. 2004) and applied to Ni-NTA agarose pre-equilibrated in binding buffer. The agarose was washed with increasing concentrations of imidazole (binding buffer containing 10–60 mM imidazole), and protein was eluted in 250 mM imidazole. A second Ni-NTA purification was conducted if necessary to achieve $\geqslant 90\%$ purity. Purified samples were pooled and then buffer exchanged into phosphate-buffered saline (PBS, pH 7·4). Protein concentrations were determined by colorimetric detection using a bicinchoninic acid (BCA) assay kit (Pierce), and the purified proteins were stored at 4 °C for short periods or at −20 °C for extended periods.

After initial expression, we learned that amoebapores from *E. histolytica* dimerize via a likely head-to-tail mechanism, requiring a 'flush' C-terminus without affinity tags (Lee *et al*. 2002; Hecht *et al*. 2004). Therefore, we re-engineered *Ac-slp-1* and *slp-2* into a pMelBac expression plasmid, modified to contain a C-terminal TEV protease cleavage site, V5 epitope, 6 × His tag and stop codon (in that order) between the existing *Hin*d III and *Sal* I sites. The modified plasmid was constructed in our laboratory by M. Smout and was termed pHotWax. Additional 3′-primers without the nucleotides encoding the 6 × His tag were designed to clone *Ac-slp-1* and *slp-2* into pHotWax; the 5′-primers were the same as those used for cloning into pMelBac. Amplicons were produced (as described above) and double-digested with *Sac* I/*Hin*d III, cloned into the double-digested pHotWax vector and transformed into *E. coli* (Top10 cells). The transfection, expression and purification of recombinant proteins in insect cells were as described above. To address potential dimerization of the hookworm SLPs, we conducted Western blots using mouse antisera against the recombinant SLPs to probe soluble extracts of *A. caninum* (adult worm and L3), under denaturing and non-denaturing conditions (Don *et al*. 2004). Haemolysis assays using recombinant SLPs were conducted as described previously (Don *et al*. 2004).

Antibody production and immunolocalization

Serum antibodies against recombinant *Ac*-SLP-1 and *Ac*-SLP-2 were raised in female BALB/c mice, as described previously (Tran *et al*. 2006). Pre-immune sera were taken by tail bleed 2 days prior to the first injection. Ten mice were injected subcutaneously with 25 μg of protein emulsified in Freund's Complete Adjuvant for the first immunization and Freund's Incomplete Adjuvant for 2 subsequent boosts. Injections were given at 2-week intervals, and mice were necropsied 2 weeks after the last boost. Whole blood was collected via cardiac puncture, and serum was separated by incubation at 37 °C for 1 h, centrifuged at 10 000 ***g*** for 10 min and then stored at −20 °C. Immunolocalization by fluorescence microscopy was performed on paraformaldehyde-fixed sections of adult *A. caninum* cut using a microtome (7·0 μm). Sections were rehydrated in PBS for 15 min before blocking in PBS-0·1% Tween 20 (PBST)/5% skimmed milk powder (SMP) for 1 h at room temperature (RT). Sections were washed in PBST 3 × 5 min, probed with serum diluted 1 : 25 in PBST/5% SMP for 1 h at RT, followed by 3 × 5 min washes with PBST. Sections were reacted firstly with α-mouse-Cy3 (Jackson ImmunoResearch) diluted 1 : 500 in PBST/5% SMP for 1 h at RT, washed 3 × 5 min in PBST and then reacted with 4′,6-diamidino-2-phenylindole (DAPI) (Sigma), diluted 1 : 50 000 in PBS for 30 min at RT. Sections were then washed 3 × 5 min in PBS, mounted, a cover-slip applied, allowed to air dry for about 1 h and the edges sealed with nail polish. Slides were examined using a fluorescence microscope (Axioskop2, Zeiss) and then stored at 4 °C after viewing.

A. caninum L3 were fixed and processed for microscopy as described previously (Lustigman *et al*. 1992). Briefly, the L3 were fixed for 1 h at RT in 0·25% glutaraldehyde in 0·1 M phosphate buffer, pH 7·4, containing 1% sucrose. They were then washed in the same buffer, treated with 50 mM ammonium chloride to quench any remaining aldehydes, dehydrated and embedded in LR-White Resin (Electron Microscopy Sciences). Thin sections of embedded parasites were mounted on parlodion-covered nickel grids, blocked in 2% bovine serum albumin (BSA) and probed with mouse anti-SLP-1 and anti-SLP-2 sera (diluted 1 : 50) overnight at 4 °C. Sections were then washed and incubated with goat-anti-mouse IgG(Fc) coupled to gold particles (15 nm) (Amersham Biosciences; 1 : 20 dilution). Pre-immunization serum was used as the control. After staining with aqueous uranyl acetate, sections were examined using a Philips 410 electron microscope (Holland).

RESULTS

cDNAs encoding 2 SAPLIPs from A. caninum

The predicted protein sequence of clonorin (from *C. sinensis*) was used as the query in a BLASTx search of the nematode EST dataset at WUBlast. Homologues from *A. caninum* were identified and 2 were selected for further analysis: EST AW488306, termed *Ac-slp-1* and EST BM077625, termed *Ac-slp-2*. The ORF of *Ac-slp-1* comprised 104 amino acids (including a signal peptide of 17 residues) with a predicted molecular mass of 11 424 Da and isoelectric point (pI) of 4·69. The ORF of *Ac-slp-2* was 98 amino acids (including a signal peptide of 18 residues), with a predicted molecular

https://doi.org/10.1017/S003118200600148X Published online by Cambridge University Press

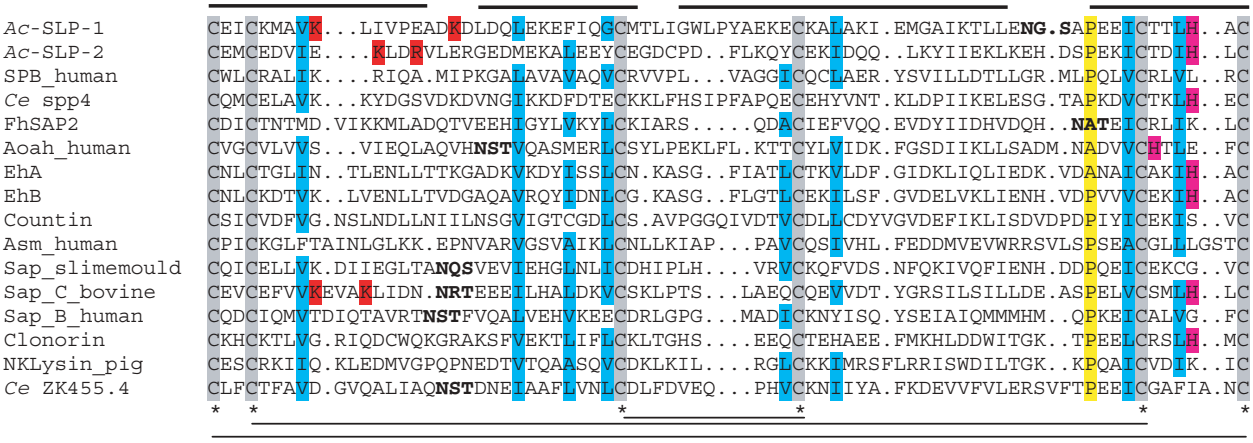

Fig. 1. Sequence alignment of *Ac*-SLP-1 and SLP-2 with other saposin-like proteins (SAPLIPs). Only the SAPLIP domain from each protein has been used for the alignment. SPB_human – saposin B (GenBank Accession number P07602); *Ce* spp4 – *C. elegans* SAPLIP protein family 4 (AAA81416); FhSAP2 – *F. hepatica* SAP2 (AF286903); Aoah_human – *Homo sapiens* acyloxyacyl hydrolase (BAD97196); EhA – *E. histolytica* amoebapore A (M83945); EhB – *E. histolytica* amoebapore B (CAA54226); countin – *Dictyostelium discoideum* AX4 countin (XP_643887); Asm_human – *H. sapiens* acid sphingomyelin phosphodiesterase 1 (BAD93012); Sap_slimemould – *D. discoideum* saposin A (BAA32237); Sap_C_bovine – *Bos taurus* saposin C (S21770); Sap_B_human – *H. sapiens* saposin B (NP_001035930); clonorin – *C. sinensis* clonorin (AF421960); NKLysin_pig – *Sus scrofa* NK-Lysin (CAA59720); *Ce* ZK455.4 – *C. elegans* hypothetical protein (CAA91493). The 6 conserved cysteine residues are boxed in grey with predicted disulphide bonding patterns shown underneath (1–6, 2–5, 3–4). Blue denotes conserved hydrophobic residues; yellow highlights the conserved proline in saposins or alanine in SAPLIPs and pink indicates the histidine predicted to be involved in dimerization of amoebapores. Red boxes in bovine saposin C denote positively charged residues involved in its anchoring to the cell membrane; positively charged residues of *Ac*-SLP-1 and SLP-2 in this vicinity are also highlighted in red. Predicted alpha-helices of amoebapore A are shown as solid bars above the alignment. Putative *N*-linked glycosylation sites are in bold. Gaps have been introduced to improve the alignment.

mass of 11 400 Da and pI of 4·65. The amino acid sequences of both predicted proteins contained features of the SAPLIP family (Bruhn, 2005), including the distribution of hydrophobic amino acids and the 6 cysteine residues involved in disulfide bond formation (Fig. 1). Both proteins contained a His residue situated 2 amino acids N-terminal to the last Cys residue; in amoebapores this His is predicted to be responsible for dimer formation (Andra and Leippe, 1994). SLP-1 has a predicted *N*-linked glycosylation site between the fourth and fifth Cys residues in the same region as the predicted glycosylation site in FhSAP2 (Fig. 1). Ac-SLP-1 and SLP-2 had positively charged residues in the vicinity of Lys 13 and Lys 17 of mammalian saposin C, 2 residues that are implicated in its anchoring to the cell membrane (Liu *et al*. 2005). Both *A. caninum* SAPLIPs had a Pro situated 4 residues N-terminal to the fifth Cys residue, confirming their putative identities as SAPLIPs rather than true saposins which usually contain an Ala in this position.

ESTs encoding SAPLIPs from parasitic nematodes

ESTs encoding a SAPLIP domain were identified from the 4 major clades of animal-parasitic nematodes (clades I, III, IV and V) (Blaxter *et al*. 1998) (Fig. 2). Again, these nematode SAPLIPs share little sequence identity other than the 6 cysteines and the hydrophobic residues making up the charged surfaces, making it difficult to infer functional inferences based on sequence identities.

Transcription in different developmental stages

Ac-slp-1 was detected in RNA from all developmental stages of *A. caninum*, but was most abundant in L3 and L3SS. *Ac-slp-2* was detected equally in L1, L3, L3SS and adult stages (Fig. 3). Both transcripts were identified by PCR in the cDNA from adult *A. caninum* gut tissue extracted by laser capture microdissection (Ranjit *et al*. 2006) (not shown).

Expression and analysis of recombinant proteins

Recombinant *Ac*-SLP-1 and SLP-2 (in the pMelBac plasmid) were both expressed and secreted by *Sf*9 and Hi5 insect cells. The yields of purified recombinant proteins were ~0·2 mg/l for *Ac*-SLP-1 and ~0·3 mg/l for *Ac*-SLP-2 when expressed in Hi5 cells (Fig. 4A). Recombinant SLP-1 migrated as a doublet, probably representing glycosylated and non-glycosylated forms (SLP-1 has 1 predicted *N*-linked glycosylation site). No expression of recombinant proteins was achieved using the pHotWax expression vector (where the C-terminal purification tags can be removed by proteolysis with TEV protease), and protein was not detected in either *Sf*9 or

https://doi.org/10.1017/S003118200600148X Published online by Cambridge University Press

```
Ac-SLP-1                CEICKMA.VKLIVPEADKD..LDQLEKEFIQ.GCMTLIGWLP.YAEKECK..ALAKIEMGAIKTLLE...NGS.APEEIC
AC-SLP-2                CEMCEDV.IEKLDRVLERGE..D.MEKALEE.YCEGDCPDF...LKQYCE..KIDQQ.LKYIIEKLK...EHD.SPEKIC
A. caninum BM077873     CEMCENL.IKKVDEVLEKGG..D.VEEAVDE.FCREDVPSF...LVEYCE..KIISKNLKYIIEKLK...EHD.PPEQIC
A. caninum BF250532     CDECQFA.AKELKTLVEDKEKQQAIREFFSQNVCKHIPR.....YQGMCD..MLVEQFLPEMFQELD...SLLQDPKKAC
A. caninum AW700518     CDECQFA.AKELKTLVEDKEKQQAIREFFSQNVCKHIPR.....YQGMCD..MLVEQFLPELFQELD...SLLQDPKKAC
A. caninum BE352419     CTRCQDI.IKEIDSAIESE....QLKNDALK.LCEAALSNPG..ILQLCK..TVVEETLGVLYKQLK...KFE.PKPELC
A. ceylanicum BQ289559  CDICKMA.VKLIAPAADKG..LDQLEKEFIQ.GCMTLIGWLP.YAEKDCK..ALAKIEIGAIKTLLE...NGS.APEEIC
H. contortus CB012778   CAACRGA.VKFIASMLDQG..ALKIEEAFVN.QCVKFLDFMP.FPRVQCR..FIAKLKLGPIKKSLE...AGD.TPETVC
H. contortus CA994847   CTLCKEA.VEIVVRMLDKS..LPEIENIFVL.QCKEMLKFLP.YGDKECE..AIAARDIPAIKGFLE...SGH.APETVC
H. contortus CB332247   CTFCKET.VDIIAPELDKS..YPEIEKAFLI.ACKHFFSFLP.DGDKQCE..EIANKVIKPIKEALE...QGV.TSEDVC
T. circumcincta BM052087 CVVCKEA.VKIMLPLLEET..TARIVEEFVV.KCKEYIHYLP.FAESICR..TFANREIAKIKAALE...AGA.NPDAVC
T. circumcincta CB043603 CTLCKEA.VEIVVRMLDKS..LPEIGNIFVL.QCKEMLKFLP.YGDKECE..AIAARDIPAIKGFLE...SGH.APETVC
T. circumcincta CB0435   CEMCENL.IKKIDEVLEQGG..D.VEKAVDQ.FCREDLPEF...LVEYCE..KIVAKNLKYIIEKLK...DHD.PPEKIC
T. circumcincta BG734380 CEKCEEVTCNDCEYMMNVYKVMVKSNLPVLEDKCEHEDP....CFQKMCE.......KFNPIVHMVK...TNS.SPKEIC
O. ostertagi BM897130   CEFCVAV.IKKLKERHSTE...ADFEKNMQE.ECWKHNG.......TDCDDGNVCELINKVALQRLR...DDE..PQKTC
N. brasiliensis BM278855 CQLCRAT.VDVVAPKLNAS..IPELEQLFIQ.KCTEFLGALP.LAAEECK..LLAEREIEPLKKELE...GGV.TAEEVC
N. brasiliensis BQ529325 CILCQRL.LRDIKEKLDRN...DDLEEVIMD.SCKNKAHG...FLTPFCQ..MFVNKIIEARVELQG...EDS..PREKC
P. pacificus CN442627  CDPCLRI.VGGIEGAVEKGE.PG.LEQLAES.ICDDTLGHIG.DLAQQCK..DWVEKDFQKIMDKLN...SDW.SAESIC
P. pacificus CK928175  CEPCLRI.VGGIEADVEAGA.PA.LLKLAEN.ICDDTLGHIG.DLAQQCK..DWVVKDFQKIMDKLN...SDW.SAESIC
P. trichosuri BG661407  CKECTDI.IQKIDDDLDNGI.TY.VEGDIDK.ICN.DLGKT...LGKECT..KIVNENLDKIIDKIN...SGL.SPQKIC
P. trichosuri BG661509  CTTCEDV.IKSVEDLGEDAL.KDGLEKAIDD.ACK.LLG.P...LKKECE..KEFDKLIDELIDLIN...QDL.SPEEVC
P. trichosuri BI863762  CTLCPDV.VKTLEDYLNSH..EGNIDGAADY.VCDKVTGGI.SILDQLCK..TLMTDEINQLIDGIE...NKE.TPEHIC
P. trichosuri BM346771  CGMCKML.VQELETDVENK..EPDIEKKANG.YCDKFFQKE.KLLDGACK..MMIDKEIDNIIQKLE...NDE.KPDAIC
P. trichosuri BM346355  CTMCLML.VQEVETDVANG..ESDIDRKAEA.NCDKMFSN..SFLDAMCK..TLVDEEIDNIIKRVE...NKD.TPPVIC
P. trichosuri BM346611  CKACEQF.VGDLETDLEND..EPDLKSKAEG.YCIKYFGS..GVLGDTCK..LFVDEELDNVVAKIK...NGD.TPDVIC
P. trichosuri BM346302  CKVCEQF.VGDLETDLEND..EPDLKSKAEG.YCTKYFGS..GLLGDTCK..LFVDEELDNVVAKIK...NGD.TPDVIC
P. trichosuri BI322174  CPICEMI.VKQSEIWIGKE..GEERENAVIA.LCDKELSSLGVYGKMFCE..AFVKDELTNIFLHMENSGDESKDANKVC
S. ratti BQ090866       CTLCLDV.VKELEDYLNAH..EGNIDGAADY.ICKKVTGGI.IILDQLCE..TLMTDEINQLIKGIE...AKE.TPEHIC
S. ratti CD525422       CELCLEV.VKLVEEYLINN..EGVINRDLSS.LCDKLSGKL.AALDQPCK..NPLINEVDNIIQGIK...QRD.EPEVIC
H. glycines CB375630    CEECQLA.ARELAREVSEPKFQQYVEEWLQGNVCVHLGT.....LKSSCE..QMLDEFLPEMWRELE...DILSDQKKFC
A. suum CA954558        CEECQFA.AGELKSLVDQEQSQEKLKKFLSEEVCSRLGK.....YQGSCD..LMLDQFIPELFEELG...KLLNNTKQFC
A. suum BM516707        CSLCSQV.VSATKDLIIDH..EAEIDEVLEQ.VCYKLFNRHP.SEEAACE..ALIKQELPYIVSLIK...KGA.QPTQIC
B. malayi AA255372      CNECKML.ISRFAEAIKDPRKVTELKDLLRI.LCHETP......YEDECR..LLVNQ.LDHFIEKLE...PYLENPEKVC
B. malayi AA275508      CESCQMF.INGINNVIEQA..FDWVTQEMDD.FCDDHFAYNS.XATMTCK..AKVDKVVEKIRDFVV...LED.ASEMIC
W. bancrofti CK850766   CNECKML.ISRFAEAIKDPRKVTELKDLLRI.LCHETP......YEDECR..VLVNQ.LDHFIEKLE...PYLENPEKVC
O. volvulus AI130133    CNECKML.ITRFAEAIKDPKKIAELQDLLRL.LCHETP......YEDECR..VLVNQ.LDHFIEKLE...PYLKNPEKVC
X. index CV510466       CSVCEGL.MNELKTVLDQPGLMDQVKSSLKQ.FCQATGP.....YQQQCE..DSIDNAVNSVVQMGQ.......NPEQSC
T. spiralis prosap_3A   CVLCKYG.TKIYVDYFSRHY.LDALTNEVIE.MCNHFSSD....WSAQCA..DFMQIYFKVFIQSIL....NNFNPTEAC
T. spiralis prosap_2A   CDECLFL.TNEAASILSDEEYQRQLIQTAKG.ICRVIPQ.....VKEECE..TAIDNYAPTLFQDLL...DYLSNPKPVC
T. spiralis prosap_1A   CELCKSY.IMELRDIINNPEKLAQLKKLLKA.ACMAAT......QRAECE..AAVEG.LDLILENMK...ESLENPENVC
T. spiralis prosap_4A   CESCELM.ADYIMDDIEELELEEQLSTELIR.GCESMS....FFKKHECY..ANVRSLMTKLMNHLK...EEL.FTSKTC
T. muris BM174347       CTVCHVA.MELLQLALKELRFEANTLSLLQS.LCSYLPAE....VHQDCS..DFVKTNVQELVEKMI....EYLGPDHIC
```

Fig. 2. Multiple sequence alignment of parasitic nematode expressed sequence tags encoding SAPLIP domains. GenBank Accession numbers are listed next to the species name. *Trichinella spiralis* prosaposin (*T. spiralis* prosap_1–4: GenBank accession number AAR83123) full-length cDNA sequence was included in the analysis – it contains 4 SAPLIP domains; hence, each domain was included as a separate entry. Grey boxes denote the 6 conserved Cys residues. Species are grouped according to the phylogeny of Blaxter *et al*. (1998) and colour coded as follows: brown – clade V; green – clade IV; red – clade III; blue – clade I.

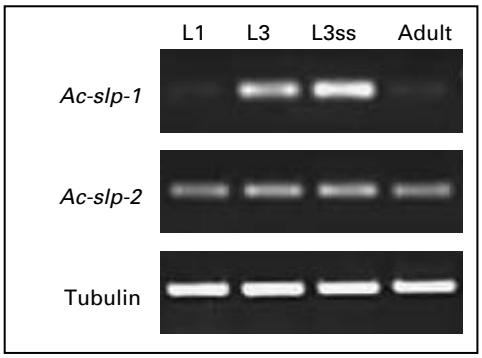

Fig. 3. Transcription profiles of *Ancylostoma caninum* SAPLIP mRNAs. Reverse transcription (RT)-PCR analysis for mRNA transcription levels of *Ac-slp-1* and *slp-2* in *A. caninum* L1, L3, serum stimulated L3 (L3ss) and adult worms. Beta-tubulin mRNA (AF077870) was used as a constitutively expressed positive control. Controls lacking cDNA template did not reveal any bands (not shown).

Hi5 insect cells for either *Ac*-SLP-1 or SLP-2 (not shown), precluding studies aimed at determining whether head-to-tail dimerization occurs in the presence of native termini free of purification tags. Both recombinant proteins were detected by their homologous mouse antiserum on Western blots (Fig. 4B). Antisera to recombinant SLPs were used to probe *A. caninum* extracts electrophoresed under denaturing and non-denaturing conditions (to address dimerization of native SLPs), but bands were not detected using either method (not shown). The antisera did, however, detect proteins in immunohistochemistry, perhaps reflecting differential accessibility of epitopes when the proteins were electrophoresed in a polyacrylamide gel compared with parasite tissue sections. Therefore, we cannot comment with any degree of certainty on whether these proteins dimerize in their native states.

Immunolocalization

Using specific antisera, both *Ac*-SLP-1 and SLP-2 were immunolocalized to the intestine of adult *A. caninum* (Fig. 5). Intense staining was detected in small punctate 'vesicles' beneath the brush border

https://doi.org/10.1017/S003118200600148X Published online by Cambridge University Press

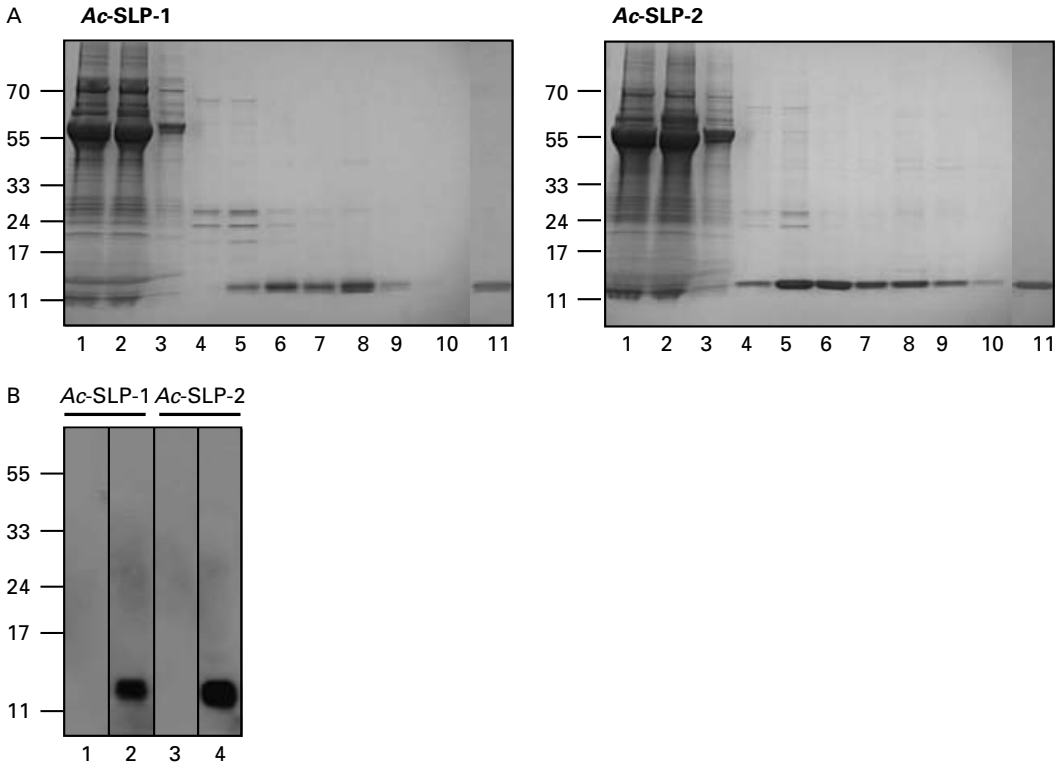

Fig. 4. Expression and purification of recombinant *Ac*-SLP-1 and *Ac*-SLP-2 proteins in baculovirus. (A) Coomassie Brilliant Blue-stained SDS-PAGE gel depicting the purification of recombinant *Ac*-SLP-1 and SLP-2 by nickel-affinity chromatography. Lane 1, culture supernatant in nickel binding buffer; lane 2, unbound material after initial chromatography; lanes 3–6, washes with increasing concentrations of imidazole (10, 20, 40, 60 mM respectively); lanes 7–10, eluate fractions (1·0 ml) of purified protein in 250 mM imidazole; lane 11, recombinant protein after a secondary round of purification on Ni-NTA agarose to remove contaminating bands seen in lanes 7–10 after primary purification. (B) Western blot showing recognition of recombinant proteins by homologous antisera. Lanes 1 and 2, *Ac*-SLP-1; lanes 3 and 4, *Ac*-SLP-2; lanes 1 and 3 probed with normal mouse serum; lanes 2 and 4 probed with mouse anti-SLP-1 and anti-SLP-2, respectively.

epithelium of the intestinal cells. Some fluorescence, albeit weak and dispersed, was also displayed on the microvillar brush border of the intestinal cells. Pre-immunization mouse serum did not bind to any structures in sections. Using electron microscopy and immunogold labelling, *Ac*-SLP-1 was localized to vesicles within the intestinal cells of the L3 (Fig. 6), supporting the findings for the transcription and expression of these molecules in the intestinal cells of adult *A. caninum*. Antibodies to *Ac*-SLP-2 bound to the same structures in the L3 as did anti-SLP-1 sera (not shown), consistent with the punctate vesicles observed in the adult worm.

Haemolysis

No haemolytic activity was observed for recombinant *Ac*-SLP-1 or SLP-2 containing C-terminal purification tags (expressed in pMelBac – not shown). Also, no dimer formation of recombinant proteins was detected when proteins were subjected to electrophoresis on native-PAGE gels under non-reducing conditions, despite dimerization of a positive control recombinant protein (thioredoxin peroxidase) (not shown). Insufficient quantities of recombinant proteins were generated by insect cells infected with virus containing the pHotWax plasmid (i.e. where the purification tags could be cleaved with exogenous protease).

DISCUSSION

All members of the SAPLIP family (with the exception of granulysin) share a conserved placement of 6 cysteines, predicted to have a similar disulfide cross-linkage: 1–6; 2–5; 3–4 (Leippe, 1997; Zhai and Saier, 2000; Bruhn, 2005). The disulfide bonds confer remarkable stability to the peptides, particularly against heat and other environmental factors (Kishimoto *et al*. 1992; Zhai and Saier, 2000; Lee *et al*. 2002). Apart from the conserved cysteines, there is very little sequence homology among SAPLIPs. Therefore, function may not be reflected in sequence similarity, and is most likely due to similar tertiary structure (Bruhn, 2005). The crystal structure has been elucidated for amoebapore A (Leippe *et al*. 1992; Hecht *et al*. 2004) and NK-lysin (Liepinsh *et al*. 1997), and forms the backbone upon which

https://doi.org/10.1017/S003118200600148X Published online by Cambridge University Press

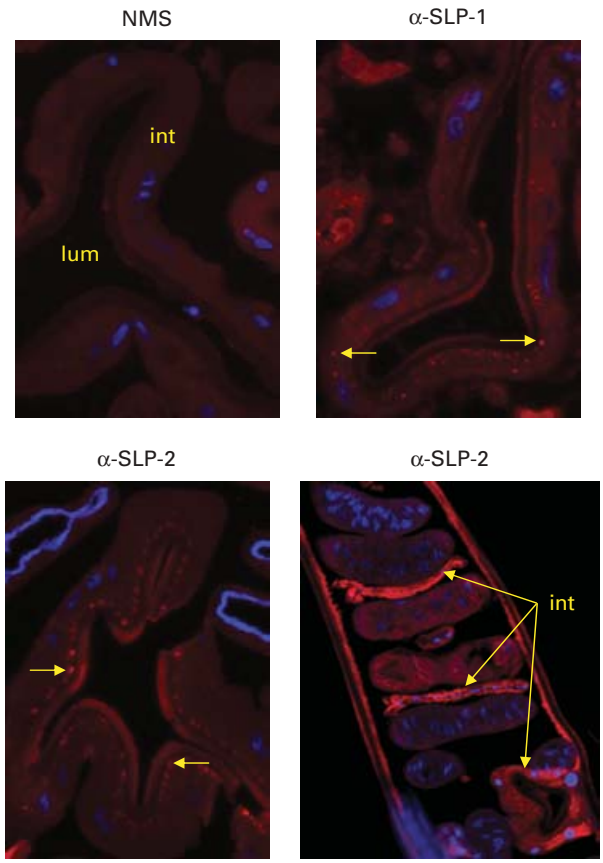

Fig. 5. Immunostaining of tissue sections from *Ancylostoma caninum* adult worms using mouse antibodies raised to recombinant SLP-1 or SLP-2 or pre-immunization mouse sera, followed by Cy3-conjugated anti-mouse Ig (red colour). Sections were counterstained with DAPI to highlight the nuclei (blue colour). Both proteins were localized to the intestine, with intense staining seen in small punctate 'vesicles' (arrows) and less intense, sporadically distributed staining in the microvillar brush border of the intestinal cells. NMS, normal mouse serum; int, intestinal cell; lum, intestinal lumen.

other SAPLIPs can be modelled. Four or 5 alpha helices are usually present in SAPLIPs, and the surface charge distribution of these proteins is the basis of their membrane binding capacity and ability of some to form pores. The function of SAPLIPs is to bind to lipids in cell membranes (Zhai and Saier, 2000; Bruhn *et al*. 2003) and then to perform a range of functions, including transporting lipids within and between membranes (e.g. saposins) (Munford *et al*. 1995), cytolysis (e.g. amoebapores) (Leippe *et al*. 1991) and antimicrobial activity (e.g. NK lysin) (Leippe, 1995). Both *Ac*-SLP-1 and SLP-2 contain the 6 conserved cysteines as well as several hydrophobic residues representing the protein core of the SAPLIP superfamily (Bruhn, 2005).

Dimerization is common in SAPLIPs, but the mechanisms by which dimer formation occurs are variable. Amoebapore A forms head-to-tail homodimers which are predicted to insert as pores into lipid membranes and, in turn, facilitate the subsequent lysis of the cells (Hecht *et al*. 2004). This dimer formation hinges on the presence of a histidine near the C-terminus of the protein (Andra and Leippe, 1994). Similarly, clonorin from *C. sinensis* has a histidine in the same position as amoebapore A, and is predicted to form dimers when active (Lee *et al*. 2002). Both *Ac*-SLP-1 and SLP-2 have a C-terminal histidine between cysteine residues 5 and 6 which might mediate dimer formation. Interestingly, recombinant clonorin is unable to lyse erythocytes when it contains a C-terminal 6 × His tag and an enterokinase cleavage site, but does lyse cells after proteolytic removal of the tags, implying that extra residues at the C-terminus interfere with dimer formation (Lee *et al*. 2002). Recombinant *Ac*-SLP-1 and SLP-2 produced in pMelBac contained a C-terminal 6 × His tag which could not be removed; these proteins were neither haemolytic nor did they dimerize. Therefore, an attempt was made to express them in pHotWax (containing a cleavable 6 × His tag), but insufficient yields of recombinant protein prevented the pursuit of dimerization studies.

Both *Ac*-SLP-1 and SLP-2 are localized to the intestines of adults and L3 of *A. caninum*, indicating that they could play a role in parasite feeding. Clonorin is haemolytic and localized to the intestine of adult *C. sinensis* (see Lee *et al*. 2002), further supporting a role for *Ac*-SLP-1 and SLP-2 in the lysis of ingested host cells by L3 and adult stages. Both proteins displayed a unique expression site within the intestine of *A. caninum*, localizing to punctate vesicles/granules within the intestinal cells. A similar pattern of localization has been shown for an intestinal SAPLIP of *Fasciola gigantica* (see Grams *et al*. 2006) which is orthologous to a haemolytic protein from *F. hepatica* (see Espino and Hillyer, 2003). SAPLIPs might be secreted from these 'vesicles' and transported to the intestine where they bind to and, subsequently, lyse erythrocytes, releasing haemoglobin into the intestinal lumen where it is degraded by a suite of proteases (Brindley *et al*. 2001; Williamson *et al*. 2003*b*, 2004). *A. caninum* SAPLIPs have signal peptides, and it is possible that these proteins are secreted across the intestinal epithelium into the lumen upon demand, possibly accounting for the scattered presence of fluorescence on the epithelium. Alternatively, these vesicles might be lysosome-like organelles and the final site of SLP trafficking.

Ac-slp-1 and *slp-2* mRNAs are expressed in the intestines of multiple developmental stages of *A. caninum*, suggesting multiple functions in parasite biology. *Ac-slp-1* mRNA is most abundantly expressed in the L3 (determined by non-quantitative methods), both serum-stimulated and

https://doi.org/10.1017/S003118200600148X Published online by Cambridge University Press

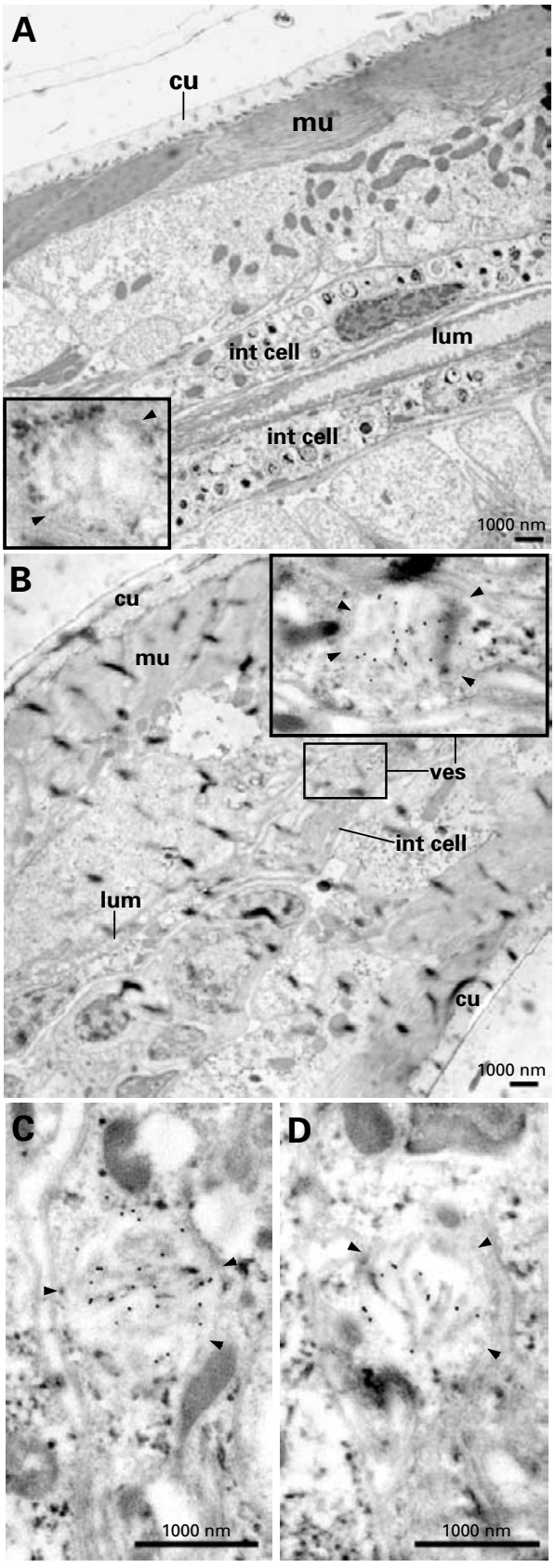

Fig. 6. Localization of *Ac*-SLP-1 in *Ancylostoma caninum* L3 using immunogold electron microscopy. Mouse anti-SLP-1 antibodies were used to localize both proteins in L3. (A) Normal mouse serum. The insert shows unlabelled 'vesicle'. (B) Mouse anti-SLP-1 antibodies recognized the protein in 'vesicles' (arrowheads) within the intestinal cells (boxed). The insert shows a larger magnification of the stained 'vesicle'. (C and D) Anti-SLP-1 antibody labelled vesicles taken from other regions of the intestinal cells. The same structures were also labelled using anti-SLP-2 antibodies (not shown). cu, cuticle; mu, muscle; lum, intenstinal lumen; int cell, intestinal cell; ves, 'vesicle'.

non-stimulated; *Ac-slp-2* on the other hand is expressed equally in L1, L3 and adult stages. Since the majority of *Ac-slp-1* is expressed in the L3 and adult worm, the SLP-1 protein could interact with host cells. SLP-2 protein might perform multiple functions, because it is expressed in L1 as well as the intra-mammalian stages. Alternatively, SLP-2 might lyse multiple cell types in the parasite's intestine – soil bacteria in the L1 and host cells in the L3/adult. SAPLIPs often lyse different cell types (Bruhn, 2005) and, in the absence of functional recombinant proteins (i.e. inability to determine whether dimerization occurs), it would be premature to comment further on the biological functions of these proteins in the gut of *A. caninum*.

Since both *A. caninum* SAPLIPs described here are found in pre-parasitic and parasitic developmental stages, they have potential as vaccine candidates. Indeed, vaccination of rabbits with recombinant FhSAP2 achieved protection against challenge infection with *F. hepatica* (see Espino and Hillyer, 2004), implying that this family of proteins is a valid target for helminth vaccines. Existing hookworm vaccine molecules are restricted in their developmental expression to either the L3 (Bethony *et al.* 2005) or adult worm (Loukas *et al.* 2005), prompting the suggestion that a bivalent vaccine may be required to attack both major developmental stages within the host (Hotez *et al.* 2006). Therefore, a molecule expressed in the gut of different developmental stages might be worthy of pursuit as a single antigen vaccine against hookworm disease. However, prior to vaccine testing, the production of a biologically active recombinant protein (which allows dimer formation, if appropriate) is needed to determine the function(s) of both hookworm SAPLIPs.

We thank Mary Duke, Mai Tran, Mark Pearson, Ben Datu, Michael Smout and Danielle Smyth for helpful discussions and the provision of reagents. We are grateful for advice and support from Drs Peter O'Donoghue and Peter Hotez. T. D. was supported by an Australian Postgraduate Award and by Queensland Institute of Medical Research. A. L. was supported by a Career Development Award from the National Health and Medical Research Council of Australia (NHMRC). The work was supported by grants from the NHMRC and The Sabin Vaccine Institute.

https://doi.org/10.1017/S003118200600148X Published online by Cambridge University Press

REFERENCES

Andersson, M., Gunne, H., Agerberth, B., Boman, A., Bergman, T., Sillard, R., Jornvall, H., Mutt, V., Olsson, B., Wigzell, H., Dagerlind, A., Boman, H. G. and Gudmandsson, G. H. (1995). NK-lysin, a novel effector peptide of cytotoxic T and NK cells. Structure and cDNA cloning of the porcine form, induction by interleukin 2, antibacterial and antitumour activity. *The EMBO Journal* **14**, 1615–1625.

Andra, J. and Leippe, M. (1994). Pore-forming peptide of *Entamoeba histolytica*. Significance of positively charged amino acid residues for its mode of action. *FEBS Letters* **354**, 97–102.

Bethony, J., Loukas, A., Smout, M., Brooker, S., Mendez, S., Plieskatt, J., Goud, G., Bottazzi, M. E., Zhan, B., Wang, Y., Williamson, A., Lustigman, S., Correa-Oliveira, R., Xiao, S. and Hotez, P. J. (2005). Antibodies against a secreted protein from hookworm larvae reduce the intensity of hookworm infection in humans and vaccinated laboratory animals. *The FASEB Journal* **19**, 1743–1745.

Blaxter, M. L., De Ley, P., Garey, J. R., Liu, L. X., Scheldeman, P., Vierstraete, A., Vanfleteren, J. R., Mackey, L. Y., Dorris, M., Frisse, L. M., Vida, J. T. and Thomas, W. K. (1998). A molecular evolutionary framework for the phylum Nematoda. *Nature* **392**, 71–75.

Brindley, P. J., Kalinna, B. H., Wong, J. Y., Bogitsh, B. J., King, L. T., Smyth, D. J., Verity, C. K., Abbenante, G., Brinkworth, R. I., Fairlie, D. P., Smythe, M. L., Milburn, P. J., Bielefeldt-Ohmann, H., Zheng, Y. and McManus, D. P. (2001). Proteolysis of human hemoglobin by schistosome cathepsin D. *Molecular and Biochemical Parasitology* **112**, 103–112.

Bruhn, H. (2005). A short guided tour through functional and structural features of saposin-like proteins. *The Biochemical Journal* **389**, 249–257.

Bruhn, H., Riekens, B., Berninghausen, O., Leippe, M., Herbst, R., Ott, C., Jacobs, T., Marti, T. and Marciano-Cabral, F. (2003). Amoebapores and NK-lysin, members of a class of structurally distinct antimicrobial and cytolytic peptides from protozoa and mammals: a comparative functional analysis of pore-forming polypeptides of the pathogenic protozoon *Naegleria fowleri*. *The Biochemical Journal* **375**, 737–744.

De Silva, N. R., Brooker, S., Hotez, P. J., Montresor, A., Engels, D. and Savioli, L. (2003). Soil-transmitted helminth infections: updating the global picture. *Trends in Parasitology* **19**, 547–551.

Don, T. A., Jones, M. K., Smyth, D., O'Donoghue, P., Hotez, P. and Loukas, A. (2004). A pore-forming haemolysin from the hookworm, *Ancylostoma caninum*. *International Journal for Parasitology* **34**, 1029–1035.

Espino, A. M. and Hillyer, G. V. (2003). Molecular cloning of a member of the *Fasciola hepatica* saposin-like protein family. *Journal of Parasitology* **89**, 545–552.

Espino, A. M. and Hillyer, G. V. (2004). A novel *Fasciola hepatica* saposin-like recombinant protein with immunoprophylactic potential. *Journal of Parasitology* **90**, 876–879.

Grams, R., Adisakwattana, P., Ritthisunthorn, N., Eursitthichai, V., Vichasri-Grams, S. and Viyanant, V. (2006). The saposin-like proteins 1, 2, and 3 of *Fasciola gigantica*. *Molecular and Biochemical Parasitology* **148**, 133–143.

Hawdon, J. M. and Schad, G. A. (1991). Albumin and a dialyzable serum factor stimulate feeding in vitro by third-stage larvae of the canine hookworm *Ancylostoma caninum*. *Journal of Parasitology* **77**, 587–591.

Hecht, O., Van Nuland, N. A., Schleinkofer, K., Dingley, A. J., Bruhn, H., Leippe, M. and Grotzinger, J. (2004). Solution structure of the pore-forming protein of *Entamoeba histolytica*. *The Journal of Biological Chemistry* **279**, 17834–17841.

Hotez, P. J., Brooker, S., Bethony, J. M., Bottazzi, M. E., Loukas, A. and Xiao, S. (2004). Hookworm infection. *The New England Journal of Medicine* **351**, 799–807.

Hotez, P. J., Bethony, J., Bottazzi, M. E., Brooker, S., Diemert, D. and Loukas, A. (2006). New technologies for the control of human hookworm infection. *Trends in Parasitology* **22**, 327–331.

Kishimoto, Y., Hiraiwa, M. and O'Brien, J. S. (1992). Saposins: structure, function, distribution, and molecular genetics. *Journal of Lipid Research* **33**, 1255–1267.

Lee, J. Y., Cho, P. Y., Kim, T. Y., Kang, S. Y., Song, K. Y. and Hong, S. J. (2002). Hemolytic activity and developmental expression of pore-forming peptide, clonorin. *Biochemical and Biophysical Research Communications* **296**, 1238–1244.

Leippe, M. (1995). Ancient weapons: NK-lysin, is a mammalian homolog to pore-forming peptides of a protozoan parasite. *Cell* **83**, 17–18.

Leippe, M. (1997). Amoebapores. *Parasitology Today* **13**, 178–183.

Leippe, M., Ebel, S., Schoenberger, O. L., Horstmann, R. D. and Muller-Eberhard, H. J. (1991). Pore-forming peptide of pathogenic *Entamoeba histolytica*. *Proceedings of the National Academy of Sciences, USA* **88**, 7659–7663.

Leippe, M., Tannich, E., Nickel, R., Van Der Goot, G., Pattus, F., Horstmann, R. D. and Muller-Eberhard, H. J. (1992). Primary and secondary structure of the pore-forming peptide of pathogenic *Entamoeba histolytica*. *The EMBO Journal* **11**, 3501–3506.

Leippe, M., Andra, J., Nickel, R., Tannich, E. and Muller-Eberhard, H. J. (1994). Amoebapores, a family of membranolytic peptides from cytoplasmic granules of *Entamoeba histolytica*: isolation, primary structure, and pore formation in bacterial cytoplasmic membranes. *Molecular Microbiology* **14**, 895–904.

Liepinsh, E., Andersson, M., Ruysschaert, J. M. and Otting, G. (1997). Saposin fold revealed by the NMR structure of NK-lysin. *Nature Structural Biology* **4**, 793–795.

Liu, A., Wenzel, N. and Qi, X. (2005). Role of lysine residues in membrane anchoring of sapsoin C. *Archives of Biochemistry and Biophysics* **443**, 101–112.

Loukas, A., Bethony, J. M., Mendez, S., Fujiwara, R. T., Goud, G. N., Ranjit, N., Zhan, B., Jones, K., Bottazzi, M. E. and Hotez, P. J. (2005). Vaccination

https://doi.org/10.1017/S003118200600148X Published online by Cambridge University Press

with recombinant aspartic hemoglobinase reduces parasite load and blood loss after hookworm infection in dogs. *PLoS Medicine* **2**, e295.

Lustigman, S., Brotman, B., Huima, T., Prince, A. M. and McKerrow, J. H. (1992). Molecular cloning and characterization of onchocystatin, a cysteine proteinase inhibitor of *Onchocerca volvulus*. *The Journal of Biological Chemistry* **267**, 17339–17346.

Munford, R. S., Sheppard, P. O., O'Hara, P. J., Kishimoto, Y., Hiraiwa, M. and O'Brien, J. S. (1995). Saposin-like proteins (SAPLIP) carry out diverse functions on a common backbone structure. *Journal of Lipid Research* **36**, 1653–1663.

Ranjit, N., Jones, M. K., Stenzel, D. J., Gasser, R. B. and Loukas, A. (2006). A survey of the intestinal transcriptomes of the hookworms, *Necator americanus* and *Ancylostoma caninum*, using tissues isolated by laser microdissection microscopy. *International Journal for Parasitology* **36**, 701–710.

Roche, M. and Layrisse, M. (1966). The nature and causes of ''hookworm anemia''. *The American Journal of Tropical Medicine and Hygiene* **15**, 1031–1102.

Tran, M. H., Pearson, M. S., Bethony, J. M., Smyth, D. J., Jones, M. K., Duke, M., Don, T. A., McManus, D. P., Correa-Oliveira, R. and Loukas, A. (2006). Tetraspanins on the surface of *Schistosoma mansoni* are protective vaccine antigens in mice and *Sm*-TSP-2 is selectively recognized by naturally resistant individuals. *Nature Medicine* **12**, 835–840.

Williamson, A. L., Brindley, P. J., Abbenante, G., Prociv, P., Berry, C., Girdwood, K., Pritchard, D. I., Fairlie, D. P., Hotez, P. J., Dalton, J. P. and Loukas, A. (2002). Cleavage of hemoglobin by hookworm cathepsin D aspartic proteases and its potential contribution to host specificity. *The FASEB Journal* **16**, 1458–1460.

Williamson, A. L., Brindley, P. J., Abbenante, G., Datu, B. J., Prociv, P., Berry, C., Girdwood, K., Pritchard, D. I., Fairlie, D. P., Hotez, P. J., Zhan, B. and Loukas, A. (2003*a*). Hookworm aspartic protease, Na-APR-2, cleaves human hemoglobin and serum proteins in a host-specific fashion. *Journal of Infectious Diseases* **187**, 484–494.

Williamson, A. L., Brindley, P. J., Knox, D. P., Hotez, P. J. and Loukas, A. (2003*b*). Digestive proteases of blood-feeding nematodes. *Trends in Parasitology* **19**, 417–423.

Williamson, A. L., Lecchi, P., Turk, B. E., Choe, Y., Hotez, P. J., Mckerrow, J. H., Cantley, L. C., Sajid, M., Craik, C. S. and Loukas, A. (2004). A multi-enzyme cascade of hemoglobin proteolysis in the intestine of blood-feeding hookworms. *The Journal of Biological Chemistry* **279**, 35950–35957.

Zhai, Y. and Saier, M. H. Jr. (2000). The amoebapore superfamily. *Biochimica et Biophysica Acta* **1469**, 87–99.

https://doi.org/10.1017/S003118200600148X Published online by Cambridge University Press