

The ABA-1 allergen of *Ascaris lumbricoides*: sequence polymorphism, stage and tissue-specific expression, lipid binding function, and protein biophysical properties

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SUMMARY

The ABA-1 protein of *Ascaris lumbricoides* (of humans) and *Ascaris suum* (of pigs) is abundant in the pseudocoelomic fluid of the parasites and also appears to be released by the tissue-parasitic larvae and the adult stages. The genes encoding the polyprotein precursor of ABA-1 (*aba-1*) were found to be arranged similarly in the two taxa, comprising tandemly repeating units encoding a large polyprotein which is cleaved to yield polypeptides of approximately 15 kDa which fall into 2 distinct classes, types A and B. The polyprotein possibly comprises only 10 units. The *aba-1* gene of *A. lumbricoides* is polymorphic, and the majority of substitutions observed occur in or near predicted loop regions in the encoded proteins. mRNA for ABA-1 is present in infective larvae within the egg, and in all parasitic stages, but was not detectable in unembryonated eggs. ABA-1 mRNA was confined to the gut of adult parasites, and not in body wall or reproductive tissues. Recombinant protein representing a single A-type unit for the *A. lumbricoides aba-1* gene was produced and found to bind retinol (Vitamin A) and a range of fatty acids, including the pharmacologically active lipids lysophosphatidic acid, lysoplatelet activating factor, and there was also evidence of binding to leukotrienes. It failed to bind to any of the anthelmintics screened. Differential Scanning Calorimetry showed that the recombinant protein was highly stable, and unfolded in a single transition at 90.4 °C. Analysis of the transition indicated that the protein occurs as a dimer and that the dimer dissociates simultaneously with the unfolding of the monomer units.

Key words: *Ascaris lumbricoides*, ABA-1 allergen, polymorphism, nematode polyprotein allergens/antigens, fatty acid binding protein.

INTRODUCTION

The ABA-1 protein of *Ascaris lumbricoides* (of humans) and *Ascaris suum* (of pigs) is abundant in the pseudocoelomic fluid of the parasites and also appears to be released by the tissue-parasitic larvae and the adult stages (Kennedy & Qureshi, 1986; Kennedy *et al.* 1987*a*, 1989). ABA-1 is probably Allergen A of *A. suum* (Ambler *et al.* 1973; Christie *et al.* 1990). It is the target of IgE antibody responses in both infected humans and rodents (Tomlinson *et*

al. 1989; Christie *et al.* 1990; Kennedy, Fraser & Christie, 1991; Fraser, Christie & Kennedy, 1993; McSharry *et al.* 1999), but it remains to be established whether its allergenic activity is an intrinsic property of the protein or merely due to generalized IgE potentiation by the infection (Christie, Fraser & Kennedy, 1992; Jarrett & Miller, 1982). There is evidence, however, that a homologue from filarial nematodes is also associated with Th2/IgE responses (Paxton *et al.* 1993; Yazdanbakhsh *et al.* 1995; Allen, Lawrence & Maizels, 1995). Only a subset of *Ascaris*-infected people produce antibody to ABA-1 (Kennedy *et al.* 1990; McSharry *et al.* 1999), which is probably due to genetic restriction of the immune repertoire, as has been formally demonstrated in rodents (Kennedy *et al.* 1987*b*; Tomlinson *et al.* 1989; Christie *et al.* 1990; Kennedy *et al.* 1991) and also applies to ABA-1 homologues in other nematode infections (Kwan-Lim & Maizels, 1990; Allen *et al.* 1995). In those humans who respond to ABA-1 there is an association between IgE antibody to ABA-1 and

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relative resistance to infection, although, as with many such studies, this remains an associative rather than a causal link (McSharry *et al.* 1999).

Homologues of ABA-1 have been described in a wide range of nematode species, and DNA sequences encoding them arise frequently from the screening of cDNA expression libraries with antibody from infected hosts (e.g. Culpepper *et al.* 1992; Poole *et al.* 1992; Tweedie *et al.* 1993; Ajuh *et al.* 1995; de Graaf *et al.* 1995; Yahiro, Cain & Butler, 1998). These proteins are unusual in being produced as large polyproteins which are post-translationally cleaved to yield multiple copies of approximately 15 kDa polypeptides (Tweedie *et al.* 1993; Selkirk *et al.* 1993; Spence *et al.* 1993; Poole *et al.* 1996). This unusual means of synthesis has led to the term Nematode Polyprotein Allergens/antigens (NPAs). The individual units within the polyprotein precursors can be highly diverse in amino acid sequence (Britton *et al.* 1995), although some species have several units with identical or very similar sequences (Culpepper *et al.* 1992; Poole *et al.* 1992; Tweedie *et al.* 1993; Ajuh *et al.* 1995; Yahiro *et al.* 1998). The NPA gene (*aba-1*) of *A. suum* is known to have two distinct classes of repeat unit; type A (9 very similar units) and type B (1 unit), whose sequences show only 50% identity (Moore *et al.* 1999).

Parasite-derived ABA-1 or recombinant forms of single NPA units from several species have been found to bind a variety of small lipids including fatty acids and retinol (Vitamin A) (Kennedy *et al.* 1995*a-c*; Moore *et al.* 1999). In the case of ABA-1, fluorescence methods have revealed subtle differences in the ligand-binding sites of type A and type B proteins, although with no fundamental disparity in their binding activities (Moore *et al.* 1999).

A. lumbricoides and *A. suum* are able to infect either humans or pigs, but cross-infection with adult worms is rare in endemic areas in which genetic studies have shown that the *Ascaris* of humans and pigs exist essentially as reproductively isolated populations and could be considered as distinct taxa (Anderson, 1995; Anderson, Romero-Abel & Jaenike, 1993, 1995; Anderson & Jaenike, 1997; Peng *et al.* 1998; Zhu *et al.* 1999). It is conceivable, however, that the tissue-invasive larval stages of *A. suum* infect humans and cause pulmonary hypersensitivity reactions. Such cross-infection may also influence the development of immunity to *A. lumbricoides*. It is therefore of interest to know whether the ABA-1 allergen differs between the two species, and whether polymorphisms exist.

Here we report on the *aba-1* gene of *A. lumbricoides*, using parasites recovered from humans at 2 different geographical locations, and show that *A. lumbricoides* ABA-1 protein is similar to that of *A. suum*, and that the gene is polymorphic. We also provide new information on the organization of the

aba-1 gene, and on the biochemical and structural properties of the protein.†

MATERIALS AND METHODS

Parasite and host materials

A. lumbricoides worms from Guatemala were a gift from Dr T. J. C. Anderson (Oxford University), and *A. lumbricoides* worms from China were kindly provided by Professor Weidong Peng (Nanchang, China); the latter were genetically typed as carrying alleles unique to human-derived *Ascaris*. *A. suum* worms were obtained from the intestines of infected pigs at local abattoirs. Eggs, 1st-stage larvae (L1), infective larvae (here designated 2nd-stage; L2) and lung-stage larvae (taken to be a mixture of 3rd- and 4th-stage larvae, L3/4) of *A. suum* were obtained by standard methods (Kennedy & Qureshi, 1986). Gut and body wall tissues were dissected from adult parasites and stored in liquid nitrogen prior to extraction of nucleic acids. *Ascaris* pseudocoelomic fluid (= *Ascaris* body fluid, ABF) and excretory-secretory (ES) materials were prepared as described previously (Kennedy & Qureshi, 1986). Antisera to infection with *A. lumbricoides* or *A. suum*, or to specific antigens were raised in mice or rabbits as described (Kennedy *et al.* 1987*a*). The sera from humans naturally infected with *A. lumbricoides* were collected from Nigeria (McSharry *et al.* 1999) or The Gambia (a kind gift of Dr Adrian Hill, Oxford University).

Plasmids

Plasmid vectors pT7 Blue and the expression vector pET-15b were purchased from Novagen. pHS10, containing a 1.2 kb of *A. suum aba-1* cDNA inserted in pBluescript vector, was constructed as described previously (Spence *et al.* 1993).

Preparation of DNA and RNA

Ascaris genomic DNA was isolated from eggs and adult worms using standard methods (Sambrook, Fritsch & Maniatis, 1989), with minor modifications. Plasmid DNA was extracted using a Magic Minipreps DNA Purification Kit (Promega). *A. suum* total RNA from various developmental stages (unembryonated eggs, L1, L2 or L3/4 stage larvae) and from different adult tissues (body wall/muscle, ovary, testis or intestine) was isolated using either the method described by Chomczynski & Sacchi (1987) or the TRIzol™ reagent (Gibco-BRL). The integrity of both DNA and RNA preparations was evaluated by agarose gel electrophoresis.

† DNA sequences from this study (but not shown) have been submitted to the EMBL, GenBank and DDBJ Nucleotide Sequence Databases under the accession codes U86091 to U86102.

Table 1. Nucleotide sequences of the oligonucleotide primers and their code names

Primer	Sequence*
HS10N	5'-ggaattcCATCATTTTCACCCTTG-3'
HS10C	5'-ggaattcCCTCCTTCGTTCGCGAAG-3'
RepN	5'-gggaattcCATACAATGGAACACTATC-3'
RepC	5'-gggaattcCCTCCTTCGTTCGATGATG-3'
Lum1	5'-GAAGAAGCAATATATCGCCG-3'
Lum2	5'-CTTGACTGAGCCATTTTCAG-3'
Lum3	5'-cgcgatccCCATCATTTTCACCCTTGAAAG-3'
Lum4	5'-gcgatccTCACGAAGTATGTGCTGCAGC-3'

* Lower cases represent restriction enzyme (*Eco*RI or *Bam*HI) site and clamp sequence.

Southern and Northern blotting

Genomic DNA (1 µg) was digested with different restriction endonucleases (Promega or Appligene) using standard methods (Sambrook *et al.* 1989), fractionated in a 0.8% (w/v) of agarose gel and transferred to a Hybond-N nylon membrane (Amersham) according to the conditions recommended by the manufacturer. Southern hybridization with the [α -³²P]dCTP-labelled probes was carried out using standard procedures. Northern dot-blot analysis was performed with RNA (20 µg) dot-blotted on to the Hybond-N nylon. Each volume of RNA sample was mixed with 2 volumes of formamide, 0.7 volume of 37% formaldehyde and 0.2 volume of 20 × SSC, and incubated at 68 °C for 15 min. Samples were then loaded on to the nylon membrane pre-wetted with DEPC-treated water and 6 × SSC. The RNA was fixed to the membrane, hybridized with ³²P-labelled probe and washed as described for Southern blot analysis.

PCR amplification and sequencing of *A. lumbricoides aba-1 repeat units*

PCR was performed on *A. lumbricoides* genomic DNA to amplify repeat units of the *aba-1* gene. The amplification was carried out for 30 cycles at 94 °C/45 s, 55 °C/45 s and 72 °C/2.5 min using a DNA Thermal Cycler (Perkin-Elmer Cetus). Six oligonucleotide primers (Table 1) were used for PCR. Primers HS10N and HS10C were designed complementary to the extreme 5' and 3' regions of a 399 bp unit (type A) of *A. suum* cDNA clone pHS10 (Spence *et al.* 1993). Primers RepN and RepC were designed complementary to the extreme 5' and 3' regions of the 402 bp divergent type B unit of *A. suum* cDNA clone pJM33 (Moore *et al.* 1999). The remaining two primers (Lum1 and Lum2) were designed complementary to the internal sequences of the pHS10 repeat unit; Lum1 was designed as a forward primer hybridizing at the 3' region (positions 324–343) of pHS10, and Lum2 designed as a reverse primer hybridizing to the 5' region (positions 37–55)

of pHS10. The amplified DNA fragments were purified from low melting temperature agarose gels (1% [w/v], SeaPlaque GTG agarose, FMC) using Wizard™ PCR Preps DNA Purification System (Promega) and cloned into the plasmid vector pT7Blue (Novagen). The constructs were subsequently used to transform *E. coli* DH5 α strain cells (Gibco-BRL). Clones containing the insert were identified by direct colony PCR and restriction endonuclease digestion. Both the coding and the non-coding strands of the DNA inserts were sequenced by the Sanger dideoxy method either using the Sequenase 2.0 Kit (US Biochemical) or the ABI Prism Dye Terminator Cycle Sequencing Ready Reaction Kit on an ABI 373 DNA Sequencing System (Perkin-Elmer Corporation). Nucleotide and deduced amino acid sequences were compared using Wisconsin GCG (Genetic Computer Group) package, version 8.0 for Unix. Similarity and identity between each sequence were determined by the GCG GAP program.

Recombinant *A. lumbricoides ABA-1 type A protein*

DNA encoding 1 repeat unit of *A. lumbricoides aba-1 (aba-1r1)*, originally amplified from DNA of a single specimen of human-derived *A. lumbricoides* from Guatemala, using primers HS10N and HS10C, was subcloned into the pET-15b expression vector (Novagen) using a PCR amplification procedure. Briefly, 2 oligonucleotide primers with *Bam*HI restriction sites (Lum3 and Lum4, Table 1) were designed complementary to the 5' and 3' ends of this *aba-1r1* sequence but omitting sequence encoding the terminal 4 arginines. The reverse primer Lum4 incorporated a stop codon. Amplification was carried out for 30 cycles at 94 °C/45 s, 55 °C/45 s and 72 °C/1.5 min. The amplified DNA was purified from a 1% (w/v) SeaPlaque GTG agarose gel. Using Wizard™ PCR Preps DNA Purification System (Promega), digested with *Bam*HI and ligated into the *Bam*HI digested and dephosphorylated pET-15b. The pET-15b construct, containing *A.*

lumbricoides aba-1r1 unit (designated as pAL2), was transformed into two *E. coli* strains. The first, NovaBlue, was used for initial cloning of target DNA into pET vectors and for maintaining plasmids; the second, BL21 (DE3), was used for target gene expression. Colonies were screened by direct colony PCR and the DNA insert was sequenced to ensure the correct sequence and orientation. Transformants containing the pAL2 clone were grown to an absorbance of 0.6 units at 600 nm, then induced with 1 mM isopropyl β -D-thiogalactopyranoside at 20 °C for 3–4 h. Cells were harvested by centrifugation at 7000 g, resuspended in binding buffer (5 mM imidazole, 0.5 M NaCl, 20 mM Tris, pH 7.9) and sonicated on ice at 10 μ m amplitude for 30 bursts of 30 s. Following centrifugation at 15000 g, affinity purification was carried out by applying the supernatant to His-Bind metal chelation resin and elution carried out according to manufacturer's instructions (Novagen). The N-terminal histidine tag was removed by overnight incubation of the fusion protein with thrombin (Sigma) at a concentration of 0.5 units per mg of protein at room temperature, the cleaved tag removed by dialysis, and the purified recombinant 15.5 kDa protein checked for purity by SDS-PAGE. The recombinant protein from the pAL2 clone was designated rAlABA-1A. Recombinant ABA-1A1 from *A. suum* was produced in an identical manner (rAsABA-1A; Moore *et al.* 1999).

Immunological assays

Detection of antibody in infected or immunized mice or rabbits, or naturally infected humans was carried out using radioimmunoprecipitation combined with SDS-PAGE and autoradiography, or immunoblotting of SDS-PAGE separated antigen preparations and visualization with an alkaline phosphatase conjugate in an ECL system (Amersham). Immunoblotting of *A. suum* pseudo-coelomic fluid was carried out using rabbit antisera and peroxidase-conjugated secondary antibody for the visualization steps.

Spectrofluorimetry and fluorescence-based ligand binding

Residual detergent was removed from solutions of rAlABA-1 by passage through an Extracti-Gel D column (Pierce). Fluorescence emission spectra were recorded at 20 °C with a SPEX FluoroMax spectrofluorimeter (Spex Industries, Edison, NJ), using 2 ml samples in a silica cuvette. Raman scattering by solvent water was corrected for where necessary using appropriate blank solutions. The fluorescent fatty acid analogues 11-((5-dimethylamino-naphthalene-1-sulphonyl)amino)undecanoic acid (DAUDA), and dansyl-DL- α -aminocaproic acid

(DACA) were obtained from Molecular Probes and Sigma, respectively. All-*trans*-retinol, oleic acid and 5-[dimethylamino]naphthalene-1-sulphonamide (dansylamide) were also obtained from Sigma, and *cis*-parinaric acid obtained from Molecular Probes. The excitation wavelengths used for DAUDA, DACA, retinol and parinaric acid were 345, 345, 350 and 319 nm, respectively. The dansylated fatty acids were stored as stock solutions of approximately 1 mg/ml in ethanol, in the dark at –20 °C, and freshly diluted in phosphate-buffered saline (PBS; 171 mM NaCl, 3.35 mM KCl, 10 mM Na₂HPO₄, 1.8 mM KH₂PO₄; pH 7.2) to 1 μ M before use in the fluorescence experiments. Competitors of fluorescent fatty acid binding were prepared as stock solutions in ethanol at approximately 10 mM and diluted in PBS or ethanol for use. Free retinol is poorly soluble and unstable in aqueous solution, so it was dissolved and diluted in ethanol immediately before use and binding to proteins was tested by addition of typically 5 μ l of this directly to a cuvette containing protein in PBS.

For estimation of dissociation constant of protein:fatty acid binding, 5 μ l or 10 μ l samples of protein were added successively to 2 ml of DAUDA at approximately 1 μ M, and the fluorescence measured at 483 nm, with $\lambda_{\text{excitation}} = 345$ nm. The concentration of the ethanol stock solution of DAUDA was checked by absorbance of a 1:10 dilution in methanol at 335 nm, using an extinction coefficient ϵ_{335} of 4400 M⁻¹ cm⁻¹ (Haugland, 1992). The concentration of retinol was estimated by absorbance of a solution of retinol in ethanol at 325 nm, with an ϵ_{325} of 52480 M⁻¹ cm⁻¹. For the titration experiments, retinol in ethanol was added in 5 μ l aliquots to 2 ml of a solution of test protein and mixed immediately. The K_d was estimated with correction for the fluorescence of free retinol added to a cuvette containing only PBS, as previously described (Cogan *et al.* 1976). The concentration of protein was estimated by absorbance at 280 nm, using an extinction coefficient of $\epsilon_{280} = 10810$ cm⁻¹ M⁻¹, based on the amino acid composition of the recombinant protein (Gill & von Hippel, 1989). Fluorescence data were corrected for dilution where necessary, and fitted by standard non-linear regression techniques (using Microcal ORIGIN software) to a single non-competitive binding model to give estimates of the dissociation constant (K_d) and maximal fluorescence intensity (F_{max}). Similar non-linear regression methods were used to analyse results of competition experiments in which test ligands were progressively added to DAUDA/protein mixtures.

Gas chromatography/mass spectrometry (GC-MS)

Purified rAlABA-1A was produced in *E. coli* as described above, but, in order to avoid loss of ligand,

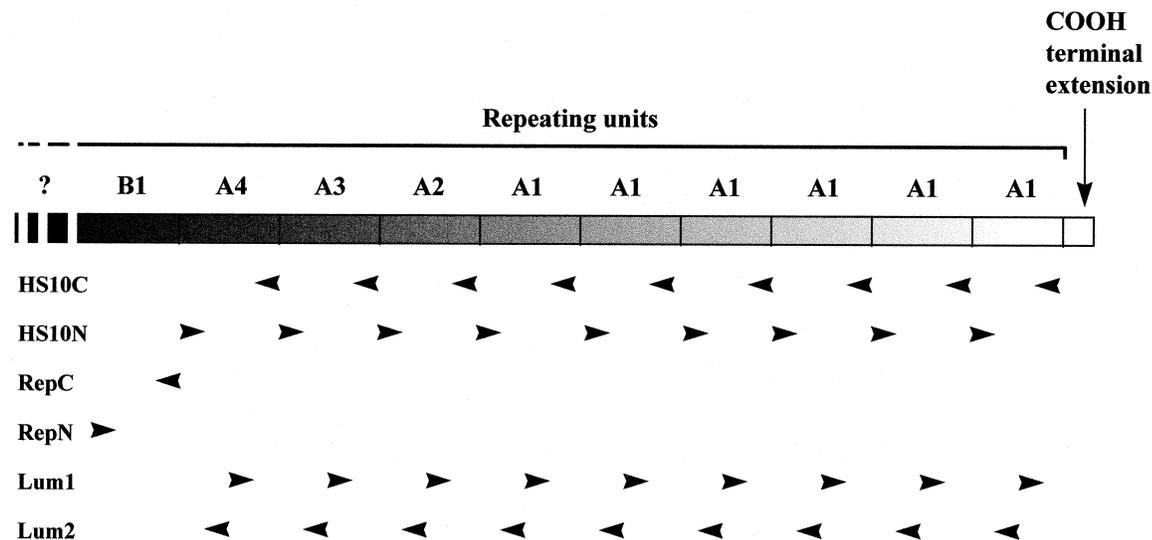


Fig. 1. Schematic representation of the known region of the *Ascaris aba-1* mRNA (derived from Moore *et al.* 1999). Also shown are the positions at which the various oligonucleotides should anneal to initiate amplification in PCR and the direction of their extension.

was not passed down the detergent removal column after elution from the affinity chromatography column. Protein in PBS (1 mg/ml) was acidified with 200 μ l of 1 M HCl and was extracted with 2 ml ethyl acetate. The extract was evaporated to dryness under a stream of nitrogen and 100 μ l of *N,O*-(bis)trimethylsilylacetamide was added, and 2 μ l of the resulting solution were injected into the GC-MS system. GC-MS was carried out using a Hewlett-Packard 5988 A machine. The instrument was fitted with an HP1 column (12 m \times 0.2 mm i.d. \times 0.33 μ m film) and the GC was programmed from 60 $^{\circ}$ C at 10 $^{\circ}$ C per minute to 320 $^{\circ}$ C. The mass spectrometer was operated in the electron impact mode and the scan range was 50–800 atomic mass units.

Differential Scanning Calorimetry (DSC)

Experiments were performed with the rAlABA-1A (1–2 mg/ml) by standard procedures (Cooper & Johnson, 1994) using a Microcal MC2-D instrument at a scan rate of 60 $^{\circ}$ C/h over a 20–110 $^{\circ}$ C range. Normalized excess heat capacity data were analysed by standard procedures, using Microcal ORIGIN software.

Semantics

The gene encoding the ABA-1 polyprotein of *A. lumbricoides* and *A. suum* is termed *aba-1*, and the general term used for the genes encoding nematode polyprotein allergens/antigens, of which *aba-1* is a member, is *npa*. Protein or gene sequences from *A. lumbricoides* are prefixed by Al, and those from *A. suum* by As. The 4 different type A unit sequences (A1, A2, A3 and A4) refer to the *protein* sequences found in *A. suum*, there being several synonymous/silent differences in the DNA sequence which were

useful in assembling the known contiguous cDNA sequence from *A. suum* (Fig. 1; Moore *et al.* 1999).

RESULTS

Fig. 1 illustrates the structure of the mRNA encoding the ABA-1 polyprotein of *A. suum* (AsABA-1). Because of the large size of NPA mRNAs, complete cDNA sequences have been difficult to obtain and are only available from one nematode species (*Dictyocaulus viviparus*; Britton *et al.* 1995). The genomic sequence is available for *Caenorhabditis elegans* (from cosmids VC5 and F27B10). For the ABA-1 of *A. suum*, the available cDNAs together represent 10 tandemly repeated units, each having a consensus processing proteinase cleavage site at the junctions (ArgArgArgArg). The units fall into 2 distinct classes, types A and B (Moore *et al.* 1999). The 6 units at the 3' end are identical in encoded amino acid sequence (AsABA-1A1), and the first 41 amino acids of each are identical to that provided by N-terminal amino acid sequencing of parasite-derived ABA-1 (Christie *et al.* 1990). The 3 units upstream are slight variants (AsABA-1A2, AsABA-1A3 and AsABA-1A4), but the next is substantially different (AsABA-1B), although all possess the conserved amino acid positions common to all NPAs (Trp15, Cys64 and Cys120 in the numbering for the type A unit in Fig. 1; Kennedy *et al.* 1995b).

Gene organization

Southern blotting and hybridization with DNA encoding a type A unit was performed to examine the organization of the *aba-1* genes of both *A. lumbricoides* and *A. suum*. Genomic DNA was digested with *Eco*R1 (which has no site within the

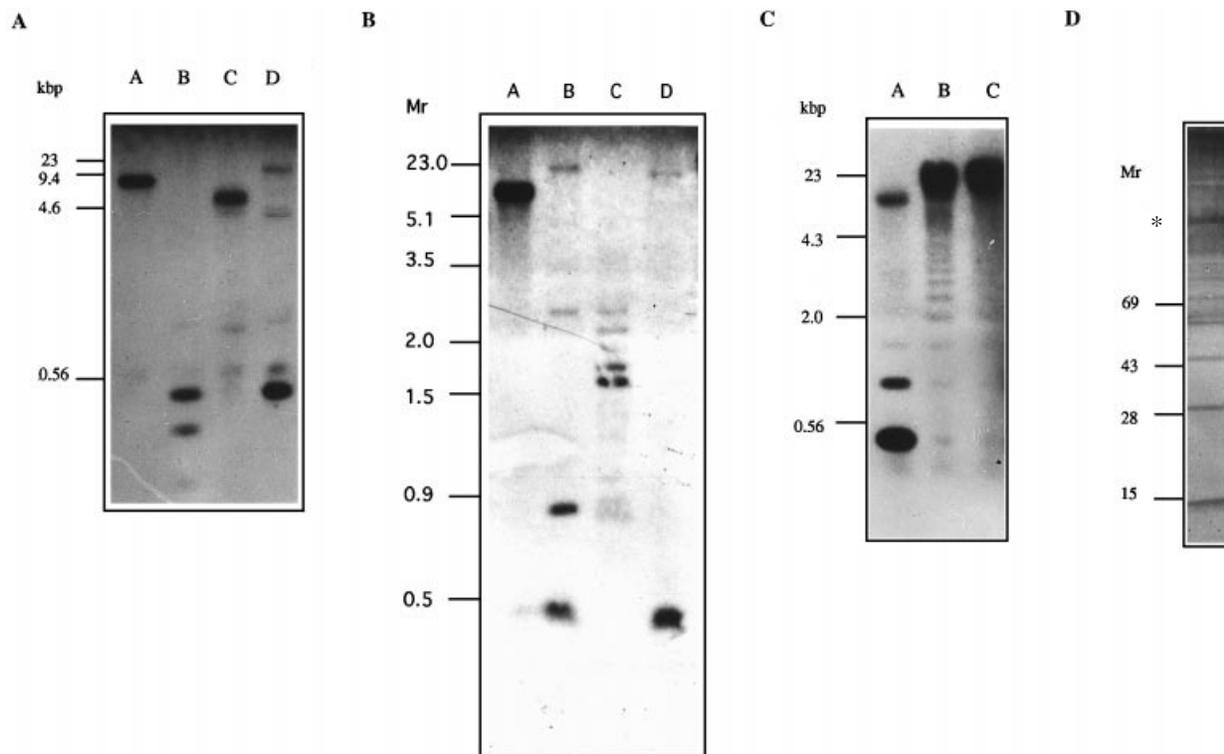


Fig. 2. Organization of the *aba-1* gene in *Ascaris lumbricoides* and *A. suum*. Southern blot analysis of *A. lumbricoides* and *A. suum* genomic DNA probed with the insert encoding ABA-1A1 of *A. suum* (clone HS10). (A) *A. lumbricoides* genomic DNA was digested with *EcoRI* (track A), *XbaI* (B), *Sau3A* (C) or *NruI* (D) and electrophoresed on a 0.8% agarose gel. The gel was capillary blotted onto Hybond N and probed with the insert encoding AsABA-1A1. (B) Similar analysis of *A. suum* genomic DNA digested with *EcoRI* (track A), *NruI* (B), *Sau3A* (C), or *XbaI* (D). (C) Progressive digestion of *A. lumbricoides* genomic DNA with the following concentrations of *XbaI* for 1 h: 5 units of enzyme (A), 1 unit (B), and 0.5 units (C). The gel was capillary blotted on to Hybond N and probed with the insert encoding AsABA-1A1. For each blot, λ *HindIII/EcoRI* markers were run on the same gel and their positions are indicated in kbp. (D) Western blot of the adult pseudocoelomic fluid of *A. lumbricoides* probed with polyclonal rabbit antiserum raised to the recombinant ABA-1A1 protein of *A. suum*. The protein sample was electrophoresed under reducing conditions on a 5–25% gradient SDS-PAGE gel. The positions of standard proteins are as indicated in kDa, and the band corresponding to relative mobility 150 kDa is indicated (*).

known repeat type A units of *A. suum*), and a Southern blot probed with DNA encoding a type A unit of *A. suum* (derived from clone pHS10, sequence AsABA-1A1; Spence *et al.* 1993) (Fig. 2A and B). In both cases, this revealed a single DNA species of approximately 9 kbp, probably representing the full or near full length *aba-1* gene in single copy. Digestion with *Sau3A* (which also has no site within the known *A. suum aba-1* sequences) produced a single 5 kbp band with *A. lumbricoides* DNA (Fig. 2A). In *A. suum* DNA, however, digestion with *Sau3A* produced a more complex set of fragments, indicative of restriction sites in unknown 5' repeat units, and/or polymorphism within the population of *A. suum* (Fig. 2B). Consistent with the latter possibility was the finding that a similar Southern blot carried out with DNA isolated from bulk cultures of infective larvae produced an even more complex pattern of bands (not shown). New *Sau3A* sites are more likely to occur through mutation than are *EcoRI* sites because they comprise 4 bp rather than 6 bp.

Digestion of *A. lumbricoides* or *A. suum* DNA with enzymes having sites within each unit of the known *A. suum* sequences (*XbaI* and *NruI*) produced dominant bands at approximately 400 bp, commensurate with DNA encoding a single unit protein (Fig. 2A and B). The closeness in size of the units in both cDNA and genomic DNA indicates that the region of *aba-1* encoding the A type units is either devoid of introns, or has extremely small introns. *NruI* also produced a fragment of 800 bp in *A. suum*, indicating the existence of at least one repeat unit in which the restriction site is absent. In addition to the dominant 400 bp fragment, *XbaI* also produced a faint band of approximately 200 bp in *A. lumbricoides* DNA, possibly indicating that a second site for this enzyme exists in a minority of the units. Both enzymes also produced fragments of considerably larger sizes which are probably due to restriction sites at the extreme ends of the *aba-1* gene or in flanking genomic sequence.

Progressive digestion of *A. lumbricoides* genomic DNA with *XbaI* (Fig. 2C) produced, first, a single

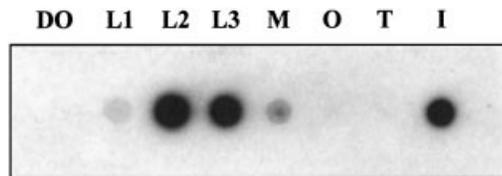


Fig. 3. Northern dot blot to show stage- and tissue-specificity of ABA-1 expression. mRNA from the different life-cycle stages and tissues of adult *Ascaris suum* worms was spotted on to a nylon membrane and probed with ^{32}P -labelled insert cDNA from clone pAL-2 and autoradiographed. mRNA was obtained from unembryonated eggs (D0), eggs containing L1 larvae (L1), eggs containing fully-developed infective larvae (L2), lung-stage larvae (L3), or the following tissues dissected from adult worms, muscle/body wall (M), ovary (O), testis (T) and intestine (I).

band of poorly digested DNA, then approximately 10 bands spaced 400 bp apart, and culminated in a dominant band at 400 bp, presumably representing a single unit length. A very similar result was obtained with *A. suum* DNA (not shown). This effect has been observed for other NPA-encoding genes, and is taken to reflect the repetitive nature of the genes (Selkirk *et al.* 1993; Tweedie *et al.* 1993).

More direct evidence that *A. lumbricoides* ABA-1 is produced as a polyprotein was gained from an immunoblot in which pseudocoelomic fluid from *A. lumbricoides* was probed with rabbit antibody raised against recombinant type A repeat protein (rAsABA-1A1) from *A. suum* and showed a ladder of bands interpretable as polyproteins at progressive stages of processing (Fig. 2D). A strong band at approximately 150 kDa was also evident, which is commensurate with the size of putative unprocessed polyprotein comprising 10 units.

The analysis therefore showed that the *aba-1* gene is similarly organized in *A. lumbricoides* and *A. suum*, and that polymorphisms exist in the *A. lumbricoides aba-1* gene.

Stage- and tissue-specific expression

Total RNA was extracted from different tissues of adult *A. suum* and Northern dot blots were probed with DNA encoding a type A unit (in this case *A. lumbricoides* Alaba-1r1). This demonstrated that the gene was transcribed at a high level in the gut, at a substantially lower level in the muscle/body wall tissue, and not at all in reproductive tissue (Fig. 3). Moreover, mRNA was not detected in unembryonated eggs of *A. suum*, but was present in developing larvae within the egg, and in the later tissue-invasive stages.

Sequence of *A. lumbricoides* ABA-1 and polymorphisms

Fig. 1 illustrates the positions at which the different oligonucleotide primers (Table 1) used for the PCR-

based isolation of NPA genomic sequences from *A. lumbricoides* will hybridize, and the direction of their extension (using the known structure of the *A. suum* mRNA for illustration). Convergent PCR primers (HS10N and HS10C) were used to amplify *aba-1* units from genomic DNA of a single human-derived specimen from Guatemala or a mixture of genomic DNA from 10 specimens from China. This provided 6 unique sequences (Alaba-1r1 to Alaba-1r6) that were slightly different from *A. suum* ABA-1A1, and are aligned in Fig. 4B. This PCR procedure will sample at different positions within this part of the array because the primers derived from type A units will hybridize and initiate amplification of DNA encoding any one of the type A units. Also because of the repeated nature of the coding sequence, amplicons of 400, 800 and 1200 bp were produced, although only those of 400 bp were sequenced.

A similar procedure was used to examine the type B unit in *A. lumbricoides* using primers designed either to amplify DNA encoding a type B repeat alone or contiguously with the type A repeat immediately downstream (Table 1). PCR using the primers RepN/RepC produced amplicons of approximately 400 bp in length, indicating the existence of a single type B unit in the *A. lumbricoides aba-1* gene (not shown). Amplicons of approximately 800 and 1200 bp were then generated using the primers RepN/HS10C, and of approximately 455, 855 and 1255 bp with primers RepN/Lum2 indicative of type A units immediately 3' of a type B unit. However, when a forward primer (HS10N or LUM1) from a type A repeat was used in conjunction with a reverse primer (RepC) from type B, no amplified product was observed, indicating that there are no A-type units upstream of the B-type (not shown). DNA fragments (455 and 855 bp from RepN/Lum2 and 800 bp from RepN/HS10C) were cloned into pT7Blue, and the inserts from 6 clones (including 3 with 455 bp insert, 2 with 855 bp insert and 1 with 800 bp insert) were sequenced on both strands. This provided six type B unit (aligned in Fig. 4A) sequences (Alaba-1d1 to Alaba-1d3 and Alaba1dr1 to Alaba1dr3) and 3 type A unit sequences (Alaba-1dr1 to Alaba-1dr3) (aligned in Fig. 4B) immediately downstream of the type B unit.

The alignment reveals the existence of 23 dimorphic sites, and one tetramorphic site in the type A and B units. The substitutions are a mixture of amino acids with similar or dissimilar properties (as indicated in Fig. 4). One region in which substitutions are particularly prevalent was evident (positions 33–38; Type A).

Recombinant *A. lumbricoides* ABA-1 allergen

In order to examine the immunological and biochemical properties of ABA-1 from *A. lumbricoides*, the Alaba-1r1 cDNA was expressed in *E. coli*.

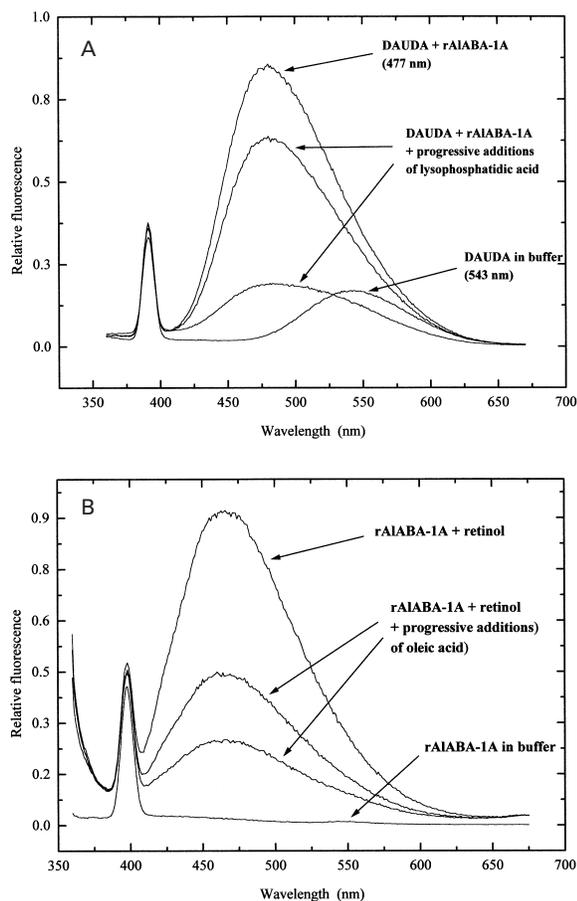


Fig. 5. Ligand binding by recombinant *Ascaris lumbricoides* ABA-1 protein. (A) Recombinant AlABA-1A protein was added to an approximately $1 \mu\text{M}$ solution of DAUDA and produced an enhancement of fluorescence and a blue-shift in emission from 543 nm to 477 nm, which is similar to that with parasite-derived ABA-1 (Kennedy *et al.* 1995b). Also illustrated is a typical result of the addition of a natural, non-fluorescent ligand (here lysophosphatidic acid) to a DAUDA:rABA-1 mixture in which the DAUDA probe is competitively displaced from the binding site into solvent, with a resultant decrease in fluorescence emission. Progressive addition of this ligand resulted in complete reversal of the change in the emission of a DAUDA:rAlABA-1 mixture and provided an estimate of the dissociation constant for rABA-1:lysophosphatidylethanolamine (not shown). Values for this and other ligands are given in Table 2. $\lambda_{\text{excitation}} = 345 \text{ nm}$. (B) Retinol binding to recombinant AlABA-1A. A solution of retinol (Vitamin A) was added to 2 ml of buffer in the fluorescence cuvette containing rAlABA-1. The effect of addition of oleic is also shown. $\lambda_{\text{excitation}} = 350 \text{ nm}$.

Table 2. Ligand binding by recombinant AlABA-1A protein from *Ascaris lumbricoides*

(The binding studies were carried out either by competitive displacement of a bound fluorescent ligand (DAUDA), or by detecting changes in the fluorescence of natural ligands (e.g. retinol, parinaric acid). The value for the dissociation constant (K_d) for rAlABA-1A:DAUDA interaction was calculated as previously described, and this value then used to estimate the apparent dissociation constant (K_{app}) for non-fluorescent compounds in competitive titration experiments (Kennedy *et al.* 1995b).

Binding	No binding
Oleic acid ($K_{\text{app}} = 0.72 \mu\text{M}$)	Tocopherol
Retinol	Tocopherol acetate
<i>cis</i> Parinaric acid	β -carotene
<i>trans</i> Parinaric acid	Mebendazole
Arachidonic acid	Albendazole
Lysophosphatidic acid	Thiabendazole
($K_{\text{app}} = 3.3 \mu\text{M}$)	Oxibendazole
Lysophosphatidyl ethanolamine	Piperazine
Lysophosphatidyl choline	Tetramisole
Platelet activating factor	Pyrantel
Lysoplatelet activating factor	DEC
Leukotrienes B ₄ , C ₄ , D ₄ , E ₄ *	Levamisole
Bilirubin ($K_{\text{app}} = 5.3 \mu\text{M}$)	
DAUDA ($K_d = 1.37 \mu\text{M}$)	
DACA	

* Binding with these compounds was weak but detectable.

Antibodies against the protein were detected in the serum of mice and rabbits infected with *A. suum* or *A. lumbricoides* using both immunoprecipitation and immunoblotting assays (not shown) and in humans naturally infected with *A. lumbricoides* (McSharry *et al.* 1999). In the latter case, it was found that only a subset of infected people produced detectable antibodies to rAlABA-1A, but that these were the same as those found to respond to parasite-derived ABA-1. In mice infected with *A. suum*, only those of the H-2^s haplotype are known to respond to AsABA-1, and serum antibody from such mice also bound rAlABA-1A (not shown).

Ligand binding

rAlABA-1A was found to bind the fluorescently tagged fatty acid DAUDA, and retinol (Vitamin A) (Fig. 5). The fluorescence emission of DAUDA was strongly enhanced and blue-shifted (543 to 475 nm) upon binding, which is similar in degree to that with parasite-derived ABA-1 (Kennedy *et al.* 1995b), and

Alaba-1dr1, Alaba-1dr2, and Alaba-1dr3. Although the entire cDNA from mRNA is not yet available for the ABA-1 polyprotein, it is likely from our previous DNA and protein sequencing work that Type A units are the most abundant form. The recombinant protein used in subsequent experiments was derived from DNA encoding the Alaba-1r1 sequence, but without the COOH-terminal four arginines. The parts of the protein which are predicted to form helical secondary structure (Kennedy *et al.* 1995b) are underlined.

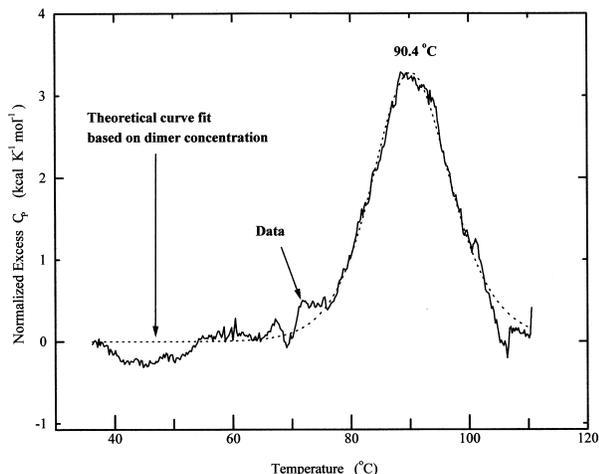


Fig. 6. Differential Scanning Calorimetry analysis of recombinant AlABA-1A. Protein solution (0.9 mg/ml, 2 ml) was scanned from 20 $^{\circ}C$ to 110 $^{\circ}C$ yielding a single cooperative unfolding transition at 90 $^{\circ}C$. Reasonable numerical agreement between calorimetric ($\Delta H_{cal} = 58.0$ kcal/mol) and van't Hoff enthalpies ($\Delta H_{vH} = 59.5$ kcal/mol) analysed assuming dimer concentration supports the idea that the protein unfolds cooperatively as the dimer.

is indicative of entry of the fluorophore into a highly apolar environment (Macgregor & Weber, 1986). DAUDA was found to be competitively displaced from the protein's binding site by natural fatty acids and related lipids, examples of which are also shown in Fig. 5. This provided an assay for screening anthelmintics and biologically important lipids for binding (Table 2). None of the anthelmintics examined could displace the fluorescent probe in this assay system. Lipids associated with inflammatory processes were found to bind, including lyso-phosphatidic acid, lyso-platelet activating factor, and certain eicosanoids (arachidonic acid, leukotrienes). Dissociation constants were estimated in fluorescence titration experiments, or indirectly in competitive titration experiments, revealing that the binding affinities were of a similar order of magnitude to that for other lipid transport proteins (Wilkinson & Winton, 1986; Thumser *et al.* 1994).

To examine the hydrophobic ligands which bind to rAlABA-1A in a biological context, a sample of the affinity-purified protein, but with no further treatment, was subjected to extraction against ethyl acetate and the extracted material subjected to GC-MS. The mass spectra revealed single ions consistent with the presence of palmitoleic, palmitic, oleic and stearic acids. No evidence for significant amounts of retinol was found, which would be consistent with the fact that *E. coli* does not synthesize retinoids.

Calorimetry

DSC showed that rAlABA-1A requires very high temperatures before unfolding ($T_m = 90.4$ $^{\circ}C$; Fig. 6)

and only a single transition was observed. Reasonable numerical agreement between calorimetric and van't Hoff enthalpies (analysed assuming dimer concentration) supports the idea that the protein unfolds cooperatively as the dimer. That is, the dimer dissociates and the monomer units unfold simultaneously, indicating that the structural integrity of the monomer units depends on intermolecular interactions in the dimer and/or that the dimer interaction depends on the structural integrity of the monomer partners.

DISCUSSION

This analysis of the *aba-1* genes of *A. lumbricoides* and *A. suum* reveals them to be similarly organized, and each encodes a polyprotein of at least 10 repeat units. This is the same number as that already known from contiguous sequence from *A. suum* (Moore *et al.* 1999). *D. viviparus* and *C. elegans* have NPA genes which contain 11 and 10 units, respectively (Britton *et al.* 1995; *C. elegans* genomic cosmids VC5 and F27B10), so it is conceivable that there are no more units to be found in *aba-1*. A notable feature of *aba-1* which *Ascaris* and *Toxocara* spp. appear to have in common with filarial nematodes, but not with *D. viviparus* and *C. elegans*, is close similarities in the sequences of several contiguous repeat units (Culpeper *et al.* 1992; Poole *et al.* 1992; Tweedie *et al.* 1993; Paxton *et al.* 1993; Yahiro *et al.* 1998). It might be relevant that the ascaridids and the filarial nematodes are in the same clade within the nematoda, and that *D. viviparus* and *C. elegans* belong to another, distantly related, clade (Blaxter, 1998; Blaxter *et al.* 1998).

The current analysis indicated that a large part or all of the coding region of the *A. lumbricoides aba-1* gene is without introns, as is thought to be the case for the homologues in other species (Tweedie *et al.* 1993; Paxton *et al.* 1993). A more direct analysis of part of the genomic sequence of *aba-1* indeed found no introns within the repeating units, except for a very large intron (4 kb) in the region encoding the short COOH-terminal extension peptide of the polyprotein (Spence, 1994). The *npa* gene of *C. elegans* has, however, very small introns predicted to interrupt several, but not all, of the units of the polyprotein (I. L. Johnstone, personal communication). Such introns are sufficiently small that they could be missed in experiments involving partial digestion of genomic DNA carried out in this and other studies (Culpeper *et al.* 1992; Poole *et al.* 1992; Tweedie *et al.* 1993). It is therefore possible that *npa* genomic DNA of all nematodes, including ascaridids and filariae, have stretches devoid of introns, but the existence of small introns cannot be excluded at this stage.

The only other known cases of polyproteins encoded in intronless genes are certain structural

proteins synthesized by terminally differentiating keratinocytes (Gan *et al.* 1990; Haydock & Dale, 1990; Rothnagel & Steinert, 1990; Presland *et al.* 1992; Yoneda *et al.* 1992), and it has been suggested that such proteins are the products exclusively of cells undergoing apoptosis (Rothnagel & Steinert, 1990). If so, then ABA-1 and its ilk are exceptional because there is no evidence that they are the products of similarly fated cells.

The polymorphisms observed in *A. lumbricoides aba-1* may also exist in the *A. suum* population, but sampling of any population of the latter remains to be carried out. Different alleles of the *npa* gene of *Dictyocaulus viviparus* have been noted (DvA-1; Britton *et al.* 1995) and further analysis has since revealed several more examples of variant sites in DvA-1 (T. J. M. Anderson, J. B. McKeand and M. W. Kennedy, unpublished observations). Whether the allelic variants of the proteins exhibit differences in biochemical activity remains to be seen.

About half of the amino acid substitutions found in the ABA-1 sequences in *A. lumbricoides* are reasonably common in proteins without alteration of function, so they can be classed as neutral (Dayhoff, Schwartz & Orcutt, 1978; Bordo & Argos, 1991), although there are several examples of radical changes. The substitutions may identify positions which are not crucial to the structure and function of the proteins, and it might be pertinent that the clustering between positions 33 and 38, 62 and 65, and 82 and 83, of the type A sequences correspond to loop regions in the predicted secondary structure of the NPAs (Kennedy *et al.* 1995*b*). The only region where deletions/insertions appear is in the C-terminal region of the ABA-1A repeats, presumably because of lessened structural/functional constraints near the C-termini of the proteins and in the proximity of the cleavage sites.

The high level of ABA-1 expression in the gut of *Ascaris* is significant given that the protein has a lipid-binding function and the protein may be involved in distribution of lipids absorbed and processed by the gut to other sites in the nematode. It might therefore be that the NPA polyproteins are synthesized, post-translationally cleaved into 15 kDa polypeptides and loaded with lipid in the gut cells, prior to release into the pseudocoelomic fluid, where they act as shuttle/transport proteins from the gut to receptors on cells requiring the ligand.

The gp15/400 of *B. malayi* is found in basal laminae separating the hypodermis, muscle cells and oesophagus, at lower levels in cells overlying the basal laminae, and absent from the cuticle (Selkirk *et al.* 1993). The NPA of *Dirofilaria immitis* is reported to occur in the cuticle (Poole *et al.* 1992), yet our immunoelectron microscopy work on *A. suum* larvae, like that for gp15/400, showed no evidence for cuticular localization (I. M. Huxham & M. W.

Kennedy, unpublished observations). Together with the abundance of ABA-1 in the pseudocoelomic fluid of *Ascaris*, it is probable that the NPAs are extracellular proteins responsible for transporting and protecting insoluble and chemically sensitive lipids. NPAs may unload their cargo upon interaction with appropriate cell surface receptors, or be briefly internalized and recycled as *apo* proteins into the pseudocoelomic fluid.

We were unable to detect any antigenic differences between the ABA-1 of *A. lumbricoides* and *A. suum*, consistent with previous work (Kennedy *et al.* 1987*a*, 1989). This is not surprising given the similarity of the A-type units between the two species, although analysis at the epitope level remains to be carried out. This means, however, that humans sensitized to ABA-1 from one species will react to the homologue from the other. So, even if adult *A. suum* were not able to establish as adults in humans, exposure to its tissue-invasive larvae might nevertheless provoke hypersensitivity reactions initiated by prior *A. lumbricoides* infection (Gelpi & Mustapha, 1967, 1968). IgE antibody responses to rAlABA-1A associate with relative resistance to *A. lumbricoides* in humans (McSharry *et al.* 1999), so exposure to the porcine parasite could affect the development of both immunity and/or pulmonary hypersensitivity syndromes. The same would apply to the type B units of the two species, which we find to be antigenically distinct from the type A units (J. Moore, H. J. Spence & M. W. Kennedy, unpublished results).

Most of the anthelmintic drugs used against parasitic nematodes are hydrophobic and poorly soluble in water. It is therefore conceivable that a protein such as ABA-1 might be involved in the absorption and distribution of these drugs to their sites of action within the nematode, or act to sequester hydrophobic drugs in resistant strains. We found, however, that none of the commonly used anthelmintics binds to rAlABA-1A (ivermectin was not examined because of the technical problems we found it to present in our fluorescence assays).

The ligand-binding properties of rAlABA-1A are essentially indistinguishable from those of parasite-derived ABA-1 in terms of binding specificity and the nature of the binding site environment (Kennedy *et al.* 1995*b*). The current finding that rAlABA-1A binds certain pharmacologically active lipids will therefore be pertinent to the release of NPAs by tissue-parasitic species and the possibility that they modulate local inflammatory and immune responses, which might in turn be material to the persistence of the parasites in the sensitized host.

We have previously used differential scanning calorimetry (DSC) not only to examine the stability of this ascaridid allergen, but also to provide information on its quaternary structure (Kennedy *et al.* 1995*b*). Using parasite-derived ABA-1, we found that the protein exists as a dimer, and that it

appeared to undergo a 2-phase unfolding process which is not the same as observed here for the recombinant AlABA-1A, or the NPA from a different species (Kennedy *et al.* 1995c). The 2-phase transition seen with parasite-derived ABA-1 might therefore have been an artifact of molecular heterogeneity in the parasite-derived preparation (which is most likely given current knowledge of the *aba-1* gene), or contamination with other types of protein.

It has previously been noted that the allergens of ascaridids are heat stable, retaining their allergenicity following cooking (Audicana *et al.* 1997) or even autoclaving (Christie, Dunbar & Kennedy, 1993). This could be due to the IgE epitopes being resistant to heat denaturation (as, for example, would be short linear peptide epitopes), and/or that the allergens are structurally stable at high temperatures or capable of refolding efficiently upon cooling. The calorimetry experiments reported here showed that rAlABA-1A is highly heat stable, and that unfolding requires temperatures in excess of 90 °C. We have also found that the NPA proteins refold efficiently, although their binding function is compromised (L. McDermott, A. Cooper & M. W. Kennedy, unpublished observations). This high degree of stability is a characteristic of 4 bundle alpha helix proteins, a class of protein to which ABA-1 possibly belongs (Kennedy *et al.* 1995a), and is a type of structure known for certain carrier proteins of invertebrates (Branden & Tooze, 1999). Of significance, calorimetry showed that the recombinant AlABA-1A exists as a dimer in solution at the concentrations used. This increase in effective size will presumably limit the protein's diffusability within, and loss from, the nematode. Moreover, existence as a homodimer means that cross-linking of IgE receptors on mast cells will be facilitated, even where the number of epitopes on a monomer unit is limited. We have yet to establish, however, whether NPA proteins naturally exist as homodimers or as heterodimers with other members of the array from which they are derived, and whether dimerisation is necessary for their ligand binding activities.

The organization of the gene encoding the ABA-1 polyprotein of *A. lumbricoides* appears to be similarly organized to that for *A. suum*, and the encoded proteins have virtually indistinguishable biochemical activities. The polymorphisms of the gene found here in the former may also occur in *A. suum*, but it is too early yet to say whether any divergence between the 2 taxa is reflected in differences in the ABA-1 proteins which are unique to either. It is likely, however, that the ABA-1 allergens of *A. lumbricoides* and *A. suum* are for all intents and purposes immunologically indistinguishable. In terms of the biology of the parasite, the new ligand-binding data contribute to the idea that NPAs secreted by nematode parasites could modulate their

tissue and immunological environments through their binding of pharmacologically active lipids.

Note added to proof

A recent paper by P. L. Geenen *et al.* (*Journal of Parasitology* (1999) **85**, 616–622) has convincingly settled the debate as to the developmental stage of the infective stage larva in the egg of *Ascaris* – it is the third stage (L3), and not the L2 stage as used in our paper. The convention used in our paper follows the previously standard nomenclature, and is thus incorrect, but the naming and source of the various larval stages used is detailed in Materials and Methods and in the legend to Fig. 3.

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