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Evidence that a 16-Kilodalton Integral Membrane Protein Antigen from *Schistosoma japonicum* Adult Worms Is a Type A2 Phospholipase

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Type A2 phospholipase (PLA2) activity has been observed in integral membrane protein extracts of *Schistosoma japonicum*. Antiserum raised against bee venom PLA2 recognized a single 16-kDa band in the parasite extracts; it also localized to antigen in the gut lining of fixed adult schistosomes as shown by immunofluorescence techniques. Evidence was obtained that the molecule was expressed at low levels in comparison with other integral membrane proteins and was weakly immunogenic in rabbits. Two oligonucleotide probes were constructed on the basis of highly conserved regions between the nucleotide sequences of rat, bovine, rattlesnake, and bee venom PLA2; these probes were used to isolate *S. japonicum* genomic DNA phage clones. A 1.8-kb *Fnu*D2 fragment was shown by Southern blot analysis to strongly hybridize with the 5′ ^{32}P-labeled PLA2 oligonucleotides in both *S. japonicum* genomic DNA and DNA from one of the phage clones. The nucleotide and predicted amino acid sequences of this fragment revealed homology with the C terminus of PLA2s from different species.

In the search for vaccines against schistosomes, one approach is to identify parasite molecules that play a pivotal role in the essential and ingenious immune avoidance mechanisms displayed by these well adapted multicellular parasites (13). In this context, phospholipases are important enzymes concerned with lipid metabolism; phospholipase A2 (PLA2) catalyzes the specific hydrolysis of ester bonds linking the acyl chain to the C-2 position of 1,2-diacyl-3-*sn*-glycerophospholipids (20). PLA2 has been one of the most well studied phospholipid-degrading enzymes, partly because it is a major and important component of snake and bee venoms (9, 22) and of pancreatic digestive secretions from which it can be obtained in large quantities (15, 22). Surprisingly, even though PLA2s from different organisms show little conformity in terms of their molecular size and their substrate and pH requirements, considerable homology has been observed between these enzymes at both the amino acid and nucleotide levels (5, 21, 22). This feature is thought to be typical of proteins with a common evolutionary origin (5).

Recent work has shown that membrane-bound forms of PLA2 are involved in membrane lipid homeostasis and are particularly active against highly cytotoxic phospholipid hydroperoxides (19) which can be generated in membranes by the release of superoxide radicals from activated leukocytes (7). Considering the importance of this enzyme in protection against the lethal effects of membrane lipid peroxidation (18, 19), and as an adjunct to our recent preliminary characterization of *Schistosoma japonicum* integral membrane proteins (16), the present studies were undertaken to determine whether adult schistosomes possessed membrane-bound PLA2.

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MATERIALS AND METHODS

Parasites and host. A Philippine strain (Sorsogon) of *S. japonicum* and a Puerto Rican strain of *Schistosoma mansoni* were maintained in the laboratory; *S. japonicum* was passaged through *Oncomelania hupensis quadrasi* snails and either rabbits or mice, while *S. mansoni* was passaged through *Biomphalaria glabrata* snails and mice by previously published methods (23). Adult schistosomes were harvested at 6 to 8 weeks postinfection and were carefully washed three times in ice-cold phosphate-buffered saline (PBS).

Sera. Antisera were raised in rabbits against sodium dodecyl sulfate (SDS)-denatured bee venom PLA2 (Boehringer Mannheim, North Ryde, NSW, Australia), *Clostridium welchii* phospholipase C (type XIV; Sigma, Poole, United Kingdom), or homogenates of whole adult *S. japonicum* or *S. mansoni* worms as described previously (16). Serum was also collected from a rabbit harboring a 55-day *S. japonicum* infection.

SDS-polyacrylamide gel electrophoresis and Western blotting (immunoblotting). Adult worms were homogenized in PLA2 buffer (see below) by 50 passes in a Dounce homogenizer and then extracted with Triton X-114 as documented previously (16). For some experiments, the integral membrane protein fraction was recovered, resuspended in 400 μl of reducing sample buffer (1% SDS, 50 mM Tris-HCl, 10% β-mercaptoethanol, 2 mM phenylmethylsulfonyl fluoride, 1 mM EDTA, 1% glycerol, 0.01% bromophenol blue [pH 7.5]), and boiled for 2 min. Samples were then electrophoresed on 13% polyacrylamide gels, and the separated proteins were transferred onto nitrocellulose filters (2). Nitrocellulose filters were blocked for 1 h in 5% skim milk powder (Blotto) in PBS (pH 6.8), and then strips were cut and incubated overnight at 4°C with 1/200 dilutions of the appropriate antiserum. The nitrocellulose strips were then washed three times with Blotto-PBS and probed with ^{125}I-labeled protein A (5×10^8 cpm ml^{-1}) for 15 min at room temperature.

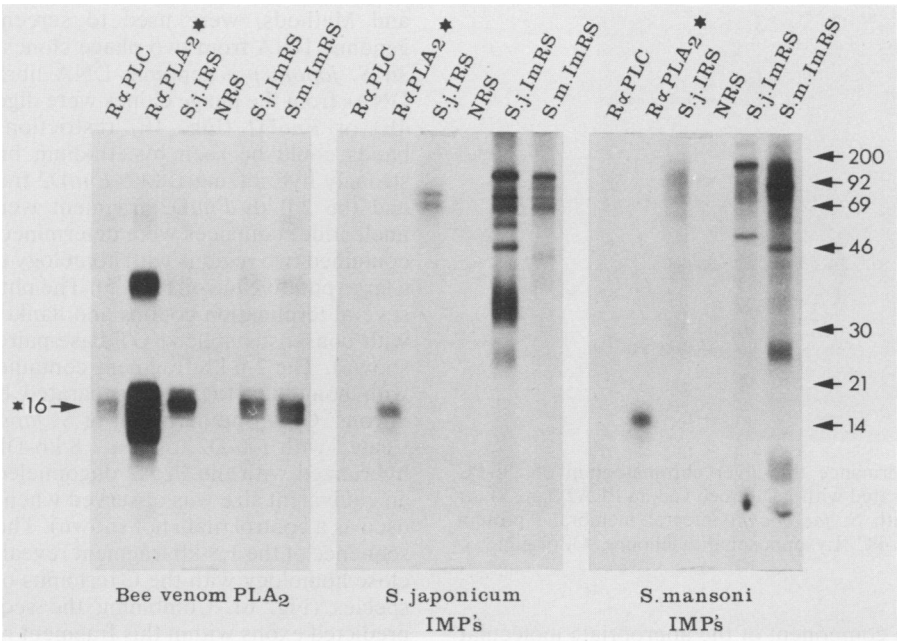

FIG. 1. Western blot analysis of sera from rabbits immunized with bee venom PLA2 A2 (RαPLA2*), *C. welchii* phospholipase C (RαPLC), and extract from whole adult *S. mansoni* (S.m. ImRS) or *S. japonicum* (S.j. ImRS). Serum was also taken from rabbits infected with *S. japonicum* (S.j. IRS). NRS, Normal rabbit serum. The antigens used were bee venom PLA2 (Boehringer Mannheim) (left panel) and integral membrane proteins (IMP's) from *S. japonicum* and *S. mansoni* (middle and right panels, respectively).

Finally, the strips were washed extensively in PBS for 1 h and set up for autoradiography.

Enzyme assay. PLA2 activities in integral membrane protein extracts of whole adult worms (prepared as described above) and in a commercial enzyme preparation were measured by a modification of published methods (4, 8). Briefly, L-3-phosphatidylcholine-1,2-di[1-^{14}C]oleoyl (Amersham, International plc, Aylesbury, United Kingdom) (PC) was evaporated to dryness under oxygen-free nitrogen and resuspended in 25 mM sodium deoxycholate in 80% ethanol to give 12 nmol of lipid per 100 μl of solvent. A 100-μl volume of the labeled lipid was then added to 100 μl of PLA2 buffer (0.1 M Tris [pH 9], 1 mM calcium chloride, 100 mM sodium chloride, 1 mM sodium deoxycholate) containing 0 to 20 U of bee venom PLA2 per ml (Boehringer Mannheim) or 100 μl (5 μg) of integral membrane protein fraction from *S. japonicum* adult worms. An 800-μl volume of PLA2 buffer was added, and the reaction was allowed to proceed for 1 h at 37°C. The reaction was then stopped by the addition of 50 μl of chloroform-methanol (2:1, vol/vol), 50 μl of chloroform, and 50 μl of 4 M potassium chloride. The organic and aqueous phases were separated by centrifugation at 10,000 × *g* for 5 min. The lower (organic) phase was then recovered, and 20 μl was spotted onto a silica-gel high-performance thin-layer chromatography plate (Merck, Darmstadt, Federal Republic of Germany) and chromatographed in chloroform:methanol-acetic acid-water (100:60:16:8, vol/vol) to separate parent phospholipid from the major product of PLA2 digestion.

Histochemical analyses. Adult schistosomes were fixed lightly in paraformaldehyde, dehydrated in graded alcohols, embedded in JB4 methacrylate resin (Biorad Microscience Ltd., Hemel Hempstead, United Kingdom), and sectioned. Sections (1 μm) attached to glass slides were incubated with antibody to bee venom PLA2 or normal serum, washed with PBS, and then incubated with a fluorescein-labeled second antibody as described elsewhere (14). Washed sections were mounted under glass coverslips and examined with a Zeiss microscope by using epifluorescence.

Molecular cloning and sequencing. Two oligonucleotides were constructed by use of an Applied Biosystems 381A DNA synthesizer and were based on regions of strong homology between rat, bovine, rattlesnake, and bee venom PLA2s. A single inosine was placed in each oligonucleotide at a point where there was considerable amino acid variation and where degeneracy could not be applied. The nucleotide sequence for the two oligonucleotides was as follows: for PL35-1, 5′-GCCGCCATGTGCTTCTCCAAGGTCCC(I)TACACCAA-3′, 35-mer; for PL34-2, 5′-TGGACAGGTGCTGCCAGAG(I)CATGACCACTGCTA-3′, 34-mer. An *S. japonicum* EMBL3 genomic DNA library was screened with 5′ ^{32}P-labeled (~10 cpm μg^{-1}) PL35-1 and PL34-2 oligonucleotides (10) by using the Benton-Davis method (1), and a number of phage clones were identified. Southern blots of *S. japonicum* and rabbit liver genomic DNA or the purified *S. japonicum* EMBL3 genomic DNA clones, digested with *Eco*RI and *Fnu*D2 restriction enzymes, were probed with the 5′ ^{32}P-labeled oligonucleotides. Nylon Hybond filters (Amersham) were washed in 4× SET (0.1 M NaCl, 1 mM EDTA, 10 mM Tris-HCl [pH 8])–0.1% SDS at 42°C. The complete nucleotide sequence of a 1.8-kb *Fnu*D2 restriction fragment from one of the genomic EMBL3 clones was deduced by dideoxynucleotide nucleotide sequencing (Sequenase; United States Biochemical Corp.) as well as the sequences of smaller *Dra*I, *Alu*I, *Hin*dII, and *Nla*IV restriction enzyme fragments.

RESULTS

Identification of a 16-kDa membrane-bound putative PLA2. Serum from rabbits immunized with SDS-denatured bee venom PLA2 (Fig. 1, RαPLA2*) strongly recognized this enzyme on Western blots (Fig. 1, left panel) but also

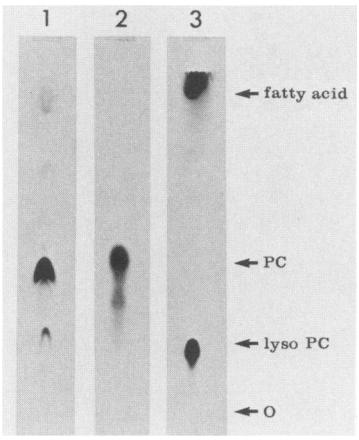

FIG. 2. High-performance thin-layer chromatogram of ^{14}C-PC (lane 2), ^{14}C-PC incubated with 1 U of bee venom PLA2 (lane 3), or ^{14}C-PC incubated with *S. japonicum* integral membrane protein fraction (lane 1). lyso-PC, Lysophosphatidylcholine; O, origin.

recognized a single component of the appropriate molecular weight in integral membrane protein fractions isolated from *S. japonicum* and *S. mansoni* adult worms (Fig. 1, middle and right panels). A second antiserum raised against phospholipase C from *C. welchii* (Fig. 1, RαPLC) showed weak cross-reactivity towards the bee venom PLA2 and did not recognize any band in the schistosome integral membrane protein fractions (Fig. 1). Interestingly, *S. japonicum*-infected rabbit serum (Fig. 1, S.j. IRS) and both *S. japonicum*- and *S. mansoni*-hyperimmune rabbit sera (Fig. 1, S.j. ImRS and S.m. ImRS) contained antibodies which recognized the bee venom PLA2 (Fig. 1, left panel) but did not recognize the 16-kDa component in the integral membrane protein fraction as a major band (Fig. 1, middle and right panels). A 16-kDa band was visible, however, after a much longer exposure period (data not shown).

PLA2 activity in the integral membrane protein fraction from *S. japonicum* was examined by incubation with PC and analysis of the reaction products by high-performance thin-layer chromatography (Fig. 2). Incubation of the labeled PC with bee venom PLA2 resulted in the generation of lysophosphatidylcholine and free fatty acid (Fig. 2, lane 3). Partial cleavage of labeled PC into the appropriate reaction products was observed after incubation with *S. japonicum* integral membrane proteins (Fig. 2, lane 1), indicating trace levels of a PLA2 activity in this extract. Similar results were observed with *S. mansoni* (data not shown). Labeled glycosyl-inositol phospholipid of *Leishmania major* promastigotes (a generous gift from M. McConville), which has a single ester-linked acyl chain in the 2 position, was cleaved upon incubation with either *S. japonicum* or *S. mansoni* integral membrane protein fractions.

Histochemical analyses. In the histochemical analysis experiments, rabbit antiserum against PLA2 and fluorescein isothiocyanate-conjugated goat anti-rabbit immunoglobulin G antiserum was incubated sequentially with semithin JB4 methacrylate sections prepared from adult schistosomes. Microscopic examination of the sections clearly showed that the brightest fluorescence was associated with the parasite gut lining and that no fluorescence was observed in or on the tegument (Fig. 3).

Isolation and characterization of a genomic DNA clone. ^{32}P-labeled PLA2-specific oligonucleotides (see Materials and Methods) were used to screen a Southern blot of genomic DNA from two phage clones (Fig. 4) isolated from an *S. japonicum* genomic DNA library. The 15-kb insert DNAs from the phage clones were digested with *Eco*RI (Fig. 4E) or *Fnu*D2 (Fig. 4F) restriction enzymes (numerous bands could be seen by ethidium bromide staining). The strongly hybridizing 1.8-kb *Fnu*D2 fragment (Fig. 4, arrow) and the 2.0-kb *Fnu*D2 fragment were isolated, and their nucleotide sequences were determined. The 1.8-kb fragment contained two regions with homology to PLA2s separated by a large putative intron (Fig. 5). The putative intron contained several termination codons and flanked the predicted exons with consensus spliced GT base-paired junctions (data not shown). The 2.0-kb fragment contained some small regions with homology to PLA2 separated by numerous putative introns. On a Southern blot of *S. japonicum* genomic DNA treated with *Fnu*D2, only a 1.8-kb DNA fragment strongly hybridized with the PLA2 oligonucleotides and no band of an equivalent size was observed when rabbit liver DNA was used as a control (data not shown). The predicted amino acid sequence of the 1.8-kb fragment revealed two regions of very close homology with the C terminus of PLA2 from different species (Fig. 6). Combining the sequences of these two predicted exons within this fragment revealed an amino acid sequence that is homologous to the sequences of PLA2 from bee venom and from the tiger snake (residues 89 to 102). In addition, the schistosome sequence shares strong homology from residues 103 to 119 with the sequences of mammalian PLA2. Some of these shared residues include alanine (residue 103), cysteine (residue 105), proline (residue 110), and asparagine (residue 112). Whether this sequence is a portion of a gene that encodes the 16-kDa putative PLA2 protein remains to be determined.

DISCUSSION

Parasite surface integral membrane proteins are likely to play an important role in molecular exchange at the interface between schistosomes and their mammalian hosts (17), in the numerous strategies by which blood flukes evade potentially lethal immune responses (3, 13), and in other adaptations necessary for survival in their harsh intravascular habitat (6). In the present study, we have documented preliminary evidence that adult schistosomes possess a membrane-bound form of PLA2, an important enzyme for lipid metabolism.

Two compelling lines of evidence led to the identification of this enzyme in adult blood flukes. Firstly, by using a rabbit polyclonal antiserum raised against bee venom PLA2, we identified a single 16-kDa integral membrane protein from *S. japonicum* and *S. mansoni* adult worms that had a molecular weight compatible with bee venom PLA2 on Western blots. Moreover, we were able to demonstrate that the integral membrane protein fraction from both schistosome species contained PLA2 activity by employing an appropriate enzyme assay. The possibility that the 16-kDa molecule had been acquired from the host was excluded by the fact that anti-PLA2 antibody did not recognize any band on Western blots of rabbit whole-blood extract (data not shown).

Immunohistochemical investigations with our anti-PLA2 antiserum on adult worm tissue sections revealed unexpectedly that the putative PLA2 was localized to the membrane of the gut lining and yet, under the conditions of the experiment, could not be identified at the parasite surface. Moreover, we were unable to demonstrate PLA2 activity in

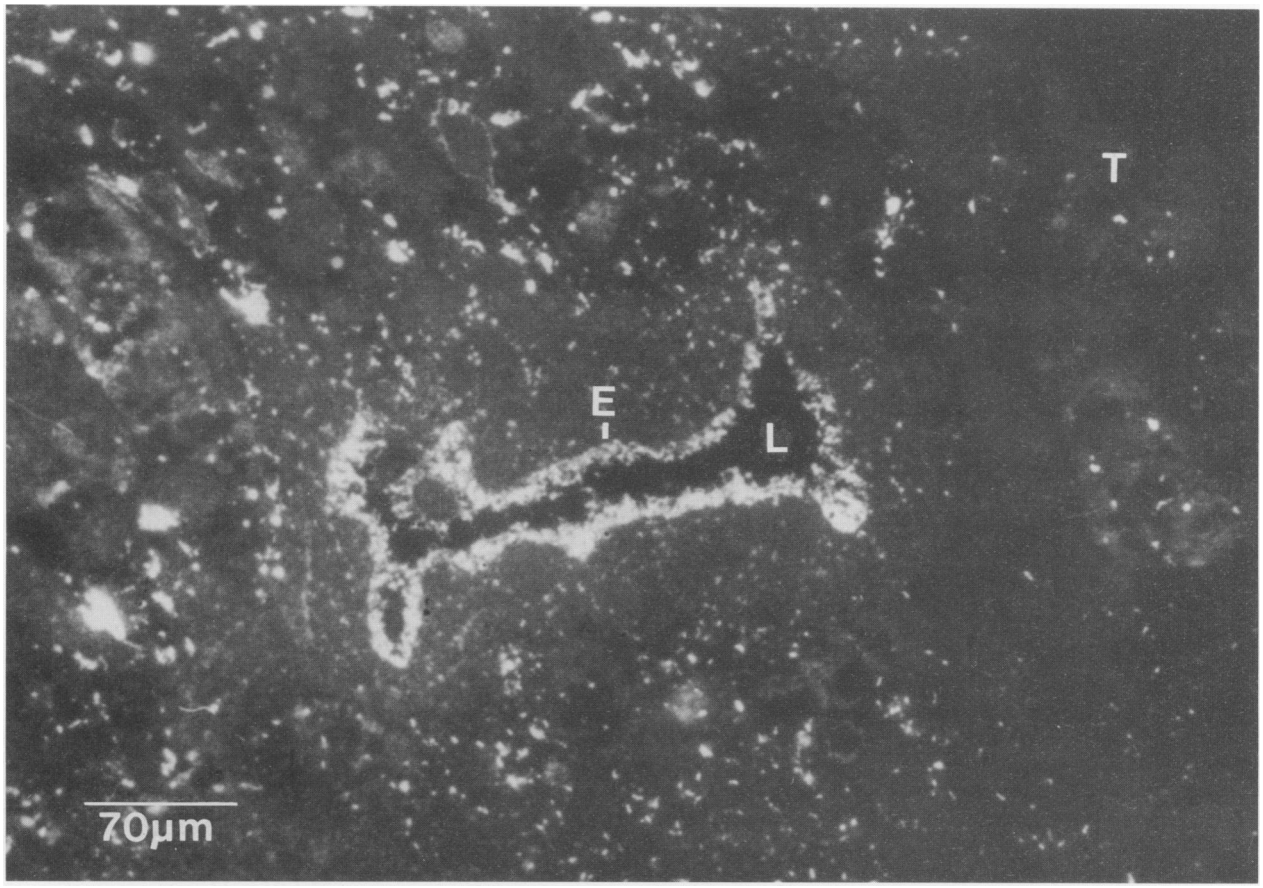

FIG. 3. Fluorescence micrograph of rabbit anti-PLA2 binding to a JB4 methacrylate section of an adult schistosome. T, Tegument; E, gut lining; L, gut lumen.

purified tegumental membrane preparations (data not shown). These observations are compatible with the fact that there has been no evidence that the surface tegumental membrane can internalize and/or metabolize host-derived lipids or is capable of undertaking repair of lipid peroxidation damage for which PLA2 would be required other than by outer membrane sloughing (13).

Localization of PLA2 to the gut lining raises many questions concerning the role of the molecule at this particular site. In this context, it is unlikely that the PLA2 performs a digestive function on ingested host cells, since it is membrane bound and does not seem to be expressed in high levels (unlike PLA2 from mammalian digestive secretions [15]). The schistosome gut lining which is delineated by a single trilaminate membrane may, however, be prone to generation of lipid peroxides after release of superoxide

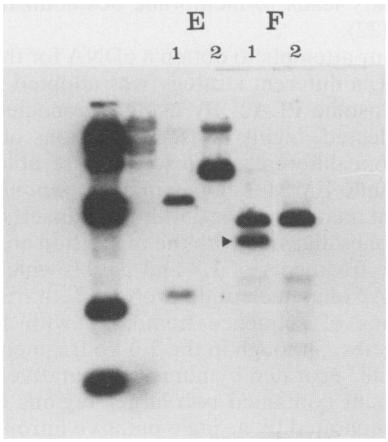

FIG. 4. Oligonucleotide Southern blot showing genomic DNA from two *S. japonicum* phage clones (columns 1 and 2) treated with *Eco*RI (E) or *Fnu*D2 (F). ^{32}P-labeled *Hin*dIII-cut lambda DNA size markers are included in the left track. The 1.8-kb *Fnu*D2 fragment (arrow) was subjected to nucleotide sequence analysis.

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K  Y  P  L  D  S  V  V  D  Q  E  V  M  T  A  L
*AAGTATCCCCTCGATAGCGTGGTCGACCAGGAAGTGATGACCGCCCTT
600                   620                   640

C  T  S  K     G  P  K  N  K  E  K  S  D  L  D
TGTACTTCTAAA*/*GGTCCCAAGAACAAAGAAAAGTCAGACTTGGAC*
      655                1140
```

FIG. 5. Nucleotide and predicted amino acid sequence of two regions of the 1.8-kb *Fnu*D2 genomic DNA fragment with significant homology to the C terminus of PLA2 from different species. These regions are flanked by what is assumed to be putative introns (*); */* comprises 470 nucleotides. Other smaller regions of the 1.8-kb *Fnu*D2 fragment also shared homology with PLA2 (data not shown).

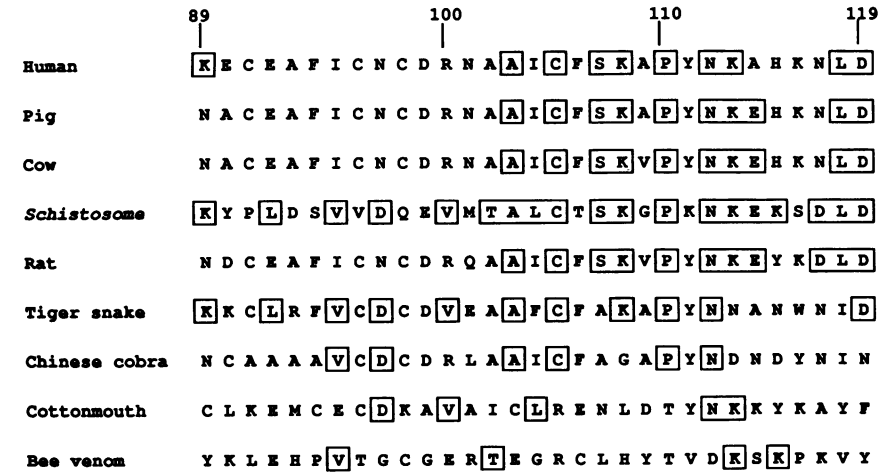

FIG. 6. Predicted C-terminal amino acid sequence of *S. japonicum* PLA2 from residues 89 to 119. This sequence is compared with the same region of PLA2s from different species.

radicals from ingested leukocytes. In other systems, it is known that lipid peroxides disrupt membrane integrity and are highly cytotoxic; however, they do enhance the activity of membrane-bound PLA2 (19). Released fatty acyl hydroperoxides are then rapidly hydrolyzed by glutathione-dependent peroxidases (18). It is tempting, therefore, to speculate that the gut PLA2 performs an important role in assuring membrane integrity.

As already mentioned, the parasite enzyme is present in trace amounts but nonetheless appears to be antigenic, since antibody which recognized bee venom PLA2 was present not only in the sera of rabbits immunized with whole-worm homogenate but also in sera from rabbits harboring a patent schistosome infection. We have further observed that human infection sera contain antibody to bee venom PLA2 (data not shown). How this reactivity with PLA2 actually arises in infected individuals is not clear and will be examined in more detail in the future. These investigations highlight the fact that there are antibodies of exceptionally low titer in sera of infected individuals, directed against potentially interesting parasite antigens, which can only be demonstrated by using a high concentration of appropriate antigen. In this context, we were able to detect antibody on Western blots to endogenous schistosome PLA2 only in sera from patients or rabbits exposed to schistosomes or in sera from rabbits immunized with whole-worm homogenate after long periods of autoradiography. This was undoubtedly a result of the relatively low titers of PLA2 antibody in these sera and the trace quantities of the enzyme in parasite extracts.

The localization of PLA2 to the schistosome gut epithelium would provide some constraints on consideration of it as a prospective vaccine component. The main problems for antibody efficacy in this environment would be degradation by numerous proteolytic enzymes (11) and that antigen-antibody interaction would be impeded by the suboptimal pH of 5. Nevertheless, marked immunoglobulin G responses arise against schistosome gut antigens in human schistosomiasis, although their role in immunity has yet to be defined (11). As a precedent, serum from guinea pigs immunized and thrice boosted with highly irradiated *S. mansoni* cercariae has been shown to passively protect naive recipient animals from a challenge infection. Electron microscope analysis of the challenge parasites revealed extensive antibody-mediated gut damage with little or no damage to the tegument (12). It is not inconceivable, therefore, that the production of high-titered antibody by appropriate immunization strategies with strong affinity for antigen(s) located in the gut epithelium may prove to be efficacious against adult blood flukes.

Having made our initial observations, further characterization of schistosome PLA2 proved difficult for the following reasons: firstly, its low expression militated against purification; secondly, we were unable to identify any clones from an *S. japonicum* cDNA library by using our high-titered rabbit anti-PLA2 antiserum. A possible explanation for this is that mRNA encoding for PLA2 was either absent or present in low amounts when the library was constructed. Cell-free translation and dot blotting with RNA isolated from adult worms confirmed this possibility (data not shown). Our findings are compatible with the low level of membrane PLA2 expression observed in many other systems, a phenomenon linked to the fact that there must be very strict controls on the levels of this enzyme, as unchecked PLA2 activity rapidly leads to membrane destabilization and cellular injury (22).

Because our attempts to obtain a cDNA for this gene were unsuccessful, a different strategy was adopted for the cloning of schistosome PLA2. By using oligonucleotide probes based on selected, highly conserved regions of PLA2 gene sequence from different species, we were able to identify several genomic EMBL3 clones in an *S. japonicum* library. Southern blot analysis of the 15-kb DNA inserts from two of the phage clones digested with the restriction enzyme *Fnu*D2 revealed two fragments of 1.8 and 2.0 kb which hybridized with the PLA2 oligonucleotide probes. Both fragments contained regions of sequence homology with PLA2s from different species, although in the 2.0-kb fragment these were very small and separated by numerous putative introns. The 1.8-kb fragment contained two larger regions of homology with PLA2 separated by a single putative intron. This intron contained numerous stop codons and was flanked by consensus spliced junctions. In addition, on a Southern blot of *S. japonicum* genomic DNA treated with *Fnu*D2, a 1.8-kb band strongly hybridized the PLA2 oligonucleotides, implying that the 1.8-kb fragment from the phage clone is present

in the schistosome genome. The sequence of the predicted exons from the phage clone fragment exhibited very striking homology with the C termini of PLA2 from different species.

In the future, we intend to carry out the inordinate task of intron boundary distinction and complete nucleotide sequence analysis along with other experiments to clarify whether the product of this gene accounts for the membrane-bound PLA2 activity observed in this investigation.

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