Characterization of [³H]palmitate- and [³H]ethanolamine-labelled proteins in the multicellular parasitic trematode *Schistosoma mansoni*

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Biosynthetic labelling experiments with cercariae and schistosomula of the multicellular parasitic trematode *Schistosoma mansoni* were performed to determine whether [³H]palmitate or [³H]ethanolamine was incorporated into proteins. Parasites incorporated [³H]palmitate into numerous proteins, as judged by SDS/polyacrylamide-gel electrophoresis and fluorography. The radiolabel was resistant to extraction with chloroform, but sensitive to alkaline hydrolysis, indicating the presence of an ester bond. Further investigation of the major 22 kDa [³H]palmitate-labelled species showed that the label could be recovered in a Pronase fragment which bound detergent and had an apparent molecular mass of 1200 Da as determined by gel filtration on Sephadex LH-20. Schistosomula incubated with [³H]ethanolamine for up to 24 h incorporated this precursor into several proteins; labelled Pronase fragments recovered from the three most intensely labelled proteins were hydrophilic and had a molecular mass of approx. 200 Da. Furthermore, reductive methylation of such fragments showed that the [³H]ethanolamine bears a free amino group, indicating the lack of an amide linkage. We also evaluated the effect of phosphatidylinositol-specific phospholipase C from *Staphylococcus aureus*: [³H]palmitate-labelled proteins of schistosomula and surface-iodinated proteins were resistant to hydrolysis with this enzyme. In conclusion, [³H]palmitate and [³H]ethanolamine are incorporated into distinct proteins of cercariae and schistosomula which do not bear glycopospholipid anchors. The [³H]ethanolamine-labelled proteins represent a novel variety of protein modification.

INTRODUCTION

The multicellular parasitic trematode *Schistosoma mansoni* infects humans worldwide [1]. Free-living cercariae penetrate intact skin of the definitive host, changing into parasitic larvae called schistosomula [1]. These parasites then mature over the next 6 weeks into egg-laying adult worms. Schistosomula are susceptible to immune killing *in vitro* for the first 24–48 h after transformation, and then they become resistant [2–6]. Loss of critical surface antigens of the parasite is thought to contribute to this immune evasion [6–8]. How antigens are released from the parasite's surface is not known, although upon transformation, a heptalaminate surface membrane replaces the cercarial trilaminate membrane, much of which may be lost along with elongating microvilli [9]. The above considerations make it clear that it is important to understand how proteins are anchored to, and how they may be released from, the surface of this organism. One possibility is that surface proteins are anchored to the surface membrane by glycopospholipid moieties which can be severed by appropriate phospholipases.

Such anchors and several possible corresponding phospholipases have recently been identified for a variety of eukaryotic cells, ranging from protozoa to mammals [10–15]. Chemical analysis and biosynthetic labelling experiments have demonstrated that these moieties contain ethanolamine, neutral and amino sugars and phosphatidylinositol (PI) attached via an amide linkage to the C-terminal amino acid [15–20]. Many of these proteins can be released from membranes by treatment with phosphatidylinositol-specific phospholipase C (PI-PLC) [15,21,22]. Available evidence suggests these glycopospholipid anchors are post-translationally coupled to the acceptor protein in the endoplasmic reticulum [23,24].

In the present study we therefore performed biosynthetic labelling experiments to determine if ³H-labelled fatty acids or [³H]ethanolamine are incorporated into proteins of *S. mansoni*. Covalent attachment of these precursors to amino acids would suggest the existence of glycopospholipid-anchored proteins. We have demonstrated that both fatty acids and, separately, ethanolamine, are attached to several proteins, but not, however, in the form of a glycopospholipid anchor. This novel

Abbreviations used: PI, phosphatidylinositol; PI-PLC, phosphatidylinositol-specific phospholipase C; PAGE, polyacrylamide-gel electrophoresis; BSA, bovine serum albumin; PBS, phosphate-buffered saline (0.01 M-phosphate/0.12 M-NaCl, pH 7.4).

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attachment of ethanolamine to proteins is essentially without precedent.

MATERIALS AND METHODS

Parasite preparation

Cercariae from a Puerto-Rican strain of *S. mansoni* were shed from laboratory-reared snails (*Biophalaria glabrata*) in spring water (Mineral Spring Water C; Akron). Mechanical schistosomula were prepared by a modification of the methods of Ramalho-Pinto *et al.* [25]. Briefly, cercariae were concentrated on ice, the supernatant removed, and the pellet vortex-mixed for 1 min. This procedure was repeated. Cercarial bodies were then separated from tails on a 60% (w/v) Percoll gradient centrifuged at 600 *g* for 10 min at 4 °C [26]. Parasites were washed and incubated in RPMI-1640 medium (Whittaker M. A. Bioproducts, Walkersville, MD, U.S.A.) supplemented with 2 mM-L-glutamine, 100 units of penicillin/ml and 100 µg of streptomycin/ml in a CO₂/air (2:23) incubator at 37 °C.

Biosynthetic labelling of parasites

Aliquots (30000/ml) of cercariae or schistosomula were incubated from the time of preparation with 50 µCi of [9,10-³H]palmitate (28.5 Ci/mmol; New England Nuclear, Boston, MA, U.S.A.) or [1-³H]ethan-1-ol-2-amine hydrochloride (12 Ci/mmol; Amersham, Arlington Heights, IL, U.S.A.)/ml in RPMI-1640. Parasites were washed five times in RPMI and resuspended in 50 µl of RPMI. These organisms were then added to 50 µl of concentrated boiling sample buffer [0.125 M-Tris/HCl (pH 6.8)/4% (w/v) SDS/20% (v/v) glycerol/10% (v/v) 2-mercaptoethanol] and boiled for an additional 10 min.

In other experiments parasites were disrupted with 30 strokes of a Dounce homogenizer on ice in 100 µl of 0.1% sodium deoxycholate in 10 mM-Hepes, pH 7.4, or 0.1% Triton X-100 (Sigma, St. Louis, MO, U.S.A.) in 10 mM-PBS supplemented with the following inhibitors: 10 mM-zinc acetate, 10 mM-EGTA, 1 mM-phenylmethanesulphonyl fluoride, 1 mM-tosylphenylalanyl-chloromethane and 1 mM-iodoacetamide (all from Sigma). Thereafter, the sample was sonicated four times with 15 s bursts on ice, a micro ultrasonic cell disrupter (Kontes, Vineland, NJ, U.S.A.) being used. The extract was centrifuged at 2200 *g* for 15 min at 4 °C and the supernatant maintained at -70 °C until used.

To extract the tegument, schistosomula were suspended in 100 µl of 1% Triton X-100 in PBS supplemented with the above inhibitors. Parasites were vortex-mixed every 5 min for a total of 30 min on ice and then centrifuged at 2200 *g* for 15 min at 4 °C [27]. The supernatant was mixed with 7 vol. of acetone at -20 °C and 3 µg of BSA and maintained at -20 °C for 2 h. The sample was then centrifuged at 2200 *g* for 15 min at 4 °C, the supernatant discarded, and the pellet resuspended in 40 µl of distilled water and 40 µl of concentrated sample buffer.

Surface labelling of schistosomula was performed by incubating 30000 parasites with 500 µCi of Na¹²⁵I (100 mCi/ml; Amersham) in 500 µl of Hank's basic salt solution in glass scintillation vials coated with 50 µg of Iodogen (Pierce, Rockford, IL, U.S.A.) with intermittent agitation on ice for 30 min [28].

Protein analysis and characterization

Proteins were analysed by SDS/PAGE using 12.5%-(w/v)-acrylamide gels [29]. Gels were fixed in methanol/acetic acid/water (5:1:4, by vol.) for 1 h, soaked in Enlighter (New England Nuclear) for 30 min, dried, and exposed to Kodak XAR film (Eastman Kodak Co., Rochester, NY, U.S.A.) at -70 °C for fluorography.

For electroelution of proteins, 2 mm-wide slices were excised from unfixed gels and electroeluted on a Tube Cell (Bio-Rad, Rockville Centre, NY, U.S.A.) at 80 V for 18 h according to the manufacturer's instructions. Electroeluted protein was dialysed against distilled water for 24 h at 4 °C.

For experiments involving the study of Pronase fragments, aliquots of parasites (30000) were disrupted with 30 strokes with a Dounce homogenizer in 100 µl of 0.1 M-Tris/HCl (pH 8)/10 mM-CaCl₂/0.4% Triton X-100 on ice and then sonicated for 1 min on ice. The extract was centrifuged at 2200 *g* for 15 min at 4 °C. Thereafter the supernatant was incubated with 1 mg of Pronase (Boehringer, Mannheim, Germany)/ml for 18 h at 60 °C. Pronase was preincubated for 60 min at 37 °C before addition to the extract. Control samples were incubated in a similar fashion, but with 1 mg of BSA/ml added in place of Pronase. Samples were dried on a Speed Vac concentrator (Savant; Hicksville, NY, U.S.A.).

To prepare protein fragments for gel filtration, 2 mm-wide gel slices were excised from unfixed SDS/PAGE gels at the expected position for the protein of interest (as compared with prestained molecular-mass standards). The slices were eluted with Pronase as described above and analysed on a column (1 cm × 40 cm) of Sepharose CL-6B (Pharmacia, Piscataway, NJ, U.S.A.) run at 11 ml/h at 4 °C and eluted with 20 mM-sodium phosphate (pH 7.4)/0.1% Triton X-100. Void-volume, detergent-micelle and solvent-elution volumes were marked by using 0.2% Blue Dextran (Pharmacia), free [³H]-palmitate and dinitrophenyl-lysine (Sigma) respectively. The fraction size was 0.74 ml.

Molecular masses of Pronase-digested fragments were estimated by chromatography on a column (1 cm × 40 cm) of Sephadex LH-20 (Pharmacia) in 88% (v/v) formic acid/ethanol (3:7, v/v) at 9 ml/h at 23 °C [30]. The fraction size was 0.64 ml.

To remove non-covalently associated lipids, schistosomula extracted with 0.1% Triton X-100 were mixed with chloroform/methanol/water (8:4:3, by vol.) [31], centrifuged at 800 *g* for 1 min and the chloroform phase removed. The aqueous phase was washed at least three additional times with chloroform/methanol (2:1, v/v) to remove further traces of free lipid.

To study the nature of the protein-fatty acid linkage, schistosomula labelled with [³H]palmitate for 24 h were solubilized in boiling sample buffer and analysed by SDS/PAGE. Immediately after electrophoresis the gel was fixed in propan-2-ol/acetic acid/water (5:2:13, by vol.) for 1 h, washed with distilled water (five changes of 200 ml each with agitation at 23 °C) and treated with 0.1 M-NaOH in 20% (v/v) methanol for 90 min at 23 °C. Control gels were treated in a similar fashion, but NaOH was omitted. Thereafter the gels were washed with distilled water and prepared for fluorography. BSA was electrophoresed in parallel as a standard to determine non-specific losses by hydrolytic treatment.

For identification of labelled fatty acid species, samples of electroeluted proteins were dried on a Speed Vac concentrator in 6 mm × 50 mm glass tubes. Subsequently, 100 µl of 1 M methanolic HCl was added and the tubes were sealed under N₂. Samples were methanolysed for 20 h at 65 °C. After cooling to room temperature, the methanolic phase was extracted with three 50 µl aliquots of 2,2,4-trimethylpentane. The extracted fatty acid methyl esters were analysed on a Hewlett–Packard model 5890 gas chromatograph equipped with a flame-ionization detector, a splitless injector, a 15 m 0.25 mm-internal-diameter Supelcowax 10 column (Supelco, Bellefonte, PA, U.S.A.) and a Hewlett–Packard 3392 integrator. Helium at 103.5 kPa (15 lbf/in²) was used as a carrier gas. The column was temperature-programmed from 100 to 260 °C at 20 °C/min. Samples were collected on liquid-nitrogen-cooled scintillation vials and counted for radioactivity in a Beckman LS 1801 liquid-scintillation counter. The methyl ester of free [³H]palmitate prepared as described above was run as a standard.

For ethanolamine analysis, excised gel slices corresponding to labelled proteins were digested with Pronase (1 mg/ml) for 18 h at 37 °C and centrifuged at 1000 g for 5 min. Aliquots of the supernatant were placed in 6 mm × 50 mm glass tubes with an equal amount of 6 M-HCl and 0.6% of concentrated β-mercaptoethanol (Fisher, Fair Lawn, NJ, U.S.A.). The tube was flushed with argon, sealed under vacuum, and the sample hydrolysed at 115 °C for 16 h. Thereafter, the sample was dried on a Speed Vac concentrator and analysed on a Beckman 119CL amino acid analyser, the procedure of Haas & Rosenberry [32] being followed. Three-buffer and four-buffer elution protocols were utilized.

Methylation was performed as a modification of Jentoft & Dearborn [33] and Haas & Rosenberry [32]. Samples of radiolabelled Pronase fragments were incubated with freshly prepared 10 mM-formaldehyde (MCB Reagents, Cincinnati, OH, U.S.A.), 50 mM-NaCNBH₃ (Aldrich, Milwaukee, WI, U.S.A.; recrystallized as in [33]), in 20 mM-sodium phosphate buffer, pH 7.4, for 60 min at 37 °C. To prepare dimethyl-ethanolamine as a standard, 1 mM-ethanolamine was incubated with 0.5 mM-[¹⁴C]formaldehyde (50 mCi/mmol; Irvine, CA, U.S.A.) and 50 mM-NaCNBH₃ in sodium phosphate buffer, pH 7.4, for 60 min at 35 °C. Monomethylethanolamine was prepared in a similar fashion; however, 125 mM-[¹⁴C]formaldehyde was used and the reaction was quenched with 75 µl of concentrated cold ethanolamine after 15 min at 35 °C. Samples were then hydrolysed and analysed on an amino acid analyser as described above.

Treatment with PI-PLC

Aliquots of 30000 organisms per 100 µl in 0.1% sodium deoxycholate with 10 mM-Hepes, pH 7.4, were disrupted with 30 strokes of a Dounce homogenizer. The sample was sonicated with four bursts of 15 s each on ice and centrifuged at 2200 g for 15 min at 4 °C. The supernatant was then incubated with 20 µg of *Staphylococcus aureus* PI-PLC (gift of Dr. Martin G. Low, College of Physicians and Surgeons, Columbia University, New York, NY, U.S.A.)/ml for 60 min at 37 °C. Thereafter, 7 vol. of acetone at −20 °C and 3 µg of BSA were added and maintained at −20 °C for 2 h. The sample was centrifuged at 2200 g for 15 min at 4 °C and

the pellet analysed by SDS/PAGE and fluorography. Schistosomula surface-labelled with ¹²⁵I were treated with 20 µg of PI-PLC/ml in PBS for 1 h at 37 °C, washed, and the tegument extracted with 1% Triton X-100.

To demonstrate that PI-PLC was active, 1 µl of L-α-[myo-inositol-2-³H(n)]phosphatidylinositol (6.5 Ci/mmol, New England Nuclear) was incubated with or without 20 µg of PI-PLC/ml in 50 µl of 0.1% sodium deoxycholate with 10 mM-Hepes, pH 7.4, for 60 min at 37 °C. Thereafter, the sample was extracted three times with chloroform/methanol/water (8:4:3, by vol.). The chloroform phases were pooled, dried with N₂ and counted for radioactivity, along with the aqueous phase: over 80% of the label was transferred to the aqueous phase by the enzyme treatment. Further control experiments showed that the same enzyme preparation can convert acetylcholinesterase activity of *S. mansoni* detergent lysates from a hydrophobic to a hydrophilic form. This latter result shows again that the enzyme preparation was active and raises the possibility that acetylcholinesterase in *S. mansoni* may bear a glycolipid moiety.

RESULTS

Incorporation of ³H-labelled fatty acids into proteins

The presence of acylated proteins in *S. mansoni* was investigated by incubating newly transformed schistosomula with [³H]palmitate for 24 h. Analysis by SDS/PAGE and fluorography of parasites solubilized in boiling sample buffer revealed numerous radiolabelled bands; the predominant bands have molecular masses of 14, 16, 22, 27 and 40 kDa (Fig. 1). Additional bands were encountered in selected experiments. A similar spectrum of proteins was labelled with [³H]palmitate

Fig. 1. Radiolabelling of proteins of cercariae and schistosomula with [³H]palmitate

Parasites were incubated with [³H]palmitate, solubilized in boiling sample buffer and analysed by SDS/PAGE and fluorography. Lane 1, cercariae incubated for 6 h at 23 °C; lanes 2 and 3, schistosomula incubated for 3 h or 24 h respectively at 37 °C.

Fig. 2. G.L.C. of labelled fatty acid methyl esters released from the 22 kDa protein of schistosomula incubated with [3 H]palmitate

Schistosomula were incubated with [3 H]palmitate for 24 h and analysed by SDS/PAGE. The 22 kDa protein was excised, electroeluted and subjected to methanolysis. Thereafter, the sample was analysed by g.l.c. Key: C₁₄, methyl ester of myristate; C₁₆, methyl ester of palmitate; C₁₈, methyl ester of stearate. 'Time' designates the elution time for the samples; 0.5 min fractions were collected and counted for radioactivity.

after incubation of schistosomula for 3 h or cercariae for 6 h (Fig. 1). No labelled bands were seen when cell lysates were treated with Pronase before SDS/PAGE, indicating that radiolabelled bands were indeed proteins.

Palmitate-labelled gel bands might result from non-covalent binding of the labelled fatty acid to proteins. To explore this possibility, schistosomula were incubated with [3 H]palmitate for 24 h, homogenized in 0.1% Triton X-100 and then extracted eight times with chloroform/methanol/water (8:4:3, by vol.) Analysis by SDS/PAGE and fluorography revealed that all the characteristic labelled proteins partitioned into the aqueous phase and that no bands were seen in the organic phase (results not shown). Thus [3 H]palmitate is covalently linked to the proteins.

We subsequently examined whether the incorporated label was still [3 H]palmitate itself or a metabolite of the fatty acid. Since the 22 kDa protein was a predominant and consistently labelled species, a 2 mm gel slice corresponding to this band was excised and electroeluted. The sample was then extensively extracted with chloroform/methanol/water (8:4:3, by vol.). Less than 10% of the radioactivity was extracted into the organic phase. Methanolysis was performed on the aqueous phase, and the sample was analysed by g.l.c. A single peak corresponding to the methyl ester of palmitate was seen (Fig. 2).

Ester-linked fatty acids may be released by alkaline hydrolysis, unlike amide-linked fatty acids, which are resistant [34–36]. To determine whether [3 H]palmitate is attached by an ester bond, schistosomula labelled with

Fig. 3. Effect of alkaline hydrolysis on proteins of schistosomula labelled with [3 H]palmitate

Schistosomula were incubated with [3 H]palmitate for 24 h, solubilized in sample buffer and analysed by SDS/PAGE. The gel was treated with 0.1 M-NaOH (+) in 20% (v/v) methanol for 90 min at 23 °C or, for the control (-), treated with 20% (v/v) methanol. Thereafter gels were washed and prepared for fluorography.

[3 H]palmitate for 24 h were solubilized in boiling sample buffer and analysed by SDS/PAGE. Thereafter the gel was incubated in NaOH. As Fig. 3 shows, numerous radiolabelled bands were found in untreated gels. In contrast, after treatment with 0.1 M-NaOH, the intensity of the banding pattern was dramatically diminished. No difference in intensity of Coomassie Blue-stained serum albumin was found between gels treated with NaOH and control (results not shown).

Further characterization of the 22 kDa protein focused on a labelled proteolytic fragment. Schistosomula were labelled with [3 H]palmitate for 24 h and analysed by SDS/PAGE. The gel slice corresponding to the 22 kDa species was excised, digested with Pronase and the eluate was run on a Sepharose CL-6B column equilibrated with 0.1% Triton X-100 in phosphate buffer. On this column detergent micelles were eluted well before the solvent volume [32]. Previous studies demonstrated that the glycopospholipid moiety of 125 I-labelled acetylcholinesterase [37], [3 H]palmitate- and [3 H]ethanolamine-labelled Pronase fragments of Thy-1 antigen [31] and free [3 H]palmitate emerge with the micelles. As Fig. 4(a) shows, the Pronase-digested fragment was eluted in the micellar fraction, thus indicating that the fragment does, as expected, bind detergent.

The peak fractions (nos. 27–32) from the Sepharose CL-6B column were then pooled, dried, and resuspended in formic acid/ethanol. Gel filtration under denaturing conditions on Sephadex LH-20 showed the fragment to have an apparent molecular mass of 1200 Da (Fig. 4b), as judged by comparison with a set of peptide and vitamins used for calibration. Since free [3 H]palmitate (M_r 248) emerged from the column with an apparent M_r of 512, the size estimate for the Pronase fragment

Fig. 4. Gel filtration of Pronase fragments of the 22 kDa protein from schistosomula incubated with [3 H]palmitate for 24 h

Radiolabelled parasites were analysed by SDS/PAGE and the excised gel slice digested with Pronase. The sample was then chromatographed on Sepharose CL-6B in 0.1% Triton X-100 (a). The arrow indicates elution position of free [3 H]palmitate and detergent micelles, while 'L' indicates dinitrophenyl-lysine. Thereafter the radioactive peak from the Sepharose CL-6B column was pooled and analysed on Sephadex LH-20 (b). The arrow indicates elution position of free [3 H]palmitate (M_r 248). The fraction size was 0.64 ml. Recoveries ranged from 70 to 95%. The elution position of the fragment on Sephadex LH-20 was compared with those of the following standards: glucagon, M_r 3490; gramicidin, M_r 1850; renin, M_r 1759; vitamin B-12, M_r 1355; kyotorphin, M_r 355 (all from Sigma).

must be considered no more than approximate. Elution of the Pronase fragment before free [3 H]palmitate is consistent with a palmitoylated cysteine or serine residue.

The surface membrane of *S. mansoni* limits a syncytial epithelial-cell layer called the tegument [9]. This surface layer covers the parenchyma of the parasite. On the basis of ultrastructural studies the tegument can be extracted from the body of the parasite with 1% (w/v) Triton X-100 [27]. To determine if acylated proteins are found in the tegument, schistosomula were incubated with [3 H]palmitate for 24 h and extracted with 1% Triton X-100. SDS/PAGE-fluorographic analysis of the tegumental extract revealed only three radioactive bands with molecular masses of 16, 22 and 40 kDa (results not shown).

Fig. 5. Labelling of proteins of schistosomula with [3 H]ethanolamine

Organisms were incubated with [3 H]ethanolamine for 24 h, solubilized in boiling sample buffer and analysed by SDS/PAGE and fluorography.

Incorporation of [3 H]ethanolamine into proteins

Schistosomula were incubated with [3 H]ethanolamine for 3 and 24 h, solubilized in boiling sample buffer and then analysed by SDS/PAGE-fluorography. Numerous proteins were found to incorporate [3 H]ethanolamine; the predominant bands had molecular masses of 22, 27, 40 and 50 kDa (Fig. 5). When cercariae were incubated with [3 H]ethanolamine for 6 h at 23 °C in two separate experiments, no radioactive bands were seen, despite evidence of uptake of [3 H]ethanolamine into phospholipids.

Labelling with [3 H]ethanolamine may represent incorporation of [3 H]ethanolamine itself or a labelled metabolite. To examine this point, three prominently labelled proteins (22, 27 and 50 kDa) were eluted from the gel with Pronase, acid-hydrolysed and analysed on an amino acid analyser. In each case a single radioactive peak was identified that co-migrated with free [3 H]ethanolamine (Figs. 6a–6c).

To determine whether the incorporated [3 H]ethanolamine had a free amino group, Pronase fragments from each of the three proteins (22, 27 and 50 kDa) were reductively methylated, acid-hydrolysed and analysed. As shown in Figs. 6(d)–6(f), all three fragments yielded a labelled species which co-migrated with the [14 C]dimethylethanolamine standard, indicating that the ethanolamine residues do bear a free amino group. To our knowledge, Pronase does not cleave amide bonds including the amino group of ethanolamine. Our studies of Thy-1 antigen indicate that this certainly does not occur for glycosphospholipid-anchored proteins [37].

These [3 H]ethanolamine moieties were further characterized with regard to hydrophobicity. Gel slices corresponding to the three labelled proteins were digested with Pronase and fractionated on a Sepharose CL-6B column in the presence of 0.1% Triton X-100. As shown

Fig. 6. Amino acid analysis of the 22, 27 and 50 kDa proteins from schistosomula incubated with [^3H]ethanolamine for 24 h

Excised gel slices were digested with Pronase, acid-hydrolysed and analysed on an amino acid analyser. (a), 22 kDa; (b), 27 kDa; (c), 50 kDa. The arrow represents the elution position of free [^3H]ethanolamine. To determine whether [^3H]ethanolamine has a free amino group, Pronase-digested gel slices were methylated before acid hydrolysis and analysed on the amino acid analyser. (d), 22 kDa; (e), 27 kDa; (f), 50 kDa. 'd' indicates the elution position of [^{14}C]dimethylethanolamine, and the arrow represents free [^3H]ethanolamine. In (a) a three-buffer system was used, and the remainder of the fragments were analysed in a four-buffer system [32].

in Figs. 7(a)–7(c), the [^3H]ethanolamine-labelled fragments emerged in the solvent volume, indicating that they do not bind detergent. The radioactive fractions eluted from the CL-6B columns were pooled, concentrated, and chromatographed on Sephadex LH-20. A single radioactive peak for each fragment was eluted, as shown in Figs. 7(d)–7(f). Comparison of the elution position of each peak with a calibration curve demonstrated that each fragment had an apparent molecular mass of 200 Da.

To determine whether [^3H]ethanolamine-labelled proteins were concentrated in the tegument [27], schistosomula incubated with [^3H]ethanolamine for 24 h were extracted with 1% Triton X-100 and analysed by SDS/PAGE. No significant incorporation of the radiolabel into tegumental proteins was found (results not shown).

Resistance of [^3H]palmitate-labelled and surface proteins to PLC

Many glycopospholipid moieties are hydrolysed by PI-PLC [10,15,21,22]. To determine whether proteins of

Fig. 7. Gel filtration of Pronase fragments of the 22, 27 and 50 kDa [^3H]ethanolamine-labelled proteins of schistosomula

Parasites were radiolabelled for 24 h and analysed by SDS/PAGE. Excised gel slices were digested with Pronase and chromatographed on Sepharose CL-6B in 0.1% Triton X-100. (a), 22 kDa; (b), 27 kDa; (c), 50 kDa. The arrow indicates the elution position of free [^3H]palmitate (M_r 248) and 'L' designates dinitrophenyl-lysine (M_r 480). Thereafter the radioactive peaks from the Sepharose CL-6B were pooled and analysed on Sephadex LH-20. (d) 22 kDa; (e), 27 kDa; (f), 50 kDa. A standard curve for Sephadex LH-20 was prepared as in Fig. 4. Kyotorphin (M_r 355) was eluted in fraction 31. [^3H]ethanolamine itself was eluted anomalously in fraction 33.

S. mansoni were also sensitive to this enzyme, schistosomula were incubated for 3, 10, and 24 h with [^3H]palmitate, solubilized in 0.1% sodium deoxycholate and treated with high doses of PI-PLC. No difference in radioactive banding patterns was seen between treated and control extracts (results not shown). To focus on whether surface proteins were also sensitive to PI-PLC, intact schistosomula were surface-labelled with ^{125}I by using Iodogen and treated with the enzyme. Thereafter, extracts of the tegument and samples of the medium were analysed by SDS/PAGE–fluorography. Radioactive banding patterns of tegument extracts with or without PI-PLC were similar. Moreover, no radiolabelled proteins were released into the medium by the enzyme (results not shown). Control experiments showed that the enzyme preparation was active (see the Materials and methods section).

DISCUSSION

The only published investigation of proteolipids in *S. mansoni* indicates that adult worms contain a [^{14}C]oleic

acid-labelled hydrophobic 10–15 kDa protein which is immunogenic [38]. Its labelled moiety is susceptible to mild alkaline hydrolysis [38]. Acylation of proteins of other eukaryotes has frequently been observed [39–40]. Myristate is added co- or immediately post-translationally to *N*-terminal glycine residues of several proteins by an amide linkage [41–45]. In contrast, palmitate is often post-translationally linked by a thioester bond to cysteine [34–36,46]. Palmitate can also be attached to polypeptides long after protein synthesis [39,47–49]. In certain cases, the fatty acids may help bind proteins to membranes [50–52]; however, several acylated proteins appear to be soluble in the cell sap [39,53].

We have observed that [³H]palmitate is incorporated into several proteins of cercariae and schistosomula. At least for the 22 kDa labelled protein, [³H]palmitate is not metabolized, but rather persists as the intact fatty acid and is covalently bound, apparently by an ester linkage. The location of the 22 kDa proteolipid within the multicellular schistosomula is unknown; however, limited amounts of this species are detected within the outer tegumental surface layer of this species of the parasite.

The apparent size of the Pronase-digested [³H]palmitate fragment recovered from the 22 kDa protein is estimated to be 1200 Da. This observation suggests that, in addition to palmitate and the 'linkage' amino acid, the labelled fragment may contain additional fatty acids, sugars or a Pronase-resistant peptide. Nevertheless, since the elution behaviour of fatty-acid-containing compounds on Sephadex LH-20 may be anomalous, as demonstrated by the overestimation of the molecular mass of free [³H]palmitate, the actual molecular mass may be less than 1200 Da. Thus the unit in question may be palmitoyl-cysteine or -serine.

[³H]ethanolamine was incorporated into numerous proteins of schistosomula. Characterization of three of these proteins revealed that their labelled Pronase fragments were hydrophilic and have an apparent molecular mass of approx. 200 Da. Since reductive-methylation studies indicate that the amino group of the incorporated [³H]ethanolamine is free, the ethanolamine residues are presumably linked via their alcohol function. Judging from the lability of this bond to strong acid hydrolysis, an ether is not involved. Possible linkages include an ester or phosphodiester.

The direct covalent attachment of a single residue of ethanolamine to an amino acid represents a new class of protein modification, although covalent attachment of at least two larger units including ethanolamine has been documented: (a) in a single soluble protein of lymphoma cells (E. J. Tisdale & A. M. Tartakoff, unpublished work), and (b) in glycopospholipid anchors [10,15–20]. These latter moieties are hydrophobic and, on the basis of experiments with human erythrocyte acetylcholinesterase, have an apparent *M_r* of 3100 [54]. Moreover, owing to the amide linkage, their ethanolamine residues cannot be quantitatively methylated [32]. Our observations clearly indicate that the [³H]ethanolamine-labelled proteins of *S. mansoni* do not have a glycopospholipid anchor: the Pronase fragments are hydrophilic and too small, and their [³H]ethanolamine can be quantitatively methylated. It does remain possible, however, that this organism bears minor or slowly-turning-over proteins with glycopospholipid anchors or that such anchored proteins might be synthesized before shedding of cercariae from the snail host.

The significance of palmitate and ethanolamine-containing proteins in cercariae and schistosomula of *S. mansoni* is unclear. The tegumental location of a limited fraction of the acylated proteins suggests that these fatty acids may have a role in binding proteins to the surface membrane of schistosomula [9]. In contrast, [³H]-ethanolamine-labelled proteins were found only in the body of the organisms. The presence of protein-bound ethanolamine in these and other cells, and the nature of the ethanolamine–amino acid linkage, require further investigation.

Note added in proof (received 4 July 1988)

Since this paper was accepted and sent to press, acetylcholinesterase and alkaline phosphatase have been reported to be susceptible to release from the tegumental membranes of *Schistosoma mansoni* by a PI-PLC [55].

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