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Sm25, a major schistosome tegumental glycoprotein, is dependent on palmitic acid for membrane attachment

Edward J.Pearce[3], Anthony I.Magee[1], S.Ronald Smithers[2] and Andrew J.G.Simpson[2]

Immunology and Cell Biology Section, Laboratory of Parasitic Diseases, National Institutes of Allergy and Infectious Disease, Bethesda, MD 20892, USA, [1]Laboratory of Eukaryotic Molecular Genetics and [2]Division of Parasitology, National Institute for Medical Research, London NW7 1AA, UK

[3]Present address: Department of Microbiology, Immunology and Parasitology, NYSCVM, Cornell University, Ithaca, NY 14853-6401, USA

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Sm25, a major antigen in the surface tegument of the parasitic helminth *Schistosoma mansoni*, is a 25 kDa *N*-glycosylated glycoprotein which co-purifies with isolated surface membranes and behaves as an integral membrane protein in Triton X-114 (TX-114). The deduced amino acid sequence of Sm25 shows a short C-terminal hydrophobic domain between residues 163 and 180, containing six uncharged polar amino acids and followed by a Lys181–Ser192 dipeptide. We were interested in whether or not this marginal C-terminal amphiphilic domain is responsible for the association of Sm25 with the membrane or whether a post-translational modification such as the addition of glycosyl phosphatidyl inositol (GPI) represents the membrane anchor for this molecule. We find that treatment with phosphatidyl inositol-specific phospholipase C, which cleaves many GPI anchors, does not reveal Cross Reacting Determinant (CRD) on Sm25, nor affect the association of this protein with membranes, providing no support for the addition of GPI. However, Sm25 is palmitoylated via a thioester bond to the single Cys residue, at position 168, which lies within the C-terminal hydrophobic domain. Removal of palmitate by reduction results in a marked decrease in the hydrophobicity of Sm25, as demonstrated by its partitioning into the aqueous rather than detergent phase of TX-114 and its quantitative release from membrane preparations.The hydrophobicity of several membrane proteins in addition to Sm25 is also decreased by reduction, raising the possibility that fatty acylation by thioester linkage is an important mechanism used by schistosomes to stabilize protein–membrane interactions.

Key words: anchor/membrane/palmitoylation/schistosome/thioester

Introduction

Sm25 is a major antigenic tegumental membrane glycoprotein of the important pathogenic helminth, *Schistosoma mansoni* (Karcz *et al.*, 1988; Smithers *et al.*, 1989, 1990). Evidence from three separate experimental systems is consistent with this molecule being a key target antigen of host-protective humoral immune responses (Wright *et al.*, 1988; Smithers *et al.*, 1989, 1990; El-Sherbeini *et al.*, 1990). In its native form Sm25 is *N*-glycosylated and behaves as an integral membrane protein, co-purifying with isolated surface membranes and separating into the detergent phase of Triton X-114 (TX-114) (Wright *et al.*, 1988). However, analysis of the amino acid sequence (deduced from cDNA) of Sm25 (Omer Ali *et al.*, 1991) revealed only a 17 amino acid long hydrophobic domain (residues 163–180) towards the C terminus, which might not be sufficiently long to anchor the molecule in the bilayer. In addition, this region contains six uncharged polar amino acids, including two Asn residues, which compromise its hydrophobicity and ability to interact with the bilayer in a stable fashion (see Williams and Tse, 1985). Finally, the Lys–Ser in positions 182–183 represent a very short putative cytoplasmic domain. These observations indicate that Sm25 might require co/post-translational modifications for stable interactions with the membrane.

Recent work has shown that many schistosome surface proteins are anchored to the membrane by C-terminal GPI (Espinoza *et al.*, 1989; Pearce and Sher, 1989; Sauma and Strand, 1990) raising the possibility that Sm25 is likewise modified. While a consensus signal for GPI attachment has not been identified, it now appears clear from analysis of cDNA and polypeptide sequence that proteins anchored in this manner have in common a C-terminal hydrophobic domain of 17–31 residues which proceeds to the end of the protein but is missing in the mature molecule, being replaced by GPI (see Ferguson and Williams, 1988; Doering *et al.*, 1990). These regions differ from classical peptide anchors in often including uncharged polar or charged residues, a feature shared with 163–180 of Sm25. However, unlike Sm25, unprocessed molecules destined to be anchored by GPI nearly always lack charged or uncharged polar amino acids at their absolute C termini. Nevertheless, the similarities between the C terminus of Sm25 and known GPI-anchored proteins prompted us to investigate whether or not Sm25 is modified with GPI. During the course of these studies, while attempting to label metabolically the putative lipid anchor, we found that Sm25 is not anchored to the membrane by GPI but is covalently modified with palmitic acid. This acylation is shown to be essential for the stable interaction of Sm25 with the lipid bilayer.

Results

Sm25 can be metabolically labelled with palmitic acid, but is not modified by GPI

Many GPI-anchored proteins possess a Cross Reacting Determinant (CRD), a carbohydrate epitope located within the anchor but revealed only after the hydrophobic alkyl-, acyl- or diacylglyerol moiety has been cleaved from the rest of the molecule by phosphatidylinositol-specific phospholipase-C (PIPLC) or variant surface glycoprotein lipase (Holder and Cross, 1981; Holder, 1985; Shak *et al.*, 1988).

PIPLC treatment of isolated schistosome membranes (mb-a) followed by SDS−PAGE, electrophoretic transfer to nitrocellulose and probing with anti-CRD antibodies failed to identify a 25 kDa species (Figure 1A). However, in the same sample CRD was revealed on a 200 kDa protein (Figure 1A); a GPI-anchored molecule of this mol. wt has been reported previously (Sauma and Strand, 1990). Separation of PIPLC-treated mb-a into pellet and supernatant fractions by centrifugation, followed by SDS−PAGE, blotting and probing with rabbit anti-mb-a (which recognizes the 200 kDa antigen) revealed a shift of the 200 kDa molecule from the pellet to the supernatant (Figure 1B). This is wholly consistent with the loss of the hydrophobic domain. Use of antisera from mice protectively vaccinated with isolated schistosome tegumental membranes (mb-s) revealed no similar change in hydrophobicity of a 25 kDa protein (not shown). The major specificity of mice vaccinated in this way is Sm25, and antibodies to only one molecule in the 25 kDa range, Sm25, are produced in these animals (Omer Ali *et al.*, 1991). These observations are not supportive of Sm25 being modified by a PIPLC-sensitive GPI, but do not rule out the presence of a PIPLC-resistant GPI anchor (Roberts *et al.*, 1988).

Previous work has shown a wide variety in the fatty acid composition in GPI-anchors (see Ferguson and Williams, 1988). Therefore, coincident with the attempts to identify a CRD on Sm25, we investigated whether, during culture, adult schistosomes incorporate [^{3}H]fatty acids into this molecule. Analysis of NP-40 extracts of parasites cultured in the presence of [9,10-^{3}H]palmitic acid revealed, strikingly, a major labelled species of diffuse appearance and approximate mol. wt 25 kDa (Figure 2A). Little of this protein, or other labelled proteins, remained in the NP-40-insoluble fraction (Figure 2A). The relative molecular mass of this protein and its multi-component pattern after SDS−PAGE are similar to those described previously for the major adult schistosome tegumental glycoprotein, Sm25. Characteristically, *N*-glycanase treatment of Sm25 results in a reduction of its relative mass to ~18−20 kDa (Karcz *et al.*, 1988; Omer Ali *et al.*, 1991). *N*-Glycanase treatment of the [^{3}H]palmitate-labelled preparation resulted in a similar shift in mass of the palmitoylated 25 kDa band (Figure 2B), suggesting that this molecule is Sm25. This was confirmed by incubation of the labelled detergent extract with protein A−Sepharose complexed antibodies from mice vaccinated with mb-s, which resulted in the immunoprecipitation of the 25 kDa labelled species (Figure 2C). From these observations, we conclude that the major schistosome surface antigen Sm25 is acylated.

Sm25 is palmitoylated through Cys168

All described covalent linkages of palmitate directly to proteins have involved base-labile esters with Ser or Thr or thioester bonds to Cys. It is probable that the latter is the most common form (see Sefton and Buss, 1987). To examine the nature of the Sm25−palmitate interaction, we separated [^{3}H]palmitate-labelled schistosome proteins by SDS−PAGE and incubated the gel in 0.2 M KOH in methanol or in methanol alone. Subsequent fluorography revealed that the intensity of the signal at 25 kDa was much weaker in the gel which had been exposed to base (Figure 3A, lane 1) than in the methanol treated gel (Figure 3A, lane 2) which itself was indistinguishable from an untreated sample (Figure 3A, lane 3). These results suggest that the acylation of Sm25

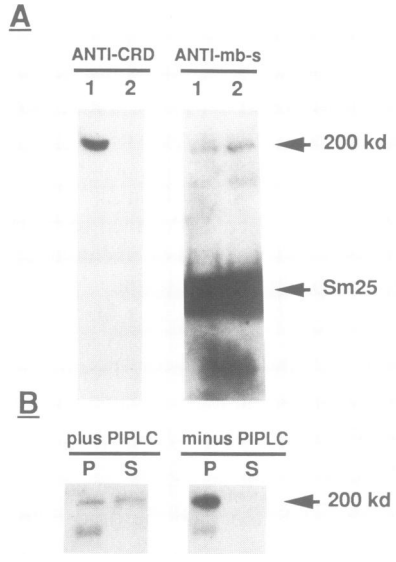

Fig. 1. Sm25 is not modified with GPI. (**A**) mb-a was treated with PIPLC (lanes 1) or sham incubated (lanes 2), separated by SDS−PAGE, transferred to nitrocellulose and probed with rabbit anti-CRD or mouse anti-mb-s antisera, as indicated, followed by [^{125}I]protein A and autoradiography to reveal bound antibody. The position of the GPI-anchored 200 kDa protein and Sm25 are indicated with arrows. (**B**) PIPLC or sham treated mb-a were separated into membrane bound (pellet, P) and soluble (supernatant, S) proteins by centrifugation and analysed by SDS−PAGE, transfer to nitrocellulose and probing with rabbit anti-mb-s antiserum followed by [^{125}I]protein A and autoradiography.

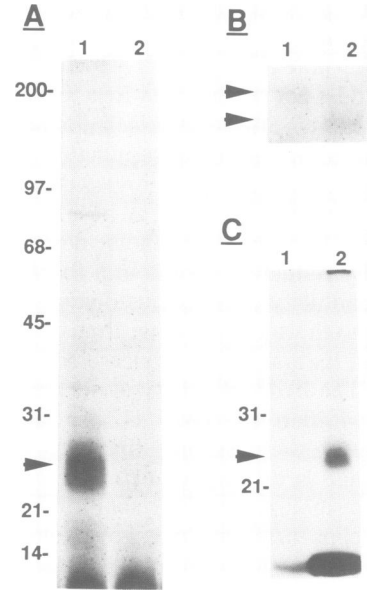

Fig. 2. Sm25 can be metabolically labelled with [9,10-^{3}H]palmitic acid. (**A**) NP-40 extract (lane 1) and NP-40-insoluble components (lane 2) of labelled schistosomes were separated by SDS−PAGE and fluorographed. (**B**) NP-40 extract of labelled parasites (lane 1) and *N*-glycanase treated extract (lane 2). (**C**) Immunoprecipitation from NP-40 extract of labelled parasites with sera from normal mice (lane 1) or from mice vaccinated with mb-s plus saponin (lane 2). In each panel, the position of Sm25 is indicated by an arrow. Mol. wt markers are shown to the left in panels (A) and (C).

occurs via a covalent (ester) bond, and confirm that it is not simply due to weak lipid–protein interaction (Magee *et al.*, 1984). Analysis by reverse-phase thin layer chromatography (TLC) of the labelled methyl ester released by the methanolic KOH treatment revealed it to possess a mobility comparable to that of palmitate standard (Figure 3B).

To define the palmitate–Sm25 linkage further, we attempted to delipidate the protein using a reducing agent; this process would be expected to remove thioester-linked fatty acid (Schmidt *et al.*, 1988). Isolated membrane preparations (mb-a) from [^{3}H]palmitate-labelled adults were solubilized in SDS–PAGE buffer containing increasing concentrations of 2-mercaptoethanol (2-ME) or dithiothreitol (DTT). Fluorography of these samples separated by SDS–PAGE revealed that essentially all of the [^{3}H]fatty acid was released from Sm25 at concentrations >1.0 M 2-ME (Figure 3C) or 0.4 M DTT (not shown). As oxygen esters are resistant to similar treatment, Cys residue(s) appear to be the palmitoylation site(s) in Sm25.

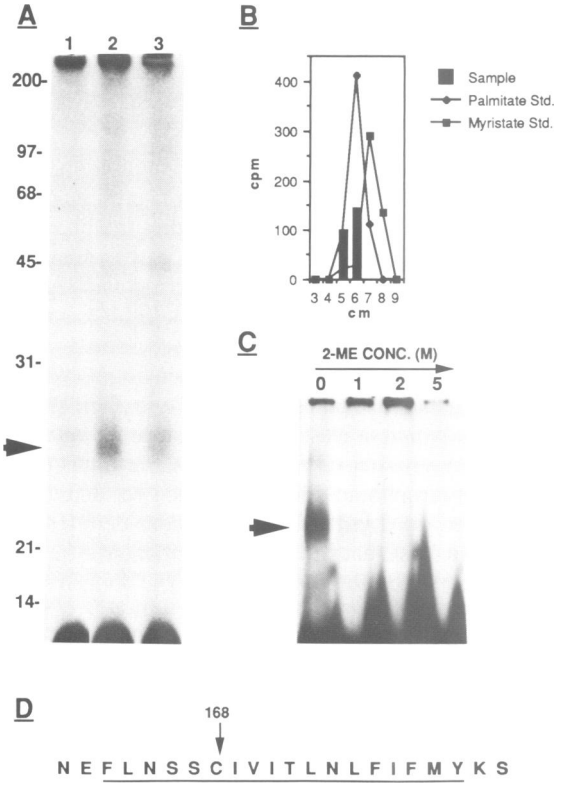

Fig. 3. Sm25 is palmitoylated through Cys168. (**A**) The palmitate–Sm25 linkage is labile in the presence of base. Equivalent samples of mb-a from [^{3}H]palmitate labelled parasites were separated by SDS–PAGE under non-reducing conditions. Lane 1 was incubated in methanol containing 200 mM KOH; lane 2 was incubated in methanol alone and lane 3 was fixed directly in acetic acid:methanol: water. All were fluorographed. (**B**) The lipid linked to Sm25 is palmitate. TLC analysis of [^{3}H]fatty acid methyl ester released from Sm25 by methanolic KOH. The position of migration of the released lipid relative to methyl-palmitate and methyl-myristate standards is shown. (**C**) Release of palmitate from Sm25 by 2-ME. Equivalent samples of palmitoylated mb-a were solubilized in SDS sample buffer containing increasing amounts of 2-ME as indicated, separated by SDS–PAGE and fluorographed. (**D**) Sm25 contains only one Cys, located in the middle of a 17 amino acid hydrophobic domain (underlined, single letter amino acid code used) followed by the short hydrophilic C-terminal sequence of Lys-Ser (Omer Ali *et al.*, 1991).

Analysis of full-length cDNA sequence for Sm25 reveals only one Cys, at position 168 (Omer Ali *et al.*, 1991), which is therefore highly likely to represent the site of palmitoylation (Figure 3D).

Palmitate anchors Sm25 to the membrane

Cys168 is located within a hydrophobic domain between amino acid residues 163 and 180, which are followed by a C-terminal hydrophilic Lys–Ser dipeptide (Figure 3D). While region 163–180 would appear to be the membrane anchor for Sm25 it is two amino acids shorter than the length considered minimal to span a lipid bilayer, and is further compromised by six uncharged polar residues. We postulated therefore that palmitoylation at Cys168 might serve to stabilize the interaction of this domain in the membrane. To test this, we subjected unlabelled mb-a to treatment with concentrations of 2-ME or DTT known to result in deacylation, followed by extraction and phase separation in TX-114, SDS–PAGE and electrophoretic transfer to nitrocellulose. Blots probed with mouse anti-mb-s (Figure 4A) or rabbit anti-recombinant Sm25 (Figure 4B) clearly showed that whereas Sm25 normally separates into the detergent layer (D), heavily reduced antigen partitions into the aqueous phase (A), suggesting a marked reduction in hydrophobicity under these conditions. Furthermore, Sm25 was found in the supernatant of mb-a exposed to 2-ME and centrifuged at 10^5 *g* (Figure 5). Since these changes in the behaviour of Sm25 occur in the presence of inhibitors of thiol-proteases and without a change in the relative mass of Sm25, it is extremely unlikely that the observed dissociation from the membrane is due to proteolysis. These results therefore indicate that covalently linked palmitate plays a critical

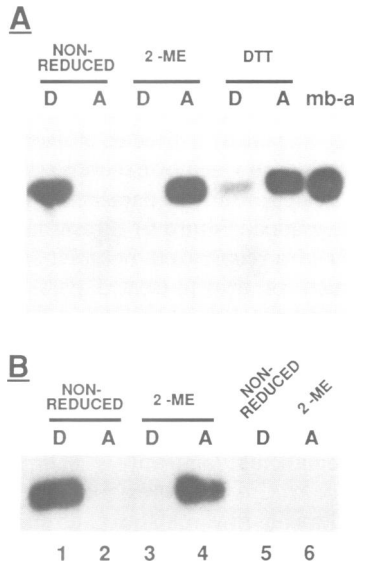

Fig. 4. Palmitate is responsible for Sm25 behaving as an integral membrane protein in TX-114. Unlabelled mb-a was treated with 2 M 2-ME or 450 mM DTT, partitioned in TX-114 into detergent (D) and aqueous (A) phases, electrophoretically separated and transferred to nitrocellulose. Blots were probed with: (**A**) antisera from mice vaccinated with mb-s plus saponin; (**B**) lanes 1–4, a rabbit antiserum raised against purified recombinant Sm25 or, in lanes 5 and 6, control antiserum from a rabbit immunized with schistosome glutathione-S-transferase (Sj26). Bound antibody was detected using [^{125}I]protein A and autoradiography.

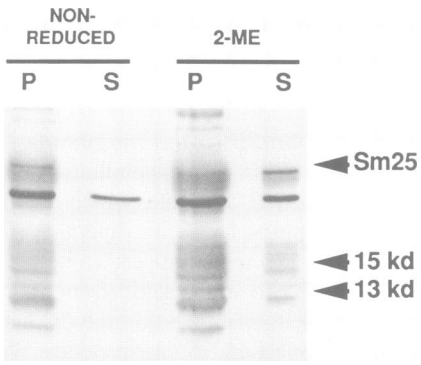

Fig. 5. Sm25 and additional integral membrane proteins of 13 kDa and 15 kDa are solubilized from the bilayer by 2-ME. Samples of mb-s and mb-s treated with 1 M 2-ME were centrifuged at 10^5 *g*. Resulting pellets (P) and supernatants (S) were electrophoretically separated, blotted to nitrocellulose, and probed with mouse anti-mb-s, followed by peroxidase labelled goat anti-mouse IgG and 4-chloro-1-napthol. Sm25 and the 13 kDa and 15 kDa proteins of interest are indicated.

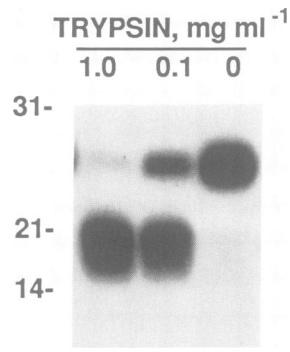

Fig. 6. Sm25 is exposed at the surface of the parasite. Living schistosomes were incubated without or with trypsin at the concentrations indicated and extracted with 0.5% NP-40. Blots of electrophoretically separated extract were probed with rabbit anti-recombinant Sm25 followed by [^{125}I]protein A and autoradiographed. Mol. wt standards are shown on the left.

role in anchoring Sm25 to the bilayer. Furthermore, the observation that Sm25 can be released from the membrane by reduction excludes the possibility that this molecule is anchored by PIPLC-resistant GPI.

In experiments where high-speed supernatants from 2-ME treated mb-a were probed with a polyclonal mouse anti-mb-s antiserum and compared with supernatants from untreated mb-a, proteins of 15 and 13 kDa were also seen to be effectively solubilized by heavy reduction (Figure 5).

Sm25 is exposed at the surface of the parasite

In mammalian cells, covalent modification with palmitate can be involved in anchoring proteins to the inside of the plasma membrane (Hancock *et al.*, 1989). While the observed *N*-glycosylation of Sm25 makes this highly unlikely, we felt it necessary to demonstrate formally that this molecule is not similarly oriented at the parasite surface. Support for this was provided by experiments in which Sm25 from trypsin-treated cultured parasites was found to be substantially degraded (Figure 6). Presumably, the diffuse band of 16–21 kDa recognized by rabbit anti-recombinant Sm25 in extracts of trypsin-treated parasites (Figure 6) represents the trypsin degradation product(s) of Sm25.

Discussion

The data presented here indicate that Sm25, a major schistosome tegumental antigen, is palmitoylated via a thioester linkage to Cys168, a residue lying within a short hydrophobic domain towards the C-terminal end of the protein. This post-translational modification is shown to be essential for the stable interaction of Sm25 with the membrane.

Interestingly, as judged by the intensity of the fluorograph signal of SDS–PAGE separated adult schistosome proteins from labelled parasites, Sm25 incorporated far more palmitic acid than did any other schistosome protein. Initially we hypothesized that this unusual pattern was due to relatively slow turnover of palmitoylated proteins other than Sm25, in which case our short labelling time, chosen to minimize the risk of palmitate degradation and conversion of label into amino acids, might be biasing the labelling process in favour of Sm25. However, a report by Sauma and Strand (1990) showed that in schistosomes cultured in [^{3}H]palmitate for 22 h the main labelled species appeared as a diffuse 25 kDa band on SDS–PAGE. This probably represents Sm25 and supports the view that Sm25 is the major palmitoylated schistosome protein.

The ability to release Sm25 from unsolubilized membranes by reductive removal of palmitate raises interesting questions on the orientation of this molecule within the bilayer. The observed physical behaviour of Sm25, partitioning into the detergent phase of TX-114 and co-purifying with tegumental surface membranes, suggests that this molecule is an integral membrane protein when fully processed. Analysis of the deduced amino acid sequence is consistent with this, revealing a putative signal peptide and two *N*-glycosylation sites, one of which at least is utilized since the molecule is sensitive to *N*-glycanase. Further, the C terminus of Sm25 contains a hydrophobic domain between residues 163 and 180 followed by Lys181–Ser182. Considered together, these data suggest that Sm25 is a component of a surface membrane with the C-terminal hydrophobic domain spanning the bilayer and Lys181–Ser182 constituting a short cytoplasmic tail. However, a transmembrane alignment of region 163–180 is difficult to reconcile with: (i) the necessity for Cys168 to be available to the acyl-transferase which catalyses its palmitoylation; and (ii) the implied passage of the two C-terminal polar amino acids across the bilayer after the delipidation of Cys168. An alternative hypothesis is that the C-terminal Lys–Ser lie outside the surface membrane with the hydrophobic domain 'dipping' into the bilayer where it is stabilized by the covalently attached palmitate. Such an orientation is unprecedented, however, and fails to accommodate the anticipated cytoplasmic location of described palmitoyl-transferases (see Sefton and Buss, 1987). Therefore, in the absence of data to support the latter argument, we propose that the polypeptide backbone of Sm25 does span the membrane and that the palmitic acid at Cys168 is necessary to stabilize an otherwise non-viable association of region 163–180 with the bilayer.

The attachment of fatty acid to a protein would be expected to lead to increase in hydrophobicity and consequently an enhanced ability of the protein to interact with lipid bilayers, a view supported by, for example, the observed association with membranes of most palmitoylated proteins in cultured

avian cells (Magee and Courtneidge, 1985). However, a role for covalently bound lipid in membrane attachment has previously been shown unequivocally only for GPI-linked proteins (see Ferguson and Williams, 1988), $p21^{ras}$ (Willumson *et al.*, 1984; Hancock *et al.*, 1989) and $pp60^{src}$ (Kamps *et al.*, 1986), the latter two of which differ from Sm25 in being otherwise essentially hydrophilic cytoplasmic proteins which associate with the inner face of the plasma membrane.

A minority of documented palmitoylated proteins are comparable to Sm25 in being glycosylated, exposed at the cell surface and possessing domains of relatively hydrophobic amino acids which putatively span the membrane. In molecules of this type, such as the human transferrin receptor, the HLA molecule and the spike proteins of Semliki Forest Virus, the site of palmitoylation appears to be similar to that observed here for Sm25, involving a Cys residue within or close to the transmembrane domain (Kaufman *et al.*, 1984; Schneider *et al.*, 1984; Schmidt *et al.*, 1988). Whether or not palmitate plays a role in stabilizing the interaction of these proteins with membranes remains unknown.

The co-purification of Sm25 with surface membrane blebs from adult schistosomes supports the view that this glycoprotein is a component of the characteristic double surface membrane complex of the schistosome tegument, a cytoplasmic syncitium which encapsulates this multicellular parasite (Hockley and McLaren, 1973). Previous reports suggested that Sm25 is integral with the inner of these two bilayers (Karcz *et al.*, 1988), in which case it presumably would not be exposed at the surface; however, this is inconsistent with its observed availability to exogenous protease (Figure 6) which suggests that it is a component of the outer membrane. We are currently using immuno-electron microscopy to address this question directly. If Sm25 is located in the outer surface bilayer, the unusually short cytoplasmic domain may represent an adaptation to the small hydrophilic zone between the outer and inner membranes.

The observation that several apparently non-palmitoylated proteins, including those of 13 kDa, 15 kDa (Figure 5), 22 kDa and 31 kDa (data not shown), are released from tegumental membranes by exposure to high concentrations of 2-ME is of interest. It is possible that some of these molecules are palmitoylated but, perhaps due to slow turnover, somehow fail to incorporate [^{3}H]palmitate over the course of the culture conditions utilized here. Indeed, a previous study which utilized a 22 h labelling period did report palmitoylated proteins of 22 and 31 kDa (Sauma and Strand, 1990). Alternatively, Sm25 may be associated with other membrane proteins in such a way that they are dependent upon this intermolecular interaction for stable attachment to the bilayer. In this context, one possibility is that region 163–180 of Sm25 interacts with the membrane spanning domains of other proteins. This hypothesis is supported by the presence of Asn165 and Asn174 within the putative transmembrane region of Sm25. It is extremely uncommon to find Asn within an anchor sequence (see Williams and Tse, 1985), it seemingly only being present in domains which associate together within the bilayer, as for example in rhodopsin (Dunn *et al.*, 1987). Interestingly, recently obtained sequence of a cDNA for the 2-ME-released 13 kDa protein shows that this molecule has an analogous C terminus to that of Sm25 (Omer Ali *et al.*, 1991) including a similarly compromised hydrophobic domain. Thus, the attachment of Sm13 to the membrane may be stabilized by an association with a firmly anchored but appropriately inter-reactive molecule such as Sm25. If this is true, it might be argued that the unusual C terminus of Sm25, rather than being a flawed transmembrane domain which requires palmitoylation for stability, represents a highly specialized structure to permit the type of inter-molecular interactions proposed above.

Materials and methods

Parasite culture, metabolic labelling

Six-week-old (adult) *S.mansoni* (NMRI strain) parasites were perfused from infected Swiss outbred mice into Dulbecco's modified Eagle's medium (DMEM) containing 100 U/ml penicillin, 100 μg/ml streptomycin and 2 U ml^{-1} heparin. After several washes by sedimentation in fresh medium the host cell-free parasites were resuspended in DMEM with 10 mM HEPES, antibiotics, 2 mM glutamine, 5 mM sodium pyruvate, fatty acid-free albumin (Sigma) and 3× the normal concentration of non-essential amino acids and transferred into a glass vial coated with [9,10^{3}H]palmitic acid (Amersham). Final culture conditions included 100 μCi/ml labelled fatty acid and an equal molar concentration of fatty acid-free albumin. Incubations were for 6 h at 37°C in 95% air/5% CO_2.

Identification of GPI-anchored membrane proteins

For membrane preparations, freshly recovered or labelled adult schistosomes were resuspended in 20 mM Tris–HCl, pH 8.0, 150 mM NaCl, 2 mM PMSF, 2 mM 1,10-phenanthroline, 10 μg/ml α_2-macroglobulin (TBS, all protease inhibitors from Sigma), frozen and thawed twice, homogenized with a motorized pestle (Kontes), diluted to 5 ml with TBS and centrifuged at 10^5 *g* for 1 h at 4°C. The resulting pellet, mb-a, was washed once in TBS and resuspended in the same buffer. To identify proteins with GPI anchors, 10 μg samples of unlabelled mb-a were diluted to 100 μl in TBS containing only α_2-macroglobulin and incubated for 1 h at 37°C with or without 1 U of PIPLC (ICN), after which the samples were diluted to 5 ml in ice-cold TBS and centrifuged at 10^5 *g*. The resulting supernatant (concentrated using a Centrico-10, Amicon) and pellet were separated by SDS–PAGE and electrophoretically transferred to nitrocellulose. Blots were probed with purified rabbit anti-CRD immunoglobulin (a gift from Dr Paul Englund, Johns Hopkins University), antisera from mice immunized with isolated tegumental surface membranes (mb-s) plus saponin (Smithers *et al.*, 1989) or polyclonal rabbit anti-mb-s antisera raised by immunizing animals with mb-s emulsified in complete Freund's adjuvant.

Immunochemical analysis of palmitoylated proteins

After culture, schistosomes were washed three times with ice-cold DMEM and extracted with 0.5% NP-40 in TBS for 15 min at 4°C, after which the parasite bodies were pelleted by centrifugation at 1.3×10^4 *g*. The resulting supernatant and pellet were analysed by SDS–PAGE under mild reducing conditions (2% 2-ME) as described previously. *N*-glycanase (Genzyme) treatment was according to the manufacturer's instructions. Labelled proteins were immunoprecipitated from the NP-40 extract using sera from normal mice or mice immunized with mb-s plus saponin, followed by protein A–Sepharose (Pharmacia), analysed by SDS–PAGE, amplified (Amersham) and fluorographed with Kodak X-Omat AR film, as described (Pearce *et al.*, 1986).

Analysis of fatty acid–Sm25 linkage

Equivalent samples of mb-a made from [^{3}H]palmitic acid-labelled schistosomes were separated by 10% SDS–PAGE under non-reducing conditions. One lane of the gel was incubated for 1 h at room temperature in methanol containing 200 mM KOH, while another was incubated in methanol alone, or fixed as usual in acetic acid:methanol:water (10:25:65); all were amplified and fluorographed (Magee, 1988).

The fatty acid covalently attached to Sm25 was identified as palmitate essentially as described by Buss and Sefton (1985). The [^{3}H]palmitate-labelled membrane preparation was separated by preparative SDS–PAGE and the area of the gel coinciding with the region of migration of Sm25 excised and incubated in methanolic KOH as above. The supernatant from this step was dried by rotary evaporation under vacuum, resuspended in methanol and analysed by ascending TLC on KC_{18} reversed-phase plates (Baker) in acetic acid:acetonitrile (1:1), which separates fatty acids according to chain length; under these conditions methyl-palmitate and palmitate behave similarly. One centimetre regions of the chromatogram were scraped from

the TLC plate into Econoscint A (National Diagnostics) and counted in a scintillation counter. The location of the labelled lipid eluted from the gel was determined relative to the location of commercial standards (Amersham; Matreya).

To investigate whether the palmitate–Sm25 linkage was susceptible to reduction, equivalent samples of labelled mb-a were solubilized in SDS sample buffer containing increasing amounts of 2-ME or DTT and separated by 15% SDS–PAGE, amplified and fluorographed (Schmidt *et al.*, 1988).

TX-114 partitioning assays

Unlabelled mb-a was preincubated for 10 min with the thiol-protease inhibitors leupeptin (5 μg/ml) and 5 mM *N*-ethylmaleimide (NEM) (Dalton and Heffernan, 1989). Preparations were then adjusted to 2 M 2-ME, or 450 mM DTT, or left untreated, and incubated at room temperature for 1 h before either: (i) centrifugation at 10^5 *g*, or (ii) solubilization at 4°C in 2% precondensed TX-114 (Sigma) in TBS containing leupeptin and NEM with or without 2 M 2-ME or 450 mM DTT. For the latter, non-soluble components were removed by centrifugation at 1.3×10^3 *g* and the resulting supernatant heated to 65°C for 5 min to permit phase separation (Bordier, 1981); 65°C rather than the more usual 37°C was found to be necessary for phase separation in the presence of high molarity reducing agents. After brief microcentrifugation to consolidate phases, the upper aqueous and lower detergent components were removed, diluted with SDS sample buffer (assuring a 7:1 final ratio of SDS:TX-114), boiled, separated by SDS–PAGE on 7–15% gradient gels and electrophoretically transferred to nitrocellulose paper. Pellets and supernatants from reduced samples not extracted with TX-114 were also electrophoresed and blotted. Blots were probed with sera from mice immunized with mb-s plus saponin or rabbits immunized with purified recombinant Sm25 produced by a partial cDNA clone in pGEX 1N (Knight *et al.*, 1989; Omer Ali *et al.*, 1991) or schistosome glutathione-S-transferase (Sj26), the protein to which the expressed product is fused in pGEX 1N. Bound antibody was detected with ^{125}I-labelled protein A (Amersham) and autoradiography using Kodak X-Omat AR film and Cronex III screens or, in some cases, with a peroxidase conjugated goat anti-mouse IgG followed by 4-chloro-1-napthol substrate (Jackson Immunoresearch) which, in our hands, proved a more sensitive technique for detecting antigens on immunoblots.

Proteolytic digestion of Sm25

Intact adult schistosomes were incubated at 37°C in HEPES buffered DMEM without or with 100 μg/ml or 1 mg/ml trypsin (Calbiochem). After 1 h the parasites were extensively washed in ice-cold DMEM and extracted for 15 min at 4°C with 0.5% NP-40 in TBS (which contained the trypsin inhibitor, PMSF). Extracted material was boiled in SDS sample buffer, separated by SDS–PAGE, electrophoretically transferred to nitrocellulose paper and probed with rabbit anti-recombinant Sm25 followed by [^{125}I]protein A. Bound antibody was visualized using autoradiography as above.

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