SMP-1, a Member of a New Family of Small Myristoylated Proteins in Kinetoplastid Parasites, Is Targeted to the Flagellum Membrane in *Leishmania*

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The mechanisms by which proteins are targeted to the membrane of eukaryotic flagella and cilia are largely uncharacterized. We have identified a new family of small myristoylated proteins (SMPs) that are present in *Leishmania* spp and related trypanosomatid parasites. One of these proteins, termed SMP-1, is targeted to the *Leishmania* flagellum. SMP-1 is myristoylated and palmitoylated in vivo, and mutation of Gly-2 and Cys-3 residues showed that both fatty acids are required for flagellar localization. SMP-1 is associated with detergent-resistant membranes based on its recovery in the buoyant fraction after Triton X-100 extraction and sucrose density centrifugation and coextraction with the major surface glycolipids in Triton X-114. However, the flagellar localization of SMP-1 was not affected when sterol biosynthesis and the properties of detergent-resistant membranes were perturbed with ketoconazole. Remarkably, treatment of *Leishmania* with ketoconazole and myriocin (an inhibitor of sphingolipid biosynthesis) also had no effect on SMP-1 localization, despite causing the massive distention of the flagellum membrane and the partial or complete loss of internal axoneme and paraxial structures, respectively. These data suggest that flagellar membrane targeting of SMP-1 is not dependent on axonemal structures and that alterations in flagellar membrane lipid composition disrupt axoneme extension.

INTRODUCTION

Eukaryotic flagella and cilia are complex surface organelles that play a key role in motility and sensory reception in many unicellular protozoa and metazoan organisms (Rosenbaum and Witman, 2002; Cole, 2003). The assembly and maintenance of the internal microtubule axoneme requires the continuous import of axoneme precursor proteins from the cytosol as well as the removal of proteins generated by turnover of axonemal structures. The import and export of these proteins appears to be largely mediated by intraglascular transport (IFT) particles that move along the axonemal doublet microtubules just beneath the flagellar membrane (Kozminski et al., 1995; Rosenbaum and Witman, 2002; Cole, 2003). IFT particles moving from the base of the flagellum to the tip (anterograde transport) are associated with plus-end directed kinesin motor proteins, whereas IFT particles moving in the reverse direction associate with dynein motor proteins and recycle kinesin and discarded axonemal proteins back to the cytosol. Current models also suggest that IFT particles may play a role in targeting proteins to the flagella membrane, which often contains a distinct protein (and lipid) composition from the rest of the plasma membrane, with which it is contiguous (Rosenbaum and Witman, 2002). However, it has recently been shown that polycystin-2, a membrane protein in kidney cilia, is still targeted to the cilia membrane of mutant kidney cells partially deficient in IFT (Pazour et al., 1998). IFT-independent mechanisms (Kozminski et al., 1993) may thus contribute to the distinctive protein composition of the flagellar membrane.

Trypanosomatid parasites are protozoa that cause a number of important diseases in humans, including African sleeping sickness (*Trypanosoma brucei*), Chagas disease (*T. cruzi*), and the (mucocutaneous and visceral leishmaniasis (*Leishmania* spp). The flagellum of most trypanosomatid developmental stages emerges from a deep invagination in the plasma membrane, termed the flagellar pocket and contains the typical 9+2 array of microtubule doublets as well as a paracrystalline structure termed the paraxial flagellar rod (Gull, 1999). Although the trypanosomatid flagellum is clearly important for parasite migration and invasion of host tissues, it may also regulate processes such as secretion and endocytosis in the flagellar pocket and may be enriched in proteins involved in environmental or nutrient sensing (Gull, 1999; Bastin et al., 2000; Landfear and Ignatushchenko, 2001; McConville et al., 2002; Hill, 2003; Vaughan and Gull, 2003). Studies on trypanosomatid flagellar membrane proteins have provided new insights into the signals that target proteins to the flagellar membrane. Specifically, sorting of the *L. enrietti* glucose transporter isoform, ISO1, to detergent-soluble membranes in the flagellum, is mediated by a peptide sequence within the N-terminal cytoplasmic tail of this polytopic membrane protein (Piper et al., 1995; Snapp and Landfear, 1999). In contrast, dual acylation of the amino termini of...
of T. cruzi flagellar calcium-binding protein, FcCaFp, is required for flagellar localization (Godsel and Engmann, 1999). Binding of FcCaFp to the flagellar membrane is Ca²⁺-dependent, indicating the presence of a novel myristoyl/palmityl switch mechanism (Godsel and Engmann, 1999).

In the present study, we describe the isolation, characterization, and flagellar targeting of a new diacylated membrane protein, termed small myristoylated protein-1 (SMP-1), in L. major. SMP-1 was initially identified in a Triton X-100-insoluble membrane fraction that contains plasma membrane components, such as the major glycosylphosphatidylinositol (GPI)-anchored proteins, free GPI glycolipids (GPI-CPLs), and monogalactosyl diacylglycerols (Denny et al., 2001; Ratlon et al., 2002). SMP-1 belongs to a new family of mono- or di-acylated SMPs that may exist in all trypanosomatids. We show that the insolubility of SMP-1 in cold Triton X-100 is due to association with membrane components rather than the flagellar axoneme and parflagellar rod proteins. Remarkably, perturbation of these membranes with inhibitors of sterol and sphingolipid biosynthesis resulted in the appearance of swollen flagella that retain the SMP-1-positive limiting membrane, but lacked prominent axoneme and parflagellar rod structures or their precursors. These findings strongly indicate that the targeting of SMP-1 to the flagellar membrane can occur independently of the axoneme or IFT and that the formation of the axoneme is critically dependent on membrane lipids.

MATERIALS AND METHODS

Cell Culture

L. major (LRC-L137, clone V121) promastigotes were cultivated at 27°C in Schneider's Drosophila medium containing 10% heat-inactivated fetal bovine serum (fBS), L. mexicana (NYC/BZ/62/M379) and L. donovani (L) promastigotes were grown in RPMI or SDM79 medium, respectively, supplemented with 0.4% (vol/vol) Tween 20. The blots were probed with the following antibodies (at 1:1000 dilution): mouse monoclonal anti-β-tubulin (Clone DM1A, Sigma), rabbit anti-SMP-1–protein A agarose were diluted 1:1000 in 1% powdered skim milk. Secondary antibodies, anti-rabbit horse-radish peroxidase–conjugated antibody were diluted 1:1000 in 1% powdered skim milk. Radiolabeled myristoylated or palmitoylated proteins were detected by fluorography after treating the gel with Amplify (Amer sham, Piscataway, NJ) and exposure to Biomax MR film (Eastman Kodak, Rochester, NY) at 70°C. Radiolabeled proteins in the Triton X-100–insoluble fraction, were recovered by boiling the pellet in sample buffer and analyzed on 15% SDS-PAGE gel, the proteins were fixed in 25% isopropanol, 10% acetic acid. The gel was washed with water and incubated with 1 M hydroxyamine in 1 M Tris, pH 7 (16 h, 25°C, Olson et al., 1985). Radiolabeled myristoylized or palmitylated proteins were detected by fluorography.

Immunofluorescence and Electron Microscopy

L. major promastigotes were fixed in 4% paraformaldehyde for 15 min and then immersed in poly-L-lysine-coated coverslips (4 × 10 cells/ coverslip). In experiments shown in Figure 6, promastigotes were extracted with 1% Triton X-100 at either 0 or 25°C before immobilization on the
Figure 1. SMP-1 is a major protein in the buoyant Triton X-100–insoluble membranes. (A) L. major promastigotes were extracted in cold Triton X-100 and lysates subjected to flotation centrifugation in a sucrose density gradient. Protein in gradient fractions were analyzed by 15% SDS-PAGE and silver staining (top panel). PSA2 (a DRM component) and α-tubulin (cytoskeleton) were detected by Western blotting (bottom panels). Fractions 1–17 encompass a step gradient of 5–60% sucrose. (B) Large format 15% gel showing the 15-kDa protein doublet in the buoyant membrane fraction (Fractions 2–4 of A). (C) L. major, L. mexicana, and L. donovani promastigotes were extracted in cold Triton X-100 and insoluble (I) and soluble (S) fractions were analyzed by 15% SDS-PAGE. Western blotting with a polyclonal antiserum directed against the unique C-terminal peptide of SMP-1, revealed a single reactive protein at 15 kDa.

Cloning and Expression of Epitope-tagged Di-, Mono-, and Nonacylated SMP-1

For episomal expression in L. major, SMP-1 constructs were cloned into the vector pX containing the neomycin resistance gene and three copies of the influenza hemagglutinin peptide epitope (HA) within the multiple cloning site (Mullin et al., 2001). SMP-1 was amplified from genomic DNA with primers 1 (TCCCCGGATGCGCGCTCCCAAC) and 2 (CGGGATCCCTTGTCCCTTCCTCGCC) containing unique BamHI and SmaI restriction sites (underlined) to allow in frame placement of the gene upstream of the triple HA epitope. The SAP-1 antisense sequence (1:500 dilution), anti-α-tubulin (cytoskeleton) and anti-GPI-anchored protein, PSA2, was largely recovered in the buoyant membrane fraction (Figure 1A, fractions 1–6). The DRM fractions also contained a prominent 15-kDa protein (Figure 1A, top panel), which was resolved as a doublet on 15% SDS-PAGE gels (Figure 1B). Mass spectrometry of the tryptic peptides released from this doublet or tryptic digests of unfractionated DRMs identified two peptides, DNGLLFR and MDALPSEEEYR, derived from a putative 131 amino acid protein encoded by the L. major genome (Figure 2A). As the N-terminal sequence of this protein contained a strong myristoylation motif (MGXXXS/T; Towler et al., 1987), this protein was referred to as small myristoylated protein-1 (SMP-1). Western blot analysis of Triton X-100–soluble and –insoluble fractions of L. major, L. mexicana, and L. donovani promastigotes using a polyclonal antibody raised against the unique C-terminal peptide of SMP-1, indicated that this protein was expressed at comparable levels in these pathogenic species of Leishmania (Figure 1C).

BLAST searches of the L. major genome suggested that SMP-1 was a member of a new family of Leishmania proteins (Figure 2A). These proteins contain a relatively well-conserved N-terminal domain and a highly divergent C-terminal domain and may differ in the extent to which they are acylated (Figure 2A). Although SMP-1 is predicted to be both myristoylated and palmitoylated at positions Gly-2 and Cys-3, respectively, SMP-2 and SMP-4 only contain myristoylation sites (Figure 2A). In contrast, SMP-3 lacks both N-terminal myristoylation and palmitoylation sites (Figure 2A). BLAST searches of the genomes of T. brucei and T. cruzi indicated that SMP-like proteins are ubiquitously encoded in other trypanosomatidae and that they share some identity with the N-terminal domain of the recently identified T. brucei cytoskeletal protein, TbCAP5.5 (Hertz-Fowler et al., 2001). TbCAP5.5 is also N-terminally diacylated, but clearly differs from the SMPs in containing two distinct indels and a large calpain protease-like domain (shown schematically in Figure 2C). Phylogenetic analysis of the trypanosomatid SMP family revealed eight clusters. SMP-1, SMP-2, and SMP-3 proteins are unique to L. major and lack any clear orthologues in T. brucei or T. cruzi. The SMP-4 cluster contains orthologues from T. cruzi and L. major united by a DD/S motif at position 22, the PY motif at 35, a Q at 166, and...
Figure 2. Sequence comparison of trypanosomatid SMPs. (A) Alignment of the L. major, T. brucei, and T. cruzi SMP family members with the N-terminus of TbCAP5.5. Accession numbers for L. major and T. brucei proteins are as follows: LmSMP1 (AY642931), LmSMP2 (AY642932), LmSMP3 (CAC24686), LmSMP4 (AY642933), TbSMP5 (CAB95480), TbSMP6 (CAB95484), TbSMP8a (CAB95485), and TbCAP5.5 (AAAG48626). Genedb numbers for the T. cruzi proteins are (prefix Tc00.104705350); TcSMP4 (6563.110), TcSMP5a (6563.170), TcSMP6a (6563.180), TcSMP7a (6563.70), TcSMP7c (9003.30), and TcCAP5.5 (9003.40). (B) Phylogenetic tree of the SMP family members. A neighbor joining distance tree rooted using the N-terminus of TbCAP5.5 is shown. Bootstrap support values for three types of analysis (neighbor joining/maximum parsimony/quartet puzzling) are shown at nodes. Bootstrap values $<50\%$ are shown as a dash. Monoacylated or diacylated proteins are indicated by single or double bullet points, respectively. (C) Schematic showing related protein domains in the SMPs, TbCAP5.5, and the calpain superfamily. Sequence identity is indicated by like-shaded cylinders. The diacylated N-terminal domain of CAP5.5 shares homology with the acylated SMPs. The central domain of TbCAP5.5 shares homology with the protease domain of the calpains but lacks other domains (domain I, III and the calcium-binding domains) typical of calpains.
a basic C-terminus. The SMP-5 cluster has representatives in *T. brucei* and *T. cruzi*, and each member has a predominate acidity, eight amino acid indel in the middle plus a relatively short C-terminus ending in F/YRL. The SMP-6 cluster also has representatives in *T. brucei* and *T. cruzi* with clear signatures such as the threonine at 100, phenylalanine at 104, asparagine at 106, and a charged C-terminus ending in AA/TT/SF. The SMP-7 cluster is restricted to *T. cruzi* but perhaps weakly related to the SMP-8 cluster of *T. brucei*. Intriguingly, there is no clear trend for mono- or di-acylation in the SMP orthologue clusters with clear-cut orthologues such as the SMP-5s predicted to be monoacylated in *T. cruzi* but diacylated in *T. brucei*. Similarly, LmSMP-4 is predicted to be monoacylated, whereas TbSMP-4 may be diacylated. It is interesting that LmSMP-3, which lacks a clear acylation motif, is nested within a cluster of acylated proteins and is not an evolutionary outlier.

**L. major SMP-1 Is Targeted to the Flagellum of Promastigote Stages**

The polyclonal antibody raised against the unique C-terminal peptide of LmSMP-1 (abbreviated to SMP-1) was used to localize this protein to the flagellum of promastigote stages (Figure 3A). Staining extended from near the basal body, juxtaposed to the kinetoplast, to the distal tip of the flagellum (Figure 3A). This contrasts with the distribution of the paraflagellar rod proteins-1 and -2 (PFR1, and -2) that only associate with the flagellum after it emerges from the flagellar pocket (Figure 3A). SMP-1 staining was not observed around the flagellar pocket or to the rest of the cell body membrane (Figure 3A). Western blotting showed that SMP-1 was expressed in all promastigotes stages containing an elongated flagellum, but not the amastigote stage that contain a highly truncated flagellum (Figure 2C, lane 3). Finally, trypsin digestion of live or detergent-permeabilized promastigotes indicated that SMP-1 was primarily localized on the inner leaflet of the flagellar membrane (our unpublished results).

**Myristoylation and Palmitoylation of SMP-1 Is Required for Flagellar Targeting**

The acylation state of SMP-1 was investigated by metabolically labeling *L. major* promastigotes with [3H]myristate or [3H]palmitate and total cellular proteins analyzed by SDS-PAGE. A major [3H]myristate-labeled protein with the same molecular weight as SMP-1 was recovered in the Triton X-100–insoluble fraction (Figure 4A). This band could be solubilized in 1% Triton X-100 at 25°C and immunoprecipitated with the SMP-1 specific polyclonal antibody (Figure 4A). The labeled band was resistant to hydroxylamine treatment, indicating the presence of an amide linkage (Figure 4A). A protein with the same properties was also labeled with [3H]palmitate (Figure 4B). Unlike the [3H]myristate label, the [3H]palmitate label was removed after hydroxylamine treatment (Figure 4B), indicating the presence of a thiol ester linkage. Interestingly, the [3H]palmitate label was lost from SMP-1 when promastigotes were solubilized in Triton X-100 at 25°C (our unpublished results), suggesting that this modification is enzymatically removed.

To confirm that Gly2 and Cys3 are indeed acylated, C-terminally HA-tagged chimeras of SMP-1 containing both residues or lacking one or the other (SMP-1::HA, SMP-1ΔMyr::HA and SMP-1ΔPalm::HA) were ectopically expressed in *L. major* promastigotes. All three chimeras were expressed at similar levels (~10-fold lower than endogenous protein) and had the expected molecular weight of ~19 kDa on SDS-PAGE (our unpublished results). As expected SMP-1::HA was targeted to the flagellum in the same way as the native protein (Figure 4C, top panel). In contrast, the two mutant SMP-1 proteins lacking the N-terminal Gly2 and Cys3 residues were targeted to the cytosol (Figure 4C, middle and bottom panels). These data strongly suggest that SMP-1 is myristoylated and reversibly palmitoylated and that both of these modifications are essential for flagellar targeting.

**SMP-1 Is Not Associated with the Flagellum Cytoskeleton**

Diacylated proteins such as *T. brucei* CAP5.5 appear to be tightly associated with the subpellicular microtubules, as well as the cell body plasma membrane (Hertz-Fowler et al., 2001). To examine whether SMP-1 is similarly associated with an underlying cytoskeleton, notably the flagellar axoneme, *L. major* promastigotes were solubilized in cold 1% Triton X-100 and the lysates were floated in sucrose density gradients. Approximately 40–60% of SMP-1 as well as externally disposed surface glycolipids (GIPLs), which are not expected to interact with the cytoskeleton (Ralton et al., 2002), were recovered in the buoyant membrane fraction (Figure 5A). This fraction lacked tubulin, indicating that the DRM fraction does not contain a tightly bound microtubule.
component (Figure 5A). To further assess whether the SMP-1 and GIPL pools that remained at the bottom of the sucrose gradient were associated with the cytoskeleton, \textit{L. major} promastigotes were extracted in 1% Triton X-100 at 25°C instead of 0°C. These conditions resulted in the total extraction of SMP-1 and GIPLs, whereas tubulin remained in the insoluble fraction (Figure 5B), indicating that all of the SMP-1 is associated with DRMs. We have previously shown that leishmanial DRM components, but not the microtubulin cytoskeleton, are more effectively extracted in cold 1% Triton X-114 than in 1% Triton X-100 (Ralton et al., 2002). As expected for a DRM component, SMP-1 was partially solubilized from \textit{L. major} promastigotes in cold Triton X-114 (Figure 5B), without detectable solubilization of cytoskeletal components (Figure 5B). Interestingly, both SMP-1 (Figure 5C) and the major GIPLs (Ralton et al., 2002) were quantitatively solubilized from \textit{L. mexicana} membranes in cold 1% Triton X-114 (Figure 5C), indicating species-specific differences in the lipid composition of leishmanial DRMs. Finally, unlike other trypanosomatid cytoskeleton-associated proteins (Vedrenne et al., 2002), the solubility of SMP-1 and GIPLs in

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cold 1% Triton X-100 was not increased by addition of high salt (Figure 5B). The absence of a strong association of SMP-1 with the flagellar cytoskeleton was further supported by immunofluorescence studies. Specifically, SMP-1 and GIPLs were poorly extracted with cold 1% Triton X-100, but were totally extracted when promastigotes were treated with 1% Triton X-100 at 25°C (Figure 6A). In contrast, the subpellicular/axoneme microtubules as well as the paraflagellar rod were not extracted under either condition (Figure 6, B and C). Collectively, these data suggest that SMP-1 is largely associated with detergent-resistant membranes and that the flagellar localization of SMP-1 is not dependent on strong associations with the microtubule axoneme or the paraflagellar rod.

Flagellar Localization of SMP-1 Is Not Affected by Changes in DRM Composition or Axoneme and Paraflagellar Rod Structure

Leishmanial DRMs are enriched in the major cellular sterol and sphingolipids, ergosterol and inositolphosphoceramide, respectively (Denny et al., 2001; Ralton et al., 2002). To investi-
gate whether the flagellar localization of SMP-1 is dependent on the lipid composition of the flagellar DRM, \textit{L. major} promastigotes were treated with myriocin, an inhibitor of serine: palmitoyl-CoA transferase, the first committed enzyme in sphingolipid biosynthesis (Miyake et al., 1995) and ketoconazole, an inhibitor of sterol 14'-demethylase (Beach et al., 1988). At the concentrations used, these inhibitors cause a reduction of 70 and 50% in inositolphosphoceramide and sterol levels, respectively (Ginger et al., 2001; Ralton et al., 2002; our unpublished results). Myriocin-treatment alone had no effect on the growth rate of \textit{L. major} promastigotes (our unpublished results), the solubility of SMP-1 and GIPLs in cold Triton X-100 (Figure 7A) or the flagellar localization of SMP-1 (Figure 7B). In contrast, ketoconazole-treatment significantly increased the solubility of both SMP-1 (Figure 7A) and the GIPLs (our unpublished results) in cold Triton X-100, demonstrating that changes in sterol composition alters the physical properties of DRMs. However, ketoconazole treatment had little effect on SMP-1 localization (Figure 7B), indicating that the targeting and/or retention of SMP-1 to the flagellar membrane is not dependent on a specific lipid composition. Interestingly, ketoconazole treatment caused the flagellum of some stationary phase promastigotes (40%) to retract, forming a rounded balloon-like structure at the anterior end of the promastigote (Figure 7B). This phenotype was induced in all (90%) promastigotes when both ketoconazole and myriocin were added together (Myr/Ket) for 3 (log) or 3 (stationary) days. Immunofluorescence microscopy of fixed promastigotes probed with the polyclonal anti-SMP-1 antibody.

**Figure 7.** Inhibitors of sterol and sphingolipid biosynthesis perturb DRM formation and flagellar structure but not localization of SMP-1. (A) \textit{L. major} promastigotes were grown in the absence or presence of 1 \mu{g}/ml myriocin (Myr), 2 \mu{g}/ml ketoconazole (Ket) or both drugs together (Myr/Ket) for 3 d and extracted in cold 1% Triton X-100. The distribution of SMP-1 in insoluble (I) and soluble (S) fraction was determined by Western blotting. (B) \textit{L. major} promastigotes were grown in the presence of 1 \mu{g}/ml myriocin (Myr), 2 \mu{g}/ml ketoconazole (Ket), or both drugs together (Myr/Ket) for 1 (log) or 3 (stationary) days. Immunofluorescence microscopy of fixed promastigotes probed with the polyclonal anti-SMP-1 antibody.

DISCUSSION

We have identified a novel family of SMPs in \textit{L. major} that appear to be part of a larger family of proteins found in all trypanosomatids. Our data show that 1) SMP-1, the first of...
These proteins to be characterized in detail, is a major flagellar membrane protein in Leishmania, 2) that the dual myristoylation/palmitoylation signal in SMP-1 is required for localization of SMP-1 to detergent-resistant flagellar membranes, 3) that flagellar targeting and/or retention is not dependent on direct interactions with the axoneme or other cytoskeletal proteins, and 4) that disruption of sphingolipid modulate the potential for myristoylation and palmitoylation to target HASPB and SMP proteins to different cellular localizations. Interestingly, the association of the diacylated protein FCaBP with flagellar membranes appears to be calcium-dependent (Godsel and Engman, 1999). In contrast, the diacylated L. major protein, HASP-B, is targeted to Triton X-100–soluble domains of the plasma and endomembranes, rather than detergent insoluble-domains of the flagellum (Denny et al., 2000, 2001). Moreover, a GFP-chimera containing the monoacylated (myristoylated) N-terminal domain of HASPB was targeted to intracellular secretory/endosomal membranes (Denny et al., 2000). Collectively, these data suggest that other factors, such as protein conformation and/or additional peptide sorting signals, modulate the potential for myristoylation and palmitoylation to target HASPB and SMP proteins to different cellular localizations. Interestingly, the association of the diacylated T. cruzi protein FCaBP with flagellar membranes appears to be calcium-dependent (Godsel and Engman, 1999). In contrast, the association of SMP-1 with the flagellar membrane was not affected by calcium-chelating agents (our unpublished results), although it is possible that other factors (pH, phosphorylation, GTP-binding or oligomerization; Tang et al., 2004) could regulate flagellar localization.

A number of trypanosomatid proteins appear to be localized to the plasma membrane of the cell body through interactions with the underlying microtubule cytoskeleton (Piper et al., 1995; Hertz-Fowler et al., 2001; Vedrenne et al., 2002). However, several lines of evidence suggest that the use of the myristate/palmitate anchor, which would result in less perturbation of the flagellar membrane than a transmembrane polypeptide domain. Searches of the L. major genome revealed the presence of three other SMPs in this parasite (Figure 2). SMP-2 and -4 contain a strong myristoylation motif, but lack a clearly defined palmitoylation signal. These proteins also differ from SMP-1, and from each other, in containing distinct C-terminal sequences (Figure 2). The fourth member of the L. major SMP family, SMP-3, differed from the other members in lacking any identifiable acylation site or a comparable C-terminal domain. Although the functions of this protein family have yet to be elucidated, their diverse subcellular localizations (our unpublished results) and the presence in other trypanosomatids (T. brucei, T. cruzi; see Figure 2) suggests that they may be involved in multiple cellular processes. In this respect, it is of interest that the SMP "module" appears to act as a membrane anchor for the T. brucei cytoskeletal protein, TbCAP5.5 (Figure 2, A and B) and related proteins in the L. major genome (Hertz-Fowler et al., 2001). By analogy, the SMP family may be involved in targeting other proteins to surface or intracellular membranes. The potential importance of SMPs and other myristoylated proteins (Denny et al., 2000) in these parasites, is underlined by a recent study showing that the myristoyl-CoA: protein N-myristoyltransferase is essential for normal growth of L. major and T. brucei (Price et al., 2003).

Although the protein and lipid compositions of eukaryotic flagellar and cilia membranes differ from the abutting plasma membrane, little is known about flagellar membrane targeting signals or the mechanisms involved. Flagellar targeting of the L. enriettii glucose transporter, ISO1, is mediated by the N-terminal cytoplasmic domain of this polytopic membrane protein (Snapp and Landfear, 1999), whereas dual acylation appears to be both necessary and sufficient for flagellar localization of T. cruzi FCaBP (Godsel and Engman, 1999). Dual acylation was also found to be essential for flagellar targeting of SMP-1, as mutation of either the myristoylation or the palmitoylation sites prevented membrane targeting (our unpublished results) and resulted in a cytoplasmic localization. Dual acylation also appeared to be essential for membrane association as SMP-1<sup>N terminus::HA</sup>, which should still be cotranslationally myristoylated (Denny et al., 2000), was localized to the cytosol and soluble fractions. In contrast, the diacylated L. major protein, HASP-B, is targeted to Triton X-100–soluble domains of the plasma and endomembranes, rather than detergent insoluble-domains of the flagellum (Denny et al., 2000, 2001). Moreover, a GFP-chimera containing the monoacylated (myristoylated) N-terminal domain of HASPB was targeted to intracellular secretory/endosomal membranes (Denny et al., 2000). Collectively, these data suggest that other factors, such as protein conformation and/or additional peptide sorting signals, modulate the potential for myristoylation and palmitoylation to target HASPB and SMP proteins to different cellular localizations. Interestingly, the association of the diacylated T. cruzi protein FCaBP with flagellar membranes appears to be calcium-dependent (Godsel and Engman, 1999). In contrast, the association of SMP-1 with the flagellar membrane was not affected by calcium-chelating agents (our unpublished results), although it is possible that other factors (pH, phosphorylation, GTP-binding or oligomerization; Tang et al., 2004) could regulate flagellar localization.

Figure 8. Ultrastructure of ketoconazole/myriocin-treated L. major promastigotes. L. major promastigotes were grown in the absence (A and B) or presence of myriocin and ketoconazole (1 and 2 μg/ml, respectively; C and D) for 3 d and fixed for electron microscopy. (B and D) Detail of the flagellar pocket and emergent flagellum. Inset in B shows detail of 9 + 2 microtubule axoneme and associated paraf lagellar rod (arrowhead): f, flagellum; k, kinetoplast; ax, axoneme; fp, flagellar pocket.

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flagellar localization of SMP-1 is not dependent on interactions with the axoneme microtubules or the paraflagellar rod. First, most of the SMP-1 floated with other DRM components in sucrose density gradients and was clearly separated from microtubules or tubulin. Although some SMP-1 remained at the bottom of the gradient, DRM components such as the GIPLs and PSA2 were similarly retained, presumably reflecting incomplete solubilization of the DRMs. Second, SMP-1 was solubilized to the same extent as the GIPLs, under a variety of detergent and high salt extraction conditions (1% Triton X-100, 25°C; 1% Triton X-114, 0°C) that left the cytoskeleton (subpellicular microtubules plus axon-
eme) essentially intact. Interestingly, these studies showed that the Triton X-114 solubility of SMP-1 and GIPL in *L. major* and *L. mexicana* differed markedly, indicating species-specific differences in the lipid composition of the DRMs. These results suggest that the Triton X-100 insolubility of SMP-1 is largely due to hydrophobic interactions, rather than interactions with the cytoskeleton (Ralton et al., 2002). Third, partial inhibition of sterol biosynthesis, which is expected to alter the properties of DRMs, increased the solubility of SMP-1 and GIPLs in cold Triton X-100. Finally, the subcellular distribution of SMP-1 was not significantly altered when promastigotes were treated with sterol and sphingolipid biosynthesis inhibitors, under conditions that caused the axoneme to completely dissociate from the flagellar membrane. Collectively, these data suggest that the flagellar localization of SMP-1 is not dependent on tight associations with the axoneme microtubules and/or the paraflagellar rod. Moreover they raise the possibility that SMP-1 is targeted to the flagellar membrane via an IFT-independent mechanism. Transport of polycistin-2 to the membrane kidney epithelia cilium also appears to occur via an IFT-independent mechanism (Pazour et al., 2002). A calcium-dependent process involving flagellar matrix phosphoproteins has been proposed to operate in regulating the directed flow of flagellar membrane proteins in *Chlamydomonas reinhardtii* (Bloodgood and Salomonsky, 1994) and related proteins could play a role in regulating SMP-1 flagellar localization in *Leishmania*.

Sterol biosynthetic inhibitors such as ketoconazole and fluconazole are used as anti-*Leishmania* drugs (Arana et al., 2001; Alrajhi et al., 2002; Davies et al., 2003). However, the precise mode of action of these drugs, in terms of cellular processes affected, remain unclear. In this study we show that sublethal concentrations of ketoconazole affect flagellar structure in stationary phase promastigotes. This effect was dramatically accentuated and observed in all growth phases when promastigotes were treated with both ketoconazole and myriocin, although the sphingolipid inhibitor had no detectable effect on flagellum morphology when used alone. Partial inhibition of sterol/sphingolipid biosynthesis induced the flagellar membrane to dilate, usually at the point at which the flagellum emerged from the flagellar pocket, and was associated with a dramatic shortening of the axoneme and the loss of the paraflagellar rod. The dilated flagellum contained electron-dense material that did not appear to comprise axoneme or paraflagellar rod precursors. This flagellum phenotype is distinct from those found *Chlamydomonas* mutants deficient in retrograde or anterograde IFT, which are characterized by swellings at the distal tip of the flagellum or production of very short flagellum assembly dynamics revealed by analysis of the paralysed trypanosome mutant snl-1. J. Cell Sci. 112, 3769–3777.

**REFERENCES**


