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Short communication

Membrane targeting of the small myristoylated protein 2 (SMP-2) in *Leishmania major*

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ABSTRACT

Leishmania parasites express three highly conserved small myristoylated proteins (SMPs) that are targeted to distinct membranes. SMP-1 is exclusively found in the flagellum, depending on myristoylation and palmitoylation. In contrast, monoacylated SMP-2 and SMP-4 are localized to the flagellar pocket and plasma membrane, respectively. Here, we demonstrate that unlike SMP-4, SMP-2 resides in detergent resistant membranes, but can be readily solubilized in the presence of high concentrations of salt. We provide evidence that in detergent resistant membranes, SMP-2 forms high molecular weight complexes *in vivo*. Association with detergent resistant membranes was abrogated in the presence of a C-terminal tag suggesting acylation independent targeting signals. In addition, the N-terminal region of SMP-2 contains sufficient information for membrane targeting, as a GFP-chimera localizes to the flagellar pocket. Thus while the core sequences of the SMPs are highly conserved, individual members have evolved different mechanisms for their diverse membrane localization.

N-myristoylation, in which a 14-carbon saturated fatty acid is added to the N-terminal glycine, is irreversible and is catalyzed by a single myristoyl-CoA:protein N-myristoyltransferase (NMT) [1]. In trypanosomatid parasites as many as 60 proteins are predicted to be co- (or post) translationally modified with myristate and NMT is the target of novel chemotherapies against parasitic diseases [2]. While myristoylation can regulate protein function, it primarily affects membrane trafficking, although other signals are required for stable membrane interactions including palmitoylation (the addition of a 16-carbon saturated fatty acid) of glycine-proximal cysteine residues [3,4]. In trypanosomatid parasites mono- and diacylated proteins are found on the endomembrane system but also on different subdomains of the plasma and flagellar membrane [5–8]. The plasma membrane is continuous with the flagellar pocket and flagellar membrane and it is thought that the acylation status is the main determinant for correct localization of acylated proteins to these lipid bilayers. For example, *Trypanosoma brucei* calcium binding proteins, calflagins, rely on diacylation for flagellum localization and are mistargeted to the plasma membrane in the absence of palmitoylation [9]. The default trafficking of diacylated proteins to the flagellar membrane may reflect the preferential partitioning of these proteins into membranes that are rich in sterols and saturated fatty acids and are inherently resistant to cold Triton X-100 solubilization [10]. Conversely, myristoylated proteins are predominantly found in detergent soluble membranes, with few exceptions [11]. However, given that the entire plasma membrane of several trypanosomatid parasites stages, including *Leishmania* promastigotes, is intrinsically resistant to cold detergent extraction suggesting enrichment in sterols/saturated fatty acids, it is not clear to what extent lipid-based sorting mechanisms regulate subcellular targeting [12–14]. In addition, not all diacylated proteins are trafficked to the flagellar membrane suggesting that additional signals may still be required for sorting to this domain, including positively charged residues, or that other signals prevent trafficking to the flagellar membrane [15–17].

Trypanosomatid parasites express a highly conserved family of small myristoylated proteins (SMPs), characterized by N-terminal

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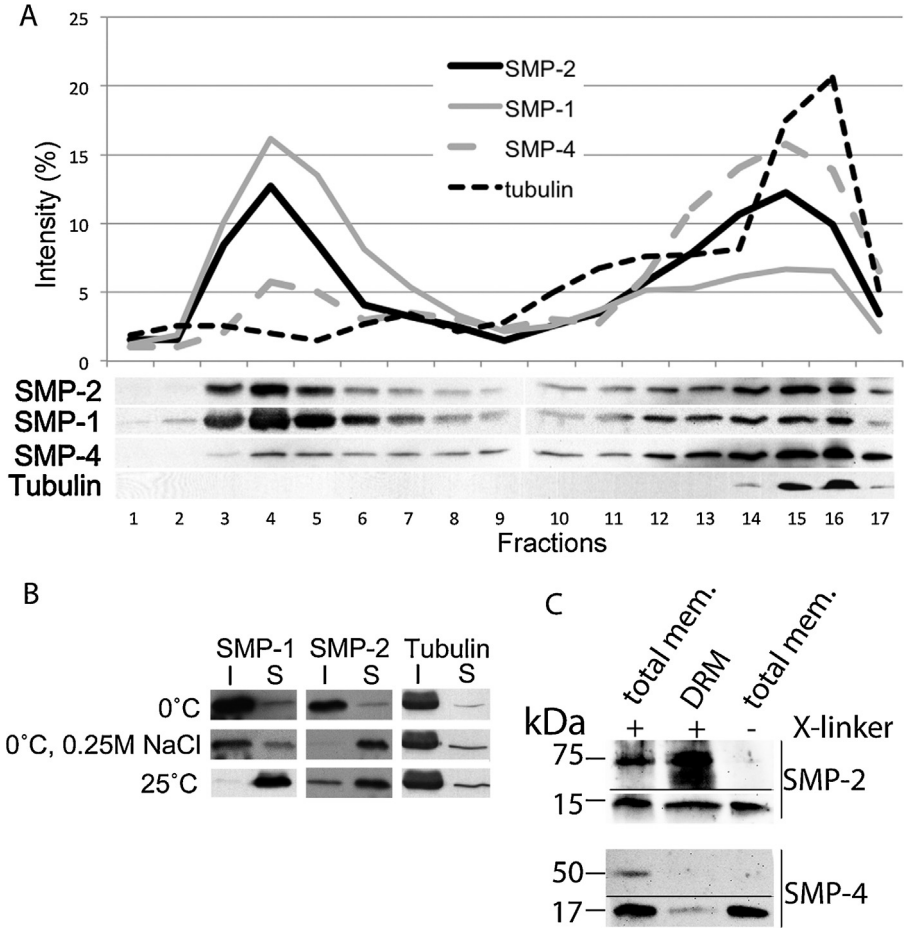

Fig. 1. SMP-2 resides in detergent resistant membranes. (A) Sucrose density flotation gradient centrifugation was performed essentially as described in [18]. *L. major* promastigotes were harvested by centrifugation (2000 × *g*, 25 °C, 10 min), washed with PBS, and extracted with 500 μl cold 1% Triton X-100 in 25 mM HEPES, pH 7.4, 1 mM EDTA, and Complete protease inhibitor cocktail (20 μl, Roche, Indianopolis, IN) at 0 °C for 30 min. The extract was adjusted to 60% sucrose, placed at the bottom of an ultracentrifuge tube and overlaid with a 5–50% continuous gradient, and centrifuged at 200,000 × *g*, at 4 °C for 20 h. Proteins from the fractions were solvent precipitated with chloroform and methanol, resolved on 15% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to PVDF (0.2 μm, Millipore) for immunodetection using anti-SMP-1, anti-SMP-4 and anti-tubulin serum, as described previously [16]. SMP-2 was detected with a peptide (R^{38}IVKRKKGHQTWAFY^{50}C) derived polyclonal antiserum from rabbits. The intensities of individual bands were determined in ImageJ. (B) *L. major* promastigotes were extracted with or without 0.25 M sodium chloride in cold or room temperature 1% Triton X-100. Insoluble (I) and soluble (S) fractions were obtained after centrifugation at 14,000 × *g* at 0 °C for 6 min and were analyzed by Western blotting. (C) *L. major* promastigotes were lysed by repeated freeze-thaw cycles, to obtain total membranes (total mem.) or were extracted with 1% Triton X-100 at 0 °C for 30 min and membrane-proteins cross-linked (X-linker) as described before [19]. Briefly, total and detergent-resistant membranes (DRM) were resuspended in PBS containing 2 mM DTSSP (Pierce) at 4 °C for 2 h. Excess DTSSP was quenched with 1 M Tris pH 7.4 buffer for 30 min. Proteins were separated on a 15% SDS PAGE using non-reducing sample buffers and analyzed by Western blotting with anti-SMP-2 and anti-SMP-4 serum.

acylation motives and unique C-terminal regions, which target different membranes [18]. The flagellated promastigote stages of *Leishmania*, which develop within the midgut of the sandfly vector, express three SMPs that are targeted to distinct membranes. SMP-1 is N-terminally diacylated (myristoylated and palmitoylated), found exclusively in the flagellum and is one of the most abundant proteins in Triton X-100 resistant membrane fractions [18]. In the absence of SMP-1, the flagellum becomes highly truncated and parasites loose the ability of directional locomotion [19]. SMP-1 contains a jelly roll β-fold, that is likely to be conserved in all SMPs and SMP-like domains, as well a distinctive C-terminal helical subdomain that would be orientated away from the membrane [19]. In contrast, monoacylated SMP-4 is localized to the plasma membrane, depending on N-terminal myristoylation and an extended C-terminal subdomain that contains a basic amphipathic α-helix [16]. Similar to SMP-4, SMP-2 shares 61% identical residues with SMP-1 and lacks a palmitoylation site, but contains only a short C-terminal tail and is primarily located to the flagellar pocket and/or surrounding membranes [19]. In addition, *Leishmania* encode a unique SMP that lacks any detectable acylation motif [18]. Both, *T. brucei* and *T. cruzi*, encode phylogenetically distinct SMPs (also termed small kinetoplastid calpain-related proteins, SKCRPs), but at least two (SMP-4 from *L. major* and *T. cruzi*) cluster together, suggesting that they may fulfill similar roles in these parasites [18]. As in *Leishmania*, the SMPs/SKCRPs in *T. brucei* contain different acylation signals and localize to different membranes. Similar to SMP-1 and SMP-4, *Tb*SMP5 (*Tb*SKCRP1.5) and *Tb*SMP8a (*Tb*SKCRP1.6) localize to the flagellum and the plasma membrane, respectively [17]. In contrast to *L. major* SMP-4, however, *Tb*SMP8a (*Tb*SKCRP1.6) is predicted to be diacylated and lacks a C-terminal tail [18]. While *Tb*SMP6 (TbSKCRP1.4) is also membrane associated via a predicted myristoylated site, it localizes to intracellular membranes that are detergent soluble [17]. Intriguingly, several *Tb*SMPs (*Tb*SKCRPs) have recently been reported to be soluble in cold Triton X-100, irrespective of the acylation status [17]. Finally, the SMP/SKCRP module is also present in several calpains and is important for their subcellular localization [17,20].

Here, we further characterized SMP-2 containing membranes and determined the major targeting signals of this SMP. Initially, promastigote membranes were separated in a continuous