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*Tb*Flabarin, a flagellar protein of *Trypanosoma brucei*, highlights differences between *Leishmania* and *Trypanosoma* flagellar-targeting signals

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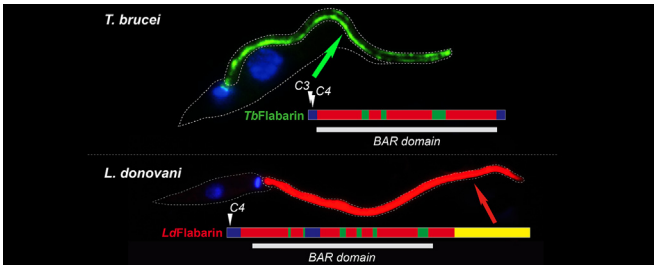
HIGHLIGHTS

- *Tb*Flabarin is a flagellar membrane protein in *Trypanosoma brucei*.
- It is mainly expressed in the procyclic form of the parasite.
- The targeting signals needs both the cysteines C3 and C4.
- The targeting signals are not recognized between *T. brucei* and *L. amazonensis*.
- *Tb*Flabarin is not needed for *T. brucei* procyclics *in vitro* proliferation.

GRAPHICAL ABSTRACT



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ABSTRACT

*Tb*Flabarin is the *Trypanosoma brucei* orthologue of the *Leishmania* flagellar protein *Ld*Flabarin but its sequence is 33% shorter than *Ld*Flabarin, as it lacks a C-terminal domain that is indispensable for *Ld*Flabarin to localize to the *Leishmania* flagellum. *Tb*Flabarin is mainly expressed in the procyclic forms of the parasite and localized to the flagellum, but only when two palmitoylable cysteines at positions 3 and 4 are present. *Tb*Flabarin is more strongly attached to the membrane fraction than its *Leishmania* counterpart, as it resists complete solubilization with as much as 0.5% NP-40. Expression ablation by RNA interference did not change parasite growth in culture, its morphology or apparent motility.

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Trypanosoma
Leishmania
Flagellum
Targeting signals

Heterologous expression showed that neither *Tb*Flabarin in *L. amazonensis* nor *Ld*Flabarin in *T. brucei* localized to the flagellum, revealing non-cross-reacting targeting signals between the two species.

1. Introduction

The development of increasingly more sensitive proteomic studies of different organisms like the protozoans *Chlamydomonas reinhardtii* (Pazour et al., 2005; Wagner et al., 2009) and *Trypanosoma brucei* (Broadhead et al., 2006; Ralston et al., 2009), higher Eukaryotes (Cao et al., 2006; Martinez-Heredia et al., 2006) and comparative genomics (Avidor-Reiss et al., 2004; Briggs et al., 2004; Inaba, 2011; Li et al., 2004; Pazour, 2004) confirmed a common architecture for eukaryotic cilia and flagella. Numerous orthologue constituents are assembled by similar mechanisms. Originally believed to only be involved in cell motility, flagella and cilia have since been recognized as essential organelles for other functions like cell division, the detection of environmental changes and signal transduction, development and morphogenesis (Avasthi and Marshall, 2012; Davis et al., 2006; Langousis and Hill, 2014; Maric et al., 2010; Merchant et al., 2007; Sengupta and Barr, 2014; Vincensini et al., 2011). As unexpected consequences, severe pleiotropic human diseases have found to be caused by mutation of the flagellar/ciliary genes (Fliegauf et al., 2007; Lee, 2011; Marshall, 2008; Ostrowski et al., 2011).

Thus, Eukaryotic flagella/cilia consist of several hundred proteins and lipids. Their targeting and assembly/disassembly depend on complex and sophisticated mechanisms like the intraflagellar transport IFT (Baldari and Rosenbaum, 2010; Cole, 2003). However, the targeting mechanisms are not extensively understood (Emmer et al., 2010; Nachury et al., 2010). Various mechanisms have been described in trypanosomatids. The flagellar localization may depend on the C-terminus amino acid sequence for the *Trypanosoma brucei* proteins *Tb*ARP and *Tb*PFR-A (Bastin et al., 1999a,b; Ersfeld and Gull, 2001) or the N-terminal region of *Tb*ADK-A (Pullen et al., 2004). Several discontinuous sequences or structural motives are found in the *Leishmania enriettii* glucose transporter *Le*ISO-1 (Nasser and Landfear, 2004). N-terminal myristoylation and palmitoylation are essential for the functioning of *Tb*Calflagin (Emmer et al., 2009), *Tb*MCA-4 (Proto et al., 2011), *Trypanosoma cruzi Tc*FCaBP (Buchanan et al., 2005; Godsel and Engman, 1999) and *L. major Lm*SMP-1 (Tull et al., 2004). We have recently characterized *Ld*Flabarin from *Leishmania donovani* (Lefebvre et al., 2013), which is a BAR domain (BAR-Superfamily, 2016) protein associated with the flagellar plasma membrane. The flagellar targeting depended on three signals: a potentially palmitoylated cysteine at position 4 and the BAR domain, for association with membranes, and the C-terminal domain for flagellar localization.

As Flabarins are found only in trypanosomatids (Group OG5_148786 (OrthoMCLDB, 2015)), they present basic biological - and possibly medical - interest. We describe here *Tb*Flabarin, the *T. brucei* orthologue of *Ld*Flabarin. *Tb*Flabarin is also a flagellar protein associated with the plasma membrane. It is mainly expressed in the insect form of the parasite, although the mammalian form is flagellated. Two potentially palmitoylated cysteines at positions 3 and 4 are both essential for flagellar localization. Contrary to *Ld*Flabarin, there is no C-terminal extension, which is therefore not needed for flagellar targeting. Heterologous expression confirms that the flagellar localization mechanisms are different, since *Tb*Flabarin in *Leishmania* or *Ld*Flabarin in *T. brucei* remain diffuse within the cell body.

2. Materials and methods

2.1. Parasites and cultures

Trypanosoma brucei 427-2913 procyclic forms were cultured at 27 °C in SDM-79 medium (Brun and Schonenberger, 1979) with 10% FCS, 30 μg/ml hygromycin and 10 μg/ml G-418. *T. brucei* 427 MITat 1.2 bloodstream forms (Cross, 1975) were cultured at 37 °C, 5% CO_2, in complete IMDM medium with 10% FCS, 5 μg/ml hygromycin and 2.5 μg/ml G-418. Both forms co-expressed the T7 RNA polymerase and the Tet repressor (Wirtz et al., 1999). Promastigotes of *Leishmania amazonensis* (MHOM/BR/1987/BA125) were cultured at 24 °C in AM medium with 7.5% FCS (Cuvillier et al., 2000; Lodes et al., 1995).

2.2. DNA and RNA technology

Conventional methods (Sambrook et al., 1989) were used for RNA, genomic DNA or small scale plasmid DNA extractions. Large scale plasmid preparations were done with Maxiprep Kits (Qiagen). Restriction and modification enzymes were from New England Biolabs. DNA fragments were amplified with the Phusion DNA polymerase (Finnzymes), gel purified, extracted with the Wizard SV Gel and PCR Clean-up System (Promega) and cloned into the pMOS vector (Blunt Ended Cloning Kit, GE Healthcare) using the *E. coli* strain XL1-Blue (Stratagene). After checking the DNA inserts sequences (GATC BioTech), the chosen fragments were transferred to expression vectors and all final constructs were sequenced prior to *T. brucei* or *L. amazonensis* transfection. Oligonucleotides were purchased from MWG-Eurofins. Northern blotting and hybridization were done as described (Mbang-Benet et al., 2014), using HybondN+ (GE Healthcare) membranes and DNA fragments labelled by nick-translation with (α-32P)dCTP (Perkin-Elmer) by the DECAprime™ II Kit (Ambion); *Tb*GPI-8 (Tb927.10.13860) was used as internal control.

2.3. Construction of the T. brucei constitutive expression vectors

The new expression vectors pLew79sot-GFPcD and pLew100sot-GFPB0cP are derived from the vectors pLew79, pLew100 (Wirtz et al., 1999) and pLew79-GFPcP (Coustou et al., 2005): the tetracycline operators were removed, leading to constitutive expression instead of tetracycline-induced expression.

To obtain the pLew79sot-GFPcD (for integration into *T. brucei* genome, conferring blasticidin resistance), the phleomycin resistance gene of pLew79-GFPcP was replaced by the blasticidin resistance gene, generating the pLew79-GFPcD. Then, given the presence of a unique *Kpn*I site upstream of the tetracycline operator 1 and of a unique *Hin*dIII site downstream of the operator 2, an upstream 252 bp fragment 1 was PCR amplified with primers Tet1dir (gggggtaccGTCATTGGGGTTAAG)/Tet1rev (gggaagcttgggggatccACCCGATAACCCCTATTTTCAAT) and cloned into pGEMTeasy (Promega), generating the plasmid pTet1; then, a downstream 122 bp fragment 2 was PCR amplified with primers Tet2dir (gggggatccTGAGTACTGAGTTTAACATGTT)/Tet2rev (gggaagcttGTGAATTTTACTTTTTGG) and cloned between the *Bam*HI and *Hin*dIII sites of pTet1, generating pTet2. Finally, the 380 bp fragment 3,