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Lysosomal degradation of *Leishmania* hexose and inositol transporters is regulated in a stage-, nutrient- and ubiquitin-dependent manner

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ABSTRACT

Leishmania parasites experience variable nutrient levels as they cycle between the extracellular promastigote stage in the sandfly vector and the obligate intracellular amastigote stage in the mammalian host. Here we show that the surface expression of three *Leishmania mexicana* hexose and myo-inositol transporters is regulated in both a stage-specific and nutrient-dependent manner. GFP-chimeras of functionally active hexose transporters, LmGT2 and LmGT3, and the myo-inositol transporter, MIT, were primarily expressed in the cell body plasma membrane in rapidly dividing promastigote stages. However MIT-GFP was mostly rerouted to the multivesicular tubule (MVT)-lysosome when promastigotes reached stationary phase growth and all three nutrient transporters were targeted to the amastigote lysosome following transformation to in vitro differentiated or in vivo imaged amastigote stages. This stage-specific decrease in surface expression of GFP-tagged transporters correlated with decreased hexose or myo-inositol uptake in stationary phase promastigotes and amastigotes. The MVT-lysosme targeting of the MIT-GFP protein was reversed when promastigotes were deprived of myo-inositol, indicating that nutrient signals can override stage-specific changes in transporter distribution. The surface expression of the hexose and myo-inositol transporters was not regulated by interactions with the subpellicular cytoskeleton, as both classes of transporters associated with detergent-resistant membranes. LmGT3-GFP and MIT-GFP proteins C-terminally modified with mono-ubiquitin were constitutively transported to the MVT-lysosome, suggesting that ubiquitination may play a key role in regulating the subcellular distribution of these transporters and parasite adaptation to different nutrient conditions.

1. Introduction

Leishmania parasites have a digenetic life cycle, alternating between flagellated promastigote stages that reside within the midgut of the sandfly vector and obligate intracellular amastigote stages that proliferate within the phagolysosomal compartment of mammalian macrophages. Both developmental stages appear to be dependent on the uptake and utilisation of glucose and other hexoses for growth (Hart and Coombs, 1982). Promastigote stages initially have access to high concentrations of glucose in the sandfly mid-gut as the bloodmeal is digested, while at later stages of infection they may have access to the sucrose-rich honeydews that the sandfly feeds upon intermittently between bloodmeals (Schlein, 1986; Schlein and Jacobson, 2001). Carbohydrate catabolism is likely to be required for the rapid expansion of the promastigote population within the midgut and the anterior migration of these stages to the foregut. Conversely, the depletion of hexose and other carbon sources in the sandfly digestive tract triggers the differentiation of dividing promastigote stages to non-dividing metacyclic promastigotes that are pre-adapted for life in the mammalian host. While less is known about the nutrient composition of the macrophage phagolysosome, recent studies suggest that this compartment is relatively sugar poor (Rubin-Bejerano et al., 2003; Naderer and McConville, 2008). However, intracellular amastigotes are still dependent on hexose uptake, suggesting that de novo hexose synthesis via gluconeogenesis is insufficient to supply all the hexose needs of this stage (Burchmore et al., 2003; Rodriguez-Contreras and Landfear, 2006; Rodriguez-Contreras et al., 2007).

A number of studies have shown that hexose uptake is developmentally regulated in all human pathogenic species of *Leishmania*. Specifically, hexose uptake is maximal in rapidly dividing promastigote stages and decreases in axenically generated and lesion-derived amastigotes (Hart and Coombs, 1982; Burchmore and Hart, 1995). Hexose uptake in both stages is mediated by broad specificity facilitative transporters in the plasma membrane (Burchmore et al.,

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2003). *Leishmania mexicana* express two high affinity hexose transporters, LmGT2 and LmGT3, that are targeted to the plasma membrane and a low affinity transporter, LmGT1, that is targeted to the flagellum (Burchmore et al., 2003). A fourth hexose transporter has recently been identified that is up-regulated in a suppressor strain of *L. mexicana* that lacks LmGT1–3 (Feng et al., 2009). While levels of LmGT2 mRNA decrease in amastigote stages, LmGT3 is constitutively transcribed in all developmental stages (Burchmore and Landfear, 1998), suggesting that the stage-specific regulation of hexose uptake is regulated by additional mechanisms.

Leishmania viability is also dependent on the uptake or de novo synthesis of myo-inositol, which is required for phospholipid and glycolipid biosynthesis (Ilg, 2002). The *Leishmania donovani* myo-inositol/proton co-transporter (MIT) was identified based on its homology to the two *Sachharomyces cerevisiae* inositol transporters, ITR1 and ITR2 (Langford et al., 1992; Drew et al., 1995; Klamo et al., 1996; Mongan et al., 2004). MIT contains several highly conserved residues and motifs from the sugar transporter superfamily that are essential for function (Seyfang et al., 1997; Seyfang and Landfear, 2000). MIT is constitutively transcribed in promastigotes and amastigotes and MIT mRNA is neither induced nor repressed in response to large fluctuations in extracellular inositol concentrations (Langford et al., 1992; Drew et al., 1995). However, glucose and myo-inositol uptake increases in response to long-term adaptation to a lack of either of these nutrients (Seyfang and Landfear, 1999), suggesting that the post-translational regulation of these transporters may be important, although the response time for increased uptake activity and the underlying mechanisms involved in enhanced nutrient uptake have not been investigated.

In this study we have investigated the role of nutrient transporter trafficking in regulating hexose uptake in *Leishmania* parasites. We provide evidence that the intracellular trafficking and surface localisation of both hexose and myo-inositol transporters are dynamically regulated by levels of expression, developmental changes in parasite growth and fluctuations in extracellular nutrient levels. Moreover, we provide evidence that the lysosomal transport and degradation of these transporters can be modulated by mono-ubiquitination.

2. Materials and methods

2.1. Cell culture

Leishmania mexicana wild-type (WT) promastiogtes (strain M379) and mutant promastigotes lacking LmGT1, LmGT2 and LmGT3 (ΔGT) were cultivated at 27 °C in RPMI containing 10% heat inactivated FBS. Axenic amastigotes (AA) were generated as previously described with minor modifications (Bates et al., 1992; Bates, 1994). Briefly, stationary promastigote (SP) stages were harvested and resuspended in RPMI supplemented with 20% iFBS, pH 5.5 (final density 1–2 $\times$ 10^6 cells/ml) and cultured continually at 33 °C. These axenic amastigotes displayed elevated cysteine protease activity, low levels of gp63 and lipophosphoglycan and were morphologically similar to intracellular amastigotes. Myo-inositol starvation experiments were performed using SP stages. Promastigotes (2–3 $\times$ 10^7 cells/ml) were centrifuged (2000*g*, 10 min), washed with PBS, and suspended in PBS containing 1% BSA and 5.5 mM glucose (2–3 $\times$ 10^7 cells/ml) with or without 5.5 mM myo-inositol. Parasites were incubated at 27 °C for 24 h prior to analysis by fluorescence microscopy, Western blotting or hexose uptake assays, as described in Sections 2.2 and 2.4–2.5.

2.2. Myo-inositol and glucose uptake assays

Uptake assays were based on the protocol previously described with some modifications (Drew et al., 1995). Briefly, parasites were washed twice in PBS and then resuspended in RPMI, pH 7.4 (for promastigote uptake), RPMI, pH 5.4 (amastigote uptake) or PBS, pH 7.5 (for starved parasites). Axenic amastigote cultures were assayed on days 5–6 of differentiation, at which stage they were still propagating (Vince et al., 2008). Cells (3×10^7) were added to uptake assay buffer (RPMI pH 7.4 or pH 5.4 or PBS containing 0.5μM myo-inositol/0.5 μCi myo-[2-^{3}H]-inositol or 100 μM glucose/0.5 μCi D-[6-^{3}H]-glucose), and layered onto a 9:1 mix of dibutyl phthalate:mineral oil. Parasites were centrifuged (14,000*g*, 30 s) after 60 s or 20 s (myo-inositol and glucose uptake, respectively), the aqueous phase removed and the oil interphase washed twice with water before removing the oil phase completely. The cell pellet was subsequently suspended in 1% SDS, boiled for 3 min, and recovered radioactivity determined by scintillation counting. BCA protein assays (Pierce, USA) were performed to standardise myo-inositol or glucose uptake to protein equivalents. The error bars shown in all uptake assays represent the S.D. from triplicate assays.

2.3. Cloning and episomal gene expression

The primers used to create the constructs for this study are listed as follows.

IT-GFP: 5′ GACTGGATCCATGCGAGCATCTGTCATGCTATGTG 3′, 5′ ATCCTTGGGCTCGTGCGGAGCTGCTTTG 3′.

LmGT2-GFP: 5′ GACTGGATCCATGAGCGACAAGTTGGAGGCG 3′, 5′ ATCCTCAGCCCTGTTGCCGCTGAG 3′

LmGT3-GFP: 5′ GACTGGATCCATGAGCGACAAGTTGGAGGCGAAC GTGCAG 3′, 5′ ATCCATTTCTTTCTTCCCGACGAATTC 3′

pX-GFP-Ub: 5′ AGCTGGATCCATGGTGAGCAAGGGCGAGG 3′, 5′ CACGAAGATCTGCATCTTGTACAGCTCGTCCATG 3′, 5’ GAGCTGTAC AAGATGCAGATCTTCGTGAAGAC 3′, 5′ GATCTCTAGATCAGCCGCCGC GCAGGCGCAGCAC 3′

MIT-GFP-Ub: 5′ GACTGGATCCATGCGAGCATCTGTCATGCTATG TG 3′, 5′ GATCGGATCCCTTGGGCTCGTGCGGAGCTGC 3′

LmGT3-GFP-Ub: 5′ GACTCCCGGGATGAGCGACAAGTTGGAGGCG AAC 3′, 5′ GTACGGATCCCATTTCTTTTTCCCGAC 3′

GRIP-GFP and GRIP-GFP-Ub: 5′ ACTGCCCGGGATGAGCTCTTTAG TTTCGCCCGAT 3′, 5′ GATCGGATCCCTTCAATGGGGGACACTGTGGA 3′

Templates used for cloning were *L. donovani* genomic DNA for MIT and *L. mexicana* genomic DNA for the glucose transporter constructs. The pX-GFP-Ub vector was created by spliced overlap PCR. Briefly, GFP was amplified from pXG′-GFP while ubiquitin was amplified from *Leishmania major* genomic DNA. These two products were then used to create GFP-Ub by spliced overlap PCR and then cloned in the pX vector to create pX-GFP-Ub. The final constructs were sequenced and then transfected into WT or ΔGT *L. mexicana* by electroporation (Robinson and Beverley, 2003), cultured in drug-free media for 24 h, and positive transfectants selected by addition of 10 μg/ml of neomycin. Following adaptation, parasites were maintained in medium containing 10 μg/ml of neomycin.

2.4. Fluorescence microscopy

Cells were prepared for live fluorescence microscopy using poly-L-lysine coverslips to immobilise parasites (Ilgoutz et al., 1999). FM4-64 uptake analysis was performed as previously described (Mullin et al., 2001). Briefly, parasites were incubated with a final concentration of 10 μM FM4-64 and examined by microscopy at designated time intervals. In some experiments, *L. mexicana* promastigotes were extracted with PBS containing 1% Triton X-100 at either 0 °C or 25 °C for 30 min and placed onto poly-L-lysine coated cover-slips within a 24 well culture plate. The parasites were dehydrated with 100% methanol (−20 °C, 5 min), washed in PBS, and incubated with 50 mM ammonium