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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. Green fluorescent protein (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-lysosome targeting of the MIT-GFP protein was reversed when promastigotes were deprived of myo-inositol, indicating that nutrient signals can over ride 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.

Keywords

Leishmania; lysosome; transporter; inositol; hexose; glucose

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

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mammalian macrophages. Both developmental stages appear to be dependent on the uptake and utilization 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 preadapted 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 (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., 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 the phospholipid and glycolipid biosynthesis (Ilg, 2002). The *Leishmania donovani* myo-inositol/proton co-transporter (MIT) was identified based on its homology to the two *Saccharomyces 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 or 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 localization of both hexose and myo-inositol transporters is 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) promastigotes (M379) and mutant promastigotes lacking LmGT1, LmGT2 and LmGT3 (Δ GT) were cultivated at 27°C in RPMI containing 10% heat inactivated foetal bovine serum (iFBS). 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 ($2000 \times g$, 10 min), washed with phosphate buffered saline (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 and prior to analysis by fluorescence microscopy, Western blotting or hexose uptake assays, as described below.

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 day 5–6 of differentiation, at which stage they were still propagating (Vince et al., 2008). Cells (3×10^7) were then added to uptake assay buffer (RPMI pH 7.4 or pH 5.4 or PBS containing 50 μ M myo-inositol/0.5 μ Ci myo-[2- 3 H]-inositol or 100 μ M glucose/0.5 μ Ci D-[6- 3 H]-glucose), and layered onto a 9:1 mix of dibutyl phthalate: mineral oil. Parasites were centrifuged (14,000g, 30s) after 60 s or 20 s (myo-inositol and glucose uptake, respectively) and 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 3min, 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.

MIT-GFP

5' GACTGGATCCATGCGAGCATCTGTCATGCTATGTG 3'

5' ATCCTTGGGCTCGTGCGGAGCTGCTTTG 3'.

LmGT2-GFP

5' GACTGGATCCATGAGCGACAAGTTGGAGGCG 3'

5' ATCCTCAGCCCTGTTGCCGCTGAG 3'

LmGT3-GFP

5' GACTGGATCCATGAGCGACAAGTTGGAGGCGAACGTGCAG 3'

5' ATCCATTTCTTTCTTCCCGACGAATTC 3'

pX-GFP-Ub

5' AGCTGGATCCATGGTGAGCAAGGGCGAGG 3'

5' CACGAAGATCTGCATCTTGTACAGCTCGTCCATG 3'

5' GAGCTGTACAAGATGCAGATCTTCGTGAAGAC 3'

5' GATCTCTAGATCAGCCGCCGCGCAGGCGCAGCAC 3'

MIT-GFP-Ub

5' GACTGGATCCATGCGAGCATCTGTCATGCTATGTG 3'

5' GATCGGATCCCTTGGGCTCGTGCGGAGCTGC 3'

LmGT3-GFP-Ub

5' GACTCCCGGGATGAGCGACAAGTTGGAGGCGAAC 3'

5' GTACGGATCCCATTCTTTTCCCGAC 3'

GRIP-GFP and GRIP-GFP-Ub

5' ACTGCCCCGGGATGAGCTCTTTAGTTTCGCCCCGAT 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 *L. major* genomic DNA. These two products were then used to create GFP-Ub by spliced overlap PCR and was then cloned in the pX vector to create pX-GFP-Ub. The final constructs were sequenced and then transfected into WT or Δ GT *L. mexicana* by electroporation (Robinson and Beverley, 2003), cultured in drug free media for 24 h, and positive transfectants selected by addition of 10 μ g/ml of neomycin. Following adaptation, parasites were maintained in medium containing 100 μ g/ml of neomycin.

2.4 Fluorescence microscopy

Cells were prepared for live fluorescence microscopy using poly-L-lysine coverslips to immobilize parasites (Ilgoutz et al., 1999). FM4-64 uptake analysis was performed as previously described (Mullin et al., 2001a). Briefly, parasites were incubated with a final concentration of 10 μ M 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 coverslips 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 chloride in PBS (10 min) to quench any auto-fluorescence. Extracted cytoskeletons were blocked (30 min) and then probed with anti-GFP (3:100 dilution, Roche), anti- α -tubulin (1:500 dilution, clone DM1A, Sigma, USA) or L3.8 anti-gp63 (1:100 dilution, kindly provided by Dr Thomas Ilg (Medina-Acosta et al., 1989)) antibodies for 30 min. Cytoskeletal extracts were then washed (4 $\times$ 5 min), and parasites incubated with Alexa Fluor 594nm or 488nm goat anti-rabbit (1:1000 dilution) or anti-mouse (1:500 dilution) secondary antibodies. Cells were viewed with a Zeiss Axioplan2 microscope equipped with an AxioCam Mrm digital camera and images processed using Axiovision V3.1 software.

2.5 Detergent extractions and western blotting

Parasites were harvested, washed with PBS and resuspended in Triton X-100 buffer (1% Triton X-100, 25mM HEPES pH7.4, 1mM EDTA and Complete protease inhibitor cocktail (Roche)) at 0°C or 25°C for 30 min. The extract was centrifuged for 20 min at 4°C to obtain a detergent insoluble pellet and detergent soluble supernatant. The supernatant protein was precipitated with acetone for western blot analysis. All other protein extracts were prepared by dissolving washed cell pellets in 1% SDS followed by boiling for 3 min. Nitrocellulose membranes with electrophoretically transferred proteins were blocked with 5% powdered skim milk in Tris-buffered saline containing 0.05% Tween-20. The blots were probed with either anti-GFP (Roche), L3.8 anti-gp63, anti-VHPPase (kindly provided by Dr. Ross Waller, The University of Melbourne, Australia), anti-ubiquitin (Sigma) or anti-BiP (kindly provided by Dr J. Bangs, The University of Wisconsin Madison, USA) and secondary antibodies conjugated to horse-radish peroxidase and detected using ECL Western detection system (Amersham).

2.6 Macrophage infection

Peritoneal macrophages were isolated by peritoneal lavage and cultured in RPMI medium at 37°C with 5% CO₂ for 24 h prior to infection. Macrophages (1×10^5) were seeded onto cover-slips and infected with 1×10^6 stationary phase promastigotes for 4 h at 33–34°C in 5% CO₂. Free parasites were removed by extensive washing ($\times 4$) in RPMI, and infected macrophages incubated until fixation. Cells were fixed in methanol, permeabilised with 0.05% saponin in PBS and detected with propidium iodide. Cells expressing GFP fusion proteins were probed with anti-GFP and FITC conjugated secondary antibodies.

3. Results

3.1 Stage-specific regulation of glucose uptake

Consistent with previous studies we found that rates of glucose uptake were maximal in logarithmically dividing promastigotes (LP), and progressively decreased in stationary promastigote (SP) and axenic amastigote (AA) stages (Fig. 1A). Glucose uptake in AA was approximately 10-fold less than in LP after normalization to cellular protein content (Fig. 1A). To investigate the extent to which this stage-specific regulation of glucose uptake was regulated at the level of protein expression and turnover, the *L. mexicana* Δ GT mutant, lacking LmGT1–3, was stably transfected with the pX plasmid encoding C-terminal GFP chimeras of LmGT2 or LmGT3. These two transporters have previously been shown to account for most of the glucose uptake capacity of *L. mexicana* (Burchmore et al., 2003). Expression of these individual transporters in the *L. mexicana* Δ GT mutant resulted in the partial restoration of glucose uptake in the promastigote stages (Fig. 1B–D), indicating that the chimeras were functionally active. Significantly, as observed in WT parasites, rates of glucose uptake were maximal during promastigote growth and were strongly down-regulated in AA stages (Fig. 1C, D). Glucose uptake experiments performed on promastigote cultures at either pH 7.5 or pH 5.5 indicated that the down-regulation of glucose uptake observed in AA growth was not due to the low pH conditions used to cultivate this stage (data not shown).

3.2 Stage-specific changes in the surface transport of LmGT proteins

To investigate the extent to which glucose uptake is regulated by alterations in the surface expression of these transporters, the subcellular localization of LmGT2-GFP and LmGT3-GFP in each stage was monitored by fluorescence microscopy in *L. Mexicana* Δ GT parasites. Both proteins were primarily expressed at the cell surface in LP growth. Surface fluorescence was restricted to the cell body and the flagellar pocket at the base of the

flagellum (Fig. 2A). Little, if any, staining was observed in the flagellum (Fig. 2A). The surface expression of both transporters remained high in SP stages consistent with the finding that both stages exhibit similar levels of glucose uptake (Fig. 1C, D, 2A). However, some labelling of a distinctive tubular structure, reminiscent of the multivesicular (MVT) lysosome was frequently observed as *L. mexicana* wild type and Δ GT parasites expressing LmGT2-GFP entered stationary growth (Fig. 2A, Supplementary Fig. S1). When *L. mexicana* promastigotes expressing LmGT2-GFP were labeled with FM-4-64, a specific marker of the early endosomes and the MVT-lysosome (Mullin et al., 2001b; Vince et al., 2008), complete overlap was observed with the internalized LmGT2-GFP chimera, while Western blotting revealed the presence of a protease resistant GFP breakdown product (Supplementary Fig. S1A, B), consistent with lysosomal transport and degradation. Strikingly, both LmGT2-GFP and LmGT3-GFP were largely internalized following the differentiation of promastigote stages to amastigotes in infected macrophages (Fig. 2B, C). After 96 h, most of the fluorescence in differentiated intracellular amastigotes was concentrated in a single vacuolar compartment (Fig. 2B, C) consistent with the lysosome compartment in this stage.

Similar changes in the subcellular distribution of LmGT2-GFP were observed when this chimera was expressed in *L. mexicana* wild type parasites. Specifically, LmGT2-GFP was primarily expressed at the cell surface of LP stages, at the cell surface and the MVT-lysosome in SP stages, and primarily in the intracellular vacuole of AA stages (Supplementary Fig. S1C, D). LmGT3-GFP was also expressed at the cell surface of wild type LP and SP stages and internalized in AA. However, in contrast to the situation when this chimera was expressed in *L. mexicana* Δ GT parasites, significant fluorescence was also observed in the MVT-lysosome and around the nuclear envelope and surrounding reticulum in LP stages (Supplementary Fig. S1C, D). This fluorescence staining, indicative of an endoplasmic reticulum (ER) distribution, was not observed in SP stages (Supp. Fig. 1C, 1D). These data suggest that overexpression of LmGT3-GFP in the presence of endogenous LmGT3, can lead to some retention or degradation of this transporter in the ER and MVT-lysosome, which is relieved when parasites reach SP stages.

Collectively, these analyses indicate that the surface transport of the GFP-tagged LmGT2 and LmGT3 protein is strongly regulated in different developmental stages and by overall levels of LmGT expression. The increased internalization of both LmGT2-GFP and LmGT3-GFP in intracellular amastigotes or AA is consistent with these stages having decreased rates of glucose uptake.

3.3 Developmental stage and nutrient regulated changes in myo-inositol uptake

Inositol uptake also appears to be regulated in a stage-specific manner. As shown in Fig. 3, uptake of *myo*-inositol was maximal in LP stages and decreased by ~50% in SP and AA stages. Inositol uptake in *Leishmania* is mediated by a *myo*-inositol/H⁺ symporter (MIT) that belongs to the same facilitative transporter superfamily as the LmGTs (Drew et al., 1995; Burchmore and Landfear, 1998). Overexpression of *Leishmania* MIT or MIT-GFP in WT parasites resulted in a significant increase in *myo*-inositol uptake (~3-fold) in LP, but not in SP stages (Fig. 3A). The MIT-GFP transporter was shown by fluorescence microscopy to be located in the cell body plasma membrane in LP stages (Fig. 3B). Strong fluorescence was also observed in the MVT-lysosome (Fig. 3B), indicating that this protein can be constitutively targeted to the lysosome or recycled to this compartment from the cell surface. Consistent with the *myo*-inositol uptake measurements, levels of expression and surface transport of MIT-GFP were both decreased in SP stages (Fig. 3B, C), while GFP staining of the MVT-lysosome was more pronounced (Fig. 3B). Similar to the LmGT2-GFP and LmGT3-GFP proteins, MIT-GFP was primarily internalized to a vacuolar compartment in AA stages that overlapped with FM 4-64 staining (Fig. 3D).

3. 4 Redirection of MIT-GFP trafficking during inositol-deprivation

To investigate whether the surface expression and retention of MIT-GFP can be regulated by the availability of its substrate, SP stages expressing MIT-GFP were suspended in PBS containing 1% BSA and 5.5mM D-glucose with or without *myo*-inositol (5.5 mM). As expected, MIT-GFP was primarily targeted to the lysosome and degraded in SP stages (Fig. 4A). After 24 h in *myo*-inositol free media, newly synthesized MIT-GFP was robustly expressed at the plasma membrane with negligible labelling of the MVT-lysosome (Fig. 4A). Some labelling of intracellular structures at the anterior end of the parasites, corresponding to the flagellar pocket and anterior endosome compartment, was observed possibly reflecting constitutive recycling of the surface transporter pools. In contrast, negligible levels of MIT-GFP expression were observed when SP stages were suspended in minimal media containing 5.5 mM *myo*-inositol (Fig. 4A). Western blotting of Triton X-100 solubilized cellular extracts showed that levels of expression of MIT-GFP were approximately 10-fold higher in the absence of exogenous *myo*-inositol than in the SP control (zero time point) or in SP suspended in 5 mM *myo*-inositol (Fig. 4B). As expected from the Western and fluorescence microscopy studies, *myo*-inositol uptake rates increased dramatically over 24 h in the absence of *myo*-inositol, but remained constant in the presence of exogenous *myo*-inositol (Fig. 4C). After 24 h in minimal media lacking inositol, *myo*-inositol uptake increased approximately 13-fold relative to control SP parasites or those incubated in minimal media with *myo*-inositol (Fig. 4D). These results show that *L. mexicana* promastigotes increase *myo*-inositol uptake during inositol deprivation by targeting MIT to the plasma membrane and/or preventing internalisation of this transporter to the MVT-lysosome.

3. 5 Plasma membrane pools of GT and MIT transporters do not show strong interactions with the subpellicular cytoskeleton

It has previously been suggested that the *Leishmania enrietti* glucose transporter, ISO2, becomes tethered to the array of subpellicular microtubules that underlie the plasma membrane (Snapp and Landfear, 1997). Such interactions could facilitate the surface retention of these transporters and regulate recycling and degradation. To investigate the extent to which *Leishmania* transporters form stable associations with the microtubule cytoskeleton, or associate with detergent resistant membrane (DRMs), LP stages expressing LmGT2-GFP, LmGT3-GFP or MIT-GFP were solubilized in cold Triton X-100 at either 0°C or 25°C. As shown previously, glycosylphosphatidylinositol (GPI)-anchored proteins such as GP63 that associate with DRMs but not the cytoskeleton are resistant to extraction at 0°C, but are quantitatively solubilised in 1% Triton X-100 at 25°C (Tull et al., 2004)(Fig. 5A). In contrast, cytoskeletal proteins, such as tubulin, are resistant to Triton X-100 extraction at both 0°C and 25°C (Fig. 5A). The three transporters, LmGT2, LmGT3 and MIT-GFP were all partially or completely resistant to extraction at 0°C, but were quantitatively extracted at 25°C (Fig. 5A), suggesting that they associate with DRMs, but not cytoskeletal components. The insolubility of these transporters at 0°C does not reflect their hydrophobicity, as the V-type H⁺-ATPase (VHPPase), with a similar polytopic membrane structure, was quantitatively extracted in Triton X-100 at 0°C (Fig. 5A). The VHPPase is primarily located in acidocalcisomes which apparently lack DRMs. Similar results were obtained when Triton X-100 extraction was performed followed by fluorescence microscopy on the remaining detergent resistant cytoskeletons. Specifically, surface pools of GP63 and the three transporter proteins were resistant to Triton X-100 extraction at 0°C, but were quantitatively extracted at 25°C. In contrast, the cytoskeletal microtubules were resistant to extraction under both conditions (Fig. 5B, C). These data suggest that unlike human GLUT1 (Bunn et al., 1999), LmGT2, LmGT3 and MIT are probably not tethered to cytoskeletal elements. However, it remains possible that weak and/or indirect interactions with the subpellicular

cytoskeleton exist or that the GFP epitope may impact on hexose transporter protein interactions.

3. 6 Ubiquitination targets LmGT3-GFP and MIT-GFP to the MVT-lysosome

In yeast and animal cells, the surface transport and/or recycling of membrane proteins can be regulated by post-translational modification of the cytoplasmic domains of these proteins with monomeric or polymeric ubiquitin (Dupre et al., 2004; Raiborg and Stenmark, 2009). Ubiquitination targets proteins in the limiting membrane of endocytic compartments into vesicles that bud into the lumen of late endosomes and are ultimately delivered to mature lysosomes for degradation. While it has been demonstrated that many yeast nutrient permeases, including both hexose and inositol transporters, undergo vacuolar targeting through ubiquitination (Belgareh-Touze et al., 2008), it is not known whether a similar pathway occurs in trypanosomatids. To examine if ubiquitin can direct polytopic membrane transporters such as LmGT3 and MIT to the lysosome, fusion proteins containing GFP and *L.major* ubiquitin at the C-terminal (LmGT3-GFP-Ub and MIT-GFP-Ub, respectively) were expressed in *L. mexicana*. Both proteins were negligibly expressed at the cell surface and were constitutively targeted to the MVT-lysosome (Fig. 6A and not shown). As expected, myo-inositol uptake levels in the MIT-GFP-Ub cell line were not increased when compared to MIT-GFP expressing cells and showed the same *myo*-inositol uptake rates in LP and SP stages as WT parasites (Fig. 6B). Ubiquitination appeared to override any other surface localization signal, as neither MIT-GFP-Ub or GT3-GFP-Ub were expressed on the surface when parasites were suspended in medium lacking myo-inositol or glucose, respectively (data not shown). These results suggest that mono-ubiquitination functions as a lysosomal targeting signal in *Leishmania* and may play a role in regulating the surface expression of hexose and inositol transporters during parasite development and in response to exogenous nutrient levels.

Proteins destined for lysosomal degradation may be ubiquitinated at the cell surface or within endocytic/secretory compartments. In particular, there is evidence that proteins can be ubiquitinated and targeted to the lysosome from Golgi and/or endosome membranes (Reggiori and Pelham, 2002; Belgareh-Touze et al., 2008). To investigate whether addition of ubiquitin to a *Leishmania* Golgi protein results in MVT-lysosome targeting, a new fusion protein containing ubiquitin fused to the C-terminal of a GRIP-GFP reporter protein was constructed. It has previously been shown that attachment of the 40 amino acid GRIP domain to the C-terminus of GFP, targets GFP to the trans-Golgi cisternae (McConville et al., 2002a). An N-terminal GRIP-GFP fusion protein was also targeted to the Golgi apparatus (Fig. 6C). The trans-face of the Golgi can be labelled with FM 4–64, which appears to cycle between the endosomes and the trans-Golgi cisternae (Fig. 6C, detail). Addition of ubiquitin to GRIP-GFP (GRIP-GFP-Ub) resulted in the re-distribution of GFP fluorescence from the Golgi to the endosomes (displaying complete overlap with FM 4–64) and the MVT-lysosome (Fig. 6C). These data show that ubiquitination of cytoplasmic proteins on the *trans*-Golgi can result in efficient transport to the endosomes and MVT-lysosome of *Leishmania* parasites.

4. Discussion

Leishmania parasites scavenge a wide range of carbon sources and essential low molecular weight nutrients from their respective sandfly and mammalian hosts. Despite increasing evidence that the uptake of different metabolites is regulated in a stage- and nutrient-dependent manner in various organisms, comparatively little is known about the regulation of nutrient uptake in these highly divergent eukaryotes. In contrast to the situation in many prokaryote and single celled eukaryotes, there is little evidence that the expression of plasma membrane transporters is regulated at the level of transcription in trypanosomatids. While

mRNA levels for LmGT2 decrease in *L. mexicana* amastigotes, both LmGT3 and MIT are constitutively transcribed in all developmental stages (Langford et al.; Drew et al., 1995; Burchmore and Landfear, 1998). The regulation of these transporters is therefore likely to occur predominantly or exclusively at the level of protein translation and/or by post-translational modification affecting protein function or turnover. In this study we present evidence that transporter targeting to the lysosome compartment can regulate stage-specific and nutrient-dependent changes in hexose and inositol uptake in *L. mexicana*.

The two *L. mexicana* hexose transporters, LmGT2 and LmGT3, are thought to be responsible for bulk hexose uptake in this species (Burchmore et al., 2003; Rodriguez-Contreras and Landfear, 2006; Rodriguez-Contreras et al., 2007). Consistent with previous reports (Ellenberger and Beverley, 1987; Burchmore and Hart, 1995), we show that hexose uptake via these transporters was maximal in LP stages and decreased progressively in SP and AA stages. Decreased hexose uptake in SP and AA may reflect the decreased growth rates and energy needs of these stages (Lahav et al., 2010). Alternatively, or in addition, they could reflect a switch to the utilization of alternative carbon sources, such as amino acids and fatty acids (Hart and Coombs, 1982). In order to determine whether the down-regulation of hexose transport in SP and AA was associated with decreased surface expression of LmGT2 or LmGT3, GFP chimeras of both transporters were expressed in WT and the Δ GT parasite line. Both transporters were shown to be functionally active and to collectively restore glucose uptake in *L. mexicana* Δ GT LP stages. While these chimeras were primarily targeted to the cell body membrane in *L. mexicana* Δ GT LP and SP stages, both transporters were directed primarily to the large vacuolar lysosome (megosome) of axenic and intracellular amastigotes. In the case of LmGT2-GFP, some lysosomal transport was also observed in the SP stages. Collectively, these data suggest that stage-specific changes in lysosomal transport of newly synthesized and/or cell surface pools of GT protein may play an important role in regulating hexose uptake.

To determine whether other plasma membrane transporters are regulated in a similar fashion, we investigated the stage-specific changes in the subcellular localization of the inositol transporter, MIT. Previous studies have shown that MIT mRNA levels do not respond to either lifecycle changes or large fluctuations in extracellular *myo*-inositol concentrations (Langford et al., 1992; Drew et al., 1995). Like the GTs, MIT-GFP was targeted to the cell body membrane of LP stages and internalized to the megosome and degraded in AA stages. Unlike the GTs, a significant fraction of the MIT-GFP protein was constitutively targeted to the MVT-lysosome even in rapidly dividing LP stages. While it is possible that the transport of this chimera to the MVT-lysosome transport reflects high levels of expression and saturation of normal plasma membrane targeting/retention mechanisms, it is notable that lysosomal targeting of MIT-GFP in SP stages was completely reversed by *myo*-inositol starvation. Under these conditions, the surface expression and activity of MIT-GFP increased ~10-fold, indicating that the surface transport of MIT is not saturated at high levels of expression. Intriguingly, the observed down-regulation of *myo*-inositol uptake and MIT-GFP surface expression in AA is associated with a marked increase in both the transcription and protein expression of INO1, a key enzyme in *de novo myo*-inositol synthesis (Ilg, 2002; McNicoll et al., 2006; Rochette et al., 2008; Rochette et al., 2009). The transcriptional regulation of INO1 represents one of the few examples of strong and consistent stage-specific regulation of a metabolic enzyme in these parasites. *Leishmania* may thus switch from scavenging *myo*-inositol from the environment to *de novo* synthesis following differentiation of promastigotes to amastigotes, consistent with the finding that INO1 is essential for the infectivity of *L. mexicana* parasites (Ilg, 2002).

Changes in the steady-state distribution of GT and MIT proteins could be regulated, at least in part, by global changes in gene transcription and/or protein synthesis relative to rates of

endocytic internalization. There is increasing evidence that mRNA and protein translation are both globally decreased during amastigote differentiation (Rochette et al., 2009; Alcolea et al., 2010; Lahav et al., 2010). A decrease in the rate of protein synthesis relative to endocytosis would be expected to lead to the gradual depletion of surface transporters and their accumulation in lysosomal compartments, providing a possible explanation for the down-regulation of GT and MIT proteins in this stage. Such a global mechanism is consistent with reports suggesting that multiple pathways of nutrient uptake, including proline (Glaser and Mukkada, 1992; Zilberstein and Gepstein, 1993), biopterin and folate (Cunningham and Beverley, 2001; Richard et al., 2004), and polyamine (Basselin et al., 2000; Hasne and Ullman, 2005), are decreased in either SP or amastigotes relative to LP stages. In the case of folate uptake, decreased uptake in SP stages was also associated with internalization and degradation of the major *Leishmania* folate transporter FT1 (Richard et al., 2004). An alternative, and possibly trivial explanation for why the GT-GFP and MIT-GFP chimeras are internalized in amastigotes is that episomal transcription is reduced relative to chromosomal transcription in this stage, leading to the selective down-regulation of the chimeras, but not native transporters. However, we believe that this is unlikely as; (i) intracellular GFP fluorescence in GT-GFP/MIT-GFP expressing parasites was detected in SP and amastigote stages for several days after differentiation, indicating continued protein synthesis, (ii) we have shown that SP and amastigote stages continue to express other GFP chimeras from the same episome (Vince et al., 2008) and (iii) MIT-GFP surface expression in SP stages is markedly increased under starvation conditions, indicating continued high levels of basal transcription in this stage despite low levels of surface expression.

The regulated internalization of the GT and MIT transporters could also be mediated by specific post-translational modifications such as ubiquitination. Mono- and poly-ubiquitination has been shown to play a key role in regulating the surface expression of nutrient transporters in a variety of other eukaryotes such as yeast (Lauwers et al., 2010), but not yet in *Leishmania*. When *L. major* ubiquitin was fused to the C-terminal of the GFP transporter fusion proteins, the resulting chimeras were constitutively targeted to the MVT-lysosome and degraded. It is possible that the ubiquitinated transporters are initially trafficked to the flagellar pocket, but then rapidly internalized into endosomes and the MVT-lysosome. Alternatively, or in addition, newly synthesized transporters may be diverted from the secretory pathway while in transit through the Golgi apparatus/early endosomes. The latter possibility was suggested by the finding that ubiquitinated GFP-GRIP was also constitutively targeted to the MVT-lysosome. GFP-GRIP associates with the cytoplasmic face of the Golgi apparatus and may cycle between the Golgi apparatus and early endosomes, but not the flagellar pocket (McConville et al., 2002a). Both pathways will deliver the transporters to the limiting membrane of late endosomes/multivesicular bodies (MVBs), and subsequently to intraluminal microvesicles that are delivered to the lumen of the MVT-lysosome (Mullin et al., 2001b; McConville et al., 2002b). The targeting of ubiquitinated proteins to the MVT-lysosome appears to be exceedingly efficient as the steady-state levels of both the ubiquitinated transporters and GFP-GRIP were very low despite the use of the high copy number plasmid expression system. Similarly we have been unable to detect the direct ubiquitination of LmGT2-GFP or MIT-GFP in SP or amastigote stages, although Western blot analysis of total cellular proteins confirmed that protein ubiquitination is up-regulated in these stages (unpublished data).

Interestingly, a parasite protein termed TOR (TOxic nucleoside Resistance), has recently been identified in *L. mexicana* that might be involved in the ubiquitination and membrane transport of a number of plasma membrane transporters. Overexpression of TOR in *Leishmania* or yeast leads to the redirection of the adenosine transporter from the plasma membrane to the MVT-lysosome and increased resistance to toxic purine analogues internalized by this transporter (Detke, 2007). When the *Leishmania* adenosine transporter

was expressed in yeast, TOR-mediated lysosomal degradation was dependent on the presence of an intact ubiquitination system (Detke, 2007). While indirect, these observations suggest that TOR, which was partially localized to the Golgi apparatus in *Leishmania*, may have a role in regulating the ubiquitination of the adenosine transporter. Intriguingly, overexpression of TOR had no effect on glucose uptake or the intracellular trafficking of LmGT1, suggesting that other proteins modulate the ubiquitination and lysosomal trafficking of LmGT2, LmGT3 and MIT. Finally, it is notable that the surface expression of a number of other *Leishmania* nutrient transporters may be primarily regulated by other mechanisms other than protein turnover. For example, increased expression of the *L. donovani* arginine transporter LdAAP3 (Darlyuk et al., 2009), and the *L. donovani* and *L.* major purine nucleoside transporters, NT1 and NT2 (Ritz et al (2010) Mol. Microbiol. 78, 108; Carter et al (2010) Mol Microbiol. 78, 92–107) in response to amino acid or purine starvation is largely inhibited by cyclohexamide treatment indicating the importance of *de novo* synthesis of new transporters is required under these conditions. Whether different mechanisms are involved in regulating the surface expression of these transporters during parasite differentiation remains to be investigated. These studies suggest that, despite the absence of transcriptional regulation in *Leishmania*, these parasites retain multiple mechanisms for regulating nutrient uptake in both a programmed and adaptive fashion.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Fig. 1. Stage-specific regulation of glucose uptake

Glucose (glc) uptake was measured in log and stationary phase promastigote (LP, SP) and axenic amastigote (AA) stages of *Leishmania mexicana* wild type (WT) (A), glucose transporter deficient (Δ GT) parasites (B) and Δ GT parasites complemented with LmGT2-GFP (C) or LmGT3-GFP (D). Error bars represent the S.D. from three replicate assays.

Fig. 2. Stage-specific changes in the subcellular localization of LmGT2-GFP and LmGT3-GFP
 A. The distribution of LmGT2-GFP or LmGT3-GFP in live log promastigote (LP) and stationary promastigote (SP) stages of *L. mexicana* Δ GT parasites was determined by fluorescence microscopy (scale bar approximately 5 μ m). B and C. Peritoneal macrophages infected with *L. mexicana* Δ GT promastigotes expressing LmGT2-GFP (B) or LmGT3-GFP (C) were fixed and probed with anti-GFP antibodies at the indicated time points post infection. Intracellular amastigotes were also identified by propidium iodide staining (red signal in merge). Scale bar approximately 3 μ m. The boxed areas at 96 h delineates a single amastigote magnified (approximately 5.5 fold) and shown in detail. DIC: differential interference contrast.

Fig. 3. Stage-specific regulation of myo-inositol uptake by transporter degradation

A. Inositol uptake in WT *Leishmania mexicana* or those overexpressing MIT-GFP or MIT was measured as described in Materials and Methods in log promastigote (LP), stationary promastigote (SP) and axenic amastigote (AA) stages. Error bars represent the S.D. of three replicate assays. B. Live *L. mexicana* parasites expressing MIT-GFP were visualised by fluorescence microscopy in LP and SP growth (left panel). The graph (right panel) shows the quantification for the % of LP or SP stages expressing cell surface localized MIT-GFP (n= greater than 100 cells examined for each growth phase). Note that most cells in LP growth showed both a surface and intracellular (lysosome) distribution of MIT-GFP, whilst surface MIT-GFP was mostly absent from the SP population. C. *L. mexicana* promastigotes

expressing MIT-GFP were harvested through LP to SP growth and total cell protein analysed by Western blotting with anti-GFP (MIT-GFP) and anti-BiP (protein loading) antibodies. D. AA growth stages expressing MIT-GFP were labelled with FM 4-64 (30–40 min) prior to fluorescence microscopy. DIC: differential interference contrast.

Fig. 4. Regulation of inositol uptake by extracellular myo-inositol levels

A. *Leishmania mexicana* promastigotes expressing MIT-GFP were harvested in stationary phase (0 hr, SP), washed in PBS, and resuspended in minimal media with (PBS + ino) or without (PBS - ino) 5.5mM myo-inositol for 24 h. The distribution of MIT-GFP in live promastigotes was monitored by fluorescence microscopy. Equal exposure times are shown for each image and are representative of the entire population. Scale bar approximately 5 μ m.

B. *L. mexicana* SP stages expressing MIT-GFP (control) were incubated in minimal media with (+ ino) or without (-ino) 5.5mM myo-inositol for 24 hr and were extracted with cold 1% Triton X-100. The pellet (P) and supernatant (S) fractions were separated by SDS-PAGE, and the levels of full length MIT-GFP and the protein loading control BiP determined by Western blotting with anti-GFP and anti-BiP antibodies, respectively.

C. Induction of inositol uptake during inositol starvation. *L. mexicana* promastigotes expressing MIT-GFP were washed and resuspended in minimal media with (- $\blacktriangledown$ -) or without (- $\circ$ -) 5.5mM myo-inositol and inositol uptake measured at the indicated time points.

D. Inositol uptake rates of the *L. mexicana* promastigote cultures shown in panel A.

Fig. 5. The glucose and inositol transporters do not associate with the subpellicular cytoskeleton under detergent extraction

A. *Leishmania mexicana* promastigotes expressing MIT-GFP, LmGT2-GFP or LmGT3-GFP were extracted in 1% Triton X-100 (TX-100) at 0°C or 25°C for 30 min and the detergent insoluble pellet (P) and soluble supernatant (S) phases separated and analysed by western blotting. B and C. *L. mexicana* promastigotes expressing MIT-GFP, LmGT2-GFP or LmGT3-GFP were solubilised with 1% Triton X-100 at 0°C (B) or 25°C (C) for 30 min and the cytoskeletal extracts probed with anti-GFP, anti-gp63 or anti-tubulin and viewed by fluorescence microscopy. Scale bar approximately 5µm.

Fig. 6. Nutrient transporters fused with ubiquitin are constitutively targeted to the MVT-lysosome for degradation

A. Localisation of MIT-GFP and MIT-GFP-Ub or LmGT3-GFP and LmGT3-GFP-Ub by fluorescence microscopy of live promastigotes. Scale bar approximately 5µm. B. Inositol uptake was measured in log phase (LP) and stationary phase (SP) of wildtype (WT) *L. mexicana* promastigotes (control) or those expressing MIT-GFP or MIT-GFP-Ub as indicated. Error bars are the S.D. from three replicate assays. C. Ubiquitin can redirect Golgi targeted GRIP-GFP to the MVT-lysosome. Live log growing *L. mexicana* promastigotes expressing GRIP-GFP or GRIP-GFP-Ub were incubated with FM 4-64 (20–30 min) and visualized by fluorescence microscopy. Scale bar approximately 5µm. DIC: differential interference contrast.