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Abstract

Leishmania are obligatory intracellular parasites that cycle between the sand fly midgut (extracellular promastigotes) and mammalian macrophage phagolysosomes (intracellular amastigotes). They have developed mechanisms of adaptation to the distinct environments of host and vector that favor utilization of both proline and alanine. LdAAP24 is the *L. donovani* proline-alanine transporter. It is a member of *Leishmania* system A that translocates neutral amino acids. Since system A is promastigote-specific, we aimed to assess whether LdAAP24 is also expressed exclusively in promastigotes. Herein, we established that upon exposing *L. donovani* promastigotes to amastigote differentiation signal (pH 5.5 and 37°C), parasites rapidly and completely degrade LdAAP24 protein in both axenic and in spleen-derived amastigotes. In contrast, LdAAP24 mRNA remained unchanged throughout differentiation. Addition of either MG132 or Bafilomycin A1 partially inhibited LdAAP24 protein degradation, indicating a role for both lysosome- and proteasome-mediated degradation. This work provides the first evidence for post-translational regulation of stage-specific expression of LdAAP24.

Introduction

Protozoan parasites of the genus *Leishmania* cause morbidity and mortality throughout large areas of the Old and New World and are the causative agents of a wide spectrum of human and veterinary diseases. The clinical manifestations of *Leishmania* infections range from lesions of the skin and mucous membranes to lethality, the latter caused by visceral species [1].

Leishmania exhibit a digenetic life cycle that includes extracellular promastigote and intracellular amastigote forms. Extracellular promastigotes develop in the alimentary tract of sand flies. Following transmission to humans, promastigotes differentiate into obligatory intracellular amastigotes within macrophage phagolysosomes [2, 3]. This differentiation process in the host can be mimicked in axenic culture by shifting promastigotes from an insect-like (26ºC, pH 7) to an intra-lysosomal-like environment (37ºC, pH 5.5 and 5% CO_2) [4-6]. Axenic differentiation of *L. donovani* has been extensively characterized over the last two decades, establishing that this is a genetically programmed process [7-10].

During their life cycle, *Leishmania* move between relatively alkaline environments rich in sugar and amino acids (AAs) and acidic environments rich in fatty acids and AAs [3, 11-13]. High AA abundance is a feature common to both environments and accordingly, the parasites display adaptive mechanisms that favor utilization of such compounds [12, 14, 15]. In particular, *Leishmania* exhibit large intracellular AA pool, of which alanine and proline are the main components [15-17]. The parasites use these AAs as osmolytes and as alternative sources of carbon.

In 1999, we biochemically showed that *L. donovani* take up proline via three systems named A, B and C [18]. Systems A and B are active only in promastigotes whereas system C is amastigotes-specific. Similarly, proline transport in *T. cruzi* was considered to be mediated by

multiple transporters, each possessing distinct biochemical properties [19]. More recently, we established that the low affinity, high capacity proline transporter in *L. donovani* (system A) is encoded by two identical copies of *LdAAP24* (*LinJ.10.0760* and *LinJ.10.0770*)[16]. Notably, we discovered that due to distinct *trans*-splicing sites, the two gene copies produce mRNAs and corresponding proteins that differ in size and function [20]. The protein encoded by *LinJ.10.0770* (named LdAAP24.2) transports both alanine and proline, whereas the one encoded by *LinJ.10.0760* (named LdAAP24.1) is 18 amino acids shorter and transports only proline. LdAAP24 plays a critical role in maintaining intracellular AA homeostasis and in responses to hypotonic stress [20].

To date, our analyses of LdAAP24 activity and expression were carried out in promastigotes. Previous biochemical studies suggested that amastigotes import proline via transport systems distinct from that of promastigotes [18]. This prompted us to examine LdAAP24 expression in both intracellular and axenic amastigotes. We found that once promastigotes were exposed to conditions that initiate differentiation into amastigotes, rapid degradation of LdAAP24 protein was induced. In macrophages, down-regulation of both variants (e.g. LdAAP24.1 and LdAAP24.2) was slower than in axenic conditions. In contrast, hamster-derived amastigotes do not express LdAAP24 protein at all. Changes in protein expression did not parallel mRNA abundance indicating posttranslational regulation of stage-specific expression of LdAAP24.

Materials and Methods

Materials

Ampicillin, G418, Hygromycin B, Phleomycin, Blasticidin, medium-199 (M-199), MG-132 and Bafilomycin A1 were from Sigma. Fetal bovine serum (FBS) was from Biological Industries Ltd.. n-dodecyl β-D-maltoside (DDM) was from Calbiochem and magnetic beads-bound anti-HA were from Medical&Biological Laboratories Co., Ltd. (MBL). All other reagents were of analytical grade.

Axenic Leishmania cell culture

L. donovani, MHOM/SD/00/1S cell line was used [21]. Promastigotes and amastigotes were grown and maintained in axenic cultures. Differentiation was performed as previously described [5]. Briefly, late log promastigotes were transferred from promastigote medium at 26°C and pH 7.4 to amastigote medium at 37°C, pH 5.5 in 5% CO_2 environment.

Western blot analysis and dot blot analysis

Western blot analyses of proteins were carried out as previously described [17]. Briefly, each lane was loaded with 10μg of cellular protein. Following transfer to Protean BA-83 nitrocellulose paper (Whatman ltd.), parasite proteins were exposed to Rabbit anti LdAAP24 (1:2000 serum dilution) or anti HSP83 (1:2000) antisera [16, 21]. LdAAP24 band density relative to that of HSP83 was calculated using TINA2.0 software.

In vitro macrophage infection with L. donovani

THP1 macrophage maintenance and infection were carried out as previously described [22]. Briefly, macrophages were grown on a plate at 37°C in a 5% CO_2 incubator at a density of 10^7 cells/plate. Late log-phase *L. donovani* promastigotes were added to the culture for 4 hours at 1:10 macrophage to parasite ratio. Plates were then washed three times with phosphate buffered saline (PBS) and incubation continued for 48 hours. To confirm infection, round cover-slips (18mm) were added to the dishes before addition of macrophages, stained with Giemsa after the experimental procedure, and viewed in an electron microscope.

Purification of macrophage- and splenic-derived amastigotes

THP1 macrophages were grown on 8 plates at 37°C at a density of 1×10^7 cells/plate and then infected with mid-log phase *L. donovani* promastigotes as above. 48 hours after infection, macrophages were collected using a scraper and washed with ice-cold PBS for 4 minutes at 48Xg. Pellets were finally suspended in 1 ml PBS and transferred to microcentrifuge tubes. Separation of amastigotes was facilitated initially by passing the pellet repeatedly through a 1 ml syringe with a 27G needle. Lysates were then centrifuged for 15 minutes at 2500Xg at 4°C. The pellet was then suspended in 45% Percoll, which built the middle layer of the Percoll gradient as described in [22]. Cells were then isolated from the interface between the layers and subsequently centrifuged at 3500Xg for 60 minutes at 4°C as previously described [23]. Spleen-derived amastigotes were isolated from 100 days *L. donovani*-infected hamsters as described previously [24].

Confocal immunofluorescence microscopy

Immunofluorescence of LdAAP24 in axenic *L. donovani* promastigotes and amastigotes was carried out as in [16] using Zeiss LSM 510 META and Zeiss LSM 700 microscopes. For immunofluorescence of parasite-infected macrophages, cover slips were stained with anti-LdAAP24 and DAPI employing the protocol used for promastigotes [16].

Northern Blot

Total RNA from *L. donovani* promastigotes (either starved for arginine or non-starved) was prepared and subjected to Northern blotting of *LdAAP3* as described before [21]. Probes were amplified using the following primers: LinJ.31.900 AAP3 Forward: 5'-ATCATGAATTCATGAGCAAGCCCAGCAAGT-3'. Reverse: 5'-GCTTAGTCGACCGGAAGATGATGTTGCGC-3'.

Results

Differentiation signal induces rapid degradation of LdAAP24

Exposing axenic *L. donovani* promastigotes to amastigote differentiation signal induced rapid disappearance of the two LdAAP24 variants (Fig. 1A). As indicated by western blot analysis, within three hours only traces of the transporter proteins remained. Time course analysis determined the rate of degradation at $t_{1/2}$ = 35±3.3 minutes (Fig. 1B). Moreover, both short (8 weeks) and long (25 weeks) passages of axenic promastigotes [24], representing virulent and highly attenuated parasites, down-regulated LdAAP24 equally (Fig. 1C). These observations are consistent with our previous report that proline transport rate in axenic amastigotes is 5 times slower than in promastigotes, likely mediated by a different transport system [16, 18].

Further analysis of whether protein abundance down-regulation depends on the differentiation signal indicates that neither exposure to pH 5.5 nor to 37ºC alone initiated degradation (Fig. 2). Only subjecting promastigotes to the full differentiation signal initiated complete LdAAP24 protein degradation in axenic culture. Note that exposing *L. donovani* promastigotes to pH 5.5 alone caused minor degradation of the heavier variant of LdAAP24 (LdAAP24.2). However, degradation was complete only in cells exposed to both 37□C and pH 5.5.

The rapid and complete degradation of LdAAP24 at the onset of *L. donovani* axenic differentiation prompted us to assess whether the same happens *in vivo*, during macrophage infection. Human THP1 monocytes were incubated with *L. donovani* promastigotes for four hours and then incubated for additional 48 hours [22]. Intracellular amastigotes were then purified from the infected macrophages and assayed for LdAAP24 protein abundance. As shown

by western blot analysis (Fig. 3A), while axenic amastigotes lacked visible level of LdAAP24, macrophage-derived amastigotes expressed LdAAP24 protein, though, at lower level compared to promastigotes. This indicated that exposing parasites to phagolysosome environment induced LdAAP24 down-regulation, but not to complete disappearance as in axenic parasites.

Indirect immunofluorescence analysis validated the western blot data that whereas axenic amastigotes do not express LdAAP24 at all (Fig. 3B and C), amastigotes obtained from infected THP1 cells at 48h post-infection did express visible level of this protein (Fig. 3D). To further assess whether LdAAP24 is expressed in long-term intracellular amastigotes, they have been isolated from spleens of 100 days infected hamsters. Western blot analysis clearly demonstrated (Fig. 1C) that long-term hamster-derived *L. donovani* do not express LdAAP24 protein. The analysis of axenic and intracellular *L. donovani* indicate that in the latter LdAAP24 degrade much slower than in the host-free culture, likely due to intracellular element(s) that slow transporter degradation.

Degradation of LdAAP24 is lysosome-mediated and dependent on proteasome activity

Vince et al. [25] previously provided evidence for posttranslational regulation of stage-specific expression of the glucose and inositol transporters in *L. mexicana*. Exposure to differentiation conditions, in both axenic culture and macrophages, induced lysosome-mediated degradation. Using axenic amastigotes they showed that mono-ubiquitination triggered degradation and targeting to lysosomes. As shown in Fig. 4, addition of 10 μM of the proteasome inhibitor MG132 to axenic *L. donovani* differentiation medium at time zero partially (57%) inhibited LdAAP24 degradation. Bafilomycin A1 is a specific inhibitor of lysosome vacuolar

ATPase that blocks acidification of this organelle [26]. Adding 1 μM Bafilomycin A1 to amastigote differentiation medium also partially (76%) inhibited degradation of LdAAP24.

To assess whether the changes in LdAAP24 protein expression correlate with similar change in mRNA abundance, we performed northern-blot of LdAAP24 open reading frame. No significant change in mRNA was observed after exposing promastigotes to differentiation signal (Fig. 5).

Discussion

This study aimed to molecularly validate the previous biochemically-based observation that *L. donovani* transport system A is active only in promastigotes [18]. In amastigotes another low affinity and specificity system (system C) translocates several amino acids including proline [18]. Analysis of protein and mRNA abundance in axenic amastigotes, promastigotes and intracellular amastigotes confirmed that system A, represented by LdAAP24 is expressed and active only in promastigotes. Exposing *L. donovani* promastigote to amastigote differentiation signal initiated rapid degradation of LdAAP24 protein. In axenic promastigotes degradation was rapid ($T_{1/2}$ =35 minutes), while in intracellular amastigotes degradation was slower, LdAAP24 protein was visible 48 hours after macrophage infection, but not in splenic amastigotes recovered after100 days of infection.

LdAAP24 stage-specific expression is regulated at the level of protein abundance (post-translational) only. We showed that degradation was susceptible to treatment with both the proteasome inhibitor MG132 and the V-ATPase inhibitor Bafilomycin A1, suggesting that LdAAP24 degradation is mediated by both the lysosome and proteasome.

Usually, membrane protein degradation is mediated via the lysosome pathway. Vince et al. [25] showed that upon *L. mexicana* differentiation into amastigotes the promastigote-specific myo-inositol transporter D1 actively degrades via the lysosomes-mediated pathway. Similarly, the dolichol-phosphate-mannose synthase [27], the multidrug resistance protein 1 [28] and the folate transporter 1 [29] are all stage-specific whose abundance is down-regulated by the lysosome mediated pathway. Likewise, in the present study, inhibition by Bafilomycin A1 indicates a role for the lysosome in LdAAP24 degradation.

This work provides the first evidence for post- translational regulation of stage-specific expression of LdAAP24. It also provides an additional evidence that axenic *Leishmania* resemble intracellular parasites.

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Figure Legends

Figure 1. Rapid down-regulation of LdAAP24 abundance during first phase of *L. donovani* axenic differentiation.

[A] Western blot analysis of LdAAP24 degradation during axenic differentiation in wild type (WT) promastigotes. Time indicates hours after exposing promastigotes to differentiation to amastigotes signal. **[B]** Time course of LdAAP24 down-regulation during axenic differentiation. Analysis was carried out using western blot analysis at indicated hours after exposing promastigotes to differentiation signal. This figure is representative of five independent repeats. **[C]**. Western blot analysis of LdAAP24 in splenic amastigotes and axenic *L. donovani* promastigotes derived from the splenic amastigotes and maintained for 8 (8W) and 25 weeks (25W) in culture. Proteins were extracted from 100 days old splenic amastigotes or axenic promastigotes (at 0 and 3 hours after differentiation signal). In all panels LdHSP83 was used as loading control

Figure 2. Promastigote to amastigote differentiation signal, not acidic pH or elevated temperature alone, initiate LdAAP24 degradation.

Western blot analysis of LdAAP24 degradation during exposure of the parasites to either pH 5.5, 37^0C or both ('complete differentiation signal'). Time indicates hours after exposure to indicated signal components. Parasite actin was used as loading control.

Figure 3. LdAAP24 undergoes partial degradation during first 48 hours of macrophage infection.

[A] Western blot analysis of LdAAP24 degradation in promastigotes, mature axenic amastigotes and intracellular parasites 48h after THP1 macrophage infection with promastigotes. Anti

LdHSP83 was used as loading control. **[B-D]** Confocal immunofluorescence microscopy (Zeiss LSM 700) of LdAAP24 expression in axenic promastigotes, axenic amastigotes and infected THP-1 macrophages, respectively, was determined by anti- LdAAP24 primary antibody, with fluorescent CY3 anti rabbit as secondary antibody (red). DAPI was used to stain the nucleus and kinetoplast (blue).

Figure 4. LdAAP24 degradation is lysosome and proteasome- dependent.

Axenic promastigotes were subjected to 3-hours differentiation with (+) or without (-) MG-132 and/or Bafilomycin A1 (Baf A1). Anti-LdAAP24 was used to determine LdAAP24 expression-levels and LdHSP83 was used as loading control (upper panel). Densitometry of each band relative to HSP83 is illustrated in the lower panel.

Figure 5. mRNA levels of LdAAP24 do not change during axenic differentiation.

Total RNA was extracted from *L. donovani* promastigotes and at 0.5, 1 and 3 hours after exposure to promastigote to amastigote differentiation signal and subjected to northern analysis as described in Materials and Methods. LdAAP24 open reading frame was used as a probe. rRNA was used as loading control. This experiments is representative of three independent repeats.

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Figure 1

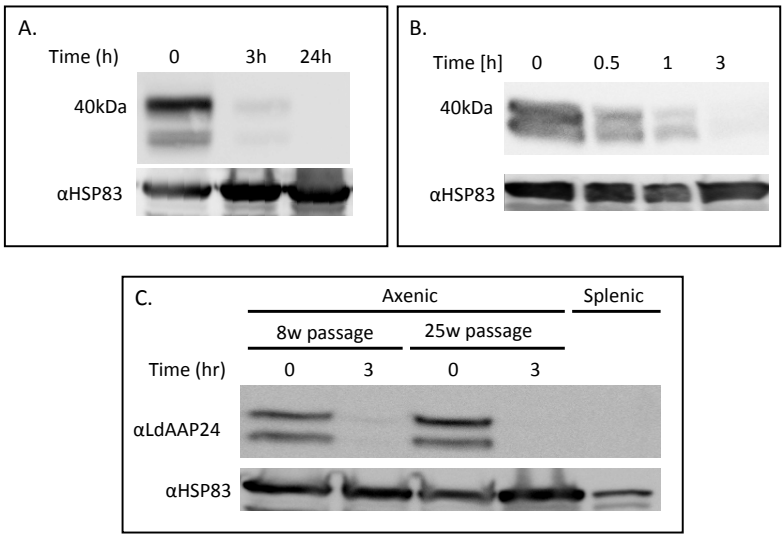

Fig. 1. Rapid down-regulation of LdAAP24 abundance during first phase of*L. donovani* axenic differentiation.

Figure 2

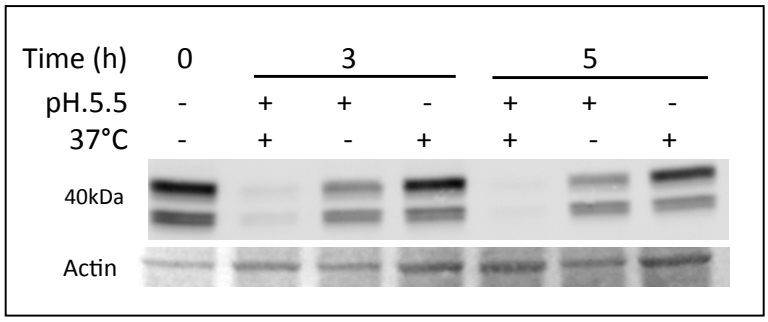

Fig. 2. Promastigote to amastigote differentiation signal, not acidic pH or elevated temperature alone, initiate LdAAP24 degradation.

Figure 3

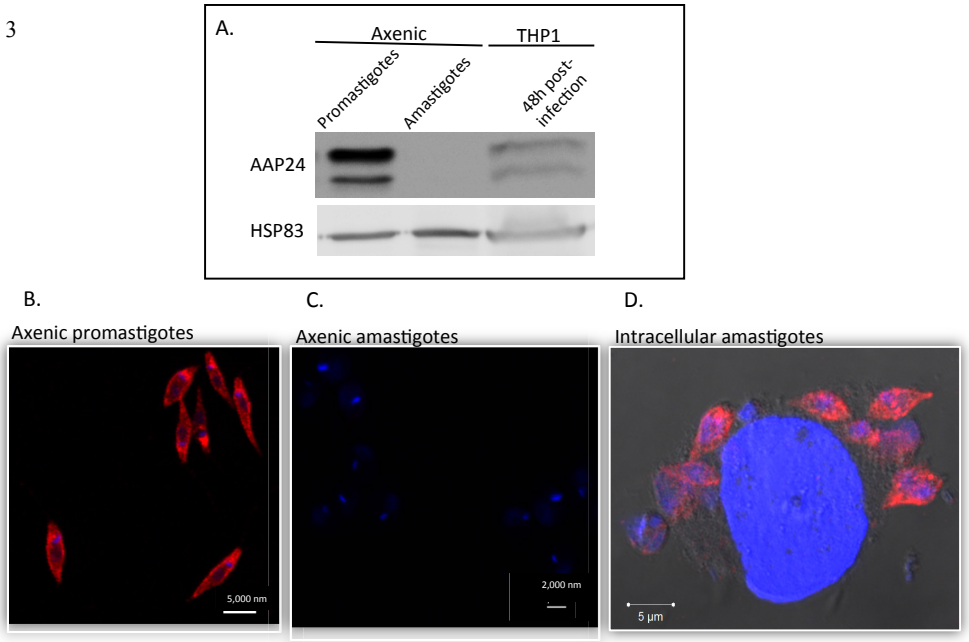

Fig. 3. LdAAP24 undergoes partial degradation during first 48 h of macrophage infection.

Figure 4

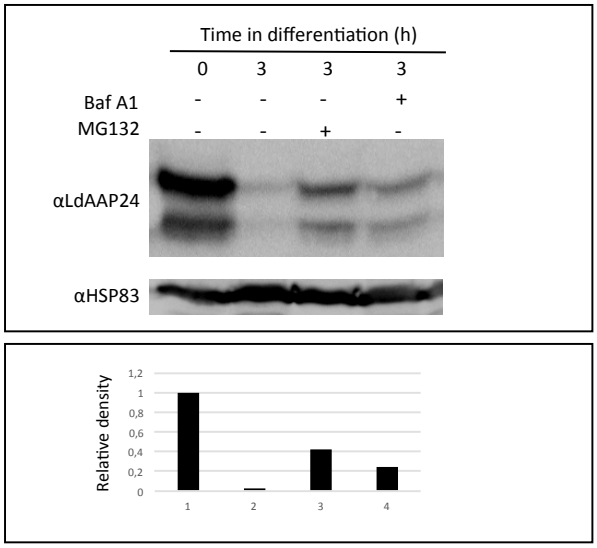

Fig. 4. LdAAP24 degradation is lysosome and proteasome-dependent.

Figure 5

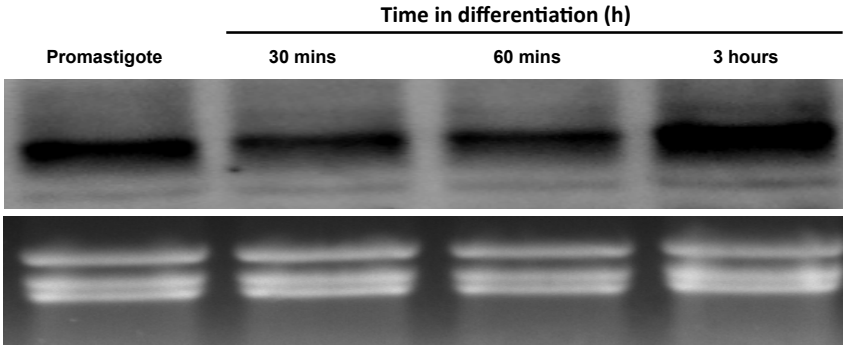

Fig. 5. mRNA levels of LdAAP24 do not change during axenic differentiation.