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ORIGINAL ARTICLE

Lysine transporters in human trypanosomatid pathogens

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Abstract In previous studies we characterized arginine transporter genes from *Trypanosoma cruzi* and *Leishmania donovani*, the etiological agents of chagas disease and kala azar, respectively, both fatal diseases in humans. Unlike arginine transporters in higher eukaryotes that transport also lysine, these parasite transporters translocate only arginine. This phenomenon prompted us to identify and characterize parasite lysine transporters. Here we demonstrate that *LdAAP7* and *TcAAP7* encode lysine-specific permeases in *L. donovani* and *T. cruzi*, respectively. These two lysine permeases are both members of the large amino acid/auxin permease family and share certain biochemical properties, such as specificity and *K*m. However, we evidence that *LdAAP7* and *TcAAP7* differ in their regulation and localization, such differences are likely a reflection of the dissimilar *L. donovani* and *T. cruzi* life cycles. Failed attempts to delete both alleles of *LdAAP7* support the premise that this is an essential gene that encodes the only lysine permeases expressed in *L. donovani* promastigotes and *T. cruzi* epimastigotes, respectively.

Keywords Lysine transport · Amino acid transport · *Leishmania* · *Trypanosoma*

Introduction

Parasites infect hundreds of millions of people every year and collectively represent one of the principal causes of human misery. Among the protozoa, the Trypanosomatidae family comprises a large number of species responsible for diseases such as sleeping sickness (*Trypanosoma brucei*) and Leishmaniasis (*Leishmania* spp) (Barrett et al. 2003). *Leishmania donovani* and *Trypanosoma cruzi* are obligatory intracellular parasites that cause Kala Azar and Chagas disease in humans, respectively, killing thousands of patients annually (Barrett et al. 2003; Singh et al. 2006). These organisms cycle between insect vectors and mammalian hosts (Herwaldt 1999). Thus, these parasites encounter dramatic environmental changes in temperature, pH and nutrients. The parasites respond to these changes by differentiating into forms that are highly adapted to each environment (Rosenzweig et al. 2008; Mukkada et al. 1985).

Amino acids play a vital role in the life cycle of these parasites, some serving as alternative carbon sources and energy reserves (Mukkada et al. 1974; Opperdoes and Coombs 2007; Pereira et al. 2000) as well as precursors for biosynthesis of key molecules (Gaur et al. 2007; Roberts

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et al. 2004) in addition to participating in osmoregulation (Blum 1996). Hence, there are certain amino acids essential to *Leishmania* and *Trypanosoma* but non- or semi-essential to the host, which consequently represent potential targets for new drugs (Opperdoes and Coombs 2007). Amino acid permeases supply parasite cells with amino acids and accordingly, are key players in the mechanisms underlying adaptation to vector and host environments. Indeed, many permeases involved in such processes became essential during parasite evolution as transport systems supplanted biosynthetic pathways (Ginger 2006).

Earlier studies indicated that biochemically homologous transporters of specific amino acids are found across the various genera of Trypanosomatidae, suggesting that the transporters serve an evolutionarily conserved function (Mazareb et al. 1999; Pereira et al. 1999; Silber et al. 2006; Silber et al. 2002; Zilberstein and Gepstein 1993). Subsequent genomic analyses identified several members of this amino acid/auxin permease (AAAP, TC 2.A.18) gene family in *T. cruzi* and *L. donovani* (Bouvier et al. 2004; Akerman et al. 2004). Further detailed analyses revealed the significance of gene rearrangements during the evolution of these molecules by transpositive duplication, tandem duplication and descent (Jackson 2007).

Previously, we cloned and characterized a member of the AAAP family from *L. donovani* (*LdAAP3*) and *T. cruzi* (*TcAAAP411*) that functions as a high affinity arginine-specific transporter (Shaked-Mishan et al. 2006; Carrillo et al. 2010). Arginine transport is regulated by its availability and by metabolic pathways that require arginine as a precursor (Pereira et al. 2002; Darlyuk et al. 2009). Notably, the response of *Ld*AAP3 to amino acid availability is identical to that reported for the mammalian cation amino acid transporter 1 (CAT1). However, CAT1 transports cationic amino acids in general (lysine and arginine) whereas *Ld*AAP3 and *Tc*AAAP411 translocate only arginine (Hatzoglou et al. 2004; Kandpal et al. 1995; Shaked-Mishan et al. 2006; Carrillo et al. 2010). Lysine is an essential amino acid for most eukaryotes. To date, only plants have been shown to synthesize this amino acid from aspartic acid (Azevedo et al. 1997; Stepansky et al. 2005; Galili et al. 2005). Indeed, trypanosomatids lack the key lysine synthesis enzymes found in plants (Opperdoes and Coombs 2007) and therefore must acquire lysine from their environments via a transporter. In light of our discovery that trypanosomatids, unlike their mammalian or vector hosts, separate lysine from arginine transport, we hypothesized that this parasite feature could play an important role in its life cycle and moreover, provide a new therapeutic approach to managing trypanosomatid pathogens. With these issues in mind, we have identified and characterized high affinity lysine-specific transporters in *L. donovani* and *T. cruzi*. Our present study represents the first characterization of a lysine transporter gene in parasitic protozoa.

Materials and methods

Materials

^{3}H-labeled amino acids and $^{32-}$P-dCTP were from Amersham. The antibiotics G418 and Hygromycin B as well as medium 199 were from Sigma; fetal calf serum was from Biological Industries, Inc. Rabbit polyclonal anti-HA IgG was from Santa Cruz and the fluorochrome-conjugated secondary antibodies were from Jackson. All other reagents were analytical grade.

Phylogenetic analysis

The phylogenetic tree was produced using three different programs. Probcons with default options produced the initial multiple alignment (Do et al. 2005), which was subsequently fed into the FastTree phylogenetic software (Price et al. 2009). Local support values were computed using FastTree to indicate whether each split in the inferred topology is correct. Then tree visualization was done using Dendroscope (Huson et al. 2007).

Parasites culture

A cloned line of *L. donovani* 1SR was used in all experiments (Saar et al. 1998). Cultures were maintained by inoculating growth medium with single colonies of promastigotes from medium 199 agar plates. Promastigotes were grown at 26°C in medium 199 supplemented with 10% fetal calf serum. *T. cruzi* epimastigotes of the CL Brener strain (starting with 10^6 cells per mL) were cultured at 28°C in plastic flasks (25 cm^2) containing 5 mL of LIT medium supplemented with 10% fetal calf serum, 100 U/mL penicillin, and 100 µg/mL streptomycin (Camargo 1964). The parasites were subcultured every 7 days and counted using a hemocytometric chamber.

Yeast strains and growth conditions

*S*train 22Δ6AAL (Fischer et al. 2002) was transformed with pDR195, pDR195-*LdAAP7* and pDR195-*TcAAP7* according to Dohmen et al. 1991. Plasmid expression was selected by growth on minimal medium (0.17% yeast nitrogen source without amino acids and without ammonium sulphate, 2% glucose) with 1 g/L urea as the nitrogen source and 1 g/L 'Lys-Asp' (rich media) or 100 µM lysine (selective media). *S. cerevisiae* strain 22Δ7AA was used for lysine transport

assays (Fischer et al. 2002). This strain was grown in minimal media supplemented with 1 g/L urea.

DNA and RNA work

S. cerevisiae complementation assays

PCR-amplified *LdAAP7* or *TcAAP7*-ORF (GeneDB systematic IDs LinJ32_V3.2800 and Tc00.1047053511545.80, respectively) was cloned into the yeast expression vector pDR195 (Rentsch et al. 1995) between *Xho*I and *Not*I or *Spe*I and *Nsi*I, respectively.

Northern blot analysis

Total RNA was extracted and subjected to northern blotting as described in Barak et al. (2005). Membranes were probed with $^{32-}$P dCTP-PCR amplified *LdAAP7* and *TcAAP7* ORFs (primer sequences in Table 1).

AAP7 over expression

For *LdAAP7* over expression, the *LdAAP7* ORF was cloned into the pNUS HnN expression vector (Tetaud et al. 2002) between the 5′ *Xho*I and 3′ *Kpn*I sites (Table 1). Expression plasmid was transfected into *L. donovani* 1SR using standard electroporation conditions (LeBowitz et al. 1990; Jiang et al. 1999). Transfected colonies were selected on medium 199 agar plates containing 50 μg/mL G418. For *TcAAP7* overexpression, the entire coding sequence of *TcAAP7* (1392 bp) was cloned into pTREX (Vazquez and Levin 1999) or was fused to the 3′ end of the GFP gene present in the pTREX-GFP expression vector. The expression plasmids were transfected into *T. cruzi* as follows: 10^8 parasites grown at 28°C in LIT medium were harvested by centrifugation, washed with PBS, and resuspended in 0.35 mL of electroporation buffer (PBS containing 0.5 mM $MgCl_2$ and 0.1 mM $CaCl_2$). This cell suspension was mixed with 50 μg of plasmid DNA in 0.2 cm gap cuvettes (Bio-Rad Laboratories). The parasites were electroporated using a single pulse of (400 V, 500 μF) with a time constant of about 5 ms. Stable cell lines were achieved after 30 days of growth in the presence of 500 μg/mL G418 (Calbiochem).

LdAAP7 gene replacement

861 bp of the gene 5′ flanking region was cloned upstream to the hygromycin resistance cassette between the 5′ *Sal*I and 3′ *Hin*dIII sites in pKOH plasmid (Ruepp et al. 1997). In addition, 929 bp of the gene 3′ flanking region was cloned downstream of the same hygromycin resistance

Table 1 Primers

PCR target	Primers	Restriction enzymes	Plasmid (reference)	Purpose of reaction
LdAAP7 ORF	Fw:ACTCCGCTCGAGATGAGCGGCGCTAACCACC Rv:ATAAGAATGCGGCCGCTCAAGGGATCTCGCTGAAGA	5′-*Xho*I 3′-*Not*I	pDR-195 (Rentsch et al. 1995)	Expression in *S. cerevisiae*
LdAAP7 ORF with HA tag	Fw:AAAAAACTCGAGATGTACCCATACGACGTCCCAGA CTACGCTATGAGCGGCGCTAACCACC Rv:CGGCGGGGTACCTCAAGGGATCTCGCTGAAG	5′-*Xho*I 3′-*Kpn*I	pNUS-HnN (Tetaud et al. 2002)	Localization and over expression of *LdAAP7*
LdAAP7 5′ flank	Fw:TATACCGGGTACCATAATCGCTCCCCTCTATC Rv:ATACCCAAGCTTCCCGATTGTGCGAAGAGG	5′-*KpN*I 3′-*Hin*dIII	pKON and pKOH (Ruepp et al. 1997)	Homologous recombination of *LdAAP7*
LdAAP7 3′ flank	Fw:AACGCGCGGATCCTTTCTTTCTGTCTCTCTCTC Rv:AACTAGATCTAGAGCGCGCTTAGAAGCAAGAAC	5′-*Bam*HI 3′-*Xba*II		
G418 ORF downstream to LdAAP7 3′ flanking region	Fw:GACCCATGGCGATGCCTG Rv:TGACCAACGTCAACATCGC			Validation of homologous recombination
Hyg ORF downstream to LdAAP7 3′ flanking region	Fw:CGGGCGTATATGCTCCGC Rv:TGACCAACGTCAACATCGC			
TcAAP7 ORF	Fw:ACTAGTATGTATGACAACGTCAATGAGG Rv:ATGCATATCATCAGCCATGGGCTT	5′-*Spe*I 3′-*Nsi*I	pDR-195 (Rentsch et al. 1995)	Expression in *S. cerevisiae*
TcAAP7 ORF with GFP	Fw:ATGTATGACAACGTCAATGAGG Rv:GTCGACTCAGCCATGGGCTTCG		pTREX and pTREX-GFP (Vazquez and Levin 1999)	Localization and over expression of Tc*AAP7*

cassette between the 5′ *Bam*HI and 3′ *Xba*I sites. The fragment containing these *LdAAP7* 5′ and 3′ flanking regions surrounding the hygromycin resistance cassette was amplified by PCR and subsequently electroporated into *L. donovani* promastigotes. Transfected colonies were selected on medium 199 agar plates containing 50 μg/mL hygromycin B. A second fragment containing 5′ and 3′ flanking regions surrounding a G418 resistance cassette was created the same way using pKON plasmid (Ruepp et al. 1997). Heterrosygous colonies from step one were electroporated with the second construct and doubly transfected colonies selected on medium 199 containing 50 μg/mL G418 and hygromycin (see Table 1 for primers). Insertion of the antibiotic resistant markers at the correct location on the genome was validated by PCR in which the reverse primers are targeted downstream to the inserted 3′UTR (see Table 1 for primers and Fig. 5a, b).

AAP7 cellular localization

Localization in *L. donovani* was pursued using immunofluorescence technique as follows: The N terminus of the *LdAAP7* ORF was fused to a hemagglutinin tag. This chimera was cloned into the pNUS-HnN expression vector (Tetaud et al. 2002) between the 5′ *Xho*I and 3′ *Kpn*I sites. *L. donovani* 1SR was transfected with the plasmid using standard electroporation conditions (LeBowitz et al. 1990; Jiang et al. 1999). Transfected parasites were selected on medium 199 agar plates containing 50 μg/mL G418. Mid-log *L. donovani* promastigotes expressing pNUS HnN HA-AAP7 were washed twice with PBS, fixed in 1% formaldehyde/PBS on a slide for 10 min and then permeabilized by exposure to 0.2% Triton X-100/PBS for 10 min. Then blocking solution (10% milk/PBST) was added and the cells were incubated for 30 min at room temperature. Then cells were incubated for 1 h at room temperature with polyclonal rabbit anti-HA (1:200) and a further 1 h in the dark with secondary polyclonal goat anti-rabbit IgG Dy-light 549 (1:500; red, Jackson Inc.). Finally, cells were washed with PBST and 5 μl of 0.5 μg/mL supplemented DAPI (Fluka). Fluorescence analyses were carried out using a fluorescent microscope (Axiovert 200 M-Zeiss).

Cellular localization in *T. cruzi* was done as follows: Freshly grown *T. cruzi* epimastigotes transfected with pTREX-GFP::TcAAP7 were washed twice with PBS. Cells were allowed to settle for 30 min at room temperature onto poly-L-lysine coated coverslips before fixation with 3% formaldehyde in PBS at room temperature for 15 min. DAPI (1.5 μg/mL) was supplemented to visualize the DNA. Cells were observed using an Olympus BX60 fluorescence microscope. Images were recorded using an Olympus DP71 camera and processed using the Olympus DP software.

Transport assays

S. cerevisiae transport studies

Logarithmically growing *S. cerevisiae* strain 22Δ7AA cells were harvested at an OD_{600nm} of 0.8, washed twice with water and resuspended in 0.6 M sorbitol to a final OD_{600nm} of 8. Before transport measurements, the cells were supplemented with 100 mM glucose and 50 mM potassium phosphate pH 4.5 and incubated for 5 min at 30°C. To start the reaction, 130 μL of this cell suspension was added to 130 μL of the same buffer (0.6 M sorbitol, 100 mM glucose, 50 mM potassium phosphate pH 4.5) containing 37 to 92.5 kBq labeled ^{3}H L-lysine and appropriate amounts of unlabelled lysine. Samples were removed after 30, 60, 120, 180 and 300 s, transferred to 4 mL of ice-cold potassium phosphate buffer (50 mM, at appropriate pH), filtered onto glass fiber filters and washed with 9 mL of the same buffer. The amount of tritium on each filter was determined by liquid scintillation spectrometry. The transport activity of *S. cerevisiae* mutants transformed with the empty vector pDR195 served as background and was subtracted from the observed transport measurements. At least three independent repeats of each transport measurement were performed and the mean calculated. When investigating pH dependence, the pH was adjusted prior to the transport experiment; cells resuspended to a final OD_{600nm} of 8 in 0.6 M sorbitol and diluted to a final OD_{600nm} of 6 by adding 0.33 vol of 200 mM phosphate buffer (pH 4.5–7.5) containing 0.6 M sorbitol.

L. donovani transport studies

Logarithmically growing promastigotes were washed twice in ice cold Earl's buffer and concentrated to 10^8 per mL. This cell suspension was mixed with reaction mix (Earl's buffer, 5 mM glucose, 10 mM Tris and 10 mM succinate) at the appropriate pH to a final volume of 570 μl. Cells were pre-incubated for 10 min at 30°C before transport was started. Transport startled by supplementing 30 μl of lysine buffer (^{3}H L-lysine plus non-labeled L-lysine at the appropriate concentration) at the appropriate pH. 100 μl samples were taken at 30, 60, 120, 180 and 300 s and placed directly on 24 mm GF/C glass microfiber filters (Whatman 1822 024). Filters were washed twice with ice cold earl's buffer at the appropriate pH and soaked in scintillation liquid. The amount of tritium on each filter was determined by liquid scintillation spectrometry.

T. cruzi transport studies

Epimastigotes (10^7 parasites) were grown for the indicated periods, harvested by centrifugation at 8,000×*g* for 30 s,

Table 2 Specificity of lysine transport

	S. cerevisiae 22Δ7AA		*L. donovani* promastigotes	*T. cruzi* epimastigotes
Concentration added Addition	100 μM Transport (%)	500 μM	50 μM	200 μM
None[a]	100	100	100	100
Alanine	ND	ND	99 ± 10	99 ± 5
Arginine	128 ± 39	83 ± 14	97 ± 19	98 ± 7
Asparagine	ND	ND	99 ± 7	111 ± 11
Aspartate	76 ± 10	81 ± 19	87 ± 9	94 ± 7
Cysteine	122 ± 23	ND	106 ± 28	60 ± 4
Glycine	ND	ND	91 ± 3	72 ± 3
Histidine	105 ± 26	ND	93 ± 5	88 ± 1
Lysine	10 ± 4	ND	ND	20 ± 4
Methionine	116 ± 54	93 ± 18	95 ± 3	81 ± 2
Proline	76 ± 17	102 ± 13	94 ± 20	78 ± 1
Serine	121 ± 24	ND	98 ± 19	98 ± 11
Threonine	100 ± 40	106 ± 36	110 ± 14	75 ± 2

For the yeast strain 22Δ7AA expressing LdAAP7, transport rates are initial transport of 10 μM ^{3}H-lysine. For *L. donovani* promastigotes and *T. cruzi* epimastigotes, transport rates are initial transport of 5 μM ^{3}H L-lysine and 20 μM ^{3}H L-lysine, respectively

[a] 100% transport corresponds to 4.12 ± 1.3 pmol of L-lysine per minute per 10^6 *S. cerevisiae* 22Δ7AA cells; 0.4 ± 0.1 nmol/min per 10^8 *L. donovani* promastigotes; and 0.021 ± 0.003 nmol/min per 10^8 *T. cruzi* epimastigotes. Assays were performed at 30°C and pH 4.5 for *S. cerevisiae*, at 30°C and pH 6.5 for *L. donovani* and at 28°C and pH 7.0 for *T. cruzi* epimastigotes. The data shown represent the mean of four independent experiments ± SD

washed twice with phosphate-buffered saline (PBS) at pH 7.0 and resuspended in 0.1 mL PBS. Transport was started by adding 0.1 mL of transport mixture that contained 300 μM (or indicated concentration) ^{3}H L-lysine (Perkin Elmer; 2 μCi per assay). Following incubation for the indicated time at 28°C, cells were centrifuged as above and washed twice with 1 mL of ice-cold PBS. Pellets were re-suspended in 0.2 mL of 0.2 N NaOH/0.1% SDS and radioactivity measured using UltimaGold XR liquid scintillation cocktail (Packard Instrument Co., Meriden, CT, USA). A control transport experiment in the presence of 10 mM L-lysine was performed to assess non-specific transport and carry over. Each transport assay was performed at least in triplicate. Cell viability was assessed by direct microscopic examination. Kinetic constants were calculated following the procedures of Lineweaver and Burk as described by Dixon and Webb (Dixon and Webb 1964).

Results

Functional expression in *S. cerevisiae* and sequence analysis

We took advantage of *S. cerevisiae* mutant strains impaired in the transport of various amino acids (see list of mutants in Table 2 of Shaked-Mishan et al. 2006) to screen and functionally characterize lysine transporters from *L. donovani* and *T. cruzi*. In the published genome of *Leishmania infantum* (http://www.tritrypDB.com), the most closely related specie to *L. donovani*, there are twenty-five putative AAPs (Akerman et al. 2004). Using *L. infantum* genome, in this work ten genes were amplified from *L. donovani*, cloned and expressed in the different mutant yeast strains. Among these genes, *LdAAP7* (LinJ32_V3.2800) and its ortholog gene from *T. cruzi* (Tc00.1047053511545.80), enabled growth only of the *S. cerevisiae* strain 22Δ6AAL, a lysine auxotroph (Fischer et al. 2002), on selective lysine concentrations (Fig. 1a). To determine transport characteristics, *LdAAP7* and *TcAAP7* were expressed in another *S. cerevisae* strain called 22Δ7AA that has an additional HIP1 mutation and in contrast to 22Δ6AAL is not a lysine auxotroph and do not require the supplementation of the dipeptide 'lys-asp' for its growth (Fischer et al. 2002). As expected, *LdAAP7* and *TcAAP7* expressing 22Δ7AA cells took up ^{3}H-L-lysine in a time dependent manner whereas cells transformed with empty vector took up negligible amounts of lysine (Fig. 1b) supporting the observation that these AAP7s function as lysine transporters. Kinetic analysis of initial lysine transport rates indicated a K_m of 7.36 ± 3.6 μM for *Ld*AAP7 (Fig. 1c) with optimal transport at pH 4.5 (Fig. 1d). Notably none of the amino acids listed in Table 2, even when supplemented at 10- and 50-fold concentrations, inhibited *LdAAP7*-mediated lysine transport significantly.

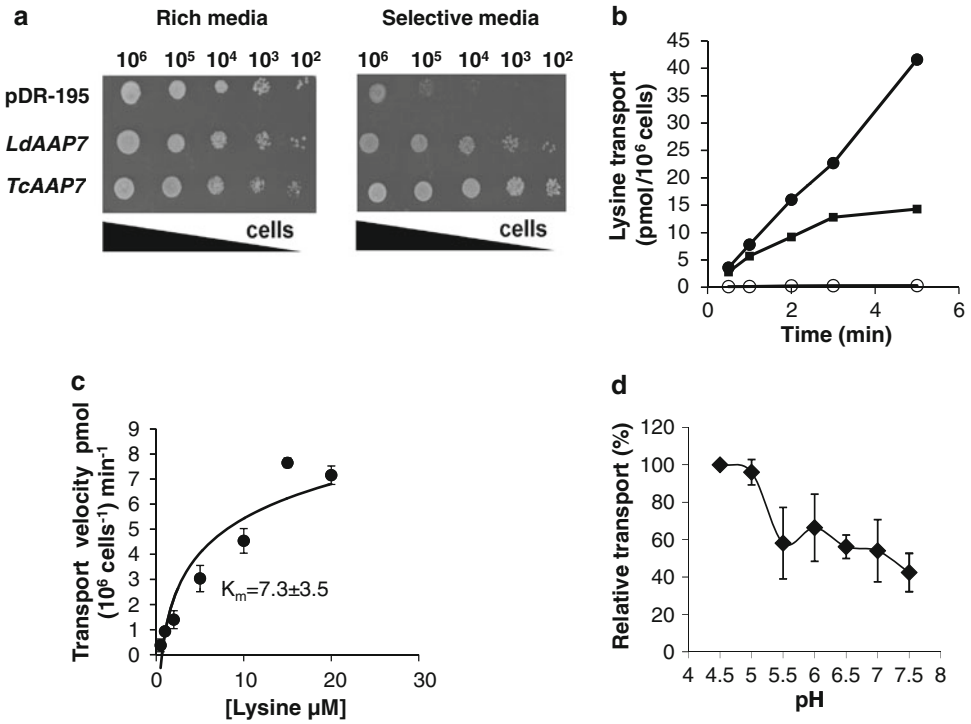

Fig. 1 *Leishmania donovani* AAP7 and *Trypanosoma cruzi* AAP7 mediate lysine transport in *S. cerevisiae*. *S. cerevisiae* strains were transformed with either pDR195-*LdAAP7* (*filled circles*), pDR195-*TcAAP7* (*filled squares*) or pDR195 (*open circles*). **a** Growth on non-selective (minimal medium with 1 g/L urea and the dipeptide 'lys-asp'—*left*) and lysine selective (minimal medium with 1 g/L urea—*right*) media (strain 22Δ6AAL). Number of cells plated is indicated above each column. **b** Time course of lysine transport (strain 22Δ7AA). Transport assays were performed with 1×10^6 cells mL^{-1} at pH 4.5 and 11 μM ^{3}H L-lysine. **c** Kinetic analysis of *Ld*AAP7-mediated initial lysine transport (strain 22Δ7AA). The data are mean values of three independent experiments ± SD. Initial transport rate was determined after 5 min of incubation, at indicated lysine concentrations, pH 4.5 and 30°C. **d** pH profile of LdAAP7-mediated lysine transport (strain 22Δ7AA). The data are mean values ± SD ($n = 3$). One hundred per cent transport corresponds to 11.2 ± 4 pmol lysine per minute per 10^6 cells. Initial transport rate was determined after 5 min of incubation at the time points indicated in panel B, and at indicated pH, 20 μM ^{3}H L-lysine and 30°C

LdAAP7 (accession number ABD64602) is a single copy gene on chromosome 32 of *L. donovani*. The encoded protein is 504 amino acids long and contains 11 predicted transmembrane domains and as such belongs to the large amino acid/auxin permease family (AAAP; TC 2.A.18;(Busch and Saier 2003; Akerman et al. 2004). According to TriTrypDB (http://www.tritrypDB.com) this gene is syntenic with *L. infantum* LinJ32_V3.2800, *L. major* LmjF32.2660, *L. braziliensis* LbrM32_V2.2900, *T. brucei* Tb11.01.7500 and *T. cruzi* Tc00.1047053511545.80. Therefore, we named these genes *LinAAP7*, *LmjAAP7*, *LbrAAP7*, *TbAAP7* and *TcAAP7* (formerly, *TcAAAP545*), respectively. The amino acid sequences of *Ld*AAP7 and *Lin*AAP7 are identical and henceforth we describe characterization of only *Ld*AAP7. Phylogenetic analysis performed using the amino acid sequences of these trypanosomatid AAP7 proteins and various plant, bacterial, yeast and mammalian lysine transporters revealed that the putative AAAPs from all members of the trypanosomatid family form a closely related group (Fig. 2). Additionally, the analysis showed that lysine transporters from *Arabidopsis thaliana* (ATF/AAAP) are contiguous to trypanosomatid AAP7s (Rentsch et al. 2007), supporting that the latter function as lysine transporters. Most of the other lysine transporters in the analysis belong to the APC super family and accordingly, appear quite distinct from the putative trypanosomatid permeases.

Over expression and subcellular localization

To confirm that *Tc*AAP7 and *Ld*AAP7 mediate lysine transport also in parasite cells, we expressed them ectopically in epimastigotes and promastigotes, respectively, and subjected the transgenic parasites to transport analysis. With regards to growth rate and morphology, the phenotypes of *TcAAP7* and *LdAAP7* over expressing parasites were identical to those of wild type parasites (data not shown). In line with the *S. cerevisiae* data demonstrating that TcAAP7 functions as a lysine permease, *T. cruzi* parasites transfected with pTREX-*TcAAP7* exhibited a transport rate about 50-fold higher than parasites transfected with empty vector (Fig. 3c). As expected, Northern blot analysis confirmed elevated *TcAAP7* mRNA

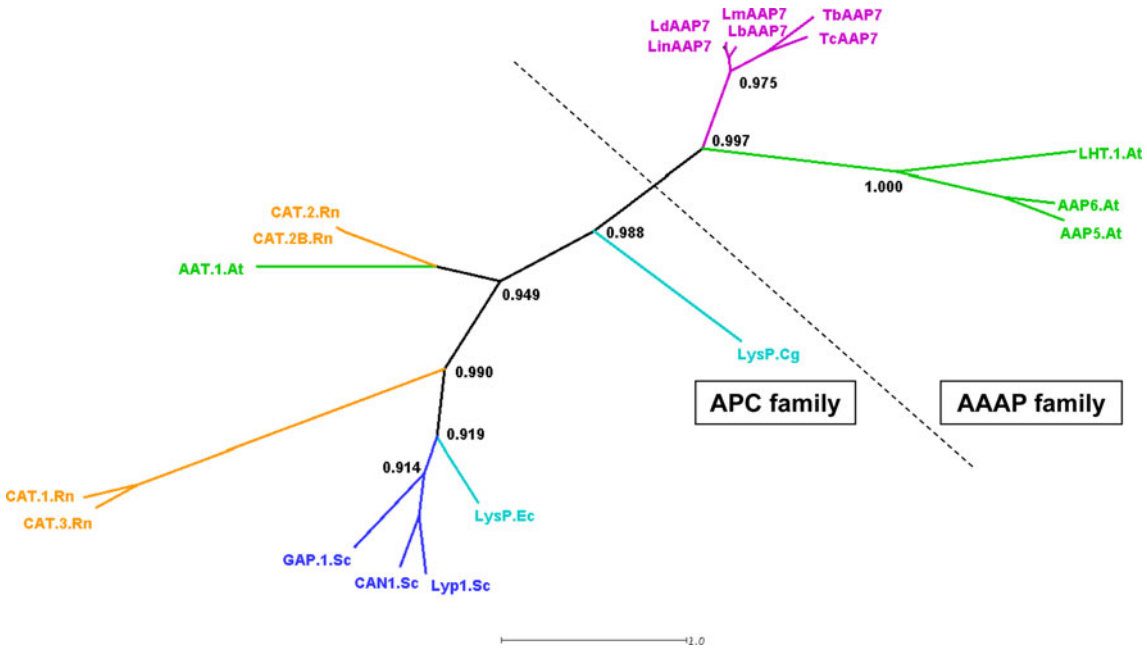

Fig. 2 Phylogenetic analysis of global lysine transporters. Radial phylogenetic tree of 19 lysine transporter genes including the putative amino acid permeases from Trypanosomatidae. Trypanosomatid transporter sequences with the following ID numbers were taken from GeneDB (http://www.genedb.org): *LinAAP7-LinJ32_V3.2800*, *LmAAP7- LmjF32.2660*, *LbAAP7- LbrM32_V2*.2900, *TcAAP7-Tc00.1047053511545.80* and *TbAAP7- Tb11.01.7500*. Other lysine transporter sequences with the following accession numbers were taken from Genbank (http://www.ncbi.nlm.nih.gov): from *S. cerevisiae*, Gap1- P19145, Can1- XP_714306.1 and Lyp1- CAA47729; from bacteria, LysP (*E. coli*) -NP_416661.1, LysP (*Corynebacterium glutamicum*)- NP_600195.1; from *A. thaliana*, LHT1- AAC49885.1, AAT1- NP_193844.2, AAP5- NP_175076.2, AAP6- NP_199774.1; from mammals (*Rattus norvegicus*), CAT1- XP_859317.1, CAT2 -AAD40315.1, CAT2B- NP_072141.2 and CAT3 NP_058913.1. *Colors* indicate different phyla; trypanosomatids are colored *violet*, plants *green*, bacteria *cyan*, yeasts *blue* and mammals are colored *orange*. Local support values are shown for major clusters. See text for details (color figure online)

expression (Fig. 3a). In contrast, ectopic expression of *LdAAP7* in *L. donovani* promastigotes grown in medium 199 did not increase lysine transport activity despite the elevated *LdAAP7* expression evidenced by Northern blot analysis (Fig. 3b, c). We reasoned that this could be due to saturating levels of lysine in the growth medium (that contains 0.35 mM lysine) and therefore repeated the experiment under reduced lysine conditions. However, no significant change in lysine transport was observed in *L. donovani* promastigotes over expressing *LdAAP7* grown in medium 199 containing no lysine and dialyzed fetal bovine serum (not shown).

Next, we confirmed the substrate specificity of lysine transport in wild type *L. donovani* promastigotes and *T. cruzi* epimastigotes (Table 2) and in *T. cruzi* parasites over expressing *TcAAP7* (data not shown). Twelve amino acids were assayed as competitors in transport experiments, but none inhibited lysine transport significantly in either parasite. Notably, *TcAAP7* over expressing parasites displayed the same substrate specificity as wild type epimastigotes. In summary, these data show that *Ld*AAP7 and *Tc*AAP7 function as lysine-specific permeases in *L. donovani* promastigotes and *T. cruzi* epimastigotes, respectively. Notably, the identical amino acid specificity of LdAAP7 both in yeast and promastigotes strongly suggest that in promastigotes, LdAAP7 is the sole lysine transporter.

To investigate the subcellular localization of these AAP7 proteins, HA-tagged *Ld*AAP7 was expressed in *L. donovani* promastigotes and GFP-tagged *Tc*AAP7 in *T. cruzi* epimastigotes. In *L. donovani* promastigotes the transporter was localized to the surface membrane and flagella (Fig. 4a). However, in *T. cruzi* epimastigotes the transporter was localized mainly to a membrane-bound structure or invagination close to the kinetoplast, the latter corresponding to the flagellar pocket or associated structures, such as the cytostome or contractile vacuole (Fig. 4b, c). These data are consistent with the recently published localization of a putrescine-cadaverine transporter (Hasne et al. 2010). Of note, parasites over expressing GFP-tagged *Tc*AAP7 were demonstrated to exhibit elevated lysine transport levels similar to parasites over expressing *Tc*AAP7, confirming the functionality of the GFP-tagged permease (data not shown).

LdAAP7 is essential for *L. donovani* survival

To determine if *Ld*AAP7 is essential, we carried out gene replacement as described originally by Cruz et al. (Cruz et al.

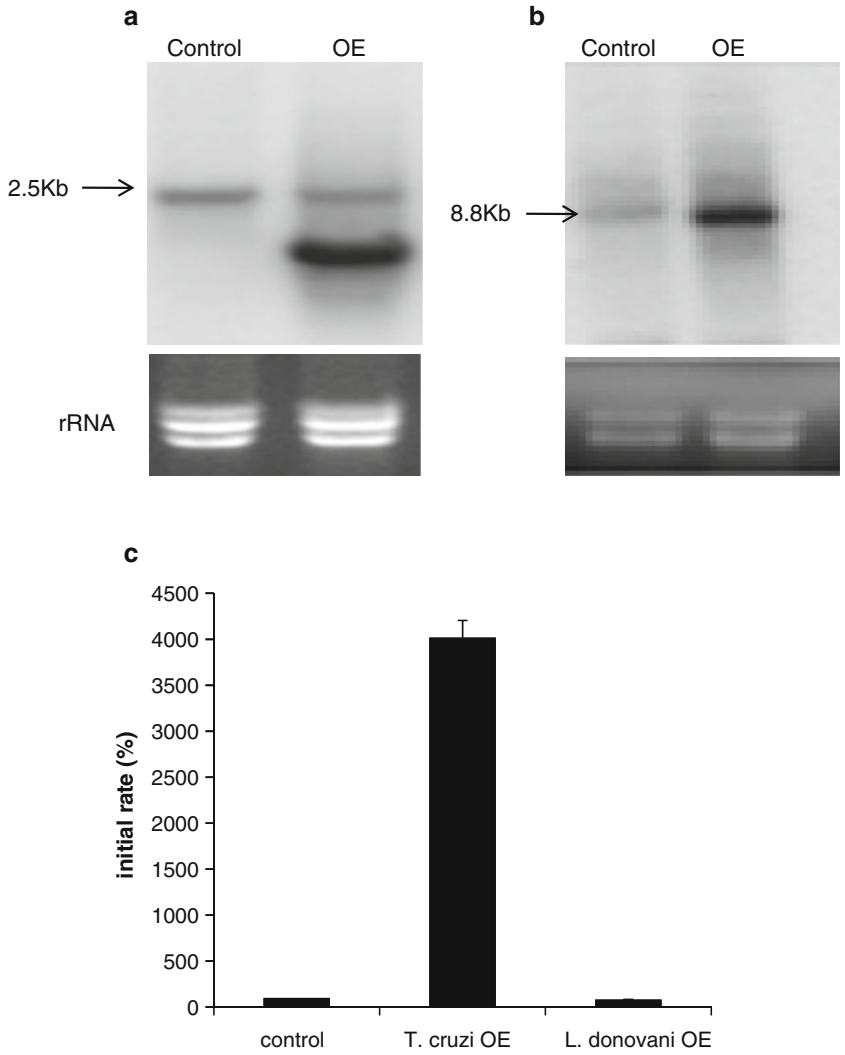

Fig. 3 *Ld*AAP7 and *Tc*AAP7 mediate lysine transport in *L. donovani* promastigotes and *T. cruzi* epimastigotes, respectively. **a** Northern blot analysis of total RNA samples obtained from wild type *T. cruzi* transfected with pTREX (*control*) or pTREX-TcAAP7 (*OE*). The upper band in both lanes is the endogenous *TcAAP7* mRNA whereas the lower band present only in lane 2 is pTREX-*TcAAP7* mRNA. **b** Northern blot analysis of total RNA extracted from wild type *L. donovani* promastigotes transfected with pNUS HnN (*Control*) or pNUS HnN-AAP7 (*OE*). Transcript sizes are indicated. The three ribosomal RNA bands serve as loading controls. **c** Initial rate of lysine transport in log phase epimastigotes and promastigotes versus AAP7 over expressing parasites. For *T. cruzi*, transport experiments were performed over 20 min at 150 μM ^{3}H L-lysine, pH 7 and 28°C. 100% transport corresponds to 0.022 ± 0.0021 nmol/min per 10^{8} cells. For *L. donovani*, transport experiments were performed over 5 min at 10 μM ^{3}H L-lysine, pH 5 and 30°C. 100% transport corresponds to 0.9 ± 0.2 nmol/min per 10^{8} cells

1991). This procedure involves two steps, as the gene must be deleted from each *Leishmania* allele (Fig. 5a). The *LdAAP7* ORF present on one allele was replaced with the gene coding for hygromycin resistance to generate heterozygous mutants. Subsequently, the second allele was replaced with the gene coding for G418 resistance. This step yielded only 10 colonies that were resistant to both antibiotics. PCR was employed to confirm insertion of both antibiotic resistance genes in the expected orientation within the *L. donovani* promastigote genome (Fig. 5b). This notwithstanding, an *LdAAP7* ORF could still be amplified by PCR (Fig 5b) and Northern blot analysis evidenced *LdAAP7* RNA expression, though at significantly lower levels than that observed in wild type parasites (Fig. 5c). Analysis of Solexa sequencing runs performed using genomic DNA from *L. donovani* 1SR (the strain used in this work) has indicated trisomy of a few *L. donovani* chromosomes, in particular chromosome 31 (Myler, P.J., personal communication). However, such data indicates there are only two alleles of chromosome 32 that encodes *LdAAP7*. Therefore, we suspect that our gene replacement procedure induced duplication of the region coding for this transporter, suggesting that *LdAAP7* is indeed an essential *Leishmania* gene. In line with this premise, the duplicated gene was found to be functional,

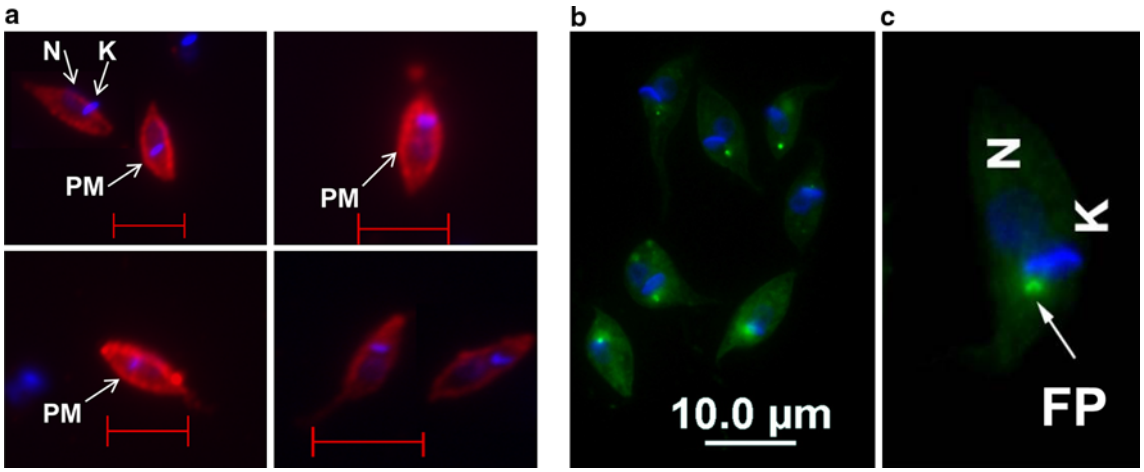

Fig. 4 Cellular localization of AAP7 in *L. donovani* and *T. cruzi*. **a** Immunofluorescence images of *L. donovani* promastigotes expressing HA-tagged LdAAP7. Cells were stained for HA (*red*) and with DAPI (*blue*). Scale indicates 10 µm. **b**, **c** Fluorescence images of *T. cruzi* epimastigotes expressing GFP-tagged TcAAP7 (*green*). Cells were stained with DAPI (*blue*). *Arrow* indicates the flagellar pocket (*FP*), plasma membrane (*PM*), the positions of the nucleus (*N*) and kinetoplast (*K*) are also indicated (color figure online)

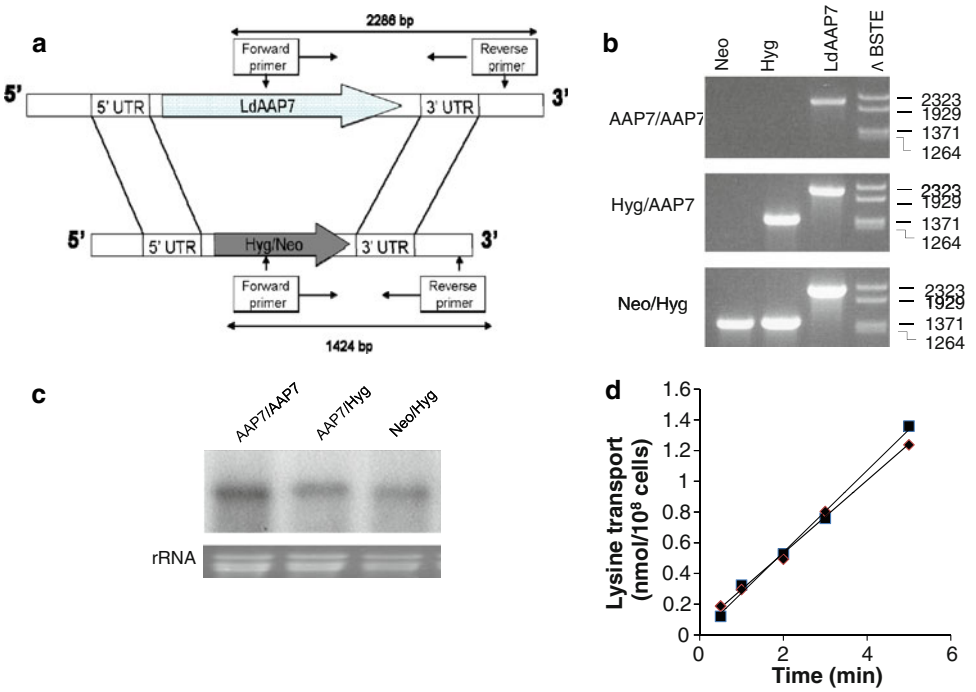

Fig. 5 Elimination of *LdAAP7* from *L. donovani* genome. **a** Strategy of knockout (*KO*) procedure. Primer locations in the *LdAAP7* ORF and in the G418/Hyg fragments replacing the *LdAAP7* ORF are indicated as well as expected PCR fragment lengths. **b** PCR on genomic DNA extracted from wild type promastigotes (AAP7/AAP7), promastigotes after the first step (AAP7/Hyg) or promastigotes after the second step (G418/Hyg). Reaction made with primers targeted to: forward: middle of G418 resistance gene and reverse: downstream to the 3′ flanking region (*Left*), Forward: middle of hygromycin, reverse: downstream to the 3′ flanking region (*Middle*) and forward: middle of LdAAP7 ORF, reverse: downstream to the 3′ flanking region (*right*). **c** Northern blot analysis on total RNA extracted from same cells. Membrane was probed with *LdAAP7* ORF. **d** Lysine transport of logarithmic phase wild type promastigotes (*filled squares*) and promastigotes after the first step of the knock out (*filled diamonds*). Transport assays were carried out at 10 µM ^{3}H L-lysine and pH 7

with the rate of lysine transport in the mutants comparable to that observed in wild type parasites (Fig. 5d). Of note, the lower *LdAAP7* mRNA expression but almost wild type lysine transport displayed by the mutants suggests that translational or posttranslational up-regulation of *LdAAP7* expression is occurring in the mutant.

Since lysine is an essential amino acid and the aforementioned replacement study suggests that *LdAAP7* is an

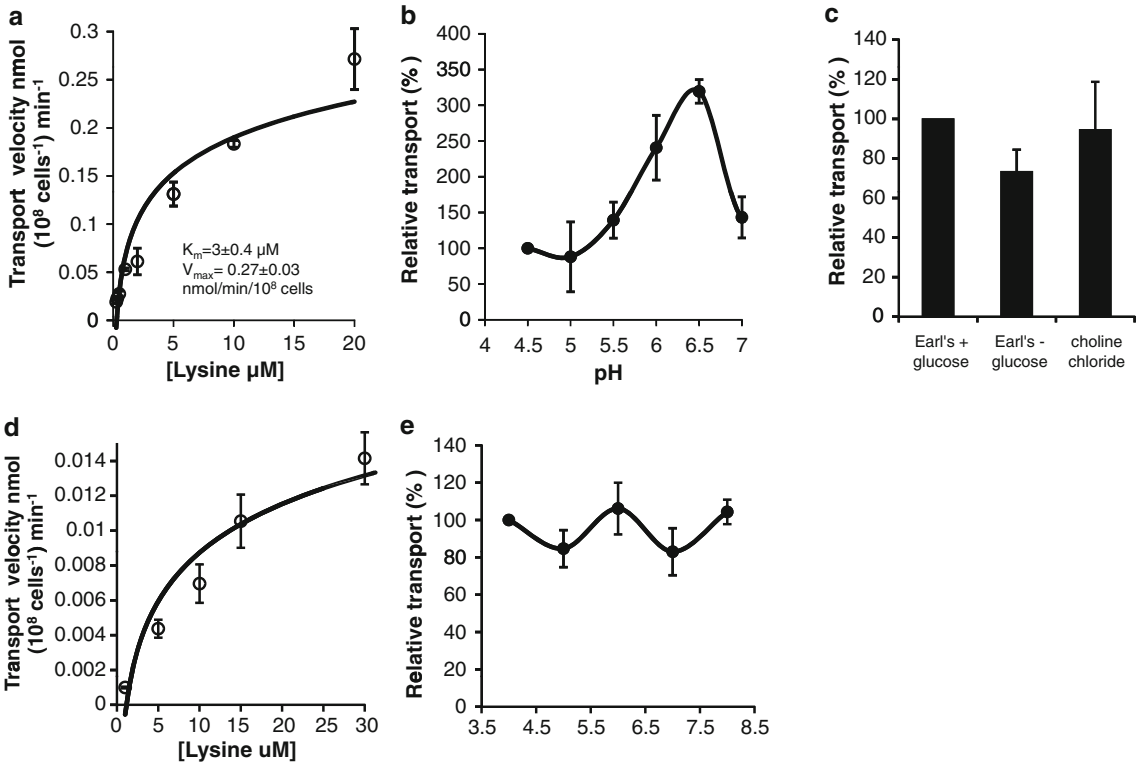

Fig. 6 Lysine transport in *L. donovani* promastigotes and *T. cruzi* epimastigotes. **a** Kinetic analysis of lysine transport in *L. donovani* promastigotes. The data are mean values of three independent repeats ± SD. Initial transport rate was determined over 5 min of incubation, at the indicated lysine concentrations, pH 7 and 30°C. **b** pH optimum of L-lysine transport in *L. donovani* promastigotes. Initial transport rate was done as indicated for panel B. Transport assays were carried out at 5 µM ^{3}H L-lysine, pH 4.5–7 and 30°C. The data are mean values of four independent repeats ± SD. 100% corresponds to 0.4 ± 0.12 nmol/min per 10^8 cells. **c** The effect of glucose and cations on lysine transport in *L. donovani* promastigotes. 100% transport corresponds to lysine transport in Earl's buffer containing 5 mM glucose. Transport assays were carried out at 5 µM ^{3}H L-lysine and pH 7. Results indicate the mean values of three independent repeats ± SD. **d** Kinetic analysis of lysine transport in *T. cruzi* epimastigotes. Initial rates of lysine transport (V_0) were measured as a function of lysine concentration in the range 1–30 µM. The data are mean values of three independent repeats ± SD. **e** pH optimum of lysine transport in *T. cruzi*. Initial transport velocities (V_0) were measured at pH ranging from 4 to 8. 100% transport corresponds to 0.023 nmol/min per 10^8 cells. Obtained values were evaluated using a one-way ANOVA followed by a Bonferroni's multiple comparison test between all pHs and all the comparisons P values were non-significant ($P < 0.001$)

essential gene, we conclude that *Ld*AAP7 is the sole lysine transporter in *L. donovani* promastigotes. Hence, lysine transport analysis in intact parasite cells reflects *Ld*AAP7 activity. When performing the same gene replacement procedure with *T. cruzi* epimastigotes, the first *TcAAP7* allele was successfully replaced by a G418 resistance marker but replacement of the second allele failed.

Regulation and kinetic analyses of lysine transport

Assuming that LdAAP7 is the only lysine permease in *L. donovani*, we carried out more detailed functional analysis of *Leishmania* lysine transport. Transport of lysine by *L. donovani* promastigotes was found to increase linearly with time (Fig. 5d). The K_m of lysine transport in intact promastigotes was 3 ± 0.4 µM with a V_{max} of 0.27 ± 0.03 nmol/min per 10^8 cells (Fig. 6a); this matches the K_m value determined using *S. cerevisiae* expressing *LdAAP7* (Fig. 1c). However, in the range tested, the optimum pH for lysine transport by promastigotes was pH 6.5, which differs from that determined using *S. cerevisiae* expressing *LdAAP7* (Figs. 6b, 1d, respectively). To ascertain if glucose influences lysine transport we suspended promastigotes in Earl's salt solution with and without 5 mM glucose. No significant effect of glucose on lysine transport was observed (Fig 6c). Then, to study the role of cations (Na^+ and K^+) in lysine transport, promastigotes were suspended in a solution at pH 7 in which both potassium and sodium had been replaced by choline (Mazareb et al. 1999). The initial transport rate was unchanged in the choline solution indicating that *Ld*AAP7 is a cation-independent lysine transporter (Fig. 6c).

In the insect vector, *Leishmania* promastigotes exist in two forms, proliferating non-virulent procyclic

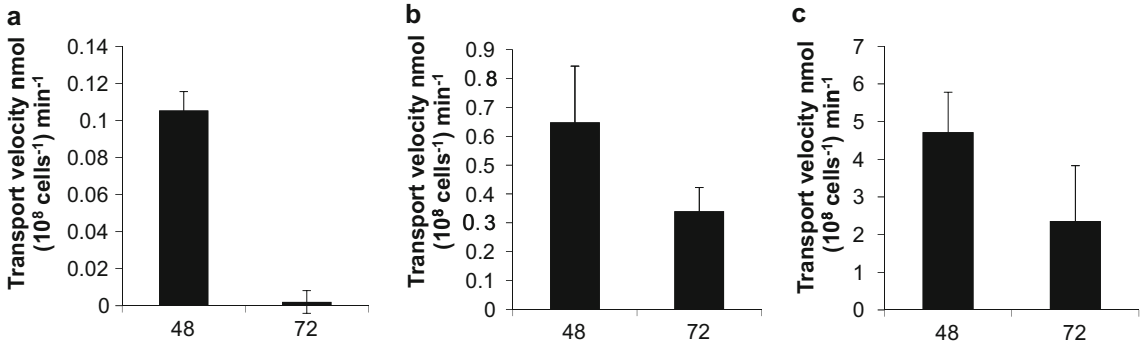

Fig. 7 Effect of culture age on lysine transport in *L. donovani* promastigotes. Initial transport rates were measured after 48 h (log phase) and 72 h (stationary phase) of growth for **a** 1 μM ^{3}H L-lysine, **b** L-arginine, and **c** L-proline at pH 7 and 30°C. Promastigote initial cell density was 5 × 10^5 cells/mL

promastigotes and non-dividing virulent metacyclic promastigotes. In axenic culture, log phase cells correspond to procyclics whereas metacyclics develop during late stationary phase (da Silva and Sacks 1987; Sacks and Perkins 1984). To assess if lysine transport changes during promastigote development we determined the transport rate of cultures at different stages of axenic growth. Accordingly, a culture was inoculated at 5 × 10^5 promastigotes per mL and subsequently lysine transport assayed after 48 and 72 h. We have shown previously that after 4 days such a culture reaches stationary phase as indicated by expression of the metacyclic-specific gene *SHERP1* (Saxena et al. 2007). Thus, after 48 and 72 h the cultures contain mid- and late log phase cells, respectively. Lysine transport decreased dramatically after 72 h, indicating that lysine transport is indeed influenced by development (Fig. 7a). For comparison, we checked the influence of development on arginine and proline transport and found that transport of these amino acids was reduced as cultures aged but less so (Fig. 7b, c, respectively).

Although we did not manage to establish if *TcAAP7* is the only lysine permease in *T. cruzi*, we proceeded to characterize lysine transport by this parasite. Unlike what we observed for *L. donovani*, in *T. cruzi* epimastigotes lysine transport increased linearly with time only for the first 10 min, the transport rate dependent on lysine concentration but saturating at lysine concentrations over 100 μM. V_{max} and K_m values, 0.024 ± 0.001 nmol/min per 10^8 cells and 23.4 ± 2.3 μM, respectively, were generated from Lineweaver–Burk plots of concentration-dependent initial influx rates (V_i) (Fig. 6d). Lysine transport was assessed in epimastigote cells after starvation for 2 h in PBS and in similarly starved cells but when the PBS had been supplemented either with glucose, lysine, arginine, proline or glycine (Table 3). We found that lysine transport increased 140% after 2 h of starvation in PBS and about 400% when the PBS contained glucose. Of note, parasites starved in PBS-lysine exhibited lysine transport rates decreased by fourfold supporting the existence of lysine sensing mechanisms; this effect was not observed for the other amino acids tested. Next, we examined if pH influences *T. cruzi* lysine transport. In accordance with previous studies concerning the pH dependence of amino acid transport in *T. cruzi* epimastigote cells (Canepa et al. 2004; Canepa et al. 2005; Canepa et al. 2009; Pereira et al. 1999; Silber et al. 2006; Tonelli et al. 2004) and unlike what we observed for *L. donovani*, lysine transport was constant between pH 4 and 8 (Fig. 6e). Finally, in a similar manner to that described for *L. donovani* above, we examined if *T. cruzi* lysine transport is influenced by development. As seen with *L. donovani*, lysine transport rates decreased with culture age; lysine transport was 11-fold lower on culture day 14 relative to day 4 (Table 3).

Table 3 Regulation of lysine transport in *T. cruzi*

Treatment/condition	Transport % (±SD)
Control	100 ± 8.1
Starved (2 h)	141 ± 12.1
Starv. w/GLC	405 ± 27.8
Starv. w/LYS	25 ± 5.6
Starv. w/ARG	80
Starv. w/PRO	138 ± 24.1
Starv. w/GLY	79 ± 18.4
Cultured day 4	100 ± 5.2
Cultured day 7	48 ± 3.1
Cultured day 14	9 ± 2.4

Starvation constituted a 2 h incubation in PBS. Where indicated, the PBS was supplemented with 10 mM glucose, 10 mM arginine, 10 mM proline, 10 mM lysine or 10 mM glycine. Transport rates are initial transport of 150 μM ^{3}H L-lysine. Where indicated transport was measured using parasite samples in different growth phases, early logarithmic (day 4), late logarithmic (day 7) and stationary phase (day 14), with transport on day 4 taken as 100%

In summary, in *L. donovani* promastigotes lysine transport increases linearly with time and is mono specific,

pH dependent, glucose independent and influenced by developmental stage. In contrast, in *T. cruzi* epimastigotes lysine transport is pH independent, glucose dependent and influenced by developmental stage.

Discussion

Lysine is used mostly for protein synthesis and is an essential amino acid for non-plant eukaryotic cells. Accordingly most eukaryotic cells, including *Leishmania* and *Trypanosoma*, acquire it from their environments using specific permeases. Here we identified and characterized genes that encode lysine permeases in *L. donovani* and *T. cruzi*, *LdAAP7* and *TcAAP7*, respectively. Both proteins are mono-specific, high affinity, and low capacity transporters. *Ld*AAP7 translocates lysine against its concentration gradient in a K^+ and Na^+ independent mechanism; with $[\text{lysine}]_{out} = 25\ \mu M$ and $[\text{lysine}]_{in} = 3.8\ mM$ (Darlyuk et al., 2009). The specificity of these trypanosomatid permeases for lysine supports our original premise that, unlike higher eukaryotes, *Leishmania* and *Trypanosoma* separate lysine from arginine transport completely (Shaked-Mishan et al. 2006). Mammalian cells, including macrophages that host *Leishmania*, translocate both amino acids through cation amino acid transporters (Christensen 1990; Closs et al. 1993; Kim et al. 1991).

*Leishmania donovani Ld*AAP7 is syntenic with *L. infantum Lin*AAP7, *L. major Lmj*F32.2660 and *L. braziliensis Lbr*M32_V2.2900 (http://www.tritrypDB.com). Thus we suggest that these latter proteins are also lysine transporters. This is corroborated by our finding that *Tb*AAP7 mediates lysine transport (Inbar et al. unpublished). Further support for the premise that trypanosomatid lysine transporters differ from those in other eukaryotes is provided by our phylogenetic analysis, which highlights that trypanosomatid permeases belong to the AAAP family whereas the permeases of other eukaryotes are members of the APC family.

Our attempts to delete *LdAAP7* from the *L. donovani* promastigote genome failed due to gene duplication; Cells after two gene replacements were resistant to both G418 and hygromycin. PCR indicates both antibiotics at the correct orientation with an additional allele that contains AAP7 ORF. Moreover, Solexa sequencing analyses indicate that the *L. donovani* 1SR chromosome 32 that carries *LdAAP7* is present at only two alleles (Myler P.J., personal communication). Therefore, we surmise that the *LdAAP7* duplication observed in the knockout experiments suggests that this gene is essential for parasite survival and is the only lysine transporter expressed in promastigotes. Of note, this finding that *L. donovani* promastigotes express only one lysine transporter represents another difference in lysine transport between these parasites and their hosts. For in general, higher eukaryotes encode at least two distinct transporters that can translocate lysine; for example, Lyp1p, and Gap1p in *S. cerevisiae* and CAT 1, 2 and 3 in mammalian cells (Ito and Groudine 1997; Reviewed in Malandro and Kilberg 1996). We suspect that arginine and not lysine is the priority substrate of the arginine/lysine transporters of higher eukaryotes and this could explain why these organisms evolved to encode more than one such transporter.

Several attempts to delete both copies of *TcAAP7* in *T. cruzi* epimastigotes failed. This failure could be interpreted as preliminary evidence for the uniqueness and essential nature of the *T. cruzi* lysine permease; further studies must validate this interpretation. This notwithstanding, another indication that the *T. cruzi* lysine transport system is unusual is its pH-independence. Moreover, an excess of lysine in starvation medium inhibited lysine transport whereas glucose promoted transport. These observations point to the existence of energy-dependent substrate-sensing mechanisms that regulate *T. cruzi* lysine transport. It is notable that the emerging phenotype of *T. cruzi* lysine transport resembles glucose-dependent arginine homeostasis in *L. donovani* (Darlyuk et al. 2009). A further indication of the unique nature of *TcAAP7* is its location next to the flagellar pocket. The flagellar pocket constitutes an invagination of the plasma membrane where the flagellum exits the cytoplasm and is involved in exocytosis, endocytosis, cell polarity and cell division (Field and Carrington 2009). Several flagellar pocket-associated proteins have been identified, however, this is the first report of an amino acid permease concentrated close to this structure. The specific cellular location of TcAAP7, which contrasts with the uniform distribution of LdAAP7, raises the possibility that metabolite intake from the extracellular media is somehow facilitated by this membrane structure in *T. cruzi* epimastigotes.

Overexpressing *TcAAP7* in *T. cruzi* epimastigotes increased lysine transport almost by 50-fold, validating its function as a lysine transporter in parasite cells. In contrast, *L. donovani* promastigotes over expressing *LdAAP7* exhibited no increase in lysine transport. These findings suggest that *Leishmania* promastigotes tightly regulate their cellular pool of lysine, which accords with the observation that the concentration of cellular lysine remains stable in these parasites even during starvation (Darlyuk et al. 2009) and development (Goldman, Rentsch and Zilberstein, unpublished). Plants also control tightly cellular lysine concentrations. The critical nature of this lysine regulation is confirmed by the abnormal phenotypes exhibited by transgenic plants engineered to have high levels of free lysine in vegetative tissues (Galili et al. 2005). Given that *L. donovani* appear to control cellular lysine levels more tightly than *T. cruzi*, it is perhaps

surprising that our kinetic analysis data indicate that *Ld*AAP7 and *Tc*AAP7 have similar biochemical characteristics, namely similar K_m and specificity. However, as described above, *T. cruzi* lysine transport does appear to exhibit some unusual regulatory and localization properties that could account for the global differences in lysine pool regulation between the two parasites.

Notably, both *T. cruzi* epimastigotes and *L. donovani* promastigotes reduced lysine transport as cultures aged. We observed that stationary phase parasites transported less proline and arginine as well but lysine transport in particular was almost completely shut down. It was shown in microorganisms such as yeast and bacteria that the level of general protein synthesis declines when cells enter the stationary phase (Boucherie 1985; Braun et al. 1996; Goldberg and St John 1976). Unlike other amino acids such as proline and arginine (Darlyuk et al. 2009), lysine does not appear to be involved in other processes beyond protein synthesis. Thus, if lysine transport would not be reduced in stationary phase its cellular levels will rise. Given the toxic effect of high lysine concentrations on numerous organisms, we suspect that stationary phase cells have evolved ways to shut down lysine transport.

In summary, lysine is an essential amino acid for trypanosomatids and its transport is mediated by lysine permeases belonging to the AAAP family. We show here that Trypanosomatids, unlike other eukaryotes, possess transporters dedicated to lysine. A potential ramification of this finding is that parasite lysine transporters could serve as vehicles for selective drug delivery or drug targets.

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