Leishmania RAB7: characterisation of terminal endocytic stages in an intracellular parasite

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Abstract

Leishmania species are intracellular parasites that inhabit a parasitophorous vacuole (PV) within host macrophages and engage with the host endo-membrane network to avoid clearance from the cell. Intracellular Leishmania amastigotes exhibit a high degree of proteolytic/lysosomal activity that may assist degradation of MHC class II molecules and subsequent interruption of antigen presentation. As an aid to further analysis of the endosomal/lysosomal events that could facilitate this process, we have characterised a Leishmania homologue of the late endosomal marker, Rab7, thought to be involved in the terminal steps of endocytosis and lysosomal delivery. The Leishmania major Rab7 (LmRAB7) protein is expressed throughout the life-cycle, shows 73 and 64% identity to Trypanosoma cruzi and Trypanosoma brucei Rab7s (TcRAB7 and TbRAB7), respectively, and includes a kinetoplastid-specific insertion. The recombinant protein binds GTP and polyclonal antibodies raised against this antigen recognise structures in the region of the cell between the nucleus and kinetoplast. By immunoelectron microscopy of axenic amastigotes, Leishmania mexicana Rab7 (LmexRAB7) is found juxtaposed to and overlapping membrane structures labelled for the megasomal marker, cysteine proteinase B, confirming a late-endosomal/lysosomal localisation. © 2002 Published by Elsevier Science B.V.

Keywords: Small GTPase; Lysosome; Endocytosis; Rab7

1. Introduction

During the life cycle of Leishmania, parasites exist either as intracellular amastigotes within a specialised phagolysosome of vertebrate macrophages, or as extracellular promastigotes in the digestive tract of their sandfly vector [1]. Within the sandfly, promastigotes undergo metacyclogenesis, differentiating from non-infective procyclics to infective metacyclics [2,3], a process essential for parasite survival within the vertebrate host. Whereas procyclics are sensitive to both complement-mediated lysis and the microbicidal environment of the phagolysosome, the morphologically distinct metacyclics are resistant to complement and preadapted for survival within macrophages and differentiation into amastigotes [4].

The amastigote resides and replicates within a parasitophorous vacuole (PV) resembling a late endosomal/lysosomal compartment [4]. Studies of intra-macrophage Leishmania mexicana amastigotes suggest that molecules are imported into the PV from the host cytosol via two independent mechanisms. Macromole-

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Abbreviations: conA, concanaavalin A; DAPI, 4',6-diamidino-2-phenylindole; DIC, differential interference contrast; EST, expressed sequence tag; FITC, fluorescein isothiocyanate; GAP, GTPase activating protein; GST, glutathione S transferase; HASPB, hydrophilic acylated surface protein B; MHC, major histocompatibility complex; MVT, multivesicular tubule; NMT, N-myristoyl transferase; PV, parasitophorous vacuole; TRITC, tetramethylrhodamine isothiocyanate.

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molecules enter by subversion of the host recycling machinery (autophagy), while small anionic molecules (e.g. Lucifer Yellow) utilise an active transport mechanism attributed to the vacular membrane organic anion transporter of the host cell [5]. Once delivered to the PV, macromolecules are endocytosed by the amastigote via the flagellar pocket [6,7]. The PV is similar to MHC class II compartments of antigen-presenting cells, in which peptide-class II complexes are formed prior to their exposure at the cell surface. Significantly, in Leishmania infected cells, amastigotes endocytose and degrade MHC class II molecules and the class II cofactor H-2M [8], thus interrupting the antigen presentation process and subsequent parasite killing. Concomitantly, proteolytic activity is upregulated in Leishmania amastigotes as compared with promastigotes, and this is thought to correlate with the appearance of multivesicular megasomes and the ability to internalise and probably degrade MHC molecules [10].

Leishmania promastigotes possess a novel multivesicular tubule (MVT) that functions as an endosomal/lysosomal compartment and stretches from the flagellar pocket region (the sole site of exo-/endocytosis) towards the posterior end of the cell [11–13]. However, the Leishmania endocytic machinery has not yet been described at the molecular level. As a starting point, and as mammalian and yeast Rab7s are known to regulate late endocytic processes [14,15], we have characterised Rab7 homologues from Leishmania major (LmRAB7) and L. mexicana (LmexRAB7).

2. Materials and methods

2.1. Parasites

L. major (MHOM/IL/81/Friedlin) promastigotes were maintained as previously described [16]. L. major amastigotes were cultivated by inoculation of 10^6 metacyclics into BALB/c mouse footpads and subsequent parasite harvesting from lesions. L. mexicana (MNYC/BZ/62/M379) promastigotes were maintained and differentiated according to published protocols [17]. In vitro infection of peritoneal macrophages from BALB/c mice was carried out as described [18].

2.2. Cloning of LmRAB7

The N-terminus of a putative L. major Rab protein (EMBL T97244) was identified during an analysis of L. major expressed sequence tags (ESTs) [19]. Following determination of the full 1197bp cDNA sequence, an open reading frame (ORF) was deduced (Q9N2P5) encoding a 223 amino acid protein sharing identity with known Rab7 proteins. The LmRAB7 gene is contained within cosmID L2647 of the Leishmania Genome Network cLHYG cosmid library [20]. BLAST searches were conducted using wu-blastp (Gish, W. (1996–2001) http://blast.wustl.edu) and sequence alignments using clustalw [21]. Phylogenetic analyses were performed on the edited alignment using maximum parsimony (Protpars, Felsenstein, J. 1993. Phylogeny Inference Package (phylip, version 3.5c); distributed by the author, Department of Genetics, University of Washington, Seattle, USA).

2.3. Expression of LmRAB7

For antibody production, the LmRAB7 ORF was amplified using Pfu polymerase (Stratagene), cloned into pGEX-2TK (Pharmacia), expressed as a GST fusion protein in Escherichia coli DH5α cells and purified on glutathione-Sepharose 4B (Pharmacia). After quantitation by SDS-PAGE and Coomassie blue staining, 50 μg of GST-fusion protein was inoculated into each of several TO outbred mice with Freund’s incomplete adjuvant (Sigma), a procedure repeated four times. Specific antibodies were affinity-purified from sera using recombinant protein coupled to CNBr-activated Sepharose 4B (Pharmacia). The resulting eluate was negatively selected by passage through a GST column (Pharmacia). Denatured parasite lysates were separated and immunoblotted as described [16]. Filters were probed with anti-LmRAB7 polyclonal primary antibody at 1:1000, anti-NMT at 1:1000 or anti-HASPB at 1:1000 (ab336; [22]) followed by horse-radish peroxidase-conjugated secondary antibodies (Sigma) and complexes were detected using the ECL system (Amersham Pharmacia).

For GTP binding assays, the LmRAB7 ORF was cloned into pET15 (Novagen), expressed in E. coli BL21 (DE3) cells and purified according to the manufacturer’s instructions. Recombinant protein was separated by SDS-PAGE, blotted, renatured and a GTP overlay assay performed with [35P]-GTP in the presence and absence of competitor nucleotides at 1 mM [23]. All expression constructs were verified by DNA sequencing.

2.4. Immunofluorescent microscopy

Cells were washed in PBS, fixed in 3% paraformaldehyde PBS, pH 7.5 for 20 min at room temperature, washed further and attached to polylysine-coated slides (Sigma). Following permeabilisation in 0.2% Triton X-100 in 5% FCS/PBS at room temperature for 30 min, cells were stained with anti-LmRAB7 antibody at 1:100 for 1 h at room temperature. Anti-mouse FITC secondary antibody (Sigma) was used according to the manufacturer’s protocol. Slides were prepared for microscopy and images obtained/processed as previously described [16].
For concanavalin A uptake assays, L. major promastigotes were washed in serum-free DMEM and incubated with TRITC-labelled concanavalin A (Sigma) at 50 μg ml⁻¹ on ice for 10 min, before transfer to 26 °C for 30 min [24], prior to processing for immunofluorescence as described above.
2.5. Immuno-electron microscopy

Parasites were fixed in suspension by incubation with an equal volume of 8% paraformaldehyde/0.4% glutaraldehyde in PBS for 10 min on ice, followed by 4% paraformaldehyde/0.2% glutaraldehyde for further 1 h. The cells were washed in PBS, infiltrated overnight in 2.3 M sucrose in PBS at 4 °C, attached to a specimen stub and plunge-frozen in liquid nitrogen. Ultra thin cryosections (50 nm) were cut on a Leica UCT ultramicrotome with EM FCS cryoattachment. Sections were mounted on grids with formvar support, blocked with 0.02 M glycine in PBS followed by 10% FCS in PBS (diluent for all following reagents). Sections were stained with anti-LmRAB7 mouse antibody at 1:20 for 1 h, then goat anti-mouse FAB fragment-10 nm gold (TAAB Laboratories Ltd) for 1 h. Sections were counter-stained with anti-cysteine proteinase B rabbit antibody at 1:200 for 1 h, then 15 nm Protein A gold (Department of Cell Biology, University Medical School, Utrecht). Contrast was achieved by incubation with uranyl acetate in methyl cellulose on ice for 10 min.

3. Results and discussion

3.1. Identification of a Rab7 homologue in L. major

The role of the Rab small GTPase subfamily in endomembrane dynamics is the focus of intense study in Trypanosoma brucei [25] but little is known about this protein family in Leishmania, particularly in intracellular parasite stages. Thus far, only a L. major homologue of mammalian Rab1A has been characterised and localised to the Golgi apparatus [26].

A putative RAB gene was identified during a screen of Leishmania ESTs. BLAST analysis identified the ORF encoded by this gene as a Rab7 homologue and this protein has been designated LmRAB7 (Q9N2P5). The lmRAB7 gene maps to chromosome 18 of the L. major Friedlin molecular karyotype (data not shown). The deduced amino acid sequence of LmRAB7 is 83% similar and 73% identical to its nearest homologue, the kinetoplastid T. cruzi TcRAB7 [27]. The other available kinetoplastid sequence, that of T. brucei TbRAB7 (TRYP9.0.000912; retrieved from the Pathogen Se-
quencing Unit database, Sanger Institute), is 78% similar and 64% identical. The Rab7s from other eukaryotes are less closely related, as expected: human HsRab7 is 64% similar and 50% identical to LmRAB7, while S. cerevisiae Ypt7 is 63% similar and 47% identical. Mammalian Rab7 and yeast Ypt7 are both involved in late endosomal trafficking [14], which suggests that LmRAB7 may also have a role in transport at the terminal stages of the endocytic pathway. An alignment of these and other Rab7 homologues, together with LmRAB1 is shown in Fig. 1. The highly conserved Rab domains involved in GTP binding and hydrolysis, and protein–protein interactions are depicted: GTP/GDP binding domains (G1–G4) and the GTPase-activating protein (GAP) binding domain or effector (Eff). The presence of these domains, together with a C-terminal prenylation motif, indicates that LmRAB7 is a functional Rab protein. Interestingly, all three Rab7 sequences from the Kinetoplastida, TcRAB7 [27], TbRAB7 and LmRAB7, contain an insertion of 20 residues preceding the G4 domain.

To facilitate phylogenetic study of Rab7, the alignment (Fig. 1) was analysed using maximum parsimony via the PHYLIP package, designating LmRAB1 as an outgroup. The resulting phenogram (Fig. 2) places LmRAB7 with TcRAB7 and TbRAB7 as divergent Rab7s, distinct from higher multicellular organism Rab7s and yeast Ypt7. Bootstrap values from 100 replicates are shown. Hs, H. sapiens; Rn, R. norvegicus; Dm, D. melanogaster; At, A. thaliana; Gm, G. max; Sp, S. pombe Ypt, Rab7 homologue; Sc, S. cerevisiae Ypt, Rab7 homologue; Lm, L. major; Tc, T. cruzi; Tb, T. brucei; LmRAB1-outgroup. Accession numbers as in Fig. 1.

Fig. 2. An artificially rooted phenogram-like tree diagram created from a maximum parsimony analysis of the edited alignment in Fig. 1. LmRAB1 is the designated outgroup. The tree groups LmRAB7, TcRAB7 and TbRAB7 as divergent Rab7s, distinct from higher multicellular organism Rab7s and yeast Ypt7. Bootstrap values from 100 replicates are shown. Hs, H. sapiens; Rn, R. norvegicus; Dm, D. melanogaster; At, A. thaliana; Gm, G. max; Sp, S. pombe Ypt, Rab7 homologue; Sc, S. cerevisiae Ypt, Rab7 homologue; Lm, L. major; Tc, T. cruzi; Tb, T. brucei; LmRAB1-outgroup. Accession numbers as in Fig. 1.

Fig. 3. (A) GTP overlay assay of SDS-PAGE fractionated, blotted and renatured recombinant LmRAB7. Recombinant LmRAB7 (35 kDa) bound [32P]-GTP (control). However, [32P]-GTP binding was competed out by an excess (1 mM) of GTP (GTP) or GDP (GDP), but not by 1 mM ATP (ATP), indicating that LmRAB7 is a guanine-specific nucleotide binding protein. (B) Expression of LmRAB7 and LmexRAB7 through the Leishmania life cycle. Immunoblotting using polyclonal anti-LmRAB7 identified L. major LmRAB7 and L. mexicana LmexRAB7 as 25 kDa proteins that are expressed in procyclic (P) and metacyclic (M) promastigotes, and in lesion or axenic amastigotes (A) of L. major and L. mexicana, respectively. Using the defined lifecycle stages of L. mexicana, it is clear by comparison with the LmexNMT loading control that LmexRAB7 is expressed at similar levels in procycyles and amastigotes, but is reduced in quiescent metacycyles.

ancient, predating eukaryotic speciation, and that Rab7 function in the terminal part of the endocytic pathway is evolutionarily conserved.
3.2. LmRAB7 is a GTP binding protein expressed throughout the Leishmania life cycle

To confirm that LmRAB7 is a functional GTP binding protein, purified recombinant His-tagged LmRAB7 was used in a GTP-binding overlay assay [23] (Fig. 3A). In this analysis, recombinant LmRAB7 bound $^{32}$P-GTP and this binding was competed by addition of either 1 mM GTP or 1 mM GDP, but not with 1 mM ATP, indicating that LmRAB7 binds specifically to guanine nucleotides. Together with the sequence analysis shown in Fig. 1, these data strongly suggest that LmRAB7 is an active GTPase.

Proteolytic activity is highly up-regulated in Leishmania amastigotes, relative to insect stage promastigotes, and it has been proposed that this correlates with the appearance of multivesicular megasomes and the ability to internalise and probably degrade MHC molecules [10]. To determine whether this increase in proteolytic/endocytic activity correlates with Rab7 expression (as may be predicted from its role in late endocytic transport), whole cell lysates of L. major and L. mexicana procyclics, metacyclics and amastigotes were immunblotted with anti-LmRAB7 (Fig. 3B). Both species were used because, unlike L. major, L. mexicana promastigotes can be induced to undergo metacyclogenesis and differentiate into axenic amastigotes [17]. Thus homogeneous parasite populations can be generated in vitro, while those from L. major are heterogeneous and, in the case of amastigotes, purified from lesions. Anti-LmRAB7 recognises LmRAB7 and the L. mexicana RAB7 homologue (LmexRAB7) as 25 kDa proteins, expressed throughout the lifecycle (Fig. 3B). A polyclonal antibody against the constitutively expressed Leishmania N-myristoyl transferase (NMT; Price et al., submitted) was used as a loading control for both species, while antibody against the infective stage specific protein, HASPB, was used to monitor parasite differentiation [22]. From this analysis, it is clear that L. mexicana metacyclics express lower levels of Lmex-
RAB7, as might be expected in this quiescent non-replicative form. However, axenic amastigotes do not express more Rab7 than procyclics, suggesting that higher levels are not required to facilitate any potential increase in lysosomal traffic in the intracellular lifecycle stage.

3.3. LmRAB7 localises to the Leishmania late endosomal/lysosomal region

To establish the sub-cellular localisation of LmRAB7 in promastigotes, *L. major* procyclic parasites were labelled with concanavalin A (ConA) and ‘chased’ for 30 min to allow uptake and labelling of the cellular endocytic network. Cells were then processed for immunofluorescence and stained with anti-LmRAB7 (Fig. 4A). LmRAB7 is shown to reside in structures largely located between the kinetoplast and the nucleus, where the majority of endocytic compartments are found in trypanosomatids [25]. The partial co-localisation observed between ConA and LmRAB7 is consistent with LmRAB7 localisation to an accessible endocytic compartment, most likely the Leishmania equivalent of the mammalian late endosome/lysosome.

To determine the subcellular localisation of LmRAB7 in intra-macrophage amastigotes, peritoneal macrophages were isolated from BALB/c mice and incubated with stationary phase *L. major*. After infection was
established (48–72 h), the macrophages were fixed and stained with anti-LmRAB7. In intracellular amastigotes, LmRAB7 localises to a body near the kinetoplast/flagellar pocket, again presumably a late endosomal/lysosomal compartment (Fig. 4B). To further characterise this amastigote compartment, we performed immuno-electron microscopy on *L. mexicana* axenic amastigotes and were able to detect reproducible low level reactivity against LmexRAB7 (Fig. 5). Co-staining with the megasomal marker, cysteine proteinase B (CPB) [28], demonstrates that LmRAB7-labelled material lies close to and within CPB-labelled structures. This localisation supports the proposal that *Leishmania* Rab7 functions in endocytic transport from late endosomes to lysosomes, as proposed for mammalian Rab7 [15,29].

### 3.4. Conclusions

Despite the likely importance of the endomembrane network in intracellular stages of *Leishmania*, its role, regulation and complexity in both exo- and endocytic traffic remain largely unstudied. As a first step to the elucidation of these pathways, we have cloned a *L. major* Rab7 homologue, LmRAB7. Analyses of sequence data and nucleotide binding studies strongly suggest that LmRAB7 is a functional Rab7 homologue that is expressed throughout the parasite life cycle in both *L. major* and *L. mexicana*. Immunofluorescence and immuno-EM show that LmRAB7 is located in endocytic/lysosomal compartments in both promastigotes and amastigotes.

Mammalian Rab7 has been implicated in endocytic transport from early to late endosomes [30,31] and from late endosomes to lysosomes [29,31]. A similar detailed delineation of late endosomal compartments has not yet been made in kinetoplastid species. Further analyses of the *Leishmania* endo-membrane network may facilitate study of amastigote–macrophage interactions and provide insight into how this pathogen manipulates its environment in order to evade the immune response of the host.

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### References


